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Integrin-linked Kinase (ILK) Expression in Moderately Differentiated Human Oesophageal Squamous Carcinoma Cell Lines – Growth Factor Modulation, Activity and Link to Adhesion



Glenn Alan Driver

A thesis submitted to the Faculty of Science, University of the Witwatersrand, in fulfillment of the requirements for the degree of PhD

Johannesburg, 2007

Declaration

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Philosophy of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

_____

Glenn Alan Driver

_____ day of _____ 2007

Mom and Dad
Thank you for all your support and encouragement

Abstract

Integrin-linked Kinase (ILK) is an integrin-associated protein kinase, which regulates growth factor-signalling pathways and cell-ECM adhesion events. Abrogated ILK expression or activity has been implicated in contributing to oncogenic transformation. We examined the role played by ILK in growth factor-stimulated and integrin signalling events in five human oesophageal squamous cell carcinoma cell lines (HOSCCs), known to overexpress the EGF receptor. Western blot analysis revealed the presence of ILK (59kDa) in all the moderately differentiated HOSCC lines. ILK1 was confirmed as being the predominant isoform. Densitometrically analysed Western blots showed that, per unit of protein, ILK is expressed uniformly across the cell lines under standard culture conditions. Following EGF (10 ng/ml) and TGFβ1 (1 ng/ml) treatment, ILK expression increased in all five HOSCCs. Indirect immunofluorescence microscopy showed the majority of ILK to localise at a cytoplasmic/nuclear level, with a proportion of ILK localising at the membrane, which resembled the distribution pattern of the β_3 integrin subunit. This membranal distribution most likely follows that of the adhesion plaques although lesser, and variable, amounts were also identified throughout the cytoplasm. The functionality of the ILK1 kinase domain was demonstrated using myelin basic protein (MBP)-based kinase assays. EGF and TGFβ1 treatment produced an increase in ILK activity in the WHCO3 cell line of 3.5 fold, but a decrease in activity in the other cell lines, which are suggested to involve the actions of PTEN. The identification of nuclear ILK was surprising, and the mechanism for nuclear ILK localisation was suggested to involve a caveolae-associated protein, caveolin-1. Cell adhesion assays revealed that KP-392-mediated inhibition of ILK resulted in a nonsignificant reduction in cell adhesion to collagen and fibronectin. These data provide distinctive evidence for the influence of growth factors on ILK expression, but a duality in the effect on ILK activity. This apparent dichotomy is noteworthy and may be of particular relevance in HOSCC. It is further suggested that KP-392-induced ILK inhibition destabilises the αβ integrin heterodimer and that PI3K acts upstream of ILK-mediated cell adhesion events in HOSCCs. This suggests that ILK mediates integrin associated processes in human oesophageal SCC cell lines.

List of Associated Publications and Presentations

Local Conferences

Driver GA and Veale RB. Integrin Linked Kinase (ILK) Modulation by Growth Factors and its Link to Cell-ECM Adhesion in Human Oesophageal Squamous Cell Carcinomas. Accepted for presentation at the *18th Congress of the South African Society of Biochemistry and Molecular Biology. Molecules of Life UP-North, University of Pretoria*. 6-9 July 2003.

Driver GA, Jones LJ and Veale RB. Crosstalk between β-catenin and ILK signalling cascades in human oesophageal squamous cell carcinoma of the oesophagus. Accepted for presentation at the *SASBMB XIX th Conference, Stellenbosch University.* 16-20 January 2005.

Driver GA and Veale RB. The Redistribution of ILK/Caveolin-1 in Human Oesophageal Carcinoma Cells Following Exposure to EGF. Accepted for presentation at the *SASBMB XX th Conference, Molecular Meander in the Midlands. University of Kwazulu Natal Pietermariztburg*, 2-6 July 2006

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International Conferences

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List of Abbreviations

ANOVA	analysis of variance
AP-1	activating protein complex
APS	ammonium persulphate
BAD	Bcl-2 associated death promoter
Bcl-2	B-cell lymphoma 2
bp	base pair
BSA	bovine serum albumin
Ca^{2+}	calcium
CAM	cell adhesion molecule
cAMP	cyclic adenosine monophosphate
Cdc42	cell division cyclin 42
Cdk	cyclin dependent kinase
CH-ILKBP	calponin homology domain-containing ILK-binding protein
CHO	chinese hamster ovary cells
CREB	cAMP-responsive element binding protein
dH_2O	distilled water
DMEM	Dulbecco's modified eagles medium
DNA	deoxyribonucleic acid
EC	extracellular
ECM	extracellular matrix
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular-signal-regulated kinase
E2F	E2F transcription factor 2
FA	focal adhesion
FAK	focal adhesion kinase
FC(B)S	foetal calf (bovine) serum
FITC	fluoroscine isothiocyanate
Fn	fibronectin
g	gram(s)

GSK3β	glycogen synthase kinase-3β
HCl	hydrochloric acid
HOS	human osteogenic sarcoma
HOSCC	human oesophageal squamous cell carcinoma
HRP	horseradish peroxidase
IEC	intestinal epithelial cells
Ig	immunoglobulin
Ig-SF	immunoglobulin superfamily
ILK	integrin-linked kinase
IRS-1	insulin receptor substrate-1
JNK	*c-Jun* NH2-terminal kinase
kDa	kilodalton
LD	leucine-aspartate repeat
Lef	lymphoid enhancer factor
M	molar
mA	milliampere(s)
mM	millimolar
Mr	relative molecular weight
MAPK	mitogen activated protein kinase
MBP	myelin basic protein
ml	millilitre(s)
MLC	myosin light chain
MMLV	Moloney Murine Leukemia Virus
MNNG	N-methyl-N'-nitro-N-Nitrosoguanidine
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NaAc	sodium acetate
NaOH	sodium hydroxide
NF-γB	nuclear factor of kappa light chain gene enhancer in B-cells
ng	nanogram(s)
nM	nanomolar
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PDGF	platelet derived growth factor
PH	pleckstrin homology domain
PI3K	phosphoinositide-3-OH-kinase
PINCH	particularly interesting new calponin-homology-domain-containing protein
PKB	protein kinase B
PMSF	phenyl-methyl-sulphonyl fluoride
PTEN	phosphatase and tensin homologue deleted on chromosome ten
$P130^{CAS}$	p130 Crk-associated substrate
RhoA	Ras homolog member A
RIA	radioimmunoassay
RNA	ribonucleic acid
SA	South Africa
SCC	squamous cell carcinoma
SDS	sodium dodecyl sulphate
SF	stress fibre
SH2	Src homology domain
SOS	Son of Sevenless
TCA	trichloro-acetic acid
TCF	T-cell factor
TE	Trypsin/EDTA
TEMED	*N,N,N',N'*-tetramethylethylene-diamine
TGFβ1	transforming growth factor β1
Tris	tris(hydroxymethyl)aminomethane
μCi	microcurie
μg	microgram(s)
UK	United Kingdom
μl	microlitre
uPA	urokinase plasminogen activator
USA	United States of America
VEGF	vascular epithelial growth factor
WHCO-	Wits Human Carcinoma of the Oesophagus-

Chapter 1

The Importance of ILK in Cell Signalling

1.1 Cell Adhesion in Tissue Maintenance

In virtually every multicellular organism, cell adhesion exists as a fundamental mechanism that provides a framework which allows for the communication between cells and their surrounding tissues. The information that cells receive from adhesion contacts must be integrated to regulate diverse processes such as protein synthesis and cell growth, motility, cell architecture and polarity, differentiation, and programmed cell death. The signalling pathways that regulate these processes are not isolated from one another but are interconnected to form complex signalling networks (Martin, 2003). From a cell biology perspective, disturbances in these regulatory mechanisms of cell adhesion are crucial to the progression of cancer. While covering the entire scope of the intricacies of cancer is a near impossibility, this literature review attempts to clarify these regulatory mechanisms, which play a pivotal role with regards to cancer progression.

Before an understanding of the process of cell adhesion can be developed, one must appreciate that although cancer invasion and metastasis are intricate processes, the initial steps of metastasis involve the detachment of neoplastic cells from the primary tumour and eventual establishment at distant tissue sites (Kurschat and Mauch, 2000). This metastatic cascade stems from the dysregulation of cell-cell and cell-extracellular matrix interactions (Nair *et al*., 2005).

Multiprotein complexes which consist of cell adhesion molecules (CAMs), extracellular matrix (ECM) proteins and cytoplasmic plaque proteins serve primarily to mediate adhesion of cells either to the extracellular matrix (ECM), or to other cells. This determines the overall structure of tissues (Hynes, 1999; Grashoff *et al*., 2004; Wu, 2004; Nair *et al*., 2005). CAMs function in a dynamic and highly regulated fashion and are necessary in orchestrating the three-dimensional structure of tissues (Edelman and Crossin, 1991; Gumbiner, 1996). As is discussed above there are three general classes of proteins which make up the multiprotein complexes that form the functional units of

cell adhesion, these are i) the cell adhesion molecules/adhesion receptors, ii) the extracellular matrix (ECM) proteins, and iii) the cytoplasmic plaque/peripheral membrane proteins (Jawhari *et al*., 1997; Grashoff *et al*., 2004; Nair *et al*., 2005).

Cell adhesion falls into two principle categories i) cell-cell adhesion where physical bonds are formed between adjacent cells, and ii) cell-matrix adhesion where these same physical bonds bind cells to adhesive proteins in extracellular matrices (Ruoslahti and Öbrink, 1996; Watt, 2002; Reddig and Juliano, 2005; Su *et al*., 2005; Vespa *et al*., 2005). The cell adhesion receptors are transmembrane glycoproteins that mediate binding interactions at the extracellular (EC) surface and determine the specificity of cell-cell and cell-ECM recognition. They include members of the integrin, cadherin, immunoglobulin, selectin, and proteoglycan superfamilies (Albelda and Buck, 1990; Gumbiner, 1996; Malik and Parsons, 1996; Hynes, 1999; Stewart *et al*., 2004; Nair *et al*., 2005). The five primary adhesion molecules will now be discussed in greater detail, but emphasis will be placed on the cadherin and integrin families since these receptors are the major regulators of epithelial cell adhesion. Epithelial cells have a particular morphology, which is maintained by a characteristic organisation of the cytoskeleton as well as the adhesion molecules (Braga, 2000; Perez-Moreno *et al*., 2003).

The nervous system and immune system consists of many molecules with one or more Ig domains (Hynes, 1999; Nair *et al*., 2005). It is in these areas that the immunoglobulin superfamily (Ig-SF) adhesion receptors dominate. The Ig-SF receptors are characterised by the presence of varying numbers of Ig-related domains (Hynes, 1999; Peggs and Allison, 2005). These domains consist of two β sheets held together by homophilic self-association, as is the case with the cadherins (Ruoslahti and Öbrink, 1999; and discussed below). However, although Ig-SF members are involved in homophilic associations certain Ig-SF members interact utilising heterophilic associations, such is the case with integrins (e.g. NCAM) or with ECM proteins (e.g. DCC-netrins) (Hynes, 1999). The immunoglobulin superfamily has also been correlated with tumour progression in certain instances. For example, melanomas are known to express a diverse range of cell adhesion molecules. These include neural cell adhesion molecules such as L1 (a ligand of $\alpha_5\beta_3$, NCAM and CD57) (Johnson, 1999).

The selectins are the second well-studied group of cell adhesion receptors. They will be considered briefly, as they are not expressed in epithelia and are thus not pertinent to this study. Although the selectins are not extensively expressed they do have crucial roles in cells of the vertebrate circulation (endothelium and blood cells) (Hynes, 1999). They are carbohydrate-specific receptors, which utilise heterophilic interactions for functioning (Rosen, 2004). For example, P selectin binds to its counter-receptor, a heavily glycosylated protein (PSGL-1). The C-type lectin domain, which mediates the association, recognises specific carbohydrate groupings in the counter-receptor/ligand (Hynes, 1999; Middleton *et al*., 2002). The selectins are not isolated molecules and are known to be crucial in the adhesion of leukocytes to endothelium, by cooperating with integrins and Ig-SF receptors (Lasky, 1995; Hynes, 1999; Witz, 2006).

So far we have examined two sets of adhesion molecules, both of which play pivotal roles in the maintenance of tissue architecture. However, when considering cell-cell and cell-ECM interactions, the remaining two adhesion receptors are crucial (Hynes, 1999). The structure and functioning of the cadherin and integrin class of adhesion receptors has been comprehensively studied and they are known to be vital in the regulation of many cellular processes (O'Toole *et al*., 1994; Gumbiner, 1996; Wijnhoven *et al*., 2000; Perez-Moreno *et al*., 2003; Su *et al*., 2005). Not only are they important with regards to normal cellular functioning, but they also play a pivotal role during the metastatic process in many cancers (Felding-Habermann *et al*., 2001). This occurs when the intricate processes of cell adhesion go awry in cells where mutations, changes in expression or localisation, and functional alterations of these molecules have been observed (Somasiri *et al*., 2000; Wijnhoven *et al*., 2000).

The cadherin family that mediates Ca^{2+}- dependent cell-cell adhesion, are transmembrane glycoproteins, that play crucial roles in both developmental and disease processes (Hinck *et al*., 1994; Ramburan and Govender, 2002; Christofori, 2003; Foty and Steinberg, 2004; Nair *et al*., 2005). Cadherins are genetically distinct from the integrin and immunoglobulin superfamily, and in the presence of Ca^{2+}, bind cells tightly by homophilic interactions (Shiozaki *et al*., 1996; Goodwin and Yap, 2004). There are three common cadherins, which are named according to their tissue distribution; these include E- (epithelial), N- (neuronal) and P- (placental) cadherins (Gumbiner, 1996; Wijnhoven *et al*., 2000; Christofori, 2003; Goodwin and Yap, 2004; Chan, 2006). Of

these, E-cadherin is probably the best studied and most relevant to this particular study. E-cadherin forms the key functional component of adherens junctions between epithelial cells (Jamora and Fuchs, 2002). During the induction and maintenance of polarised and differentiated epithelium during embryonic development, the normal expression of E-cadherin is especially important (Wijnhoven *et al*., 2000; Perez-Moreno *et al*., 2003). For example, absence of E-cadherin in epithelia results in cells being incapable of maintaining a tight association (Gumbiner, 1996). Furthermore, the many other cell adhesion and cell junction proteins that occur in epithelial cells are by themselves unable to support intercellular adhesion (Gumbiner, 1996).

In the maintenance of cell-cell adhesions, cadherins associate with cytoplasmic proteins such as α-catenin and β-catenin (Goodwin and Yap, 2004). The cytoplasmic domain of E-cadherin complexes with β-catenin or plakoglobin (also called γ-catenin). Plakoglobin and β-catenin also interact with α-catenin, which is thought to directly or indirectly link the complex composed of cadherin, β-catenin and α-catenin to the actin cytoskeleton (Caca *et al*., 1999; Kaibuchi *et al*., 1999; Braga, 2000; Jamora and Fuchs, 2002). Indeed, deficiency of α-catenin in mice results in deficits of epidermal intercellular tightness and numerical reduction of adherens junctions (Vasioukhin *et al*., 2001). Although cell-cell adhesions are generally static processes, dynamic rearragements of cell-cell interactions can occur during various cellular processes. For example, epithelial cell scattering, dispersal of cancer cells and early embryonic cell migration all require perturbation of cell-cell contacts (Kaibuchi *et al*., 1999).

β-Catenin is known to have a dual function in cells influencing both cell adhesion as well as intracellular signalling via the canonical Wnt/Lef pathway (Korswagen *et al*., 2000; Young *et al*., 2003; Bienz, 2004; Brembeck *et al*., 2006). Thus the association of E-cadherin with β-catenin may allow for E-cadherin-mediated signal transduction events. For instance, cadherin-mediated cell-cell adhesion can affect the Wnt signalling pathway (Polakis, 2000). β-catenin (as well as γ-catenin) is usually sequestered by cadherins in the cadherin-catenin complex. Overexpression of the tyrosine kinases Src and Fer and activation of the transmembrane tyrosine kinases epidermal growth factor receptor (EGFR) and c-Met all downregulate E-cadherin-mediated adhesion, and are responsible for the tyrosine phosphorylation of β-catenin (Lilien *et al*., 2002; Lilien and

Balsamo, 2005). Upon loss of E-cadherin function, non-sequestered, free β-catenin is usually phosphorylated by glycogen synthase kinase 3β (GSK3β) and subsequently degraded by the ubiquitin-proteasome pathway (Chan, 2006). Cadherins themselves lack enzymatic activity, and other than functioning as structural cell adhesion components, the cadherins obviously have further roles to play in cellular signalling and tumour growth (Nair et *al*., 2005; Chan, 2006).

Cadherins are important with respect to tumour biology, as aberrant levels of cadherins correlates with growth, invasion and metastasis (Hinck *et al*., 1994; Nieman *et al*., 1999; Wijnhoven *et al*., 2000; Peinado *et al*., 2004; Chan, 2006). For example, human invasive carcinomas and carcinomas *in situ* have been reported to show aberrant levels of E-cadherin expression in comparison to their related normal tissue (Jawhari *et al*., 1997; Valizadeh *et al*., 1997; Bailey *et al*., 1998; Wijnhoven *et al*., 2000). Further studies have demonstrated that patients with prostate cancer have reduced expression levels of E-cadherin (Davies *et al*., 2000), while other research on prostate cancer cells, which had a fibroblast-like morphology, were shown to be invasive having lost E-cadherin expression (Wijnhoven *et al*., 2000). Similarly, >85 % of all breast carcinomas display reduced E-cadherin expression (Berx *et al*., 2001; Cowin *et al*., 2005). Decreased E-cadherin expression has also been observed in gastric carcinoma in a number of different studies (Chan, 2006). In fact most carcinomas demonstrate reduced E-cadherin expression, which is directly associated with induction of an epithelial to mesenchymal transition (EMT). EMT is frequently observed during carcinoma invasion (Thiery, 2002; Peinado *et al*., 2004).

The adhesion receptors that have been discussed thus far have been examined individually for the sake of clarity. However, it must be remembered that a large degree of ‘crosstalk’ occurs between adhesion molecules. These associations are not yet fully understood, but the next section of this discussion will attempt to clarify somewhat briefly what is probably the most fundamental interaction in epithelial cells. It is the crosstalk that exists between the cadherins and the integrin class of receptors, which requires further scrutiny.

Considerable crosstalk is thought to exist between the integrin and cadherin class of adhesion receptors (von Schlippe *et al*., 2000; Kawano *et al*., 2001), suggesting that the

processes of cell-cell and cell-ECM adhesion are more closely intertwined than originally thought. Evidence of this crosstalk between these adhesion receptors includes experiments involving integrin-blocking antibodies, which were shown to induce the formation of multicellular spheroids in breast carcinoma cell lines expressing E-cadherin (von Schlippe *et al*., 2000). Similarly, a breast cancer cell line has been shown to upregulate the migratory activity mediated by α_v integrins following the introduction of a dominant negative E-cadherin construct.

The integrins are capable of mediating both cell-cell and cell-ECM interactions (Qian *et al*., 1994; Zutter *et al*., 1998; Dedhar *et al*., 1999; Lim *et al*., 2001; Cabodi *et al*., 2004; Brockbank *et al*., 2005) where this broad activity makes them an extremely versatile receptor. The integrins however are more widely known for their ability to regulate cell-matrix interactions (Plantefaber and Hynes, 1989; O'Toole *et al*., 1994; Zhang *et al*., 1996; Humphries *et al*., 2004). The integrins will be discussed in this regard since several malignant carcinomas show dysregulated integrin expression (Qian *et al*., 1994; Danen *et al*., 1998; Zutter *et al*., 1998; Miller and Veale, 2001; Evans *et al*., 2003; Brockbank *et al*., 2005). It is for this reason that the integrin receptors form a critical aspect of this study. The integrin class of receptors therefore warrants a more detailed examination.

1.2 Role of Integrins in ECM Attachment and Signal Transduction

1.2.1 Cell Attachment

Although this study places more emphasis on the functional aspects of the integrin receptors, it is necessary to examine their structure briefly to fully understand how they are able to regulate the adhesion process as well as signalling cascades. Integrins consist of $\alpha\beta$ heterodimers and each $\alpha\beta$ combination has its own binding specificity and signalling properties (Giancotti and Ruoslahti, 1999; Liu *et al*., 2000; Zamir and Geiger, 2001; Watt, 2002; Mould and Humphries, 2004; Stewart *et al*., 2004). The integrin heterodimer bears a receptor site towards the amino terminal of the extracellular domain (Gui *et al*., 1997; Travis *et al*., 2003). The carboxyl end is maintained within the cell and provides a link to the cytoskeleton via cytosolic proteins such as talin, vinculin and

actin (Otey *et al*., 1993; Martel *et al*., 2000; Brakebusch and Fässler, 2003; Reddig and Juliano, 2005; Boulter *et al*., 2006). Connection of integrins to the actin cytoskeleton allows for the correct localisation of these receptors as well as influencing their role in cell spreading, migration and matrix assembly (Schwartz, 2001; Aplin *et al*., 2002; Brakebusch and Fässler, 2003; DeMali *et al*., 2003; Wu, 2004).

Integrins mediate adhesive interactions to the ECM that in turn play fundamental roles in regulating cell survival, migration, proliferation, differentiation and tumour progression. The secretion and organisation of the ECM by cells provides structural support for these processes (Pankov *et al*., 2000; Whittard and Akiyama, 2001; Travis *et al*., 2003; Ffrench-Constant and Colognato, 2004). Cells adhere to the ECM either directly to the interstitial matrix, or through adhesion to the basement membrane, which covers the surfaces of virtually all epithelial cells; surround the surfaces of muscle fibres and ensheath nerves (Gumbiner, 1996). A constituent of the basement membrane, the basal lamina, is found adjacent to cells and is composed of glycoproteins (e.g. fibronectin and laminin), proteoglycans, and collagens (Mosher *et al*., 1992) that are secreted and assembled into an organised, and unique three dimensional networks (Taverna *et al*., 1998; Badylak, 2002).

Collagen is the most abundant protein within the ECM with well over 20 distinct types having been identified (Badylak, 2002). Type I collagen is the primary structural collagen in mammalian tissues, and is a well-characterised, ubiquitous ECM protein (Vanderrest and Garrone, 1991). Collagen type IV, although expressed in much lower quantities, is an important ligand for endothelial cells, and is expressed in the basement membranes of all epithelial structures (Badylak, 2002). Type VI collagen serves as a ‘connector’ of functional proteins and glycosaminoglycans to larger structural proteins such as collagen I, giving the ECM a gel-like consistency (Badylak, 2002). This apparent diversity of collagens is responsible for the distinctive biological activity of ECM scaffolds (Badylak, 2002).

Two other important components of the ECM are the fibronectin and laminin proteins, which are expressed in large quantities, although these quantities are lower than collagen (Badylak, 2002). Fibronectin exhibits many desirable properties, including ligands for adhesion of many cell types (Schwarzbauer, 1991). Fibronectin is found in

the ECM of both submucosal and basement membrane structures (McPherson and Badylak, 1998; Badylak, 2002). Laminin is a complex adhesion protein found in the ECM, which has a prominent role in the formation and maintenance of epithelial structures (Ponce *et al*., 1999).

As key receptors for cellular attachment to the ECM, the integrin group is capable of binding more than one cellular ligand and, more often than not, individual ligands are recognised by more than one integrin receptor (Hynes, 1992; Jones and Walker, 1999; Thorne *et al*., 2000). For example the $\alpha_v\beta_3$ integrin, a vitronectin receptor, shows strong affinity for fibronectin, collagen, tenascin C, thrombospondin and fibrinogen (Boudreau and Jones, 1999; Watt, 2002). Particular ECM components that are capable of binding to more than one integrin include fibronectin which, in addition to the $\alpha_5\beta_1$ receptor, also binds at least seven other integrin heterodimers. Similarly, laminin binds $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$ integrins with high affinity. This apparent redundancy suggests that, in addition to mediating attachment to a particular ECM ligand, different integrins perform specific signalling functions (Juliano and Haskill, 1993; Boudreau and Jones, 1999).

The association of cells with the ECM initiates the assembly of specific cell-matrix adhesion sites and, by attaching to various ECM proteins, allows cells to proliferate and grow (Ruoslahti, 1996; Kumar, 1998; Brakebusch and Fässler, 2003). The ECM provides structural support for cell adhesion, migration, and tissue organisation, as well as external regulation of cellular functions (Pankov *et al*., 2000). For example, promotion or suppression of growth by the ECM is associated with either stimulation or inhibition of cell cycle mediators including cyclins and early response genes (Boudreau and Jones, 1999). A vital purpose of the integrins is to maintain a tight regulation of the interactions occurring between cells and the ECM components (Hynes, 1992; Hynes and Lander; 1992; Giancotti and Ruoslahti, 1999; Ffrench-Constant and Colognato, 2004). Although the physical attachment provided by integrins is an essential and necessary function, it does not even begin to describe the versatility and uniqueness of this class of receptors.

Among the superfamilies of cell adhesion molecules, the integrins are quite likely the most extensively studied group of adhesion receptors and arguably the most important

(Qian *et al*., 1994; Zutter *et al*., 1998; Dedhar *et al*., 1999; Lim *et al*., 2001). The integrin adhesion receptors are known to play pivotal roles in many biological processes and malignant transformation (Zutter *et al*., 1998; Brassard *et al*., 1999; Keely *et al*., 1999; Schwartz, 2001; DeMali *et al*., 2003; Mould and Humphries, 2004). Individual members of these families may serve as adhesion ligands for members of the same or other families. They may trigger events, which regulate the expression and function of other receptors and proteolytic enzymes, or they may act coordinately to mediate the same processes such as differentiation and/or cell proliferation (Brodt, 1996a).

By creating an 'integrated' link between the outside and inside of the cell (Boudreau and Jones, 1999), the integrins are perfectly located for executing the vast number of signalling processes required by the cell (Hynes, 1992; Akiyama *et al*., 1994; Giancotti and Ruoslahti, 1999; Liu *et al*., 2000; Yu *et al*., 2000; Cabodi *et al*., 2004; Boulter *et al*., 2006). What now follows is a comprehensive look into the ways in which integrins respond to the ECM.

1.2.2 Signal Transduction by Integrin Receptors

The signal transduction events mediated by integrins have continued to be defined over the past five years. Integrins serve as integrators of the ECM and cytoskeleton and these connections have far-reaching effects in the cell. Integrins control many aspects of cellular function (Boudreau and Jones, 1999; Giancotti and Ruoslahti, 1999; Coppolino and Dedhar, 2000; DeMali *et al*., 2003; Reddig and Juliano, 2005), and therefore the signal transduction cascades mediated by integrins require detailed scrutiny.

Specialised cells are surrounded by a multitude of ECM proteins and express an array of tissue-specific integrin receptors (Juliano and Haskill, 1993; Boudreau and Jones, 1999; Ffrench-Constant and Colognato, 2004). This receptor diversity effectively generates unique intracellular signals that give rise to tissue-specific phenotypes (Boudreau and Jones, 1999). Most, if not all of the signalling molecules involved in ECM-integrin interactions appear to be rather ubiquitous mediators of signal transduction (Boudreau and Jones, 1999). For example, Miyamoto *et al*. (1995) showed that at least 20 different proteins, including RhoGTPases, Raf, Ras, focal adhesion kinase (FAK), and microtubule associated protein serine/threonine kinases (MAPKs) such as extracellular-

signal-regulated kinases (ERKs), can be recruited to the ECM ligand/integrin-binding site.

Bi-directional signalling capabilities by the integrin receptors make it possible to mediate signal transduction events between ECM and intracellular compartments in either direction (Parsons, 1996; Coppolino and Dedhar, 2000; Liu *et al*., 2000; Danen and Yamada, 2001; Mould and Humphries, 2004). Thus, the extracellular binding activity of integrins is regulated from the inside of the cell (inside-out signalling), and regulates integrin ligand-binding affinity and cell adhesion (Liu *et al*., 2000; Deryugina *et al*., 2002; Vellon *et al*., 2006). A particularly good example of this kind of signalling involves the regulation of integrin activity in cells circulating in the blood. The $\alpha_{IIb}\beta_3$ integrin in platelets and the β_2 integrins of white blood cells are expressed at the cell surface, but in an arrangement that does not bind ligand (Ruoslahti and Öbrink, 1996). Cells are allowed to respond quickly to a change in environment by the activation of ligand binding. In a similar scenario, leukocytes attach to blood vessel walls through β_2 integrins that have been activated by inflammatory signals (Ruoslahti and Öbrink, 1996). On the other hand, the binding of the ECM elicits signals that are transmitted into the cell (outside-in signalling). ECM binding results in cytoskeletal re-organisation, gene expression and cellular differentiation and various processes pivotal to carcinogenesis, such as altered tyrosine phosphorylation pathways of key signalling proteins, including FAK and ILK (Akiyama *et al*., 1990; Kornberg *et al*., 1991; Giancotti and Ruoslahti, 1999; Liu *et al*., 2000; Schwartz, 2001; Deryugina *et al*., 2002; Attwell *et al*., 2003; Ffrench-Constant and Colognato, 2004; Pinkse *et al*., 2005).

It is also interesting to note that an integrin associated protein ILK, also displays ‘inside-out signalling’ and its activation leads to decreased adhesion to the ECM (Boudreau and Jones, 1999; Brakebusch and Fässler, 2003). Another example of ‘inside-out’ signalling includes talin, which in addition to linking integrins to the actin cytoskeleton, also regulates integrin activation (Brakebusch and Fässler, 2003). Talin is capable of binding to most β cytoplasmic domains and overexpression of talin is capable of activating integrins (Ginsberg *et al*., 2005). In addition, knocking down talin expression with small interfering RNAs blocks β_1 and β_3 activation (Tadokoro *et al*., 2003).

As outlined above, there are numerous protein molecules that are able to bind to the ECM/integrin binding site. The 'outside-in' signalling pathways of Rac/cell division cyclin 42 (Cdc42)/Ras homolog member A (RhoA), FAK/Shc and MAPK (Figures 1a and 1b, pg 15) however are arguably the most fundamental integrin signalling pathways, as they influence various facets of cell behaviour and function, including cell proliferation and differentiation (Giancotti and Ruoslahti, 1999; Barberis *et al*., 2000). It is for these reasons that these molecules are considered important and hence will be the focus of the following sections. Although these pathways will be considered separately to aid understanding, it must be remembered that there is a great deal of interdigitation between these signal transduction pathways, making it possible for particular pathways to elicit a variety of responses.

1.2.3 Integrin Signalling Influences Cell Migration and Cell Spreading

The importance of integrin signalling is highlighted by its impact upon organisation of the cytoskeleton (Brakebusch and Fässler, 2003; DeMali *et al*., 2003). With regards to cytoskeletal organisation and cell migration, regulation of the Rho family GTPases is important (Graness *et al*., 2005). This can generally be characterised by two phases. Firstly, early adhesion is associated with pathways that stimulate protrusion, whereas mature adhesions are associated with the development of tension (DeMali *et al*., 2003). Early phase leads to Rac and Cdc42 activation and to actin polymerisation. The later phase leads to RhoA activation, increased contractility and the transmission of tension to the sites of integrin ligation (Ridley, 2001; DeMali *et al*., 2003; Humphries *et al*., 2004). When cells attach and spread on an ECM, cells extend filopodia and lamellipodia, structures regulated by Cdc42 and Rac, respectively. Integrin-mediated adhesion activates Cdc42 and Rac (Price *et al*., 1998; Kramer *et al*., 2005), and Rac activation requires an intact β integrin subunit.

Rho family GTPases are active when GTP-bound, and inactive when bound to GDP. Activation is catalysed by guanine nucleotide exchange factors (GEFs) and inactivation is promoted by GTPase-activating proteins (GAPs) that stimulate the intrinsic GTPase activity of the Rho proteins (DeMali *et al*., 2003; Kramer *et al*., 2005). An example of a GEF activated downstream from integrin engagement is Vav2, which is widely distributed (DeMali *et al*., 2003). Although evidence suggests that Vav2 is activated

downstream of growth factors and not from integrins (Lui and Burridge, 2000; Liu *et al*., 2000; Moores *et al*., 2000), a dominant-negative form of Vav2 blocks lamellipodium formation and spreading on fibronectin, which is consistent with Vav2 having a role in Rac activation following integrin engagement (Marignani and Carpenter, 2001).

Integrin engagement leads to transient depression of RhoA activity and it is has been proposed that reduced RhoA promotes lamellipodial extension during cell migration (Arthur *et al*., 2001; Kramer *et al*., 2005). The reduction in RhoA activity requires Src, FAK and p190RhoGAP. A role for paxillin phosphorylation in mediating RhoA reduction has also been suggested. This role includes the phosphorylation of paxillin, initiated by integrin adhesion, generates a binding site for p120RasGAP, thereby displacing it from its binding partner p190RhoGAP. When p190RhoGAP is freed from p120RasGTP, it becomes activated and hence contributes to the decrease in RhoA activity (Tsubouchi *et al*., 2002). However, it has been demonstrated that a decrease in RhoA exists even when cells are in suspension that bind soluble-peptide integrin ligands (Arthur *et al*., 2000), a situation in which paxillin does not become phosphorylated (DeMali *et al*., 2003). Nevertheless, paxillin may still contribute to the depression of RhoA activity when cells attach to fibronectin (DeMali *et al*., 2003).

Integrins have been demonstrated to have differing impacts upon RhoA. In this regard, differing integrins result in different RhoA responses. It has been demonstrated that while $\alpha_6\beta_4$ results in stimulation of RhoA activity, β_1 clustering induces depression of RhoA (O'Connor *et al*., 2000). Furthermore, engagement of $\alpha_v\beta_3$ integrin on astrocytes by Thy-1 was shown to stimulate assembly of focal adhesions and stress fibres, which is consistent with RhoA activation (Leyton *et al*., 2001). Thus it is apparent that integrins are directly involved in Rac/Cdc42- and RhoA-dependent responses, which have essential consequences with regards to cell spreading and migration.

1.3 Adhesion-Based Signalling

The coordinated cellular response to ECM attachment through integrins induces a multitude of changes in cell behaviour including alterations in cell proliferation, migration and adhesion (Bill *et al*., 2004). The "outside-in" and "inside-out" signal transduction pathways that are elicited by integrin receptors are intricately related. Moreover, the interconnection between signal pathways implies that a large degree of cross-modulation occurs during cell signalling. There are many signalling components that are crucial in ensuring proper regulation of integrin-mediated signalling cascades. However, I believe that FAK, MAPK and PTEN are key players in adhesion-based signalling events. Thus, the pathways elicited by these signalling molecules warrant further investigation.

1.3.1 Consequences of FAK Signalling

Integrins lack enzymatic activity and their cytoplasmic tails are generally short (Giancotti and Ruoslahti, 1999; Liu *et al*., 2000), suggesting that in order for integrins to be able to transduce signals, they need to associate with adaptor proteins, that connect integrins to the cytoskeleton, cytoplasmic kinases, and transmembrane growth factor receptors (Giancotti and Ruoslahti, 1999; Cabodi *et al*., 2004). FAK and Shc (SH2 homology/collagen homology proteins) are the major tyrosine kinase-dependent pathways that are activated by the integrins. Both pathways include a Src family kinase as their central component (Mauro *et al*., 1999; Barberis *et al*., 2000; Schwartz, 2001; Planas-Silva *et al*., 2006) and include Src, Fyn and the Yes (Frame *et al*., 2002; Cabodi *et al*., 2004). We now have a scenario where two distinct signalling pathways share a common element, suggesting that both pathways are involved in regulating similar events. As we develop these pathways further, an understanding of this situation will ensue.

Focal adhesions are specialised adhesion sites formed at the cell surface (Malik and Parsons, 1996; Wozniak *et al*., 2004), and are the principal sites of integrin signalling (Ruoslahti and Öbrink, 1996). Focal adhesions arise as a result of ligation of ECM proteins by integrin receptors, which cause cross-linking or clustering of integrins (Katz *et al*., 2000; Carragher and Frame, 2004). These sites are involved in physical attachment of cells to external surfaces, which is essential for cell migration and tissue

formation, as well for activation of adhesion-mediated signalling events (Katz *et al*., 2000). They are comprised of protein kinases, such as FAK and ILK, in addition to non-catalytic proteins such as integrins, talin, vinculin, paxillin and zyxin (Horwitz *et al*., 1986; Turner and Burridge, 1991; Grashoff *et al*., 2004). They also contain EGF receptors (Boudreau and Jones, 1999).

Ligand binding controls the localisation of β_1- and β_3-containing integrins into focal adhesions (Akiyama *et al*., 1994; Gumbiner, 1996), which allow for intrinsic signals facilitating focal adhesion localisation (Akiyama *et al*., 1994). Both FAK and ILK have been reported to bind the β integrin subunit (Liu *et al*., 2000). ILK phosphorylation of the β_1 integrin cytoplasmic domain is further involved in the dynamic regulation of localisation of β_1 to focal adhesions (Dedhar *et al*., 1999). All β_1 and α_v integrins are able to promote the focal adhesion assembly and FAK activation (Giancotti, 1997; Kumar, 1998), as are growth factors and cytokines. These integrins are therefore capable of initiating integrin adhesion-dependent signalling pathways. Incorporation of the integrins into focal adhesions is prevented by the α subunits of the heterodimers. This inhibition is relieved by the ligand binding, and allows the β subunit cytoplasmic tail signals to recruit the integrin dimer into the focal adhesion (Gumbiner, 1996).

FAK is a 120 kDa non-receptor tyrosine kinase (Boudreau and Jones, 1999) that co-localises with integrins and occurs at cell-substratum contact sites (Martin, 2003; Wozniak *et al*, 2004; Crowe and Ohannessian, 2004). FAK is incapable of phosphorylating other substrates directly and requires autophosphorylation (integrin-dependent) (Barberis *et al*., 2000; Zamir and Geiger, 2001; Sawai *et al*., 2005), which allows it to interact with docking or adaptor proteins, including paxillin, tensin and Grb2/SOS (Son of Sevenless) (Barberis *et al*., 2000). These adaptor proteins, in turn, are able to activate downstream signalling mediators previously implicated in growth control, including Src, Ras and Raf (see Figures 1a and 1b). Regulation of the phosphorylation state and tyrosine kinase activity of FAK occurs through binding of cells to the ECM (Boudreau and Jones, 1999; Mudhopadhyay *et al*., 2005; Sawai *et al*., 2005), as well as by tyrosine phosphatases (Giancotti and Ruoslahti, 1999) such as phosphatase and tensin homolog (PTEN). The relevance of PTEN to FAK signalling will be discussed later.

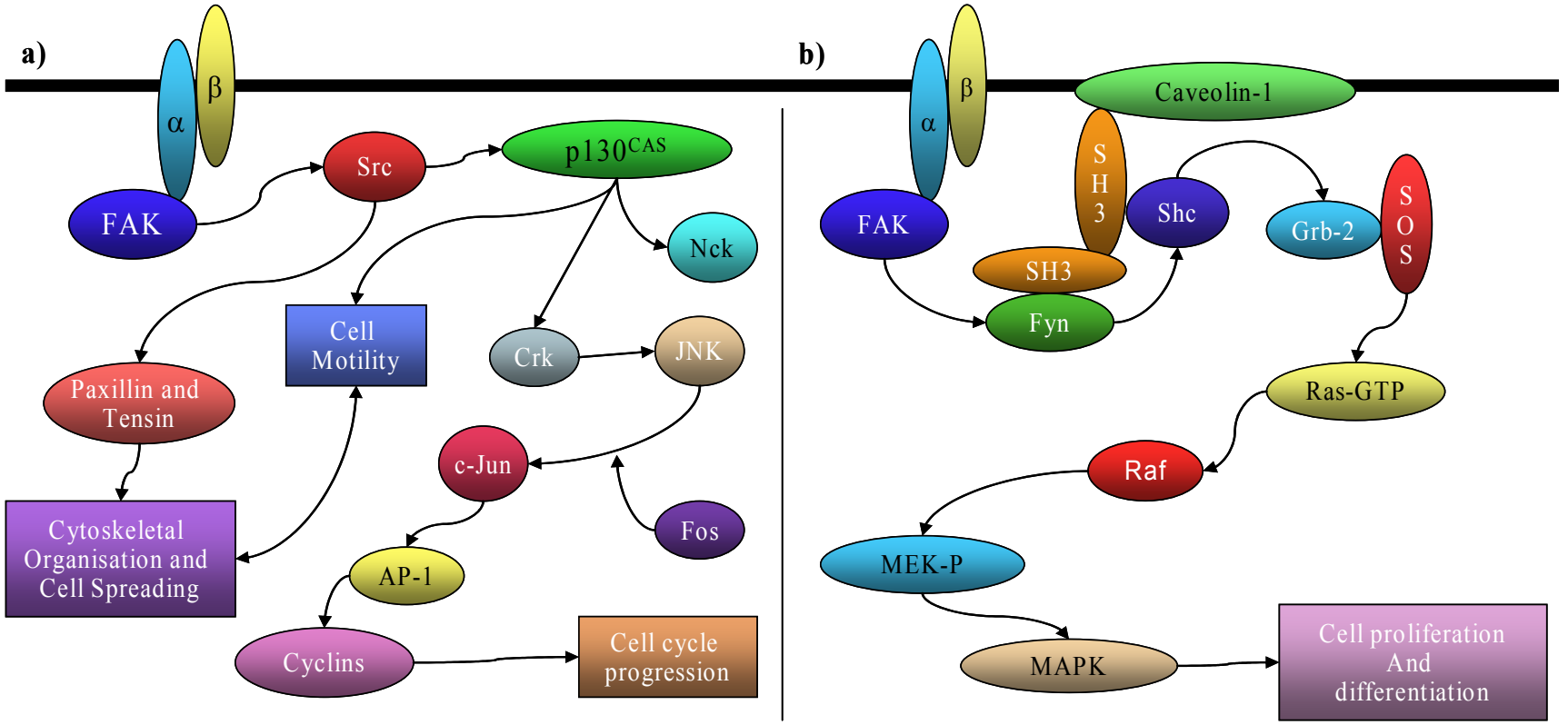


Figure 1: Focal Adhesion Kinase (FAK) Signalling Pathways.

a) The activation of FAK by the integrin receptors enables FAK to bind to the SH2 domain of Src leading to subsequent Src activation. Src in turn activates p103CAS, which recruits Crk and Nck. Crk phosphorylates JNK, which subsequently activates c-Jun. c-fos then complexes with c-jun to form the AP-1 complex, which serves to regulate cell cycle progression. In a distinct pathway the activation of Src by FAK leads to the regulation of other focal adhesion proteins such as paxillin and tensin, which influence cytoskeletal organisation and cell spreading. b) A second FAK signalling pathway involves the indirect activation of Shc via Fyn. Fyn phosphorylates Shc, which is recruited to the membrane via caveolin-1. Shc combines with the Grb-2-SOS complex and initiates the MAPK signalling cascade, which influences cell proliferation and differentiation.

In response to adhesion receptor activation and integrin clustering, tyrosine phosphorylation of FAK occurs within the focal adhesion complex (Chen *et al*., 1996; Liu *et al*., 2000; Schaller, 2004; Benlimame *et al*., 2005; Planas-Silva *et al*., 2006), thereby binding to Src or Fyn (protein tyrosine kinases) (Schaller *et al*., 1992; Juliano and Haskill, 1993; Kumar, 1998). Once activated, FAK associates with the SH2 domain (Src homology domain) of Src. The Src kinase in turn, phosphorylates paxillin and tensin and $p130^{CAS}$, a docking protein that recruits adapter proteins, including Crk and Nck (see Figure 1a and Barberis *et al*., 2000; Schwartz, 2001; Martin, 2003; Schaller, 2004; Planas-Silva *et al*., 2006). Src itself is capable of regulating FAK as transformation of FAK deficient fibroblasts with the v-Src oncogene promotes cellular motility equal to that of FAK re-expression (Crowe and Ohannessian, 2004; Wozniak *et al*., 2004).

In this pathway the MAPK c-Jun NH_2-terminal kinase (JNK) is activated via Crk, and this serves to regulate progression through the cell cycle (Giancotti and Ruoslahti, 1999). Alternatively, Crk is also able to interact with $p130^{CAS}$ which has crucial influences on cell transformation, morphology and migration (Wozniak *et al*., 2004). The Crk-$p130^{CAS}$ complex results in activation of the small GTPase, Rac, which is mentioned above, has crucial consequences with regards to cell migration (DeMali *et al*., 2003; Wozniak *et al*., 2004).

We have now reached a point where the pathways of FAK and MAPK coincide, which once again demonstrates the complexity of these signalling cascades. To continue, activated JNK enters the nucleus and phosphorylates the transcription factor c-Jun, which combines with c-Fos, to form the activating protein (AP-1) transcription factor complex. AP-1 is then able to regulate genes that are important for cell proliferation such as cyclins (see Figure 1a and Martin, 2003; Wozniak *et al*., 2004).

In a separate pathway, integrins recruit Shc via a series of sequential steps. Caveolin-1, a membrane adaptor protein, serves to couple, through its SH3 domain, the integrin α subunit to the Src family kinase Fyn (Wary *et al*., 1996; Barberis *et al*., 2000). Fyn then phosphorylates Shc and combines with the Grb-2-mSOS complex (Coppolino and Dedhar, 2000). Grb-2 serves to link FAK to the Ras pathway (see Figure 1b), and the phosphoinositide 3-OH kinase (PI3K) (Ruoslahti and Öbrink, 1996; Sanders *et al*.,

2000; Crowe and Ohannessian, 2004; Mukhopadhyay *et al*., 2005). PI3K is involved in the regulation of ILK, and is discussed later.

The consequences of FAK and Shc signalling are widespread and include amplification of various cellular responses. For instance, Shc signalling augments hepatocyte growth factor (HGF)-induced proliferation and migration in human lung adenocarcinoma (Pelicci *et al*., 1992; Pelicci *et al*., 1995 cited in Mauro *et al*., 1999). Src overexpression and enhanced activity has also been linked to the progression and metastasis of different types of cancer (Sawai *et al*., 2005).

FAK activation via integrins plays a central role in initiating a host of signals that are involved in the regulation of growth (Mukhopadhyay *et al*., 2005). For example, introduction of constitutively activated FAK leads to cell transformation, anchorage-independent growth and the suppression of apoptosis (Ilic et al., 1998; Sawai *et al*., 2005). FAK overexpression has been correlated with aggressive breast and colonic cancer as well as sarcoma and hepatocellular carcinoma (Weiner *et al*., 1993; Owens *et al*., 1995; Fujii *et al*., 2004; Lark *et al*., 2005; Schmitz *et al*., 2005). In melanoma cells, increased FAK expression is associated with increased cell motility (Mukhopadhyay *et al*., 2005; Hess and Hendrix, 2006). Both FAK and Shc contribute to the activation of the Ras-extracellular regulated kinase MAPK cascade when Shc-linked integrins bind to the ECM (see Figure 1a and Moro *et al*., 1998; Giancotti and Ruoslahti, 1999).

1.3.2 MAPK Signalling Cascades

Activation of the MAPK signalling pathway provides a common route leading to transcriptional regulation of genes that are crucial for cell growth and differentiation (Juliano and Haskill, 1993; Giancotti and Ruoslahti, 1999; Hynes, 1999). Sequential activation following transient activation of Ras GTP-binding proteins via receptor tyrosine kinases include MAPK/ERK kinase, MAPKK (also known as MEK) and ERK1 (p44)/ERK2 (p42) (Boudreau and Jones, 1999; Martin, 2003). The activation of the MAPK signalling cascade occurs in response to several stimuli including integrin engagement, activation of c-Src as well as ligand-induced activation (Carragher and Frame, 2004).

The phosphorylation of ERK1 and ERK2 results in their translocation to the nucleus, where they phosphorylate and activate a number of transcription factors associated with early response genes (see Figure 1b and Boudreau and Jones, 1999; Giancotti and Ruoslahti, 1999). MAPK activation could also occur through adhesion of cells to ECM proteins including fibronectin, vitronectin, collagen, tenascin and laminin, via ligation of $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_2\beta_1$, $\alpha_v\beta_6$ and $\alpha_6\beta_4$ (Boudreau and Jones, 1999).

MAPK signalling is able to elicit a host of responses that are crucial for normal cellular functioning. For example, upregulation of the $\alpha_1\beta_1$ collagen/laminin receptor in PC12 cells is a direct result of MAPK-dependent differentiation. An elongated morphology is often associated with differentiation of this cell type (Boudreau and Jones, 1999). Similarly, in erythroleukaemia cells, MAPK-dependent differentiation and growth arrest are accompanied by upregulation of the platelet $\alpha_{IIb}\beta_3$, the expression of which is impaired by inhibiting MAPK. Furthermore, MAPK is capable of influencing cell cycle progression for $\alpha_v\beta_3$ and a subset of β_1 integrins (Coppolino and Dedhar, 2000). In this report, Shc was shown to associate with the integrin α subunit, which promoted the MAPK pathway as well as passage through the G_1 phase of the cell cycle (Coppolino and Dedhar, 2000).

It is thought that Shc is responsible for the initial high-level activation of ERK, upon cell adhesion. FAK, which is activated more slowly, may be responsible for sustaining ERK activation (Schlaepfer and Hunter, 1998; Pozzi *et al*., 1998; Crowe and Ohannessian, 2004). FAK on the other hand, is able to limit adhesion to the ECM, which permits cells to take up a position or shape that accommodates migration and proliferation (Boudreau and Jones, 1999). This position is accomplished by cytoskeletal changes and ECM-directed morphology that co-ordinate cell receptors and intracellular signalling molecules, thereby permitting upstream effectors, including FAK, Ras and Raf to couple with their downstream targets, including MAPKs (Carragher and Frame, 2004). In situations where limited growth factor concentrations exist in cells, the need for integrins to activate ERK becomes necessary (Mainiero *et al*., 1997; Giancotti and Ruoslahti, 1999). In this setting, proliferation is likely to require co-stimulation of ERK through integrins and growth factor receptors (Martin, 2003).

The signalling complexes that are assembled by integrins are able to activate multiple signalling pathways, which serve as a class of "master regulators" of cell function. Thus it is obvious that both MAPK and FAK signalling are central to integrin signalling (Ruoslahti and Öbrink, 1996). ILK is another protein kinase influencing many aspects of integrin function such as fibronectin matrix assembly, cell cycle progression, cell adhesion processes as well as neurite outgrowth, and leukocyte recruitment (Attwell *et al*., 2000; D'Amico *et al*., 2000; Yoganathan *et al*., 2000; Tan *et al*., 2001; Friedrich *et al*., 2002; Attwell *et al*., 2003; Pinkse *et al*., 2005).

To ensure the correct biological functioning of cellular events, tight regulation is required over signalling pathways involving kinases such as MAPK and FAK. Protein kinases provide the stimuli for these signal transduction pathways, while protein phosphatases provide the necessary inhibitory regulation. One such lipid phosphatase, PTEN (phosphatase and tensin homologue deleted on chromosome ten) supplies the necessary negative regulation of these pathways and thus has particular pertinence to this study. While the following section will examine PTEN signalling in its role in regulating cell survival and growth pathways, Chapter 3 will examine the importance of PTEN signalling to ILK regulation.

1.3.3 PTEN Signalling Cascades

In the development of the majority of human cancers, it is widely established that inactivation of tumour suppressor proteins is required (Simpson and Parsons, 2001; Leslie *et al*., 2004). The tumour suppressor PTEN has been shown to play an essential role in the regulation of cellular adhesion, migration, growth and apoptosis (Marino *et al*., 2002; Suli *et al*., 2003; Kerr *et al*., 2006). The pertinence of PTEN stems from its ability to negatively regulate various signalling cascades that are critical in tumour progression. Indeed, PTEN directly exerts its effects on the MAPK, FAK and PKB signalling pathways (Gu *et al*., 1998; Cantley and Neel, 1999; Maehama *et al*., 2001; Waite and Eng, 2002; Yang *et al*., 2003). The roles of PTEN to these crucial signalling pathways will now be investigated.

Although PI3K is capable of activating numerous signalling pathways downstream of PI3K, the PKB pathway has attracted much attention due to its role in cell survival

(Cantley and Neel, 1999; Cristofano and Pandolfi, 2000; Krasilnikov, 2000; Vara *et al*., 2004; Walker *et al*., 2004). However, it is likely that the roles of PKB extend beyond that of cell survival since PKB targets substrates such as GSK3β, mammalian target of rapamycin (mTOR), insulin receptor substrate-1 (IRS-1), cyclin-dependent kinase inhibitors $p21^{CIP1/WAF1}$ and $p27^{KIP1}$, which are involved in cell cycle progression and growth (Blume-Jensen and Hunter, 2001; Gringas *et al*., 2001; Vara *et al*., 2004). However, during cancer progression, the ability of cells to evade cell death is a critical event and the PI3K/PKB pathway provides such a mechanism (Al-Khouri *et al*., 2005).

The PI3K/PKB signalling pathway is perhaps the most relevant signalling pathway that involves PTEN function (Backman *et al*., 2002; Sulis *et al*., 2003). In this model, it has been demonstrated that PTEN dephosphorylates phosphatidyl inositol triphosphate (PIP_3), which suggests a mechanism for how PTEN controls PI3K and PKB activity (Leslie *et al*., 2000; Downes *et al*., 2001; Li *et al*., 2001; Bayascas *et al*., 2005) (Figure 2, pg 23). In quiescent cells, PIP_3 levels are usually low but are rapidly increased upon stimulation by growth factors, through activation of PI3K. The accumulation of PIP_3 at the membrane allows for the recruitment of proteins which contain pleckstrin homology (PH) domains, such as PKB, which then bind to PIP_3 (Cristofano and Pandolfi, 2000). Activated PKB then exerts anti-apoptotic stimuli by preventing release of cytochrome C from mitochondria and inactivating Forkhead transcription factors (FKHR), which are known to induce the expression of genes that are critical for apoptosis (Cristofano and Pandolfi, 2000). PKB also phosphorylates and inactivates the proapoptotic factors Bcl-2 (protein originally identified in B-cell lymphoma), the Bcl-2-associated death promoter (BAD) and caspase-9 (Datta *et al*., 1999, Cristofano and Pandolfi, 2000). In this context the function of PTEN is to keep the levels of PIP_3 low. The loss of PTEN function results in an increased concentration of PIP_3 and in PKB hyperactivation, leading to protection from pro-apoptotic stimuli (Stambolic *et al*., 1998).

It is well established that the PI3K/PKB pathway are linked to human cancers via defects in PTEN. For example, in pancreatic cancer where PI3K was inhibited, PKB is constitutively activated (Stoll *et al*., 2006). Normally, PKB activity is low in the absence of growth factor stimulation. However, PTEN-deficient tumour cell lines exhibit high basal levels of PKB phosphorylation (Cantley and Neel, 1999). This is especially true in Alzheimer's disease where it has been reported that increased PKB

levels exist when PTEN levels are low (Pei *et al*., 2003; Rickle *et al*., 2004). However, PKB levels were relatively low in a glioblastoma tumour sample carrying a mutation in the extreme C-terminus of PTEN compared to tumours with a phosphatase-inactivating mutation (Downes *et al*., 2001). This suggested that deregulation of PKB is not a universal feature of tumours that carry PTEN mutations. Conversely, it has been shown that PTEN-null fibroblasts are resistant to multiple pro apoptotic stimuli. Reconstitution of wild-type PTEN restores normal PKB regulation and sensitivity to these stimuli (Stambolic *et al*., 1998). These data suggest that perturbations of the PI3K/PTEN/PKB signal transduction pathway may contribute to a large fraction of human cancers.

Even though the current knowledge regarding PI3K/PTEN/PKB is relatively well understood, many issues remain unresolved with regards to PTEN regulation of the PI3K/PKB pathway. For example, PTEN expression prevents basal activation of PKB (i.e. activation of PTEN in the absence of growth factor stimulation), yet in $PTEN^{+}$ cells, growth factors remain able to activate PKB via a PI3K-dependent pathway. This suggests that PTEN is inactivated upon growth factor stimulation or that PTEN is activated by growth factor depletion.

Although the effects of PTEN on PI3K/PKB signalling is almost certainly the most important signal transduction pathway influenced by PTEN, the effects of PTEN are also observed on the MAPK and FAK signalling cascades. Indeed, PTEN has been implicated in inhibitory regulation of these signalling cascades. It is interesting to note that in the PTEN-mediated regulation of FAK, PTEN is capable of regulating MAPK signalling. This is achieved via the PTEN-mediated dephosphorylation of FAK (Tamura *et al*., 1998; Zheng *et al*., 2003). For example, it has been reported that reintroduction of PTEN into the glioblastoma cell line U-87MG leads to direct dephosphorylation of FAK, thus inhibiting integrin-mediated cell spreading and migration (Tamura *et al*., 1998). Moreover, it has been demonstrated that PTEN is able to bind and dephosphorylate Shc, thus inhibiting the recruitment of the Grb2 adaptor and the subsequent activation of the MAP kinase cascade (Gu *et al*., 1998; Tamura *et al*., 1998). Thus, by regulating FAK and Shc phosphorylation in response to integrin ligation, PTEN is capable of modulating various aspects of cell adhesion and migration (Cristofano and Pandolfi, 2000). Furthermore, PTEN is also capable of regulating focal adhesion structure, cell spreading and motility, by controlling FAK activity (Tamura *et*

al., 1998; Cantley and Neel, 1999). The inactivation of FAK via PTEN demonstrates its ability as a tumour suppressor by inhibiting cell invasion and metastasis (Zheng *et al*., 2003). Thus, PTEN-mediated FAK regulation demonstrates that PTEN influences integrin-mediated signal transduction pathways.

The regulation of Shc via PTEN represents one mechanism for PTEN-mediated regulation of MAPK signalling. However, the involvement of PTEN on MAPK signalling can also occur through regulation of the Ras protein. The role of PTEN in regulating MAPK signalling was revealed in a study, which found that reconstitution of PTEN in a cell line devoid of this protein due to mutation markedly inhibited the Ras/ERK signal transduction pathway in response to stimulation by fibronectin or growth factors (Gu *et al*., 1998). Obviously, PTEN regulation of MAPK has crucial consequences with regards to cell growth and differentiation. This indicates that downregulation of the Ras/MAPK pathway is an important function of the PTEN tumour suppressor in its cell biological functions, which supports the function of PTEN as a tumour suppressor.

What has been discussed thus far clearly indicates that PTEN plays crucial roles within numerous signalling transduction pathways. While involvement of PTEN in MAPK and FAK signalling has important cellular consequences, the primary interest of PTEN to this study was its role in ILK activity. The details of PTEN function with regards to ILK activity will be discussed in greater in chapter 3 where the effects of growth factors on ILK activity are considered.

1.4 ILK Signal Transduction Pathways

1.4.1 ILK Signalling and Support

Several instances have clearly demonstrated protein kinases as playing key roles in signal transduction pathways and cancer, where aberrant kinase activities correlate with pathological increases in cell proliferation and resistance to apoptosis (Yoganathan *et al*., 2000; Aplin *et al*., 2002; Graness *et al*., 2005; Pinkse *et al*., 2005). The participation of cellular kinases in signal transduction pathways allows cells to respond functionally

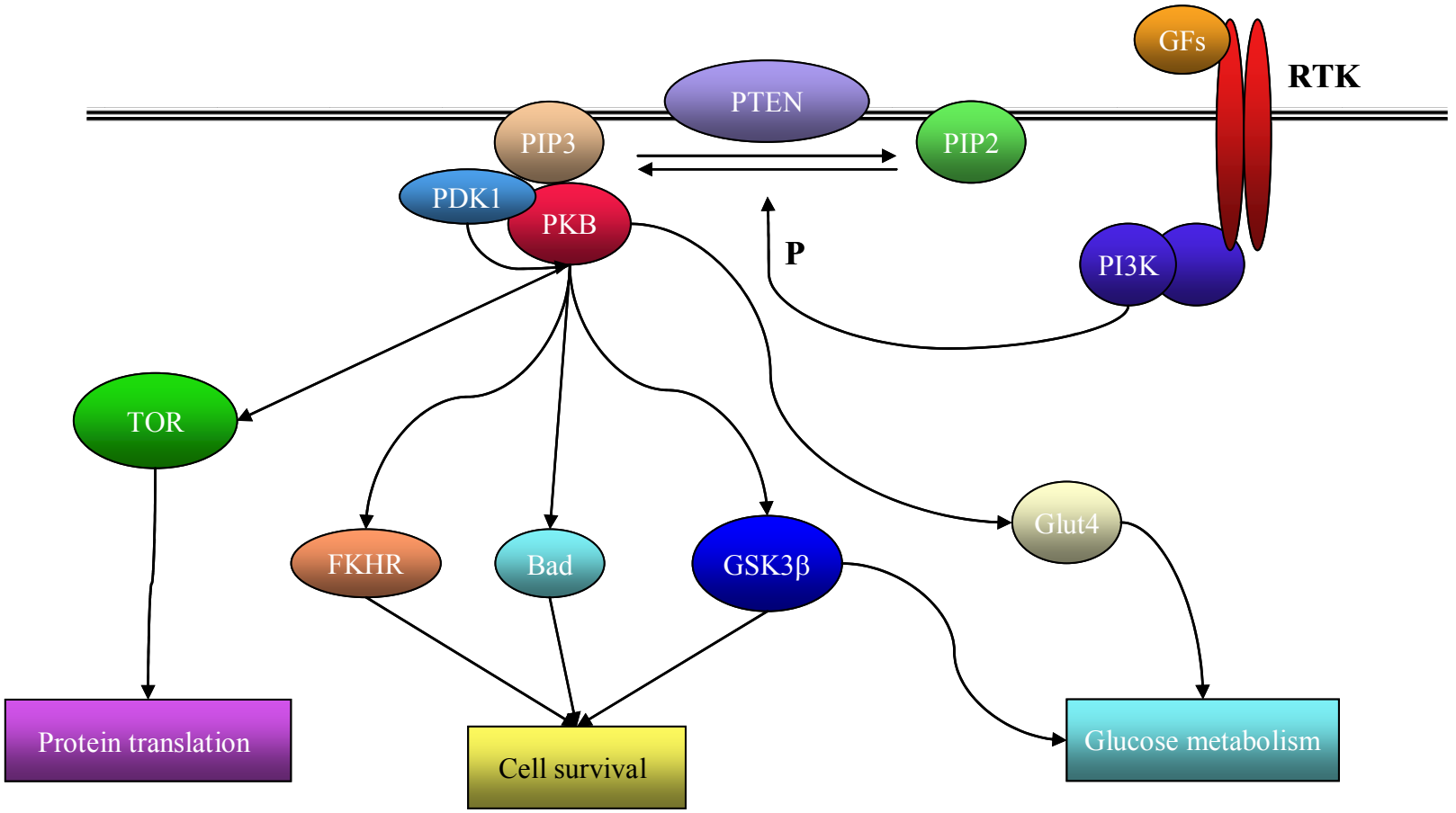


Figure 2: PTEN Signalling Pathways.

PTEN negatively regulates PKB-mediated cell survival. Binding of GFs, to RTKs activates PI3K. Activated PI3K phosphorylates PIP_2 to produce PIP_3, which recruits PKB to the plasma membrane where it is phosphorylated and activated by PDK1. PTEN prevents PKB-mediated cell survival by dephosphorylating PIP_3. PKB also regulates protein translation and glucose metabolism. Bad – Bcl-2 associated death promoter, FKHR – forkhead transcription factors, GFs – growth factors, GSK3β – glycogen synthase 3β, PI3K – phosphoinositide 3-OH kinase, PIP_2 – phosphatidylinositol (4,5) biphosphate, PIP_3 – phosphatidylinositol (3,4,5) triphosphate, PDK1 – protein dependent kinase 1, PKB – protein kinase B, PTEN – phosphatase and tensin homolog, RTK – receptor tyrosine kinase, TOR – target of rapamycin.

to external messages or to extracellular stresses (Persad *et al*., 2000; Yoganathan *et al*., 2000; Tu *et al*., 2001). ILK is a protein kinase that is critically involved in adhesion of cells to the ECM and signal transduction (Wu, 2001; Attwell *et al*., 2003; Xie *et al*., 2004; Boulter *et al*., 2006). The kinase activity of ILK can be modulated by the interaction of cells with components of the ECM, or by integrin clustering and growth factors (Delcommenne *et al*., 1998; Janji *et al*., 2000; Persad *et al*., 2000; Yoganathan *et al*., 2000; Attwell *et al*., 2003). ILK was identified in the search for proteins capable of interacting with the cytoplasmic domain of the integrin β_1 subunit. This was achieved using a yeast two-hybrid screen with the integrin β_1 cytoplasmic domain as bait (Hannigan *et al*., 1996; Dedhar *et al*., 1999). The focus of this study examines the expression and kinase activity of ILK. ILK has attracted much interest of late due to its prime location in cells for regulating integrin-mediated processes.

Extensive biochemical analysis has revealed that the structure of ILK comprises three identifiable features (Persad and Dedhar, 2003; Oloumi *et al*., 2004). The N-terminus consists of four non-catalytic ankyrin (ANK) repeats, which help target the molecule to focal adhesion complexes and link it to the growth factor signalling pathways via the adaptor protein PINCH (particularly interesting new calponin homology domain-containing protein) (Tu *et al*., 1999; Somasiri.*et al*., 2000; Kaneko *et al*., 2004; Boulter *et al*., 2006). This is followed by a pleckstrin homology (PH)-like motif that binds a lipid product of phosphatidylinositol 3-kinase (PI3K) and participates in the regulation of the kinase activity (Li *et al*., 1999; Dedhar, 2000; Persad and Dedhar, 2003; Kaneko *et al*., 2004). This motif overlaps with the extreme N-terminus of the kinase catalytic domain. The C-terminal catalytic domain contains a protein kinase catalytic site as well as a binding site for β integrin cytoplasmic domains, which is located in the extreme C-terminus of the kinase domain (Dedhar, 2000; Grashoff *et al*., 2004).

ILK was originally identified as a binding partner to β_1 integrins (Stevens *et al*., 2004), and evidence has demonstrated that ILK is important as a molecular scaffolder at cell-ECM adhesion sites. Furthermore, ILK participates in various signalling pathways involved in controlling cell survival, differentiation, proliferation and gene expression in mammalian cells (Wu, 2001; Attwell *et al*., 2003; Erdödi et al., 2003; Wu, 2004; Graness *et al*., 2005).

The regulation of ILK activation occurs either through an interaction of cells with components of the ECM or by integrin clustering (Cordes and van Beuningen, 2003; Duxbury *et al*., 2005). ILK has a somewhat unusual kinase catalytic domain, in that it requires both PIP_3 binding, as well as autophosphorylation for ILK activation (Dedhar *et al*., 1999; Lynch *et al*., 1999; Dedhar, 2000). ILK has, however, been shown to directly phosphorylate proteins such as PKB, glycogen synthase kinase 3 (GSK3), myosin light chain (MLC), and ILK-binding protein affixin (see Figure 2 pg 22 and Yamaji *et al*., 2001; Wu, 2001; Aoyagi *et al*., 2005; Pinkse *et al*., 2005; Yau *et al*., 2005). All these downstream targets of ILK will be discussed in due course. However, it is first necessary to examine the association between ILK and the actin cytoskeleton.

1.4.2 ILK Acts as a Molecular Scaffold

Since the discovery of ILK by Hannigan *et al*. (1996), much has been revealed about this fascinating protein kinase. ILK plays a versatile role in just about every aspect of cellular functioning. It has already been mentioned that integrins are involved in physical attachment of cells to cell-matrix adhesion sites, which is crucial for cellular processes such as cell migration and tissue formation, as well as for activation of adhesion-mediated signalling events (Katz *et al*., 2000; Cordes and van Beuningen, 2003; Cordes, 2004). By binding various adaptor proteins either directly or indirectly; ILK is linked to the actin cytoskeleton and is the primary regulatory kinase during these processes (Li *et al*., 1999; Janji *et al*., 2000; Somasiri *et al*., 2000; Grashoff *et al*., 2004).

Although the integrins provide the actual attachment to the ECM, they do not possess any catalytic activity of their own, and instead it is ILK which regulates their activation. This was shown in genetic model systems such as *Drosophila* where null mutations of ILK resulted in defects similar to those of integrin function loss (Zervas *et al*., 2001). Furthermore, when these mutations were examined in more detail it was revealed that actin filaments were detached from the membranes at the muscle attachment sites (Zervas *et al*., 2001). Thus, it became apparent that ILK was capable of acting as a bridge between the integrin receptors and the actin cytoskeleton. From these studies it was revealed that ILK was involved in cellular processes such as cell adhesion, cell survival, migration and angiogenesis (Yoganathan *et al*., 2000; Cordes and van

Beuningen, 2003; Dai *et al*., 2003; Fukuda *et al*., 2003; Cho *et al*., 2005). Moreover, it was demonstrated by these studies that ILK also played an important role in focal adhesion formation and actin reorganisation (Yamaji *et al*., 2002; Vouret-Craviari *et al*., 2004).

Adaptor proteins such as PINCH, calponin homology (CH) domain-containing ILK-binding protein (CH-ILKBP), paxillin, actopaxin and affixin are the primary proteins, which link ILK to the actin filaments. CH-ILKBP, paxillin, actopaxin and affixin all show a degree of sequence homology making it possible to bind to ILK (Wu, 2001; Attwell *et al*., 2003; Wu, 2004). CH-ILKBP is an actin-binding focal adhesion protein containing two calponin-homology (CH) domains. CH-ILKBP localises to focal adhesions (FAs) in response to cell-matrix attachment, but not cell-cell adhesions, which is also the case for ILK (Tu *et al*., 2001). As ILK is the only FA protein that is known to bind CH-ILKBP, ILK probably plays a crucial role in recruiting CH-ILKBP to FAs (Tu *et al*., 2001). CH-ILKBP is closely related structurally to rat actopaxin, a paxillin- and actin-binding protein (Wu, 2001). The C-terminal domain of ILK and the CH2 domain of CH-ILKBP mediate the interactions between ILK and CH-ILKBP (Wu, 2001; Fukuda *et al*., 2003; Wu, 2004; Boulter *et al*., 2006).

Although not directly associated with actin, the focal adhesion protein PINCH, comprising five LIM domains (a common protein-protein interaction motif that was originally discovered in the products of the *lin*-11, *isl*-1, and *mec*-3 genes) (Li *et al*., 1999; Velyvis *et al*., 2001; Fukuda *et al*., 2003; Boulter *et al*., 2006), is able to bind ILK with high affinity. The ILK-PINCH binding is mediated by the N-terminal ankyrin repeat domain of ILK and the second Zn finger located within the N-terminal-most LIM domain (LIM1) of PINCH (Wu, 2001, Fukuda *et al*., 2003; Persad and Dedhar, 2003; Wu, 2004, Figure 3, pg 33). The importance of the ILK-PINCH to the integrin-actin network is strengthened by reports that demonstrate that overexpression of dominant negative forms of either PINCH or ILK that disrupt the PINCH/ILK interaction impairs fibronectin matrix assembly, cell spreading and migration (Guo *et al*., 2001; Yang *et al*., 2005). As a result of these interactions a stable PINCH-ILK-CH-ILKBP tertiary complex in cells is formed (Wu, 2001). The formation of this complex facilitates the localisation of PINCH, ILK and CH-ILKBP to cell-ECM contact sites, at which they provide a crucial physical connection between transmembrane receptors such as

integrins and the actin filaments (Tu *et al*., 2001; Wu, 2001; Grashoff *et al*., 2004; Wu, 2005).

Paxillin is a multidomain adaptor protein that contains five leucine-aspartate repeat (LD) motifs at its N-terminus (Nikolopoulos and Turner, 2002). Paxillin has not been detected as part of the multicomponent (PINCH-ILK-CH-ILKBP) complex, despite its ability to interact with both ILK and CH-ILKBP through the LD1 motif (Wu and Dedhar, 2001; Persad and Dedhar, 2003). Paxillin however, binds actopaxin, which is localised to focal adhesions and binds actin and ILK (Nikolopoulos and Turner, 2002). Thus, although paxillin is not a part of the tertiary complex of ILK, it still does provide an indirect link to the actin cytoskeleton by binding to actopaxin. Interestingly, a reduction in cell adhesion/spreading on collagen has been demonstrated when a paxillin binding-defective actopaxin mutant in HeLa cells was ectopically expressed. This suggests an important role for actopaxin-paxillin interactions in integrin-dependent remodelling of the actin cytoskeleton (Nikolopoulos and Turner, 2001). Actopaxin was identified in a screen for actin-binding proteins and named parvin, defining a family of proteins with three members (α, β, γ) (Olski *et al*., 2001; Nikolopoulos and Turner, 2001). The interactions between ILK and an actopaxin family member (human actopaxin-paxillin-α-parvin-CH-ILKBP) (Nikolopoulos and Turner, 2002) provide evidence for these interactions in focal adhesion targeting of ILK and, a role for ILK in integrin-mediated signal transduction events.

It is thought that an additional connection, between integrins and the actin cytoskeleton, could be provided by the affixin-β-parvin complex (Wu and Dedhar, 2001). Affixin-β-parvin contains a tandem CH domain-containing protein and belongs to a novel family of FA proteins, together with other homologous proteins such as the actopaxin-CH-ILKBP-α-parvin complex and γ-parvin (Yamaji *et al*., 2002). Affixin is involved in the development of focal adhesions and actin stress fibres and is known to bind to ILK. It has been reported that the initial spreading of cells is inhibited by overexpression of an affixin fragment containing the ILK-binding CH2 domain (Yamaji *et al*., 2001). Extensive effort has gone into finding a direct interaction between affixin-β-parvin and actin. However, this interaction has not been detected *in vitro* (Yamaji *et al*., 2001). Recently, Yamaji *et al*. (2002) have demonstrated signalling in platelets by integrin-

ILK-affixin, thus suggesting that affixin does play an important role in linking the cytoskeleton to the integrin receptors. Thus, ILK is capable of mediating the link between the integrin receptors and the actin cytoskeleton through numerous actin-binding proteins, all of which bind ILK (Persad and Dedhar, 2003).

It is apparent that ILK is suitably placed to modulate the physical strength of the connections at the cell matrix contact sites. It is apparent that ILK provides a molecular scaffold at the cell-ECM adhesion sites (Wu and Dedhar, 2001), which requires the correct subcellular localisation of ILK, and an intact paxillin-actopaxin-ILK protein complex, which are likely to impact significantly on normal ILK signalling (Nikolopoulos and Turner, 2001; Persad and Dedhar, 2003; Wu, 2005). Besides the complexes mentioned above, there are likely protein complexes containing talin, filamin, or α-actinin, that can also physically link the ECM and transmembrane receptors to the intracellular actin cytoskeleton (Tu *et al*., 2001) to bring about specific cellular responses.

1.4.3 Intracellular signalling by ILK

ILK is suitably placed to mediate a host of cellular functions by influencing intracellular processes such as actin organisation and, extracellular processes, including cell motility and adhesion. By creating muliprotein complexes that connect ILK to the actin cytoskeleton, ILK provides a physical connection between the integrin receptors and the actin cytoskeleton, and elicits the signals that provide the communication network between the extracellular and intracellular compartments of cells (Pinkse *et al*., 2005; Vespa *et al*., 2005).

Numerous signalling pathways have been acknowledged to involve ILK, and examination of these would require in depth discussion and detail. For the sake of brevity, we will confine this discussion to two important signalling pathways involving ILK. These include the outcomes of ILK signalling on the actin cytoskeleton, as well as the Wnt signalling pathway, which is important in the regulation of many genes controlling proliferation, the cell cycle and anchorage independent growth (Wu and Dedhar, 2001).

The signals that are elicited as a consequence of the integrin-ILK-actin scaffold are able to influence actin organisation, which are mediated by affixin-β-parvin and actopaxin-CH-ILKBP-α-parvin (Wu, 2005). These signals relay changes in cell spreading and adhesion (Nikolopoulos and Turner, 2002; Yamaji *et al*., 2002; Attwell *et al*., 2003; Wu, 2004). ILK is involved in the regulation of cell migration, cell motility, and contractibility by directly phosphorylating proteins such as MLC (Deng *et al*., 2001), affixin (Wu and Dedhar, 2001; Yamaji *et al*., 2001; Attwell *et al*., 2003), as well as the Rho family members (Graness *et al*., 2005). It is thought that this occurs in the early stages of cell spreading where the phosphorylation of affixin modulates the interaction of the affixin-ILK-actin complex (Wu and Dedhar, 2001; Yamaji *et al*., 2001). Affixin and ILK are known to colocalise at FAs and, when affixin is overexpressed in CHO cells, the COOH-terminal half of affixin disrupts preformed FAs and actin stress fibres (SFs), suggesting affixin to be one of the downstream targets of ILK (Yamaji *et al*., 2001).

ILK is also an essential component connecting the clustering of integrins to the activation of the Rho proteins, Cdc42 and Rac. ILK-deficient fibroblasts are not only deficient in spreading and adhesion, but also show alterations in the expression of actin fibres compared to wild type cells. In contrast to these findings, overexpression of wild-type and mutated ILK in U2OS osteosarcoma cells hardly affected actin organisation. Furthermore, mutated ILK was correlated with increased RhoA signalling (Khyrul *et al*., 2004). A principal effector of RhoA is Rho-associated kinase (ROCK), which is capable of modulating the actin cytoskeleton through pathways involving myosin kinase (Riento and Ridley, 2003). It has been demonstrated that ILK contributes to the regulation of cytoskeletal organisation, morphology and cell migration through a ROCK-mediated pathway. In this study, the wild-type ILK-dependent effects on spreading, morphology and motility were reversed by inhibition of RhoA signalling or its downstream effector ROCK (Khyrul *et al*., 2004). It is obvious that ILK plays a major role in the modulation of the actin cytoskeleton by regulating crucial signalling molecules.

It has been mentioned above that affixin and CH-ILBP are both able to bind ILK and, furthermore this binding occurs via similar domains demonstrating that affixin and CH-ILKBP share sequence homology (Attwell *et al*., 2003). For this reason, although it has

not yet been demonstrated, it is likely that ILK is able to phosphorylate CH-ILKBP (Wu and Dedhar, 2001). On the other hand it is also likely that certain functions of ILK do not require ILK kinase activity. For example, it has been suggested that in *Drosophila*, during the regulation of integrin-actin cytoskeleton interactions, no kinase activity by ILK is required (Zervas *et al*., 2001).

To understand ILK signalling pathways, specifically the way in which ILK transduces signals, overexpression studies are often performed. The characteristics of ILK overexpression in normal epithelial cells include the loss of cell-cell adhesion, due to inhibition in the level of E-cadherin expression (Novak *et al*., 1998; Tan *et al*., 2001; Oloumi *et al*., 2004), nuclear translocation of β-catenin, as well as β-catenin stabilisation (Dedhar, 2000; Somasiri *et al*., 2001; Oloumi *et al*., 2004).

The responses that are elicited by ILK are numerous and their impacts upon cellular functioning widespread. The Wnt (cell-fate determining protein) signalling pathway is one such crucial pathway that is important in the development of vertebrates as well as invertebrates, and is also involved in the development of several human cancers (Morin *et al*., 1997; Rubinfeld *et al*., 1997; Seidensticker and Behrens, 2000). Understanding the role of ILK in this pathway is of particular interest, as ILK is able to regulate proteins in the Wnt signalling pathway (D'Amico *et al*., 2000) by inhibiting or stimulating key components of this cascade. Central to Wnt signalling is the control of β-catenin stability, which is regulated by glycogen synthase kinase-3β (GSK3β) (D'Amico *et al*., 2000). Regulation of β-catenin and GSK3β are the primary ways in which ILK directs Wnt signalling (Persad *et al*., 2000; Tan *et al*., 2001; Attwell *et al*., 2003; Persad and Dedhar, 2003, Figure 3).

β-catenin is an imperative molecule in the Wnt pathway, which forms complexes with transcription factors, such as TCF (T cell factor)/Lef-1 (leukocyte enhancing factor). These TCF/Lef-1-β-catenin complexes are often constitutively observed in tumour cell lines and tissue samples (Seidensticker and Behrens, 2000). ILK is able to induce this complex association via phosphorylation of β-catenin (Novak *et al*., 1998; Persad *et al*., 2001). The phosphorylation of β-catenin leads to an increase in the levels of β-catenin and a relocation of β-catenin to the nucleus as well as higher levels of activity for AP-1

by modulating c-Jun DNA-binding activity (D'Amico *et al*., 2000; Yoganathan *et al*., 2000). The increase in β-catenin is a direct result of inhibited GSK3β via ILK phosphorylation. Inhibited GSK3β is responsible for the degradation of β-catenin, whereas the relocalisation is a consequence of an unusual elevation of the transcription factor partner of β-catenin, Lef-1, which stimulates activity of the Lef-1-β-catenin complex (Dedhar *et al*., 1999; Persad *et al*., 2000; Wang *et al*., 2001; Attwell *et al*., 2003; Persad and Dedhar, 2003). Activation of the transcriptional complex of TCF/lef-1-β-catenin, leads to downregulation of E-cadherin expression as well as the stimulation of cell cycle control genes including c-myc and cyclin D1 (Novak *et al*., 1998; Seidensticker and Behrens, 2000; Oloumi *et al*., 2004).

The regulation of GSK3β by ILK can be either direct or indirect. Research has shown that ILK overexpression is capable of inhibiting GSK3β activity either by direct phosphorylation or indirectly, via PKB phosphorylation, which also inhibits GSK3β (Dedhar *et al*., 1999; Persad *et al*., 2000; Yoganathan *et al*., 2000; Persad and Dedhar, 2003). The phosphorylation of PKB by ILK is also an essential step for the complete activation of PKB (Dedhar *et al*., 1999). Survival pathways and glucose metabolism are regulated by PKB as well as several transcription factors, including E2F transcription factor 2 (E2F) and cyclic AMP (cAMP)-responsive element binding protein (CREB) (Yoganathan *et al*., 2000; Prolov and Dysan, 2004, Figure 3). PKB is discussed in depth later when anchorage independent growth is considered.

The aforementioned signalling pathways affected by ILK are able to control the activities of key signalling pathways, which result in either the activation or repression of genes encoding proteins involved in the regulation of cell survival, cell cycle, cell adhesion and ECM modification (Wu and Dedhar, 2001). It is thus redundant that ILK is central to many of the cellular aspects of signal transduction pathways. As well as being involved in activation and inhibition of certain molecules, ILK also plays a fundamental role in the control of cell cycle progression. This control is mediated primarily by cyclins whose expression is limited to specific points in the cell cycle (Youssef *et al*., 1997). Changes in expression of these proteins are able to alter rates of cell proliferation and, as discussed above, ILK is capable of regulating their expression levels. Associated with the induction of cyclins by ILK overexpression, is anchorage

independent growth (D'Amico *et al*., 2000). Cell proliferation is usually increased in many malignant cancers (McCormick, 1999; Giannis, 2000; Andl *et al*., 2003), and together with anchorage-independent growth, contributes to the metastatic cascade.

1.5 The Relationship between Cell Proliferation and Anchorage Dependent Growth

1.5.1 Cell Proliferation

Understanding the cell division process is essential as progression through the cell cycle increases rapidly during the metastatic process. ILK has the potential to be directly involved in influencing the rate of cell division. Cell division is a tightly controlled process regulated by the cyclin/Cdk (cyclin dependent kinase) family of protein kinase complexes (Garrett and Fattaey, 1999; Danen and Yamada, 2001; Bhadriraju and Hansen, 2004; Boonstra and Moes, 2005). Progression through the cell cycle is dependent on the presence of both growth factors and on cell attachment (Hulleman *et al*., 1999; Walker and Assoian, 2005). Several checkpoints contribute to cell cycle regulation (Amon, 1999; Donaldson and Blow, 1999; Roy and Thompson, 2006) and this fidelity is abrogated in transformed cells resulting in increased cell proliferation (Hartwell and Kastan, 1994). Ras-ERK (extracellular signal regulated kinase) activation is a consequence of ligation of $\alpha_1\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins linked to Shc signalling pathways. This in turn promotes progression through the G_1 phase of the cell cycle (Wary *et al*., 1996; Bill *et al*., 2004).

ILK influences cell cycle control directly by stimulating many molecules involved in its regulation. Studies have reported that ILK overexpression increases the expression of cyclin A, cyclin D1 and Cdk4 proteins (Radeva *et al*., 1997). Hyperphosphorylation of the retinoblastoma protein (Rb) was also shown, caused by elevated activities of both

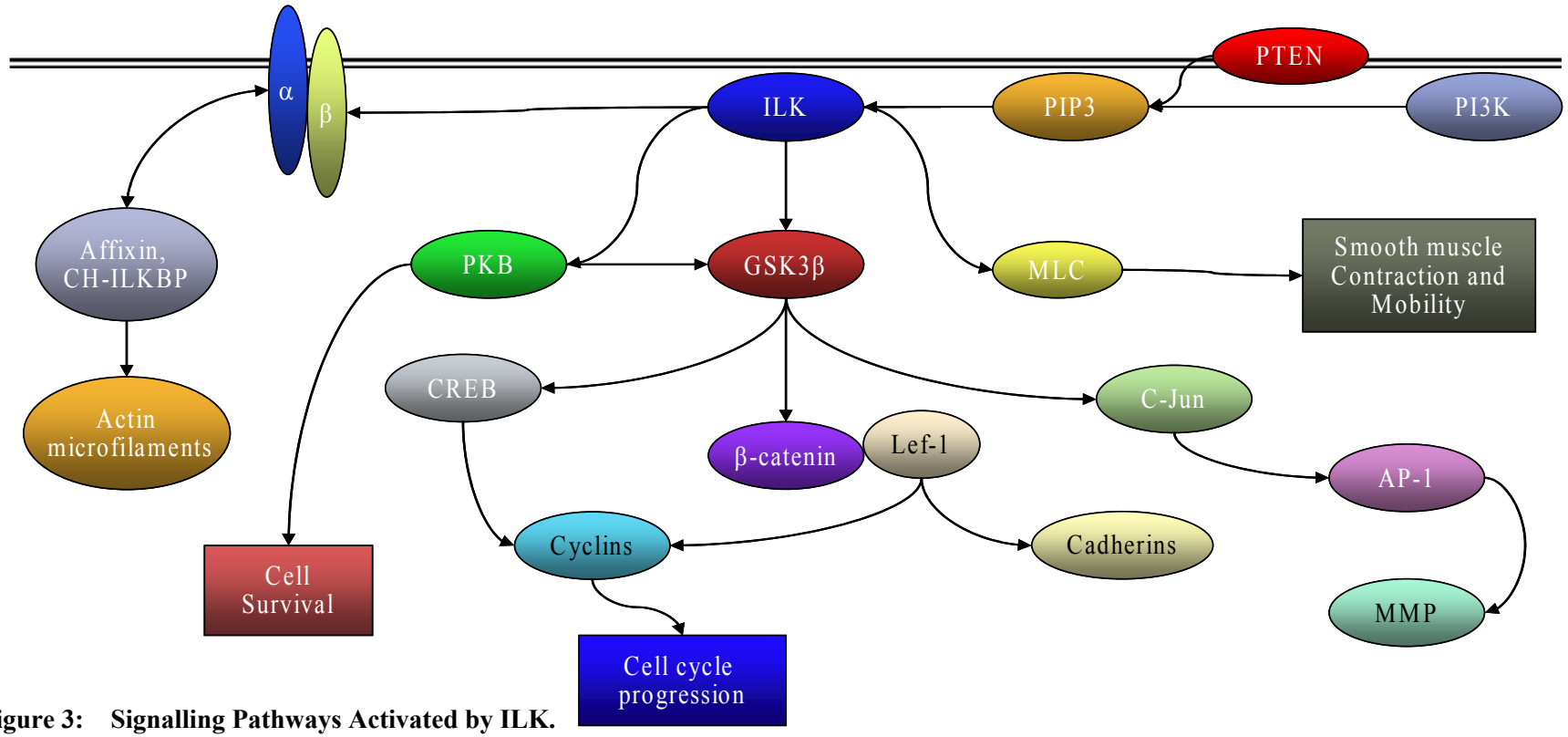


Figure 3: Signalling Pathways Activated by ILK.

ILK is activated via the lipid product of PI3K, PIP_3. ILK is inactivated by the lipid phosphatase PTEN, which dephosphorylates PIP_3. Once activate, ILK is able to phosphorylated PKB and GSK3β. Phosphorylation of PKB contributes to its activation, which suppresses apoptosis and anoikis. Phosphorylation of GSK3β leads to its inhibition, which stabilises β-catenin and stimulates AP-1 activity. β-catenin and CREB activation via ILK and GSK3β lead to upregulation of cyclin D1, whereas AP-1 activates MMPs expression, thereby controlling cell migration. ILK also phosphorylates MLC, thus regulating smooth muscle contraction and possible cell motility in muscle cells. AP-1 – activating protein-1, CREB – cAMP responsive element binding protein, GSK3β – glycogen synthase 3β, ILK – integrin linked kinase, Lef-1 – leukocyte enhancing factor-1, MLC – myosin light chain, MMP – matrix mettaloproteinase, PI3K – phosphoinositide 3-OH kinase, PIP3 – phosphatidylinositol (3,4,5) triphosphate, PKB – protein kinase B, PTEN – phosphatase and tensin homolog.

cyclin D1-Cdk4 and cyclin E-Cdk2 kinases. Important regulatory cascades involved in G1-S phase transition include the MAPK and PI3K signalling pathways (Boonstra and Moes, 2005). Activation of ILK via PI3K, leads to GSK3β inhibition, which results in the activation of the transcription factor AP-1 (Persad and Dedhar, 2003). This together with β-catenin/TCF, and the transcription factor CREB, may be responsible for ILK-mediated upregulation of cyclin D1 expression (D'Amico *et al*., 2000; Cordes and van Beuningen, 2003). All these responses lead to progression through G_1 and into the S phase of the cell cycle.

The p16-cyclin D1-Cdk4-Rb pathway is central to the regulation of the G_1-S phase transition and alterations or mutations in this pathway are encountered in most human cancers, the most prominent being cyclin D1 (Bill *et al*., 2004; Roy and Thompson, 2006). Cyclin D1 is responsible for the phosphorylation of various substrates including the Rb protein. The partial phosphorylation of Rb results in liberation of transcription factors, predominantly the ones belonging to the E2F family, which in turn activate various genes responsible for the progression through S phase (Dysan, 1998; Pankov *et al*., 2004; Walker and Assoian, 2005). Cyclin D1 has been implicated in the pathogenesis of various cancers. For example, induction of cyclin D1 in breast cancer cell lines shortens G_1 and results in an increase in the number of cells progressing through G_1 (Musgrove *et al*., 1994). In addition overexpression of cyclin D1 is correlated with progression of oral tongue and colorectal carcinoma (Wu *et al*., 2005; Wang *et al*., 2006).

Integrins have been established to have direct implications with regards to cell cycle control and cyclin D1. Studies have shown that induction of cyclin D1 requires sustained ERK activity. It has been reported that in fibroblasts cell adhesion to fibronectin and activation of $\alpha_5\beta_1$ is necessary for the prolonged ERK activity (Roovers and Assoian, 2003). While this is suggested to occur upstream of Ras other sites have been proposed to occur downstream of Ras, either between Ras and Raf (Lin *et al*., 1997), or Raf and MEK (Renshaw *et al*., 1997). Dissecting the roles of the α_2 cytoplasmic domain of the $\alpha_2\beta_1$ integrins revealed that distinct residues within the α_2 cytoplasmic tail are required for cell cycle progression in response to insulin leading to sustained ERK activity (Kiekotka *et al*., 2001; Damsky and Ilic, 2002). In another

study, integrin-mediated adhesion regulated secretion of macrophage migration inhibitory factor (MIF), which, together with growth factor receptors, sustained ERK activity, induced cyclin D1 and stimulated S phase entry (Liao *et al*., 2003). Integrin are thus capable of sustaining ERK activity, which occurs via numerous signalling pathways including FAK, Src and $p130^{CAS}$ (Crk associated substrate) (Barberis *et al*., 2000; Walker and Assoian, 2005).

Adhesion to the ECM is also required for progression of cells through the G and into the S phase of the cell cycle (Hulleman *et al*., 1999; Bhadriraju and Hansen, 2004). When this adhesion is lost, cells arrest in the G_1 phase of the cell cycle and undergo apoptosis (Danen and Yamada, 2001). However, ILK, which is capable of causing reduction in cell adhesion to ECM, as well as anchorage independent growth, is a specific correlate of tumour growth *in vivo*, may be involved directly in by-passing this apoptotic pathway.

1.5.2 Anchorage Independence

In normal cells, anchorage adhesion constraints exist in order to prevent unsuitable localisation of cells (Frisch and Francis, 1994; Valentijn *et al*., 2004). These survival restraints are breached in transformed cells, and these cells posses the ability to survive and grow in inappropriate environments *in vivo* (Wang, 2004; Redding and Juliano, 2005). It could be reasoned that transformation requires matrix-independent survival (Frisch and Francis, 1994). Upon detachment from the substratum normal fibroblasts undergo growth arrest while normal epithelial cells undergo apoptosis, a phenomenon known as ‘anoikis’ (Grossman, 2002; Wang, 2004). Without apoptosis, detached cells could possibly reattach to inappropriately localised matrices, and resume growth (Frisch and Francis, 1994). However, apoptosis occurring in normal detached cells would abrogate this escape mechanism. The ability of cancer cells to proliferate in the absence of adhesion to ECM proteins is termed anchorage independence of growth (Schwartz, 1997). Tumour cells are generally resistant to anoikis and can proliferate in the absence of anchorage to the ECM (Giancotti and Ruoslahti, 1999).

The mechanism for the induction of apoptosis in response to cell detachment from the substratum is not clear but both the mitochondria and death-receptor –mediated

activation of apoptotic caspases are likely to be involved (Grossman, 2002; Valentijn *et al*., 2004). In order to maintain appropriate cell numbers, as well as tissue organisation in certain cell types such as lymphocytes and neurons, programmed cell death (apoptosis) is vitally important (Frisch and Francis, 1994; Wang, 2004). Lack of matrix attachment could stringently restrict inappropriate growth by inducing apoptosis (Attwell *et al*., 2000).

The incorrect localisation, as occurs in metastasis, is a characteristic that distinguishes malignant from benign tumours (Schwartz, 1997). For some time anchorage independent cell proliferation has been considered as a hallmark of malignant transformation (Attwell *et al*., 2000; Wang, 2004). The migratory, invasive and anoikis-resistant capability of cancer cells provides them with the metastatic potential (Schwartz, 1997; Wang, 2004). This may explain their propensity to leave their original site and metastasise.

In order for adherent cells to survive, they must anchor to an appropriate ECM molecule, and this anchorage occurs via the integrin molecules (Valentijn *et al*., 2004; Wang, 2004). The requirement for a specific integrin as the mediator of the attachment may provide a safety factor, as it would facilitate the destruction of cells that have attached at an inappropriate tissue location (Giancotti and Ruoslahti, 1999).
Initiation of apoptosis requires caspase activation in most cells which ultimately permeabilises the outer mitochondrial membrane (OMM) (Martinou and Green, 2001). This releases a host of factors into the cytosol, whose combined effect is caspase activation (Valentijn *et al*., 2004). Cytochrome C acts as a cofactor for the recruitment of caspase-9 in to the apoptotic protease activation factor 1 (APAF-1), resulting in its cleavage and activation. OMM permeabilisation initiates the whole caspase cascade, culminating in cell death (Valentijn *et al*., 2004). In this regard, members of the Bcl-2 family contribute to OMM permeabilisation (Cory and Adams, 2002), which is necessary for cell death.

There are a wide variety of stimuli that promote the apoptotic pathway. What will be discussed next are some of the important pathways known to be involved in regulating anchorage-independent growth. These include the integrins, cadherins,

PI3K/PKB/GSK3β signalling as well as the importance of growth factor signalling. Furthermore, the relevance of ILK to apoptosis will be considered.

Interestingly, SCC cells remain anchorage-dependent and generally fail to survive in the absence of cell-ECM adhesions (Kramer *et al*., 2005). Understanding why SCCs require integrin signalling to proliferate has obvious importance, since it is difficult to explain the extensive intratumoural growth that occurs *in vivo*, where the cells do not have access to integrin engagement and FAK activation (Kramer *et al*., 2005). Activated integrins signal through a vast array of signalling cascades that are activated by tyrosine kinase growth factor receptors (Damsky and Ilic, 2002; Juliano, 2002). The activation of FAK, which, as mentioned above, is vital to many cellular processes (Kramer *et al*., 2005) may impinge upon survival pathways. Indeed, FAK is capable of activating the PI3K signalling pathway, which has important consequences in cell survival (Sawai *et al*., 2005).

The ECM is able to control anoikis in an integrin-specific manner. For example, the $\alpha_5\beta_1$ integrin binds to fibronectin and induces the expression of the anti-apoptotic protein Bcl-2 protecting cells from apoptosis (Zhang *et al*., 1995). Other integrins, including another fibronectin receptor $\alpha_v\beta_1$, do not provide this survival effect, whereas the $\alpha_v\beta_3$ integrin provides endothelial and melanoma cell survival (Stromblad *et al*., 1996; Giancotti and Ruoslahti, 1999). A study by Janes and Watt (2004) recently discovered a function of the $\alpha_v\beta_5$ integrin in promoting anoikis in H357 SCC cells. These authors demonstrate that when the $\alpha_v\beta_5$ integrin is replaced with $\alpha_v\beta_6$ integrin, anoikis is prevented. Thus it is implied that overexpression of $\alpha_v\beta_6$ may confer a survival advantage in SCCs. Other potential survival pathways include activation of Shc by the $\alpha_1\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins (Giancotti and Ruoslahti, 1999). Adhesion mediated Shc-linked integrins promote cell survival and progression through the G_1 phase of the cell cycle in response to mitogenic growth factors, whereas adhesion mediated by other integrins result in exit from the cell cycle and, in certain cases, cell death (Pozzi *et al*., 1998).

It has been proposed that cadherins and catenins are capable of regulating cell death and growth. Cell-cell contact can sometimes inhibit growth (contact inhibition of growth)

especially in normal cells (Kramer *et al*., 2005), and this has been linked to alterations in cyclin-dependent kinases (cdks). Previous work has implicated E-cadherin-mediated cell-cell adhesion in protecting head and neck SCCs from anoikis (Kantak and Kramer, 1998). There is considerable evidence that cell-cell contacts promote cell survival in the absence of cell-ECM attachment. The ability of E-cadherin to induce survival signals may depend on their association with other signalling pathways, since cadherins themselves lack enzymatic activity (Kramer *et al*., 2005). In fact, E-cadherin has been demonstrated to interact with the PI3K signalling pathway in adherens junctions (Arregui *et al*., 2000).

The PI3K/PKB signalling pathway plays a pivotal role in anchorage-independent growth. PI3K is activated in response to different mechanisms including growth factor receptors and integrin/ECM-mediated signalling (Wang, 2004). To ascertain the relevance of PI3K to anchorage-independent growth, studies revealed that the colony-forming ability of v-Ros-transformed CEF cells was inhibited upon treatment with the PI3K inhibitor LY294002 (Wang, 2004). Conversely, an activated PI3K was able to rescue the colony-forming activity of two transformation-defective mutants of v-Ros (a protein tyrosine kinase derived from an avian sarcoma retrovirus) (Nguyen *et al*., 2002; Wang, 2004).

How PI3K signalling promotes anchorage-independent growth involves the expression of various cyclins and cyclin-dependent kinases (cdks) (Wang, 2004). Indeed, cyclin A-associated Cdk activity was augmented in the v-Ros-expressing CEF, which was further found to be PI3K-dependent, since it was blocked by the PI3K inhibitor (Uttamsingh *et al*., 2003). In another study it was shown that EGF and TGFβ1 synergistically protect RIE cells from anoikis, which was also blocked by PI3K inhibitors (Wang, 2004). Thus, it is apparent that PI3K signalling is capable of blocking the apoptotic pathway and enhancing the anchorage-independent cell cycle progression at the same time.
Growth factors are also known to participate in regulating the process of anchorage-independence, since activation of MAPK and PKB was prolonged in the EGF- and TGFβ1-treated cells grown under nonadhering conditions. This leads to the phosphorylation and inactivation of pro-apoptotic proteins such as GSK3β and forkhead-related transcription factor (FKHRL1) as well as decreased levels of cyclin

inhibitor $p27^{KIP1}$ and $p16^{TNK4a}$ (Wang, 2004). It has previously been shown that inhibition of EGF signalling upregulates another proapoptotic factor, Bim. Downregulation of EGF occurs in MCF10A epithelial cells after prolonged removal from ECM (Reginato *et al*., 2003). It was found that small interfering RNA against Bim significantly reduced apoptosis over this time period suggesting that Bim may contribute to anoikis in some cells (Frisch, 1999).

Suppression of apoptosis and anoikis is one of the consequences of constitutive ILK activation in mammalian cells (Attwell *et al*., 2000; Persad *et al*., 2000). Both of these effects involve ILK-mediated activation of PKB/Akt and suppression of caspase 3 activation (refer to Figure 2 and Wu and Dedhar, 2001; Pinkse *et al*., 2005). PKB is a serine/threonine kinase (Persad *et al*., 2000; Yoganathan *et al*., 2000), which is activated in response to PI3K (Yoganathan *et al*., 2000; Vara *et al*., 2004; Yau *et al*., 2005). PKB also regulates several transcription factors including E2F, CREB, and the forkhead family member Daf-16 (Scheid and Woodgett, 2000). Furthermore, PKB is capable of directly or indirectly phosphorylating and inactivating pro-apoptotic proteins such as caspase-9, Bad and FKHRL1 (Tang *et al*., 1999; Pap and Cooper, 2002; Rossig *et al*., 2002). It also appears that the nuclear factor of kappa light chain gene enhancer in B-cells (NF-κB) family of transcription factors is a target of PKB, as shown in the Jurkat T cell line (Sizemore *et al*., 1999). The induction of NF-κB would have serious implications on T cell growth and survival (Kane *et al*., 1999; Yoganathan *et al*., 2000). It remains to be established however, whether ILK also modulates NF-κB activity through PKB (Yoganathan *et al*., 2000).

What has been discovered is that mammary gland tumours arise in ILK transgenic mice, and the tumour tissues exhibit many of the hallmarks of ILK overexpression in tissue culture cells (Wu and Dedhar, 2001). These include phosphorylation of PKB/Akt and GSK3β but also downregulation of E-cadherin expression and phosphorylation and activation of extracellular signal-regulated kinase (ERK) (White *et al*., 2001; Wu and Dedhar, 2001). As outlined above, PKB, GSK3β and E-cadherin are all possible mediators of apoptosis. Since ILK is implicated in the regulation of these molecules, emphasises how important ILK is to apoptotic events.

1.6 Aberrant ILK/Integrin Expression in Tumour Progression and Metastasis

Most cancer deaths result from the invasion of surrounding tissues and widespread metastasis to vital organs. Adhesion and detachment of tumour cells to one another and to heterologous cells may play a decisive role in metastatic spread at several levels (Qian *et al*., 1994). Cell adhesion events can therefore be considered as critical in tumour metastasis (Danen *et al*., 1998; Wang, 2004) and cell-matrix alterations are an essential prerequisite step in the metastatic cascade (Hart and Saini, 1992).

The metastatic process has traditionally been viewed as consisting of the following steps: (1) detachment of individual cells from the primary lesion, (2) invasion of local stroma, (3) entry of single cells or aggregates of tumour cells into blood vessels directly or via the lymphatic channels (intravasation), (4) arrest in the vasculature followed by extravasation, and (5) invasion into the parenchyma of the target organ site with the resulting establishment of secondary lesions (Qian *et al*., 1994; Brodt, 1996b; Kurschat and Mauch, 2000). Numerous studies have confirmed that the steps involved in metastasis entail changes in the adhesive properties of the tumour cells (Stroeken *et al*., 1998; Zutter *et al*., 1998). The cellular effects mediated by integrin expression in tumour invasion may only be empirically related to cell-matrix adhesion. Loss of integrin expression may predispose cells to tumour progression by the loss of regulatory control over growth and differentiation (Gui *et al*., 1997).

During the multi-step process of malignant transformation, cancer progression, and tumour cell invasion, malignant cells encounter and interact with components of the extracellular matrix at several crucial steps (Giancotti and Mainiero, 1994; Zutter *et al*., 1998). It was shown by Dedhar and Saulnier (1990), that treatment of human osteogenic sarcoma (HOS) cell lines with N-methyl-N'-nitro-N-Nitrosoguanidine (MNNG), a potent carcinogen, altered integrin expression. The MNNG treated cells exhibited greater invasiveness in an *in vitro* model. In this example, transformation was associated with morphologic alterations and increased invasiveness, suggesting that changes in integrin receptors might contribute to changes in cell phenotype associated with malignant transformation (Zutter *et al*., 1998). A report on colonic tumours showed that changes in the level of expression or activity of integrin receptors are thought to be involved in its progression (Pouliot *et al*., 2000). Furthermore, colonic tumours

generally exhibit altered ECM composition in their basement membranes. Thus, changes in their microenvironment and in the adhesive properties of colonic tumours are thought to contribute to the progression of the disease (Pouliot *et al*., 2000).

Altered expression levels of integrin receptors have been associated with malignant spread of many cancers. For example, reduced α_3 integrin subunit expression has been found in breast, colorectal, and pancreatic carcinomas (Zutter *et al*., 1998). Furthermore, expression of the β_4 integrin subunit was decreased in many breast, prostate, and basal cell carcinomas, but expression was increased in other subtypes of carcinoma including squamous cell carcinoma (Zutter *et al*., 1998). In squamous cell carcinoma of the larynx, it has been demonstrated that $\alpha_5\beta_1$ is upregulated (Charalabopoulos *et al*., 2005). With regards to malignant melanoma, upregulation of the α_v, α_2 and α_3 integrin subunits was reported, whereas the α_6 integrin subunit was shown to be decreased (Kuphal *et al*., 2005). Studies in mammary epithelial cells showed that loss of expression of the laminin and collagen receptor, $\alpha_2\beta_1$, is correlated with a transformed phenotype (Taverna *et al*., 1998).

In many instances, increased expression of other integrins correlates with tumour progression in many metastatic tumours such as increased $\alpha_3\beta_1$ integrin. Several other carcinomas of the lung, colon, bladder, head, and neck show increased levels of α_6 integrin, whereas $\alpha_v\beta_3$ is upregulated in breast cancer (Taverna *et al*., 1998; Felding-Habermann *et al*., 2001). In squamous cell carcinomas it has been revealed that considerable variation in integrin expression, both between tumours and in different regions of the same tumour occurs (Watt, 2002). The integrin most heavily implicated in epithelial carcinogenesis is $\alpha_6\beta_4$ (Mercurio and Rabinovitz, 2001). Overexpression of $\alpha_6\beta_4$ has been reported in human oral cancer (van Waes *et al*., 1991). Within a given tumour, $\alpha_6\beta_4$ overexpression occurs in the suprabasal layers with focal loss of $\alpha_6\beta_4$ at the tumour margin (Downer *et al*., 1993). Moreover, the $\alpha_6\beta_4$ integrin is implicated in promoting the migration, invasion, and survival of carcinoma cells (Chung and Mercurio, 2004). These data suggest that ECM molecules and their receptors can act either as suppressors, or promoters, of tumour progression.

The stimulation of intracellular signalling processes is caused by integrin engagement involving molecules such as focal-adhesion kinase (FAK), Shc, Cas and the mitogen-activated protein (MAP) kinase cascade (refer to Figure 1a and 1b, pg 15). The regulation of these signals, that are transmitted to the MAP kinase signalling pathway, are controlled by cell adhesion, which results in the alteration of the activation of MAP kinase kinase (MEK) or Raf (Schwartz, 1997). What remains unclear however, are the events proximal to the integrin receptor initiating such signals (Dedhar *et al*., 1999). ILK participates in fundamental cellular processes such as cell-ECM interactions and intracellular signal transduction pathways. By regulating relatively modest changes, such as alterations in expression or activity, ILK might contribute to the pathogenesis of cancers that involve alterations in cell proliferation and cell-ECM interactions (Attwell *et al*., 2003; Ito *et al*., 2003).

Studies have suggested that ILK is overexpressed in several human tumours, including Ewing's sarcoma, primitive neuroectodermal tumour and medullablastoma (Chung *et al*., 1998; Dedhar *et al*., 1999; Wu, 1999). Furthermore, ILK has been shown to induce an invasive phenotype in brain tumour cell lines (Troussard *et al*., 2000). ILK expression has also shown to be upregulated by constitutively active ErbB-2 (Xie *et al*., 1998), a member of the EGF receptor family that has been implicated in tumourigenesis. Furthermore, overexpression of ILK in epithelial cells induces tumour formation *in vivo* (Wu *et al*., 1998). Recently, it has been reported that ILK correlates with invasion and metastasis in gastric carcinoma as well as laryngeal squamous cell carcinomas (Ito *et al*., 2003; Wu *et al*., 2006), and expression of ILK is associated with the tumourigenesis and progression of prostate cancer (Graff *et al*., 2001). Further studies have revealed that increased ILK expression is correlated with sorter survival in non-small cell lung cancer (Takanami, 2005), and is able to promote the invasive behaviour of ovarian carcinoma (Ahmed *et al*., 2004; Rosano *et al*., 2006).

ILK function and expression are of vital importance with regard to many cellular aspects of malignant transformation and acts as a receptor-proximal effector in the crossmodulation between growth factor response and integrin signalling (Sheppard, 2005). Growth factor overexpression is known to be involved in many highly invasive squamous carcinomas (Yomamoto *et al*., 1986; Veale and Thornley, 1989; Mariotti *et*

al., 2001). Their connection with integrins and particularly ILK is vital to the understanding of cell-ECM adhesion in the multistep development of carcinogenesis.

1.7 ILK/Integrin Link to Growth Factor Signalling

The similarities that exist between integrin-mediated signalling and growth factor receptor-mediated signalling suggest the possibility of crosstalk between these signalling pathways (Coppolino and Dedhar, 2000; Janji *et al*., 2000; Persad and Dedhar, 2003; Grashoff *et al*., 2004; Sheppard, 2005). Indeed, only under appropriate cell attachment conditions are the receptors for insulin, platelet-derived growth factor (PDGF), EGF, and vascular epithelial growth factor (VEGF) optimally activated by their ligands (Giancotti and Ruoslahti, 1999). An example of this relationship comes from a study by Chung *et al*. (2004) who clearly demonstrated that VEGF expression depends on the $\alpha_6\beta_4$ integrin since it functions in concert with hypoxia to activate hypoxia inducible factor-1 (HIF-1) and stimulate VEGF transcription. It is well established that growth factors are capable of activating the Ras-Raf-MEK-Erk signal transduction pathway. Sustained Erk activity is essential for the proliferation of fibroblasts, which is activated by integrin-mediated cell adhesion to ECM (Danen and Yamada, 2001). Furthermore, growth factors are known to be associated with the metastatic process and are capable of influencing cell motility (Tong *et al*., 2000).

Numerous reports have confirmed that there is an interaction between the integrin and growth factor receptors (Delcommenne *et al*., 1998; Dedhar *et al*., 1999; Janji *et al*., 2000; Persad and Dedhar, 2003). Coppolino and Dedhar (2000) showed involvement of the $\alpha_5\beta_1$ and β_1 integrins with growth factors, where it was demonstrated that $\alpha_5\beta_1$ mediates fibronectin-induced epithelial proliferation through activation of the EGF receptors. Further evidence for the association of growth factors and integrins has been shown by the $\alpha_5\beta_3$ integrin receptor, which immunoprecipitated in complexes with the PDGF and VEGF receptors. In addition, it has been reported that the $\alpha_v\beta_1$, $\alpha_v\beta_5$ and $\alpha_v\beta_6$ integrins are capable of directly associating with the latency peptide of TGFβ. While the significance of the binding $\alpha_v\beta_1$ and $\alpha_v\beta_5$ is not known, it has clearly been shown that $\alpha_v\beta_6$ binding induces the activation of latent complexes of TGFβ (Crawford *et al*., 1998). The functional importance of this pathway is proposed to influence

fibrosis of multiple epithelial organs (Sheppard, 2005). The aggregation of the growth factor receptor results in their partial activation and enables crosstalk between integrins and growth factor receptors (Coppolino and Dedhar, 2000).

Growth factor involvement has been shown to be a central component during the progression of many cancers. For example, EGF and TGF's, which are probably the most well defined growth factors, are involved in highly malignant and invasive breast cancer (Welch *et al*., 1990; Klijn *et al*., 1992; Wang *et al*., 1998; Tong *et al*., 2000), as well as other highly invasive squamous carcinoma cells (Tennenbaum *et al*., 1996 cited in Mariotti *et al*., 2001). These include malignancies of the bladder, breast and lung (Sainsbury *et al*., 1985; Ozanne *et al*., 1986 cited in Mayer *et al*., 2000). Furthermore, EGFR and PDGFR expression are highest during the early stages of cervical cancer progression (Mayer *et al*., 2000). Mariotti *et al*. (2001) have demonstrated the necessity of EGFR activation and β_4 integrin tail phosphorylation in epithelial cell migration and invasion. This occurs by EGFR association with $\alpha_6\beta_4$ and the induction of phosphorylation of the β_4 cytoplasmic domain through the Src family kinase Fyn (Mariotti *et al*., 2001).

From these data it becomes obvious that integrins play an important role in growth factor modulation. Growth factors have been shown to affect the expression of the integrin receptors (Lai *et al*., 2000; Pouliot *et al*., 2000; Yu *et al*., 2000; Lim *et al*., 2001), but the interaction between these two classes of receptors is not direct. As ILK is known to influence both the integrin class of receptors and the growth factor receptors it is a prime candidate for mediating the link between integrins and growth factors (Delcommenne *et al*., 1998; Janji *et al*., 2000; Atwell *et al*., 2003; Pinkse *et al*., 2005).

ILK serves not only as a molecular scaffold at the cell-ECM adhesion sites, but due to its interactions with integrins, it is capable of providing a link between integrin-mediated signalling and the growth factor signalling pathways, as well as being favourably positioned to directly regulate metastatic processes. The four non-catalytic ankyrin repeats of ILK help target ILK to focal adhesion complexes and link it to growth factor receptor tyrosine kinase signalling via the adaptor protein PINCH/Unc 97 (Li *et al*., 1999; Somasiri *et al*., 2000).

It has already been noted that PINCH binding to ILK is crucial for ILK focal adhesion localisation. This occurs through the N-terminal ANK repeat domain of ILK binding to PINCH. What has not been mentioned is the fact that the ILK-PINCH interaction connects ILK to Nck-2, a SH2-SH3-containing adaptor protein (Wu, 1999). The fourth LIM domain of PINCH interacts with Nck-2 and, it is thought that Nck-2 plays a unique role in connecting the growth factor signalling pathway with the PINCH-ILK complex and thus the integrin signalling pathway (Fukuda *et al*., 2003; Wu, 2004). Both Nck-2 and PINCH are recruited to activate EGFR and PDGF receptors via the SH2 domain of Nck-2 (Figure 4 and Dedhar *et al*., 1999). Unlike ILK, PINCH does not contain a catalytic domain, and thus the primary role of PINCH is to mediate these types of intermolecular interactions (Wu, 1999; Fukuda *et al*., 2003; Grashoff *et al*., 2004; Boulter *et al*., 2006). Downstream signalling pathways involved in apoptosis, and the promotion of cell survival through PKB and GSK3β appear to be coupled to growth factors and integrins via ILK (Delcommenne *et al*., 1998; Troussard *et al*., 2000; Cordes and van Beuningen, 2003; Kaneko *et al*., 2004; Watanabe *et al*., 2005). TGF-β1 and EGF play an important role in controlling the proliferation, survival, morphology, and motility of epithelial cells (Pouliot *et al*., 2000).

In colon cancer cells, the control of the *in vitro* proliferation by EGF receptor stimulation exerts profound effects on cellular morphology (Pouliot *et al*., 2000). Integrins have been reported to form complexes with EGF (and other growth factors) receptors to modulate their functions (Miyamoto *et al*., 1995; Pouliot *et al*., 2000). Moro *et al*. (1998) have shown that integrins are capable of inducing EGF receptor tyrosine phosphorylation in the absence of EGF receptor ligands, leading to activation of a typical EGF receptor pathway that involved Shc phosphorylation and MAP kinase activation. The consequence of MAPK activation is the induction of transcription of proteases, such as urokinase-type plasminogen activator (uPA), which could affect cell motility and invasion, and transcription of proteins that induce angiogenesis, such as VEGF (McCormick, 1999; Byzova *et al*., 2000; Watanabe *et al*., 2005). Furthermore, integrin-dependent EGF receptor activation is important in anchorage-dependent cell survival (Moro *et al*., 1998). Thus, there is considerable crosstalk between the integrin and growth factor receptors, which is likely to be important in the development and progression of tumours (Pouliot *et al*., 2000; Danen and Yamada, 2001).

1.8 Relevance of ILK to Human Oesophageal SCCs

The alterations in the adhesive events that are necessary in the processes of invasion and metastasis are regulated primarily by integrins (Stroeken *et al*., 1998; Zutter *et al*., 1998). Loss of cell-ECM adhesion allows for the migration of cells and the establishment of secondary tumours at sites distant from the primary tumour. The importance of cell adhesion molecules in oesophageal carcinoma has been investigated extensively. A study by Zhao *et al*. (2003) addressing the expression of E-cadherin, α-, β-, and γ-catenin in oesophageal SCCs revealed a reduction in all these molecules which correlated closely with tumour grade. Considering integrin expression, Miller and Veale (2001) demonstrated that the α_v integrin subunit is strongly expressed in HOSCCs. Downregulation of α_2 and β_1 integrins were also reported, implying that cell adhesion may be decreased and migration promoted in HOSCCs. Another study showed that the β_1 integrin was correlated with lymph node metastasis in HOSCC (Takayama *et al*., 2003). These studies highlight the importance of CAMs such as cadherins and integrins to the development and progression of HOSCC.

Since ILK is closely associated with the integrin receptors it is highly probable that this protein kinase has a crucial influence on HOSCCs. The importance of ILK in tumour development and metastasis has been established in many other carcinomas. Upregulation of activity and expression have been shown in advanced prostate cancers (Persad *et al*., 2000; Graff *et al*., 2001), colonic polyposis, gastric, non-small cell lung cancer and colorectal cancers (Marotta *et al*., 2001; Ito *et al*., 2003; Takanami, 2005).

Even though the importance of ILK in oesophageal carcinoma has not yet been documented, its role as a diagnostic marker of tumours with primitive neural differentiation has been shown previously (Chung *et al*., 1998). What is of particular interest to us is the role of ILK in cell-ECM adhesion, with the possibility of ILK being a potential adhesion marker in carcinoma of the oesophagus.

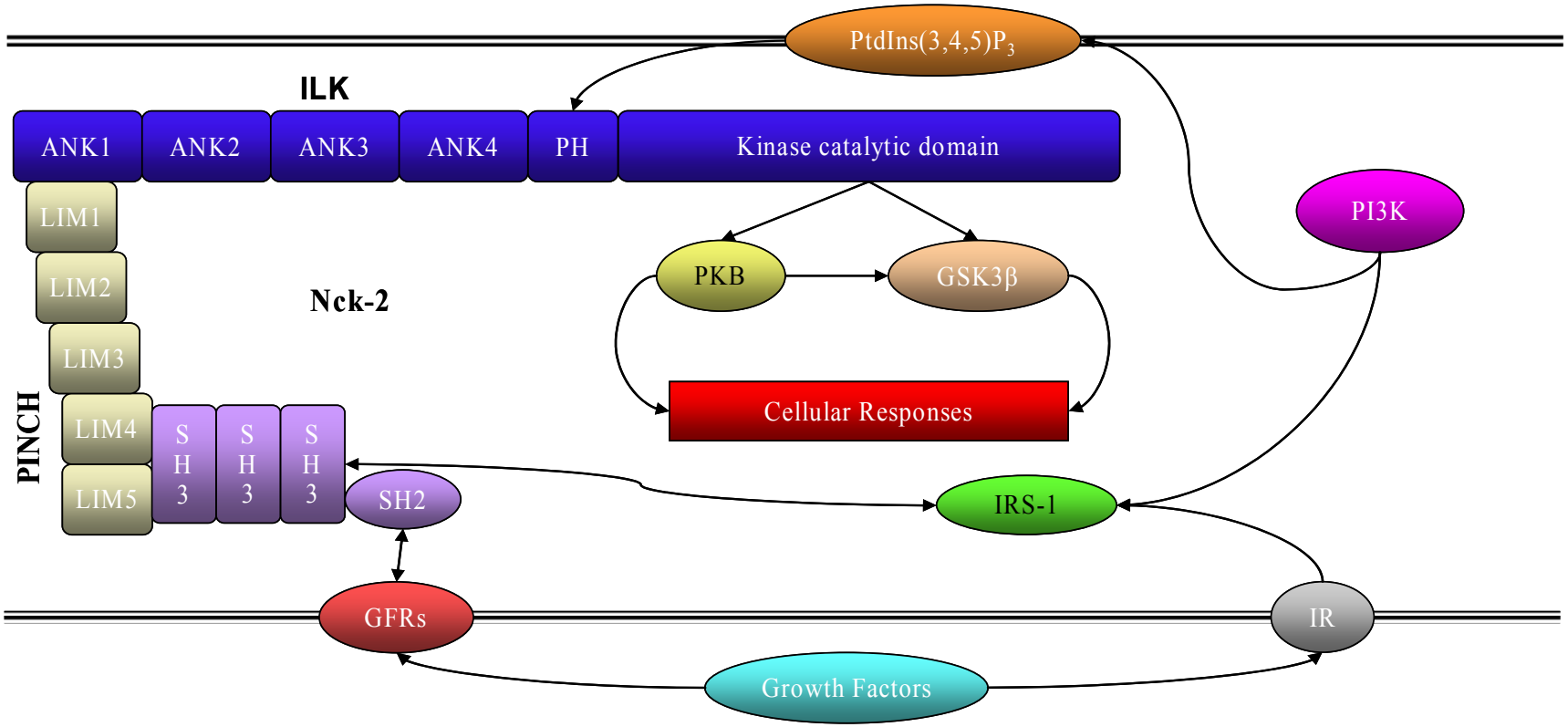


Figure 4: The Link between ILK and Growth Factor Receptor Signalling.

The coupling of ILK to the growth factor receptors or components of growth factor signalling, such as IRS-1 are mediated by the adaptor proteins, PINCH and Nck-2. PINCH mediates interactions with the SH3 domain of Nck-2 that associates with growth factors via its SH2 domain. The ILK-PINCH complex also ensures the correct localisation of ILK at focal adhesion plaques. This is accomplished via the interaction between the LIM1 domain of PINCH and the ANK1 repeat of ILK. The kinase domain of ILK is capable of phosphorylating PKB and GSK3β, which impinges cellular events such as cell survival, differentiation and cell proliferation. GFRs – growth factor receptors, GSK3β - glycogen synthase 3β, ILK – integrin linked kinase, IR – insulin receptor, PINCH – particularly interesting new calponon homology domain containing protein and PI3K – phosphoinositide 3-OH kinase, PKB – protein kinase B.

Oesophageal carcinoma remains largely prevalent amongst the black population in South Africa, predominantly in rural regions of the Transkei and Soweto (Kneebone and Mannell, 1985; Isaacson, 2005). It also shows high incidence in areas such as France, China, Japan, northern Iran, India, Thailand, and South America regions (Kneebone and Mannell, 1985; Launoy *et al*., 1997; Saidi *et al*., 2000; Lehrbach *et al*., 2003; Kazemi-Noureini *et al*., 2004; Zhang *et al*., 2004). The exposure of oesophageal cells to exogenous agents such as food, alcohol, smoke and endogenous causes (e.g. genetic) as well as inflammation of oesophagus tissue, together with racial and cultural habits have been accounted for high incidence in these geographical regions (Kazemi-Noureini *et al*., 2004). Late presentation, together with the propensity of the disease to metastasise (Quint *et al*., 1995) makes surgical resection difficult. The development of oesophageal carcinoma is a multistep, progressive process. An early indicator of this process is an increased proliferation of oesophageal epithelial cells morphologically including basal cell hyperplasia, dysplasia, carcinoma *in situ* and advanced HOSCC (Lehrbach *et al*., 2003).

Patients with oesophageal carcinoma have a very poor survival rate, with contributing factors being the delay in diagnosis, as well as patients being unaware of cancer being the cause of their symptoms (Mufti *et al*., 1997; Rothwell *et al*., 1997), and are intensified by the inaccuracy of clinical staging (Adelstein *et al*., 1997). In South Africa the 50 % mortality period are 3.6 months for males and 4.2 months for females (Walker *et al*., 1984). Over the years the prognosis has not changed significantly and remains as one of the most lethal of all cancers (Gore, 1997; Friess *et al*., 1999; Isaacson, 2005).

1.9 Aims

Since ILK has far-reaching effects in both normal and pathological conditions, and is overexpressed in numerous malignancies (Dedhar *et al*., 1999; Marotta *et al*., 2000) the aims of this investigation were:

i) Firstly, to examine the distribution of ILK in human OSCCs. This would potentially suggest the functional role of ILK.

ii) Secondly, to discern the effects of growth factors on both ILK expression and activity, and to establish the link, if any, between ILK and growth factor signalling pathways in HOSCCs, and finally,

iii) To examine the role of ILK activity in cell-ECM adhesion in moderately differentiated oesophageal squamous cell carcinoma cell lines, with a view to providing insight as to whether ILK is directly involved in integrin mediated processes.

Chapter 2

Identification and Localisation of ILK in Human Oesophageal SCCs

2.1 Introduction

Epithelial cells require that the integrin-mediated interactions of cells are tightly regulated, as it is these processes that are vital in the maintenance of tissue structure and differentiation (Gumbiner, 1996; Zhang *et al*., 2002; Khyrul *et al*., 2004; Quélo *et al*., 2004; Wu, 2004). When considering cell-ECM adhesion, the integrin class of adhesion receptors, which consist of α and β subunits are particularly important. Notably, they are directly linked to the processes of invasion and metastasis of tumours (Stroeken *et al*., 1998; Zutter *et al*., 1998; Takanami *et al*., 2005). Closely associated with the integrin receptors is ILK, which, by being in close proximity to the β integrins, is able to interact with and regulate integrin activity (Zhang *et al*., 2002; Grashoff *et al*., 2004; Khyrul *et al*., 2004; Quélo *et al*., 2004; Chun *et al*., 2005; Takanami *et al*., 2005). As a consequence ILK is thought to directly regulate the processes of cell-ECM adhesion (Dai *et al*., 2003; Pinkse *et al*., 2005; Boulter *et al*., 2006).

The presence of ILK has been identified in numerous normal tissues including kidney, lung, prostate, colon, brain, stomach and liver (Li *et al*., 1999; Janji *et al*., 2000; Persad *et al*., 2000; Kaneko *et al*., 2004; Hannigan *et al*., 2005; Takanami *et al*., 2005). Myocardiac cells and skeletal muscle fibres show highest expression levels of ILK (Chung *et al*., 1998) where it directs processes such as cell survival, cell differentiation, and cell cycle progression (Radeva *et al*., 1997; Delcommenne *et al*., 1998; D'Amico *et al*., 2000; Wu and Dedhar, 2001; Atwell *et al*., 2003; Quélo *et al*., 2004; Wu, 2004). ILK is also thought to play a central role in invasion processes since its overexpression has been reported to increase invasiveness in rat intestinal epithelial cells (Novak *et al*., 1998; Takanami, 2005). When considering ILK expression in tumours, this widespread protein kinase is consistently overexpressed in tumours such as Ewing's sarcoma, primitive neuroectodermal tumour and medullablastoma and is thus considered a marker of tumours with neural differentiation (Chung *et al*., 1998; Li *et al*., 1999; Wu,

1999). On the other hand, small round sarcomas have been found to be negative for ILK expression (Chung *et al*., 1998). Furthermore, ILK overexpression and activity has also been noted in tumours of the breast, prostate, brain and colon (Li *et al*., 1999; Persad *et al*., 2000; Troussard *et al*., 2000; Tan *et al*., 2001; Yoganathan *et al*., 2002; Aoyagi *et al*., 2005).

It is particularly noteworthy that only one previous study has considered ILK expression in oesophagus (Janji *et al*., 2000). Furthermore, since this study was conducted on normal cat oesophagus, it does not demonstrate the functional role of ILK in human oesophageal carcinoma. This, together with the fact that ILK mediates integrin-growth factor signalling events in both normal (Boudreau and Jones, 1999; Wu and Dedhar, 2001) and malignant phenotypes (Chung *et al*., 1998; Yoganathan *et al*., 2000), has prompted this investigation into examining ILK expression in human oesophageal SCC cell lines. The determination of ILK expression will allow further our understanding of the possible role/s played by ILK during invasion and metastasis.

ILK is found in focal adhesion sites (Hannigan *et al*., 1996; Mulrooney *et al*., 2000; Nikolopoulos and Turner, 2001; Chun *et al*., 2005) and fibrillar adhesion (Guo *et al*., 2001) thus placing ILK in a suitable location for the regulation of the β integrin subunits (Coppolino and Dedhar, 2000; Nikolopoulos and Turner, 2002; Quélo *et al*., 2004). A prime example of this role, is the demonstration that ILK overexpression in epithelial cells causes suppression of integrin-mediated adhesion (Radeva *et al*., 1997; Coppolino & Dedhar, 2000; Hannigan *et al*., 2001).

To fully appreciate the intricacies of ILK function in mammalian cells, and particularly in carcinoma of the oesophagus, it must be pointed out that two isoforms of ILK exist, namely, ILK1 and ILK2 (Janji *et al*., 2000). Both isoforms share characteristics such as sequence identity and protein cross-reactivity (Janji *et al*., 2000). However, a major difference between these isoforms is that the ILK1 gene contains a unique *Bam*HI restriction site and the ILK2 gene a unique *Hinc*II restriction site, which are important with regards to identification. Although extremely similar in most respects, ILK2 is not as widely expressed as ILK1, with ILK2 being found in only certain malignancies (Janji *et al*., 2000).

An important facet to ILK signalling that requires further consideration, are growth factor receptors. Growth factor receptors are able to directly impinge upon both the integrin and ILK signalling pathways. The effects of two important growth factors, namely, EGF and TGFβ1, may have important implications when considering ILK function in SCC. EGF, which serves to relay signals from the external environment to the internal cytoplasm (Mayer *et al*., 2000), affects a host of cellular responses, including cell growth, apoptosis, cell cycle progression, and cellular differentiation (Ozanne *et al*., 1986; Rosenfeld *et al*., 1987; Frish and Ruoslahti, 1997; Giancotti, 1997; Yu *et al*., 2000; Dikic *et al*., 2003). This indicates that EGF is a potent effector of key signalling molecules during normal signalling cascades. Moreover, in tumour cells, EGF expression is crucial during the progression of cancer, including metastatic spread and inhibition of apoptosis (Wells, 1999; Andl *et al*., 2003). Increased EGFR levels have also been noted in a host of epithelial malignancies, including breast, bladder, lung and more pertinently cells of squamous origin (Sainsbury *et al*., 1985; Ozanne *et al*., 1986 cited in Yu *et al*., 2000; Wang *et al*., 1998; Andl *et al*., 2003). It has been demonstrated by Mayer *et al*. (2000) that unregulated expression of EGF receptors is one of the early steps during the development of cervical carcinogenesis. This has also been suggested to be the case for oesophageal SCCs (Andl *et al*., 2003).

The significance of TGFβ1 is highlighted by the fact that a loss of response to TGFβ1 is exhibited by numerous malignancies including colon, gastric, endometrial, ovarian, and cervix, which is in term associated with malignant conversion and progression (Akhurst and Derynck, 2001; Teicher, 2001). In addition, the ability of TGFβ1 to act as both a tumour suppressor and as a major stimulator of tumour progression, invasion and metastasis further emphasises the importance of this signalling cytokine in oesophageal carcinoma (Akhurst and Derynck, 2001). Interestingly, however, TGFβ1 is generally regarded as an inhibitor of epithelial cell growth (Gotzmann *et al*., 2001; Hu and Zuckerman, 2001; Kim *et al*., 2004). Carcinoma of the oesophagus particularly, demonstrates reduced TGFβ1 expression, which is correlated with depth of invasion, lymph node metastasis and poor prognosis (Fukai *et al*., 2003).

ILK is thought to act as a mediator between growth factor receptors, such as EGF and TGFβ1, and integrin signalling pathways (Delcommenne *et al*., 1998) (see Chapter 1, Figure 2). It is known that increased numbers of EGF receptors are present on the

membranes of the human oesophageal carcinoma cell lines examined in this study (Veale and Thornley, 1989; Andl *et al*., 2003). Thus it must be borne in mind that when examining ILK expression in oesophageal SCCs, the effects of growth factor receptors may well be a major contributing factor to the way in which ILK transduces cellular signals.

This study examines and compares the expression of ILK in five cell lines derived from moderately differentiated human squamous cell carcinomas of the oesophagus. To achieve this end, it was first necessary to determine if ILK was expressed in the oesophageal SCCs examined and secondly, whether exogenous growth factors effected the expression of ILK in this group of cell lines.

2.2 Methods and Materials

2.2.1 Cell Lines

Five South African moderately differentiated human oesophageal squamous cell carcinoma cell lines WHCO1, WHCO3, WHCO5, WHCO6 (Veale and Thornley, 1989) and SNO (Bey *et al*., 1976) were obtained from the Cell Biology Laboratory, School of Molecular and Cell Biology, University of the Witwatersrand. Medium was removed and cells washed twice with pre-warmed (37 °C) phosphate buffered saline (PBS, Appendix 1.1.1). Cells were harvested using 0.05 % (w/v) trypsin and 0.01 % (w/v) EDTA (Appendix 1.1.2). Cell lines were cultured in 10 cm Nunc™ tissue culture dishes with Dulbecco's Modified Eagles Medium (DMEM)/Hams F12 (3:1), supplemented with 10 % Foetal Calf (or bovine) Serum (FCS or FBS). Cultures were maintained in a humid, 37 °C incubator, 5 % carbon dioxide (CO_2) atmosphere.

Where the experiment required, cell lines were grown to 80 % confluency and treated with 10 ng/ml EGF and 1 ng/ml TGFβ1 for a period of 0.5, 1, 3, 6 and 24 hours respectively. Cells were cultured in DMEM/Hams F12 (3:1), supplemented with 10 % FBS and maintained in a humid, 37 °C incubator with a 5 % CO_2 atmosphere.

2.2.2 Total RNA Extraction

Cells were grown to 80 % confluence in sterile 10cm Nunc™ tissue culture dishes. Cells were washed 3 times with cold (4 °C) sterile PBS. 1 ml of TRIzol® reagent (GibcoBRL) was added to the dish. TRIzol® reagent is able to maintain RNA integrity while disrupting cells and dissolving cell components. All glassware and apparatus that were used during the RNA extractions were sterilised in order to further the preservation of RNA integrity. Cells were scraped and pipetted into fresh eppendorf tubes and centrifuged at 12000 rpm for 10 minutes in a Sorvall® MC 12 V centrifuge at 4 °C. This was followed by incubation for 5 minutes at room temperature. Chloroform (200 µl) was added and the tubes shaken for 15 seconds. Samples were once again incubated for 3 minutes at room temperature, followed by centrifugation at 12000 rpm for 5 minutes and the aqueous phase being transferred to a sterile eppendorf tube.

Isopropyl alcohol (500 µl) was added and the samples incubated for 10 minutes at room temperature. This was followed by centrifugation at 12000 rpm for 10 minutes. The supernatant was decanted and the RNA pellet washed with 1 ml EtOH (75 %). The samples were centrifuged at 7500 rpm in a Sorvall® MC 12 V centrifuge for 5 minutes. After centrifugation, the supernatant was decanted and the RNA pellet resuspended in 40 µl dH_2O and incubated at 60 °C for 10 minutes. Eppendorf tubes containing RNA of interest were stored at –70 °C.

2.2.3 ILK Amplification by Reverse Transcription Polymerase Chain Reaction (RT-PCR)

2.2.3.1 Reverse Transcription

A two-step RT-PCR was carried out – this protocol has been shown to be more efficient in fragment amplification as opposed to the one-step RT-PCR method (Kawasaki and Wang, 1989; Hamaguchi, 1998). The first step, which utilised approximately 2 µg of total RNA, was the process of reverse transcription. To the RNA 2 µl of random hexamer primers (Roche, SA) was added, and incubated at 70 °C for 5 minutes. The reaction mixture was placed on ice for 5 minutes, which was followed by centrifugation at 6000 rpm in a TOMY-desktop centrifuge for 1 minute. Synthesis of cDNA was accomplished using a reverse transcription kit (Promega, USA) with the addition of RT buffer (5 µl), RNAsin (0.6 µl), dNTPs (2 µl), Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (2 µl) and dH_2O (12.4 µl) respectively. The reaction mixture was incubated at 37 °C for 1.5 hours. The reaction was stopped by placing the mixture at 90 °C for 5 minutes, placing on ice for 5 minutes and centrifuging at 6000 rpm in a TOMY-desktop centrifuge for 2 minutes. To remove any bound DNA and protein, 25 µl of a phenol/chloroform (Appendix 1.2.1) mixture was added and centrifuged in a TOMY-desktop centrifuge for 2 minutes. The aqueous phase was removed and transferred to a sterile eppendorf tube. To precipitate cDNA products 100 µl Ethanol (EtOH 100 %) and 3 µl sodium acetate (NaAc, 3 M, pH 5.2) (Appendix 1.2.2) solutions were added and the samples precipitated overnight at –20 °C. The following day, the mixture was centrifuged at 12000 rpm in a Sorvall® MC 12 V

centrifuge for 30 minutes. The supernatant was decanted, and the cDNA pellet resuspended in 10 µl dH_2O. cDNA products were stored at −70 °C.

2.2.3.2 Polymerase Chain Reaction (PCR)

The second step was standard PCR protocol. The ILK primers used were obtained from a previously published ILK cDNA sequence from Janji *et al*. (2000). These primers consisted of an antisense primer (30mer) corresponding to the published ILK nucleotide sequence 1359-1341 (Hannigan *et al*., 1996) with a *Xho*I restriction site (CTCGAG): 5'-CTCGAGCTACTTGTCCTGCATCTTCTCAAG-3'. The upstream sense primer was a 32mer corresponding to the ILK nucleotide sequence 1-21 with an *Eco*RI restriction site (GAATTC) and two additional nucleotides (GT) before the start codon ATG: 5'-GAATTCGTATGGACGACATTTTCACTCAGTGC-3'.

The cDNA was amplified for 30 cycles on a Perkin Elmer GeneAmp PCR System. The cycling program included a 95 °C denaturation step, a 1 minute annealing step at 50 °C and a 2 minute extension step at 72 °C, followed by a final elongation at 72 °C for 10 minutes. RT-PCR products were visualised on a 2 % agarose gel (Appendix 1.5.1) following ethidium bromide (EtBr, 10 µg/ml) staining. Minnie Agarose HE 33 gel units (Hoefer Scientific, USA) were used, which were filled with 1× Tris acetic acid ethylenediamine tetraacetic (EDTA) acid solution (TAE) (Appendix 1.5.3) diluted from a 20× TAE stock solution (Appendix 1.5.2).

2.2.4 Antibodies

ILK was specifically detected with a rabbit polyclonal antibody obtained from Sigma® Laboratories, USA. Polyclonal horseradish peroxidase (HRP)-bound anti-rabbit secondary antibody was used in Western blot experiments (Separations, SA). Monoclonal mouse anti-human β_3 antibody (Chemicon, USA) and Fluoroscine isothiocyanate (FITC)-conjugated anti-rabbit (or anti-mouse for β_3 detection) secondary antibody from Chappel, USA were used in the immunofluorescence experiments.

2.2.5 Triton X-100-based Extraction

A Triton X-100 extraction protocol was performed which has been shown to preserve integrin integrity (Giancotti and Ruoslahti, 1990). This extraction protocol enriches both membrane and cytoplasmic associated proteins. Cell lines were grown to 80 % confluence in 10 cm Nunc™ tissue culture dishes. To isolate ILK, which is found associated with integrins at cell membranes a Triton X-100 extraction was performed on each of the various cell lines. The cultures were washed three times with sterile PBS at 4 °C and scraped from the culture dishes with a rubber policeman in PBS containing phenyl-methyl-sulphonyl fluoride (PMSF)/Aprotinin (Trasylol® Bayer, SA) protease inhibitor stock solution (Appendix 1.3.2). The cell suspension was centrifuged at 6000 rpm in a TOMY-desktop centrifuge for 1 minute. The PBS supernatant was removed and the cell pellet suspended in 400 µl of a Tris-based Triton-X100 (pH 8.5) extraction buffer (Appendix 1.3.3) and incubated for 1 hour on ice. Thereafter, samples were centrifuged at 12000 rpm in a Sorvall® MC 12 V centrifuge for 10 minutes at 4 °C and the supernatants decanted into a sterile eppendorf and stored at −70 °C.

2.2.6 Protein Estimation

Equivalent protein concentrations were determined using the commercial BioRad protein G assay kit II (catalogue number: 500-0002). However, reliable absorbance readings could not be obtained as the Triton X-100 detergent concentration present in the extraction buffer caused interference. It is mentioned in the BioRad protocol manual that a Triton X-100 concentration of 0.1 % is compatible with the BioRad solution. Subsequent dilutions of the buffer however, resulted in precipitation of proteins. Thus, the protein estimation was accomplished utilising the Bradford assay (Bradford, 1976) following the protocol from Bramhall *et al*. (1969). Whatman® glass fibre discs were first rinsed in dH_2O for 20 minutes to remove any loose fibres, followed by 5 minute washes in 95 % alcohol, 100 % alcohol and acetone respectively, to dehydrate the discs, which were subsequently allowed to air dry. A standard curve was constructed using bovine serum albumin (BSA, Appendix 1.3.4). Following air drying, protein samples (1 µl) from each respective cell line was spotted onto the glass fibre discs and again allowed to air dry for 5 minutes. To fix proteins to the glass fibre discs, the discs were placed in 2.5 % trichloroacetic acid (TCA, Appendix 1.3.5), for 30-40 minutes with gentle agitation. Thereafter the discs were immersed in Coomassie blue stain (Appendix

1.3.6) for 5 minutes to rinse off excess TCA. The discs were then stained in Coomassie blue for 1 hour and destained in an acetic acid:methanol:dH_2O (10:12:100) solution for 2 hours, until the background levels were completely removed. Destained dots were excised from the discs and placed in 5 ml of elution buffer (Appendix 1.3.7) in a darkroom overnight. The following day absorbance readings were measured using a Beckman DU®-64 Spectrophotometer (λ=595nm). The concentration of the total protein was calculated from the BSA standard curve ($y=0.0634x-0.01$, $R^2=0.9639$, Appendix 2.1). The graphs were plotted using Microsoft® Excel.

2.2.7 Antibody Binding Assays for Primary and Secondary Antibodies

The functionality of the primary and secondary antibodies was tested using dot blots. To ensure that the primary ILK antibody was specific to ILK protein, the supplied ILK-peptide (1 µl of a 50 mg/ml solution, Zymed® Lab. Inc.), which is a synthetic peptide derived from the C-terminus of human ILK, was spotted onto a piece of nitrocellulose Hybond™-C, and allowed to air dry for 10 minutes. The nitrocellulose was blocked in a casein-based blocking buffer (BLOTTO, Appendix 1.4.1) for 5 minutes. The nitrocellulose membrane was incubated in the polyclonal rabbit anti-ILK primary antibody (1:250) for 2 hours. To ensure binding of the primary ILK antibody to HRP-bound secondary antibody, a second nitrocellulose blot was prepared containing 1 µl of preabsorbed ILK antibody blocked for 5 minutes. Both membranes were washed 6 times with ice cold PBS at 5-minute intervals and incubated with HRP-bound anti-rabbit secondary antibody (1:1500) for 1 hour. The nitrocellulose pieces were once again washed 6 times with PBS at 5-minute intervals. In conjunction with this, a third nitrocellulose blot was prepared that contained preabsorbed HRP-bound secondary antibody (1 µl). This blot was also washed 6× in PBS with 5-minute intervals and immersed in BLOTTO for 5 minutes. This reaction was performed to ensure reactivity of the secondary antibody with the Supersignal® West Pico Chemiluminescent Kit (Pierce, USA). Thereafter, all three blots were reacted with the Supersignal® West Pico Chemiluminescent Working Solution (Appendix 1.4.2) for 5 minutes. The membrane pieces were wrapped in polyethylene ‘saran wrap’. The reaction blots were exposed to hyperfilm™ MP autoradiography film (Amersham, UK) for 1.5 minutes. The film was placed in D19B developer (Appendix 1.4.3) for 5 minutes, washed in water briefly, placed in fixer (Appendix 1.4.4) for 5 minutes and washed once again.

2.2.8 Polypeptide Resolution and Western Blotting

Samples were resolved on 10 % Sodium dodecyl Sulphate (SDS) polyacrylamide gels (Appendix 1.5.4) according to Laemmli, (1970) and, resolved at a constant current of 18 milliamperes (mA) with electrophoresis running buffer pH 8.3 (Appendix 1.5.5), and visualised with Coomassie Blue protein stain (see Appendix 1.3.6). Equivalent protein concentrations (10 μg/ml) were made up to equal volumes with 1× lysis buffer (Appendix 1.5.6), boiled for 5 minutes and centrifuged briefly for 1 minute in a TOMY-desktop centrifuge prior to loading. Samples from cell lines WHCO1, WHCO3, WHCO5, WHCO6 and SNO were resolved on polyacrylamide gels, and transferred to Nitrobind nitrocellulose transfer membrane (MSI, USA), in a BioRad Trans-Blot™ Cell at 400 mA for 3 hours (4 °C) in Western Blot Transfer Buffer (Appendix 1.4.5). After transfer was complete, the nitrocellulose membranes were rinsed twice with PBS (see Appendix 1.1.1), and stored overnight at 4 °C.

Each membrane was blocked in BLOTTO (see Appendix 1.4.1) for 5 minutes to prevent non-specific antibody binding and washed 6 times in PBS. The blots were incubated in a rabbit anti-ILK primary antibody (1:250) for 2 hours. Washing was performed 6 times at 5-minute intervals with PBS to remove any residual antibody. Membranes were incubated with a HRP-bound secondary anti-rabbit antibody (1:1500) for 1 hour in the dark. Once again membranes were washed 6 times at 5-minute intervals with PBS before being exposed to the Supersignal® West Pico Working Solution (see Appendix 1.4.2) from the West Pico Chemiluminescent Substrate Kit (Pierce, USA) for 5 minutes. Blots were sealed in polyethylene ‘saran wrap’ and exposed to hyperfilm™ MP autoradiography film (Amersham, UK) for 1.5 minutes. Film was developed in D19B developer (see Appendix 1.4.3) for 5 minutes, rinsed briefly in H_2O before fixing (see Appendix 1.4.4) for 5 minutes. The experiment was repeated three times.

2.2.9 Purification of ILK PCR Fragments and Restriction Digests

ILK RT-PCR products were purified using a QIAGEN® QIAquick PCR Purification Kit (50) (Catalogue Number 28104, Germany). Buffer PB (5 volumes) was added to the PCR reaction mixture (1 volume) and adding the mixture to a QIAquick column. The

column was centrifuged for 1 minute at 12000 rpm in a Sorvall® MC 12 V centrifuge. The flow-through was discarded and 750 µl of buffer PE added to the column and centrifuged for 1 minute. The flow-through was once again discarded and the sample centrifuged for an additional minute. The column was placed in a sterile eppendorf tube, and 50 µl dH_20 was added. The column was allowed to stand for 1 minute. The column was then centrifuged for 1 minute at 12000 rpm to elute the DNA. DNA was stored at –70 °C.

Restriction digests were performed by firstly adding 10 µl of purified ILK product from each respective cell line into a sterile eppendorf tube. This was followed by the addition of buffer K (1.5 µl), dH_20 (1.5 µl) and *Bam*H1 (2 µl, 0.5 units/µg). Samples were incubated for 1.5 hours at 37 °C. Subsequent digestions were cleared of protein by the addition of 25 µl phenol/chloroform (see Appendix 1.2.1). Samples were centrifuged at 6000 rpm in a TOMY-desktop centrifuge for 2 minutes and the aqueous phase removed and transferred to a sterile eppendorf tube. Chloroform (25 µl) was added and the solution centrifuged once again at 6000 rpm for 2 minutes. The aqueous phase was decanted into a sterile eppendorf tube. Both 0.7 M EtOH (100 µl) and 3 M NaAc pH 5.2 (3 µl) (Appendix 1.2.2) were added to the aqueous phase and the samples allowed to precipitate DNA overnight at –20 °C. The following day the samples were centrifuged at 12000 rpm for 20 minutes in a Sorvall® MC 12 V centrifuge. The supernatant was decanted and the DNA pellet resuspended in 1.5 µl dH_20. Products were visualised by 2 % agarose gel electophoresis (see Appendix 1.5.1) following EtBr (10 µg/ml) staining.

Digests were also performed with the *Hinc*II restriction enzyme. 10 µl of purified ILK product was used in the reaction from WHCO1, WHCO3, WHCO5, WHCO6 and SNO cell lines. Buffer M (1.5 µl) was pipetted into the reaction mixture followed by the addition of dH_2O (1.5 µl) and *Hinc*II (1.5 µl, 0.1 units/µg). Samples were restricted for 1.5 hours. Procedure was then carried out as per the *Bam*H1 digestions above.

2.2.10 Co-immunoprecipitation of ILK and the β_3 Integrin Subunit

Cell lysates were preabsorbed with protein G sepharose beads overnight at 4 °C, and centrifuged at 3000 rpm. The supernatant was transferred to fresh eppendorf tubes and incubated with 10 µl monoclonal mouse anti-human integrin β_3 antibody (Chemicon, USA) overnight at 4 °C. The following day protein G sepharose beads (100 µl) (Amersham, SA) were added to the immune complex for 4 hours. The protein G-ILK1-β_3 complex was centrifuged at 3000 rpm in a Sorvall® MC 12 V centrifuge for 2 minutes. The supernatant was decanted, and 800 µl Tris.HCl buffer pH 8.0 added. Centrifugation was performed once again at 3000 rpm. This washing step was repeated twice more. The supernatant was decanted and an equal volume (10 µl) of double lysis buffer (Appendix 1.6.4) was added to the pellet. The suspension was boiled for 5 minutes and centrifuged for 10 minutes at 12000 rpm in a Sorvall® MC 12 V centrifuge. The product was resolved on an 8 % SDS-PAGE gel (Appendix 1.5.4) and transferred to nitrocellulose for 3 hours as in Methods and Material Section 2.2.8. The blot was probed for ILK and the β_3 integrin subunit using standard antibody concentrations. Blots were then exposed to the Supersignal® West Pico Working Solution (see Appendix 1.4.2) for 5 minutes, sealed in polyethylene 'glad wrap' and then exposed to hyperfilm™ MP autoradiography film (Amersham, UK) for 1.5 minutes. Film was developed in D19B developer (see Appendix 1.4.3) for 5 minutes, rinsed briefly in H_2O, before fixing (see Appendix 1.4.4) for 5 minutes.

2.2.11 Indirect Immunofluorescence Microscopy

The localisation of ILK and the β_3 integrin subunit was performed utilising indirect immunofluorescence microscopy. The WHCO1, WHCO3, WHCO5, WHCO6 and SNO cell lines were grown to 80 % confluency and seeded onto sterile glass coverslips. Coverslips were washed five times in cold PBS, fixed with 4 % paraformaldehyde (Appendix 1.6.1) for 30 minutes and washed again with PBS. Coverslips were immersed in 0.25 % Triton X-100 (Appendix 1.6.2) for 10 minutes to permeabilise the cells, washed twice with PBS, dipped into dH_2O, and allowed to dry partially. This allowed the DAKO® pen (Denmark) to adhere properly to the glass coverslips. Two circles or wells per coverslip were drawn with the DAKO® pen to create a fluid barrier. One well per coverslip was incubated with a polyclonal rabbit anti-ILK primary

antibody (1:250) for one hour or with a monoclonal mouse anti-β_3 primary antibody (1:250). The other well was incubated in PBS (control). This was followed by a thorough washing in PBS as before. All circled areas were incubated with an anti-rabbit Fluorescine Isothiocyanate (FITC)-conjugated anti-rabbit antibody (1:250) for ILK and FITC-conjugated anti-mouse antibody (1:250) for the β_3 integrin. Both wells were incubated for one hour in the dark. The coverslips were washed six times with sterile PBS and then mounted with Elvanol mounting agent (Appendix 1.6.3) onto sterilised glass slides. All incubations were carried out in a humidity chamber to prevent desiccation. Slides were viewed under a Zeiss LSM 410 confocal microscope (FITC excitation 490nm, emission 525nm).

2.2.12 Nuclear Extraction

Nuclear extractions were performed utilising the CelLytic™ NuCLEAR™ Extraction Kit (Sigma, USA). Cells were grown to 80 % confluency and washed twice with PBS and subsequently scraped into PBS. Cells were centrifuged for 5 minutes at 3000 rpm. The supernatant was decanted and the packed cell volume (PCV) was estimated. Thereafter, 500 µl of a hypotonic 1× lysis buffer (5 µl 0.1 M DTT, 5 µl protease inhibitor cocktail) was added to the PCV. The cell pellet was gently resuspended while avoiding foam formation. Packed cells were incubated on ice for 17 minutes, which allowed the cells to swell. A 10 % Igepal CA-630 solution was added to a final concentration of 0.6 % (i.e. 30 µl per 500 µl hypotonic lysis buffer). The cells were subsequently vortexed vigorously for 10 seconds and immediately centrifuged for 30 seconds at 12000 rpm. The supernatants, which constituted the cytoplasmic fractions, were transferred to fresh eppendorf tubes. To 98 µl of the extraction buffer, 1 µl 0.1 M DTT solution and 1 µl protease inhibitor cocktail were added. The crude nuclei were resuspended in 80 µl of the above extraction buffer. Eppendorf tubes containing the crude nuclei were incubated on ice and placed on a shaker for 30 minutes. Thereafter, the subsequent nuclear extracts were centrifuged for 5 minutes at 30000 rpm. Supernatants were transferred to fresh chilled eppendorf tubes, snap-frozen and stored at −70 °C.

2.2.13 Densitometry

Labworks ™ Image Acquisition and Analysis software (Labworks version 4.5) was used for densitometric analysis to quantitatively determine the concentration level of ILK in the Western blots. **Note:** the densitometric analysis results are an average of 3 separate repeated experiments.

2.2.14 Image Capturing

Images were captured on a Hewlard Packard Scanjet 4400c series scanner. Magnification, contrast and brightness of all images were standardised using Adobe Photoshop Version 7.

2.3 Results

2.3.1 Human Oesophageal SCCs Express ILK

Transcription of the ILK gene in the oesophageal SCC cell lines examined was elucidated by firstly extracting total RNA from the five oesophageal carcinoma cell lines. Since the TRIzol® reagent allows for rapid and efficient extraction of total RNA, intact RNA was successfully obtained for all cell lines (Figure 5a, lanes 2 and 3, blue arrow, WHCO1 and WHCO5 total RNA characteristic of all cell lines). Reverse transcription followed RNA extraction, which yielded representative cDNA across all cell lines (For precise determination of ILK mRNA expression levels real time PCR would have been necessary). The subsequent PCR analysis produced a strong ILK fragment of approximately 1360 bp for the WHCO1, WHCO3, WHCO5, WHCO6 and the SNO cell lines as indicated by the red arrow in Figure 5b, which corresponds to the size of ILK published by Janji *et al*. (2000). The β-catenin transcript was included in the experiment as a positive control and is marked in Figure 5b by the yellow arrow in lane 4. The identification of ILK mRNA provides novel evidence of active transcription processes of the ilk gene in human carcinoma of the oesophagus (Figure 5c, green arrow).

The identification of ILK mRNA in these cell lines lead to the next logical question of whether ILK mRNA was being translated to ILK protein. To answer this question the Triton X-100 protein fractions from both the pellet and the supernatant were resolved on SDS-PAGE. The polypeptide profiles from the protein fractions supernatant samples only showed a band at 59 kDa, which is corroborated by reports from Hannigan *et al*. (1996), Li *et al*. (1999), and Friedrich *et al*. (2002) (Figure 6a, lanes 3-7, blue arrow). In contrast, the pellet fractions demonstrated a banding pattern of proteins from approximately 45 kDa and lower (Figure 6b, lane 1, black arrow). The pellets comprise cytoskeletal constituents of the cell, which are insoluble in the Triton X-100 extraction buffer used. Since Triton X-100 is a non-ionic detergent, it is able to readily solubilise the hydrophilic lipid bi-layer present in cell membranes, but is unable to solubilise filamentous actin and intermediate filaments, which contain hydrophobic residues.

Before proceeding with Western blot analysis, dot blot techniques were performed to confirm antibody reactivity and binding and to ascertain optimal antibody concentrations for Western analysis. A synthetic ILK peptide (Zymed® Lab. Inc.), derived from the C-terminus of human ILK, was preabsorbed onto nitrocellulose. This peptide demonstrated strong reactivity, following incubation in the ILK antibody and HRP-bound secondary antibodies, with the Supersignal® West Pico Chemiluminescent substrate (Figure 6c). This dot blot was included as a positive control for the specificity of the rabbit anti-ILK primary antibody. The two other dot blots demonstrated that the rabbit anti-ILK primary antibody (1:250) was able to bind the HRP-bound anti-rabbit secondary antibody (Figure 6d) efficiently and that the HRP-bound secondary antibody (1:1500) was reacting with the Supersignal® West Pico Chemiluminescent substrate (Figure 6e).

Subsequent Western blotting analysis revealed the presence of an ILK band, specifically recognised by anti-ILK antibody at an apparent molecular weight of 59 kDa. ILK expression was confirmed in the supernatant fraction for all cell lines (Figure 7a, indicated by red arrow). Densitometric analysis further revealed that for standardised loading concentrations of cytoplasmic extracts, ILK protein expression levels were relatively uniform across the WHCO3, WHCO5 and SNO cell lines (per μg of total cellular protein extracted). However, the WHCO1 cell line, which displayed highest ILK expression, had ILK levels approximately 1.85 fold higher in comparison to WHCO6 cells and approximately 1.30 fold higher than the WHCO3, WHCO5 and SNO cell lines. On the other hand, WHCO6 cells showed lowest expression levels of ILK approximately 0.68 fold lower than the WHCO3, WHCO5 and SNO cell lines (Figure 7b). These experiments are representative of three separate repeats.

With this in mind, the trend suggested that ILK protein expression levels in the WHCO3, WHCO5, and SNO cell lines are not appreciably different from each other, whereas the noticeable difference in ILK expression in WHCO1 and WHCO6 in comparison to the other cell lines may well be significant. Note, due to the multi-repeats, which is essentially pseudoreplication this experiment did not lend itself to typical statistical analysis leading to a test of true significance.

As mentioned in the introduction to this chapter, two isoforms of ILK (ILK1 and ILK2) are known to exist and although they share similar characteristics with regards to structure and function, these ILK isoforms are predominantly expressed in certain tissues (Janji *et al*, 2000). Indeed, whereas ILK1 has been found in the majority of tissue types, ILK2 is only expressed in two highly metastatic melanoma and fibrosarcoma tumour cell lines and, has not been identified in normal adult tissues (Janji *et al*., 2000). In light of this, identifying isoform expression in carcinoma of the oesophagus was of interest. Restriction fragment analysis utilising the *Bam*H1 and *Hinc*II restriction enzymes showed ILK to be digested by *Bam*H1 producing two fragments of approximately 823 (Figure 8a, red arrow) and 536 bp (Figure 8a, green arrow) respectively. Digestion of the ILK fragment did not occur with the *Hinc*II restriction enzyme, where a single ILK band of 1359 bp was observed (Figure 8b, lanes 2, 4, 6, 8, and 10, yellow arrow) confirming that these oesophageal SCC cell lines only express the ILK1 protein isoform.

To summarise the data obtained thus far, this constitutes a clear demonstration that the transcriptional and translational activities of the ILK gene are intact in all cell lines tested. Furthermore, it has been shown that the ILK1 isoform is expressed in oesophageal SCCs, which although not surprising, supports the notion of ILK1 as being widely expressed in mammalian tissues. Since this study does not examine the ILK2 isoform, the ILK1 isoform will be referred to as ILK during the remainder of this report.

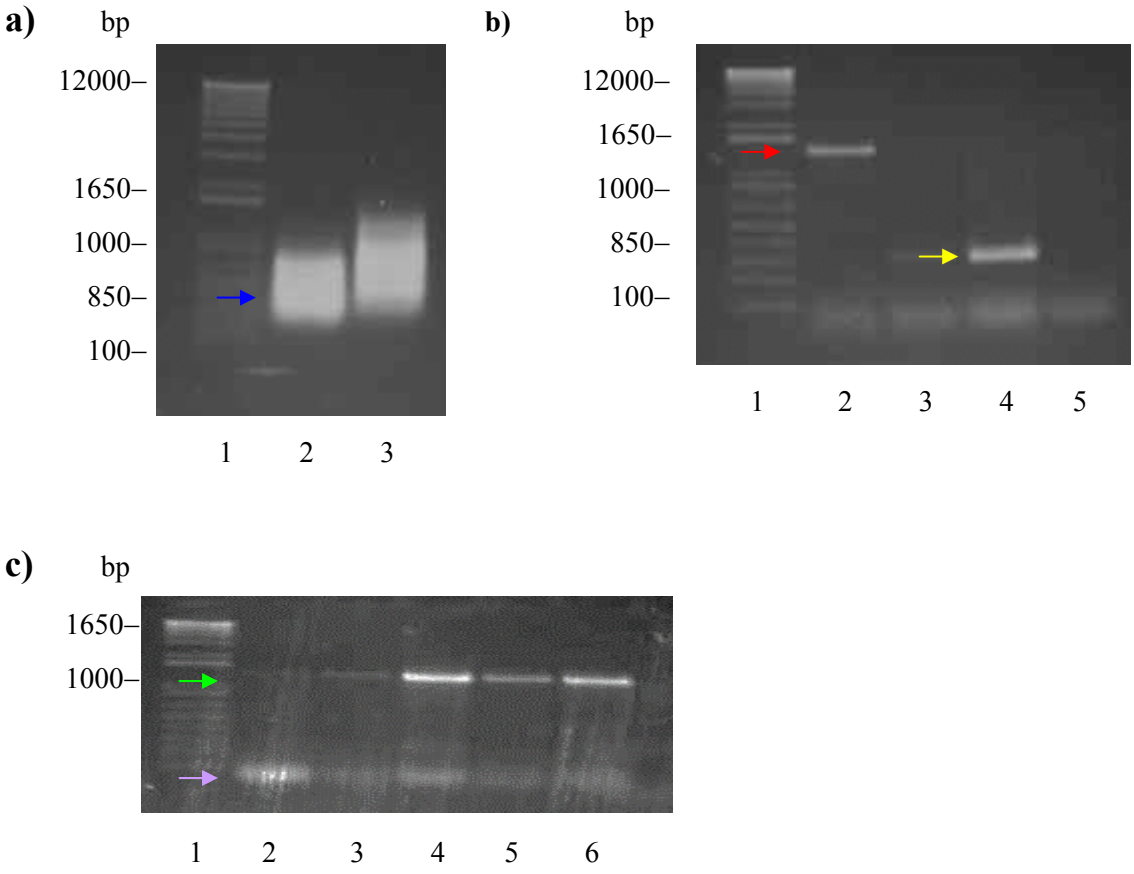


Figure 5: RT-PCR Analysis of ILK Expression in HOSSCs Cultured *In Vitro*. a) WHCO1 and WHCO5 total RNA characteristic of all cell lines extracted with TRIzol® reagent. Lane 1 = molecular weight marker (1 kb), lane 2 = WHCO1 total RNA, lane 3 = WHCO5 total RNA (blue arrow). b) ILK PCR product at a molecular weight of approximately 1360 bp. Lane 1 = molecular weight marker (1 kb), lane 2 = ILK PCR product (lane 2, red arrow), lane 3 and 4 = β-catenin positive control (yellow arrow), lane 5 = Absence of ILK fragment, negative control. c) Full ILK transcript of the WHCO1, WHCO3, WHCO5, WHCO6 and SNO cell lines. Lane 1 = 1 kb marker, lanes 2-6 = 1360 bp ILK PCR fragment (green arrow). ILK total RNA and RT-PCR products were resolved on 2 % agarose gels following EtBr staining (10 µg/ml). Note: bands indicated by purple arrow are primer dimer.

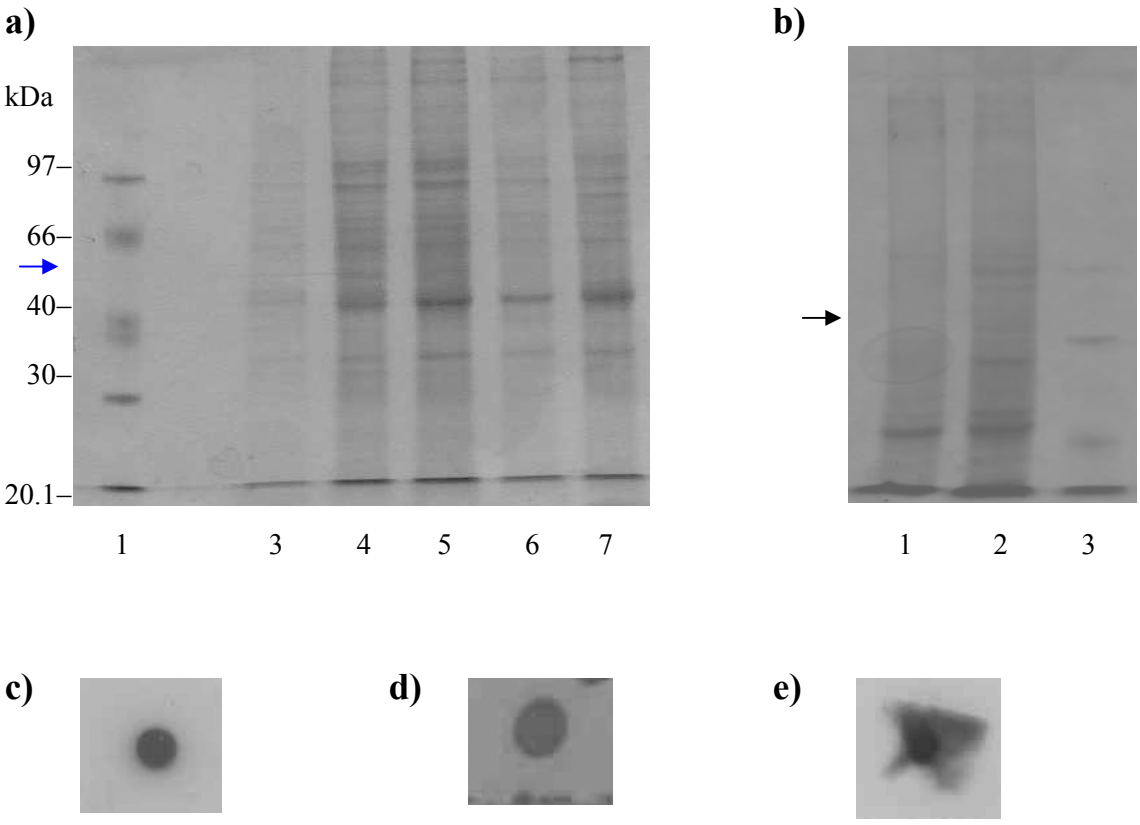


Figure 6: SDS-PAGE Resolution of Triton X-100-Based Extracts.

a) Triton X-100-based extracts of all human oesophageal SCC cell lines resolved on 10 % SDS-PAGE. Lane 1 = molecular weight protein marker, lanes 3-7 = protein profiles of supernatant lysates. The ILK protein is approximately located in the area marked by the blue arrow. It was further revealed that ILK was a component of the supernatant fraction (b - indicated by the black arrow, lane 2). Lane 1 = pellet fraction separated by SDS-PAGE, lane 2 = supernatant separated by SDS-PAGE, lane 3 = low molecular weight marker. Dot blots revealed that the rabbit anti-ILK primary antibody (1:250) was specific for ILK (c) and able to bind rabbit HRP-bound secondary antibody (d). Furthermore, the rabbit HRP-bound secondary antibody (1:1500) was shown to react with the Supersignal® West Pico chemiluminescent substrate (e). Note: due to the sensitivity of the chemiluminescent substrate a very bright reaction occurred with the HRP-bound secondary antibody, which accounts for the scatter in (e). Note: the expression levels are representative as a percentage of maximum per 10 μg of protein from concentrated fractions (included to normalise for loading variation).

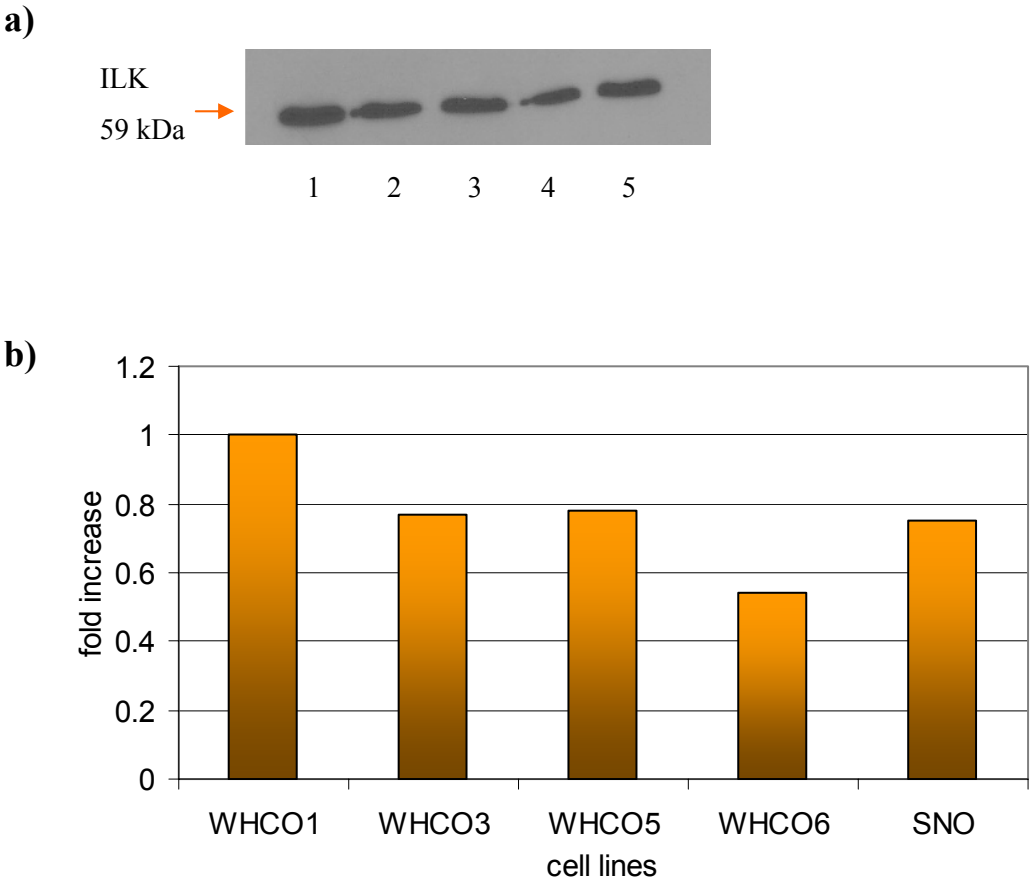


Figure 7: Western Blot and Densitometric Analysis of ILK Expression Levels.
a) Western blots utilising a rabbit anti-ILK antibody (1:1500) performed on the supernatant extracts (10 µg) revealed ILK expression at approximately 59 kDa across all cell lines (indicated by orange arrow). Lane 1 – WHCO1, lane 2 – WHCO3, lane 3 – WHCO5, lane 4 – WHCO6 and lane 5 – SNO. Note: Western blotting procedures were only performed on the supernatant extracts, which were composed of soluble components of the cell, whereas the pellets consisted of insoluble cytoskeletal elements. b) Densitometric analysis revealed a relative uniform expression of ILK between the WHCO3, WHCO5 and SNO cell lines whereas the WHCO1 and WHCO6 cell lines showed dissimilar protein expression under standard culture conditions. The WHCO1 cell line demonstrated the highest expression of ILK – 1.85 fold higher than ILK expression in the WHCO6 (lowest ILK expression) cell line and 1.3 fold higher than the WHCO3, WHCO5 and SNO cell lines. Note: the expression levels are representative as a percentage of maximum per 10 µg of protein from concentrated fractions (included to normalise for loading variation). Experiments were repeated three times.

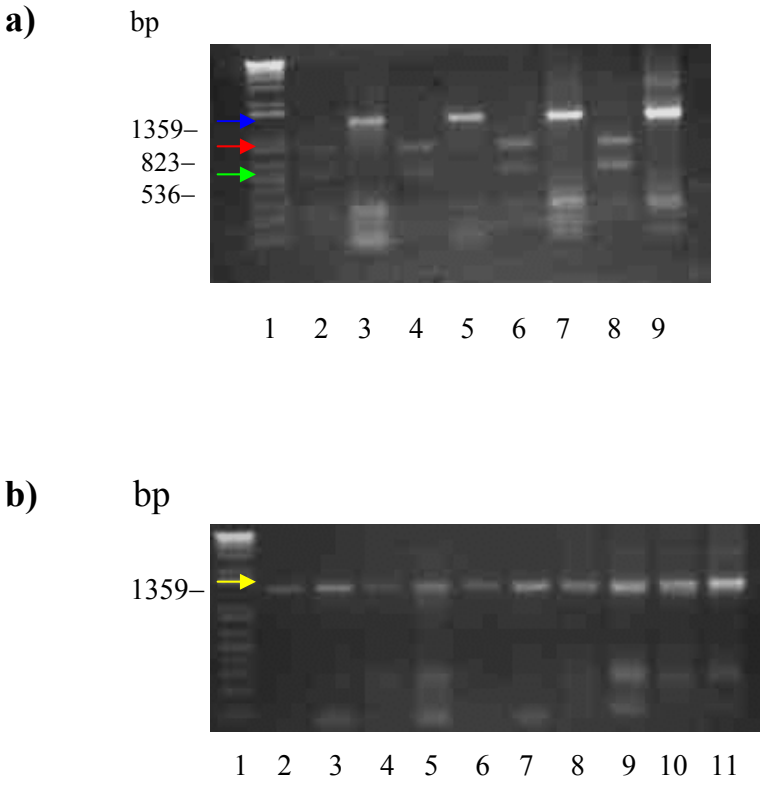


Figure 8: Restriction Fragment Analysis of the Full ILK Transcript.

a) Restriction digests using *Bam*H1 (0.5 units/µg) producing two bands of 823 (red arrow) and 536 bp (green arrow) respectively. Lane 1 – 1 kb molecular weight marker, lanes 2, 4, 6, and 8 – *Bam*HI digestions for WHCO1, WHCO3, WHCO5, and WHCO6 respectively. Lanes 3, 5, 7 and 9 – ILK cDNA (blue arrow). b) *Hinc*II (0.1 units/µg) digestion shows one unrestricted band of 1359 bp (yellow arrow). Lane 1 – 1 kb molecular weight marker, lanes 2, 4, 6, 8 and 10 – ILK DNA following *Hinc*II restriction enzyme addition for WHCO1, WHCO3, WHCO5, WHCO6, and SNO cell lines respectively, lanes 3, 6, 9 and 11 – ILK product with no addition of restriction enzymes for the respective cell lines. Restricted ILK fragments were resolved on 2 % agarose gels following EtBr staining (10 µg/ml).

2.3.2 ILK and β_3 Integrin Association

Co-localisation between the β_1 and β_3 integrin subunits and ILK has been shown previously in platelets, kidney, mammary epithelial cells and prostate carcinoma cell lines (Hannigan *et al*., 1996; Dedhar *et al*., 1999; Janji *et al*., 2000; Zervas *et al*., 2001; Yamaji *et al*., 2002) but has not been reported in carcinoma of the oesophagus. Thus, the possibility of a similar interaction between ILK and the β_3 integrin subunit was sought in oesophageal carcinoma cell lines. To ascertain if the association was occurring between the β_3 integrins and ILK would give an indication of ILK integrin regulatory functions in oesophageal SCCs. Co-immunoprecipitation analysis was performed in order to confirm this interaction. Indeed, ILK and the β_3 integrin subunit were shown to co-immunoprecipitate in all cell lines tested, indicating an ILK and β_3 integrin subunit association. A band of approximately 88 kDa for the β_3 subunit and a band of 59 kDa for ILK were obtained following protein G sepharose immunoprecipitation and Western blotting (Figure 9, β_3–purple arrow, ILK–orange arrow). As a reminder to the reader that the extract that was used for the co-immunoprecipitation analysis was preabsorbed with protein G and therefore there cannot be non-specific protein G associated products (see Methods and Meterials).

2.3.3 Cellular Localisation of ILK and Integrin β_3

The majority of cells and tissues including intestinal, spleen, kidney and thymus cells show ILK to be predominantly located at focal adhesion sites (Hannigan *et al*., 1996; Li *et al*., 1999; Nikolopoulos and Turner, 2001; Zhang *et al*., 2002). At this position it associates principally with the β_1 and β_3 integrin subunits (Dedhar *et al*., 1999; Friedrich *et al*., 2002). This pattern of ILK localisation is consistent for a role of ILK in regulating cell-ECM adhesion. Since ILK localisation has not yet been determined in oesophageal carcinoma the questions that needed to be asked were: What ILK pattern of distribution exists in oesophageal SCCs, and is there a relationship between ILK and β_3 integrin subunit localisation in these cell lines?

ILK and β_3 integrin subunit visualisation was accomplished for each of the oesophageal SCC cell lines. The distribution of ILK is at a cytoplasmic and nuclear level (Figure

10a-e, red arrows) in oesophageal SCC cell lines with a smaller proportion being distributed at the membrane (Figure 10a-e, yellow arrows). The distribution of the β_3 integrin subunit was predominantly at the membrane. To define what was considered as a membrane distribution as opposed to a cytoplasmic distribution, the following criteria were used. A distinct intercellular distribution was considered as membrane associated while distribution across the cell with a more diffuse staining was considered as cytoplasmic associated. These data support the notion obtained by co-immunoprecipitation that ILK and β_3 integrin are indeed associated in HOSCCs. The two corresponding controls where firstly, primary ILK antibody was excluded from the experiment, and secondly, ILK was preabsorbed with the ILK peptide, which both indicated negligible staining, signifying that non-specific binding is extremely low (Figure 10f).

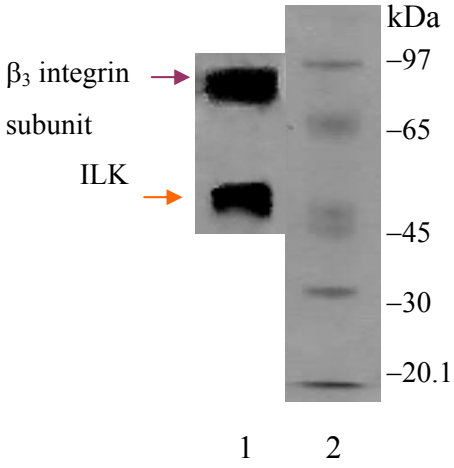
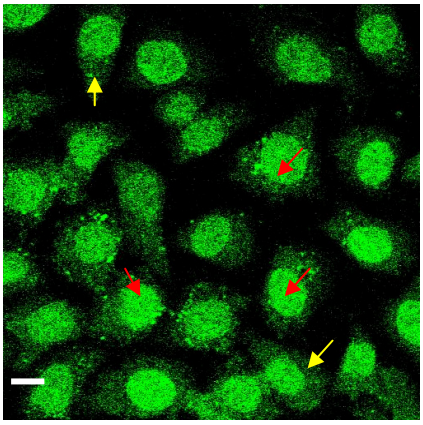
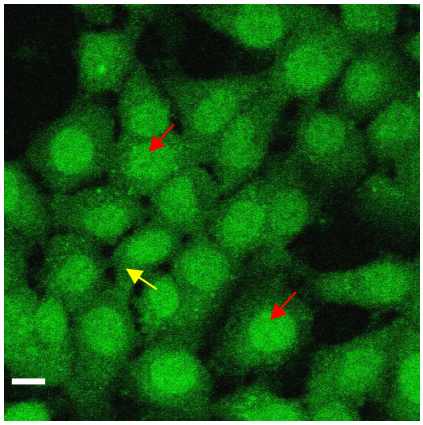


Figure 9: Co-immunoprecipitation Analysis Between β_3 Integrin and ILK.
Protein G sepharose co-immunoprecipitation between ILK and the β_3 integrin in the SNO cell line, utilising a mouse anti-β_3 antibody (10 µl) and Western blotting with mouse anti-β_3 antibody (1:1500) and rabbit anti-ILK antibody (1:250), which was characteristic of all cell lines. Lane 1 – a band of 88 kDa for the β_3 integrin subunit (purple arrow) and a 59 kDa ILK band (orange arrow) were obtained in all five cell lines, lane 2 – molecular weight marker. Note: Immunoprecipitation was also accomplished utilising *Staphylococcus aureus* cells. However, protein G sepharose is the active constituent of *S.aureus* cells and shows increased specificity towards antibodies. Furthermore, protein G sepharose beads are now the commercially accepted alternative for co-immunoprecipitation analysis and were thus the preferred choice.

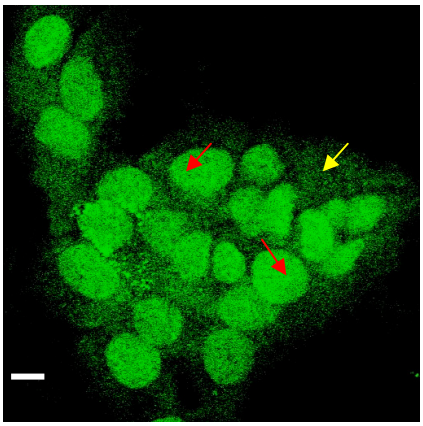
a) WHCO1



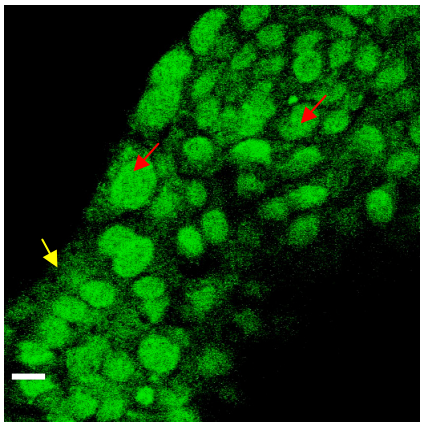
b) WHCO3



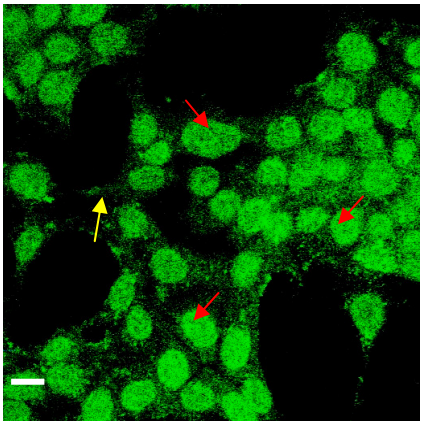
c) WHCO5



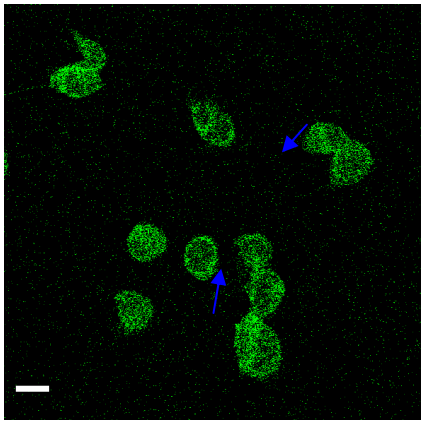
d) WHCO6



e) SNO



f) WHCO1



g) Control

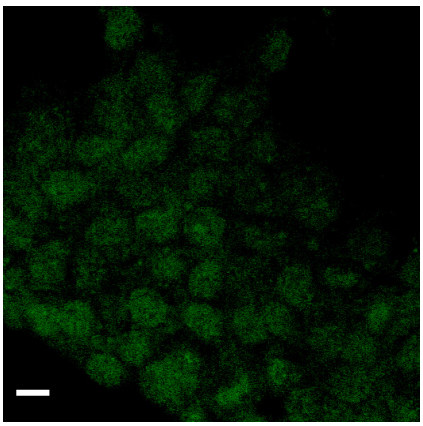


Figure 10: Indirect Immunofluorescence of ILK in HOSCC Lines Cultured *In Vitro*.

a-e) Immunofluorescence microscopy utilising a polyclonal rabbit anti-ILK antibody (1:250) or a monoclonal mouse anti-β_3 antibody (1:250) and an rabbit anti-FITC-conjugated anti-rabbit antibody (1:250) for ILK and a FITC-conjugated anti-mouse antibody (1:250) for the β_3 integrin. ILK expressed by HOSCCs showing variable amounts of ILK distribution with majority of ILK being located at a nuclear/cytoplasmic region (red arrows). A smaller proportion of ILK was also localised at the plasma membrane as indicated by the yellow arrows f) Predominant β_3 integrin subunit localisation occurring at the plasma membrane (blue arrows) in the WHCO1 cell line, which was characteristic of all cell lines examined g) Negative control (excluding FITC-conjugated antibody) demonstrating negligible diffuse staining. Bar represents 10 μm.

2.3.4 Nuclear ILK Protein Levels

To ensure that the nuclear localisation of ILK demonstrated was a true reflection of ILK distribution, we performed Western blotting analysis of nuclear extracts. Indeed, ILK was expressed at 59 kDa in nuclear fractions for the five cell lines (Figure 11a). Although it appeared from the confocal microscopy analysis that ILK was localised primarily in the nuclear/cytoplasmic region, Western analysis revealed that ILK levels in the membrane/cytoplasmic region were considerably higher than nuclear ILK protein levels per μg of total protein (densitometry). Furthermore, within the nuclear extracts, ILK levels were similar across the WHCO1, WHCO3, WHCO5 and WHCO6 cell lines. In contrast, the SNO cell line demonstrated a marked decrease in ILK expression, on average 3 fold lower by comparison to the other four cell lines (Figure 11b).

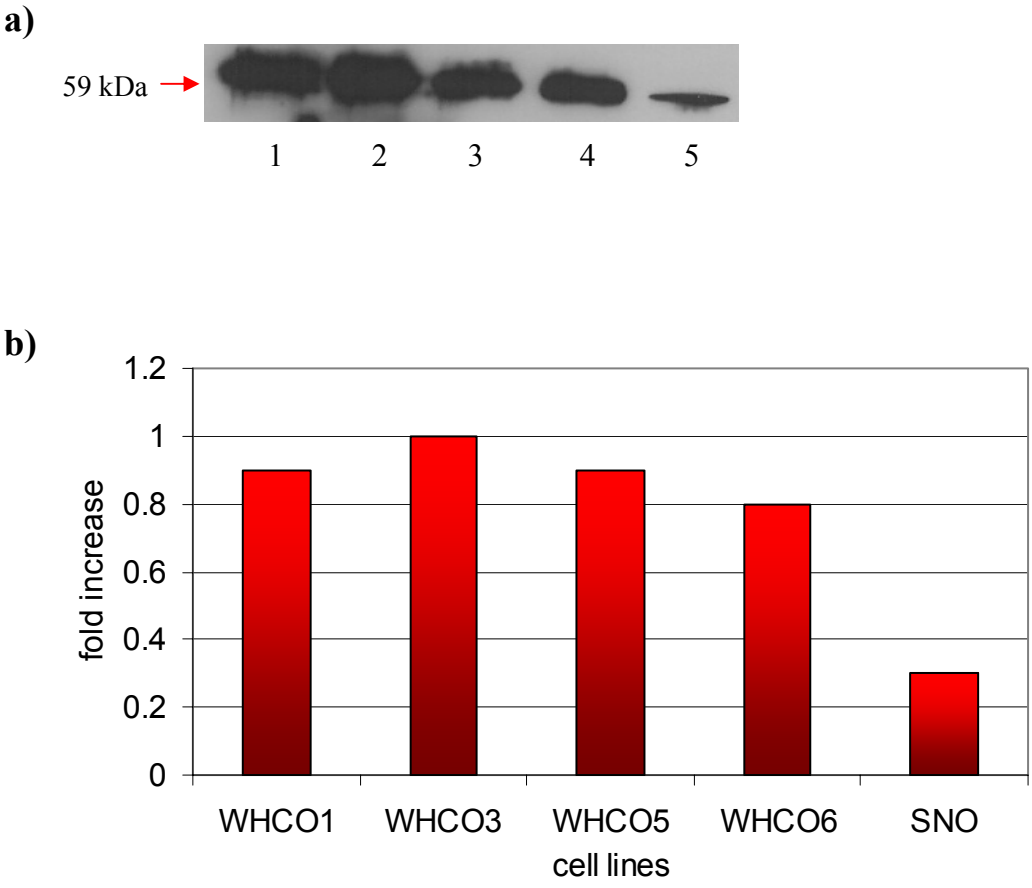


Figure 11: Nuclear ILK Protein Levels.

a) Western blot analysis utilising a rabbit anti-ILK antibody (1:1500) revealed that ILK is expressed in nuclear extracts (10 µg) at a molecular weight of 59 kDa. Lane 1 – WHCO1, lane 2 – WHCO3, lane 3 – WHCO5, lane 4 – WHCO6 and lane 5 – SNO. b) Nuclear ILK concentration levels as analysed by densitometry. Expression levels were relatively uniform across the WHCO1, WHCO5, and WHCO6 cell lines. Lowest nuclear ILK concentration was observed in the SNO cell line, on average 3 fold lower than the WHCO1, WHCO3, WHCO5 and WHCO6 cell lines. Note: the expression levels are representative as a percentage of maximum per 10 µg of protein from concentrated fractions (included to normalise for loading variation). Experiments were repeated three times.

2.3.5 ILK Expression is Stimulated by EGF and TGFβ1 Growth Factors

2.3.5.1 Effect of EGF on ILK Expression

Overexpression of the EGFR is a common occurrence in squamous tumours and has been demonstrated in malignancies of the neck, lung, cervix, skin (Ozanne *et al*., 1986) and more pertinently, squamous carcinoma of the oesophagus (Ozanne *et al*., 1986; Veale and Thornley, 1989; Andl *et al*., 2003). With the knowledge that a link exists between the growth factor receptor activation and integrin receptors (Giancotti and Ruoslahti, 1999), and due to increased EGFR expression, specifically in theses SCCs, EGFR was an important molecule to consider when elucidating the role of ILK in HOSCCs. Thus it became necessary to explore EGF effects on ILK expression in these human oesophageal squamous carcinoma cell lines.

To clarify the response of ILK expression to EGF, cells were exposed to EGF (10 ng/ml) for a period of 0.5, 1, 3, 6 and 24 hours respectively (Figure 12a). It must be remembered that equivalent protein concentrations (10 μg) were loaded onto SDS-PAGE gels prior to Western blotting. The data obtained, following densitometry, is indicative of a vast increase in the expression levels of ILK in oesophageal SCC cell lines following treatment with EGF. In interpreting the growth factor data, we considered a 1.2 fold increase (equivalent to 20 %), to be an appreciable increase in ILK expression levels. All subsequent fold increases are compared to an untreated control, unless otherwise stipulated.

Exposure to EGF generally produced an increase in ILK expression in the five oesophageal carcinoma cell lines, although there was some variation in the increases observed. Similar ILK expression levels were observed in the WHCO1, WHCO5 and SNO cell lines, whereas the levels in the WHCO3 and WHCO6 cell lines each responded somewhat differently to EGF treatment (Figure 12b).

In the WHCO1 cell line, highest ILK expression was noted after 1 hour EGF exposure where a increase of 1.8 fold was noted. These ILK levels were maintained through to 24 hours where a 1.3 fold increase was observed. Similarly, the WHCO3 and WHCO5 cell lines produced similar trends in ILK expression following time dependent exposure to

EGF. In the WHCO3 cell line, an increase in ILK expression was noted after 0.5 hours of EGF exposure reaching a 3.3 fold increase by 3 hours (Figure 12b). Thereafter, ILK expression levels continued to increase, reaching a substantial increase of 4.5 fold after 24 hours EGF treatment. Similarly, in the WHCO5 and WHCO6 cell lines, a large increase was observed following 0.5 hours EGF treatment of 3 and 2.7 fold respectively. Increased ILK expression levels remained constant and highest ILK expression was observed following 24 hours EGF exposure of 3.4 and 2.9 fold respectively (Figure 12b).

Interestingly, in the WHCO3 and WHCO5 cell lines, EGF treatment caused ILK expression levels to fluctuate depending on the exposure period. This was apparent specifically at the 1 and 6 hour EGF exposures for both cell lines. In the WHCO5 cell line, ILK expression levels were considerably decreased in comparison to the 0.5 hour, 1.3 fold higher than 1 hour exposure and 3 hours, 1.6 fold higher than 6 hour exposure treatment periods. A similar observation was made in the WHCO3 cell line, although the fluctuations were not as substantial. At the 0.5 hour time point, ILK levels were 1.1 fold higher than the 1 hour exposure. A comparable fold increase was noted at the 3 hour exposure period in comparison to the 6 hour EGF exposure.

The WHCO6 cell line was also somewhat outstanding in the sense that ILK expression levels tended to fluctuate in the presence of EGF. ILK expression increased at 0.5 hours EGF treatment (1.4 fold), decreased at 1 hour (0.6 fold), but increased again at 6 hours (Figure 12b). It is to be noted that these data are the average of three separate experiments.

In the WHCO6 cell line, ILK expression increased by 1.5 fold following EGF exposure for 0.5 hours. Thereafter, the results were somewhat unusual in that a large decrease in ILK expression of 1.9 fold occurred at 1 hour in comparison to the 0.5 hour treatment period. The more expected trend of increased ILK expression following EGF treatment re-emerged at 3 and 6 hours with an increase of 1.3 fold. By 24 hours an increase of 2.9 fold had been achieved (Figure 12b)

The SNO cell line was somewhat outstanding in that this cell line elicited greatest ILK expression of 2.2 fold following 0.5 hours EGF exposure. Thereafter, a less substantial

increase was noted at 1 hour of 1.5 fold, which was maintained through to 24 hours (Figure 12b).

2.3.5.2 The Effect of TGFβ1 on ILK Expression

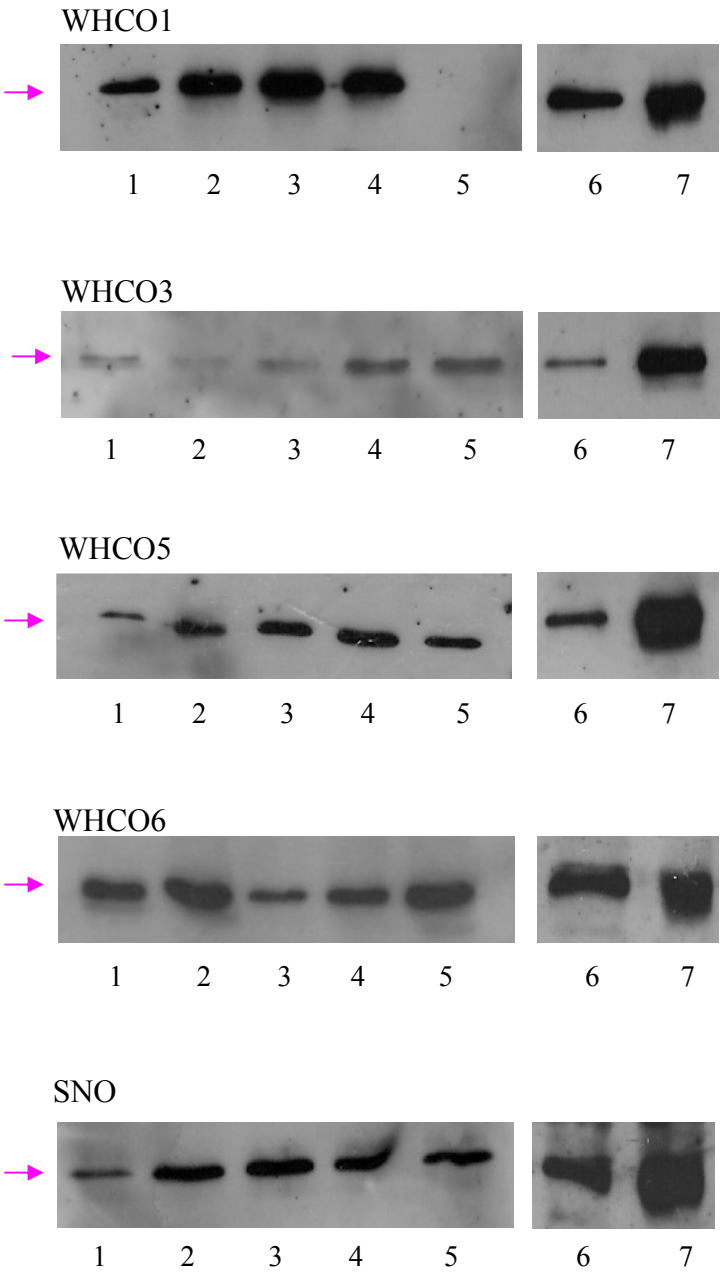
The second growth factor considered in this study was TGFβ1, which has been shown to play both an inhibitory/stimulatory role in tumour progression (Akhurst and Derynck, 2001; Vogelmann *et al*., 2005). Treatment with TGFβ1 produced an overall an increase in ILK expression (immunoblotting) not unlike the effect of EGF (Figure 13a). However variations were noted in the WHCO1 and SNO cell lines where fluctuations in ILK expression were observed (Figure 13a). After 0.5 hours ILK expression had increased by 2.5 fold for both cell lines. By 1 hour ILK expression decreased, but reached an increase of 1.5 (WHCO1) and 2 (SNO) fold by 3 hours. Although a reduction was observed at 6 hours in comparison to the 3 hour exposure period, ILK expression levels were still comparatively higher than the untreated control. By 24 hours ILK expression levels had diminished and were 1.1 and 1.9 fold higher in the WHCO1 and SNO cell lines respectively (Figure 13b).

The WHCO3, WHCO5 and WHCO6 cell lines demonstrated a similar trend in increasing ILK expression in response to TGFβ1 treatment. The WHCO3 cell line produced a substantial increase in ILK expression after exposure to TGFβ1 for 0.5 hours of 4.3 fold. Treatment for 1 and 3 hours produced an even greater increase in ILK expression where a 7 fold increase was reached and maintained. Surprisingly, exposure to TGFβ1 for 6 hours did not produce a higher increase in ILK expression, even though ILK expression was still 5.6 fold higher than the untreated control. Although treatment for 24 hours elevated ILK levels they were comparatively lower than that seen over the 0.5 – 6 hour exposure time course. With this said however, expression levels of ILK were still 4.4 fold higher than the untreated control (Figure 13b).

Exposure to TGFβ1 for 0.5 hours in the WHCO5 and WHCO6 cell lines caused an increase in ILK expression of 2.5 and 2.4 fold respectively. TGFβ1 treatment for longer periods of time resulted in a further increase in ILK expression with increases of 2.8 and 3.9 fold for the WHCO5 and WHCO6 cell lines after 3 hours TGFβ1 exposure. ILK expression reached peak levels at 6 hours of 5 fold for both cell lines. ILK expression

levels subsequently decreased following 24 hours TGFβ1 exposure as ILK levels were 2.9 and 1.9 fold higher than the untreated control for the WHCO5 and WHCO6 cell lines respectively (Figure 13b). All data presented here are an average of three separate experiments.

a)



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b)

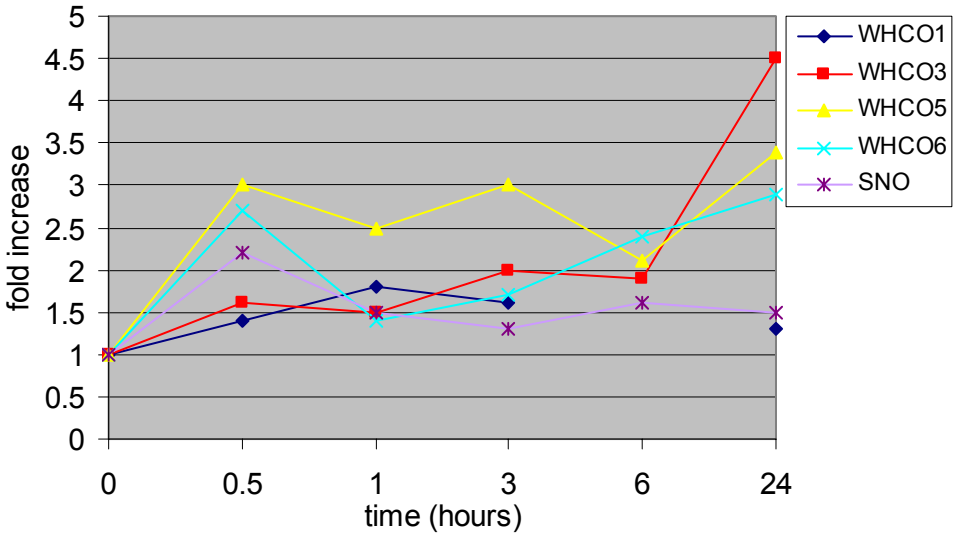
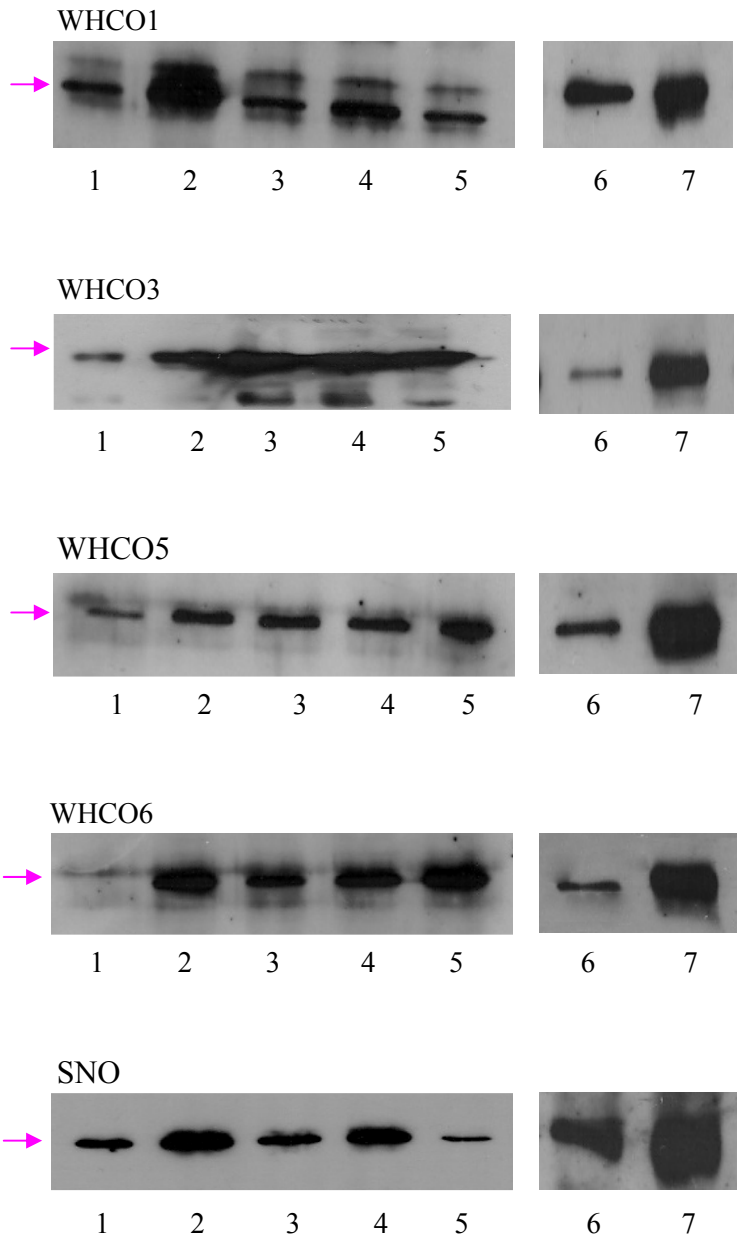


Figure 12: ILK Expression Following EGF Exposure.

a) Western blot analysis utilising a rabbit anti-ILK antibody (1:1500) of ILK expression following EGF (10 ng/ml) treatment for 0.5, 1, 3 and 6 hours respectively across all cell lines. Lane 1 – 0 hours EGF, lane 2 – 0.5 hours EGF, lane 3 – 1 hour EGF, lane 4 – 3 hours EGF, lane 5 – 6 hours EGF. Lane 6 – 0 hours EGF and lane 7 – 24 hours EGF b) Densitometric analysis indicated by a fold increase in ILK expression levels following addition of EGF in the HOSCCs. An increased trend in ILK expression was apparent, although variations in expression were noted at certain instances. Note: the expression levels are representative as a percentage of maximum per 10 μg of protein from concentrated fractions (included to normalise for loading variation). Experiments were repeated three times.

a)



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b)

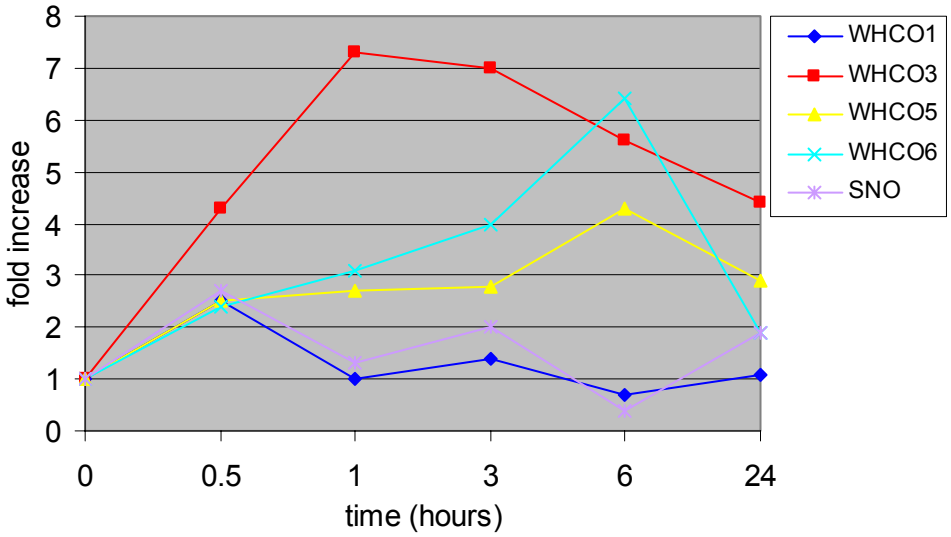


Figure 13: ILK Expression Levels Following TGFβ1 Exposure.

a) Western blot analysis utilising a rabbit anti-ILK antibody (1:1500) of ILK expression following TGFβ1 (1 ng/ml) treatment for 0.5, 1, 3 and 6 hours respectively across all cell lines. Lane 1 – 0 hours TGFβ1, lane 2 – 0.5 hours TGFβ1, lane 3 – 1 hour TGFβ1, lane 4 – 3 hours TGFβ1, lane 5 – 6 hours TGFβ1, lane 6 – 0 hours TGFβ1 and lane 7 – 24 hours TGFβ1 b) Densitometric analysis of the expression levels (fold increase) of ILK in the HOSCCs demonstrated an increased trend in ILK expression, although variations in expression were noted at certain instances. Note: the expression levels are representative as a percentage of maximum per 10 μg of protein from concentrated fractions (included to normalise for loading variation). Experiments were repeated three times.

2.4 Discussion

The intrinsic processes of cell-ECM adhesion are widely regarded as playing a pivotal role during both normal signalling and tumourigenesis. Cell-ECM adhesion is mediated primarily by integrins, and ILK is directly involved in regulating the integrins and thus cell-ECM adhesion. A major role of ILK is its ability to regulate cell-ECM adhesion by phosphorylation of the β integrin subunit (Dedhar, 1999; Attwell *et al*., 2000; Mulrooney *et al*., 2000; Pasquet *et al*., 2002). ILK is also capable of transducing signals throughout the cell that regulate gene expression, cell proliferation, and cell migration (Delcommenne *et al*., 1998; Li *et al*., 1999; D'Amico *et al*., 2000). Overexpression of ILK correlates with malignancy in several tumour types, including breast, prostate, brain and colon carcinomas (Persad *et al*., 2000; Yoganathan *et al*., 2002), indicating that ILK is also involved in tumourigenesis. Surprisingly, information regarding ILK expression by oesophageal SCCs is currently nonexistent in the literature and this prompted an investigation into ILK expression levels. The present study aimed to determine if similar levels of ILK were expressed in oesophageal squamous carcinoma cell lines derived from moderately differentiated tumours. It was also reasoned that determining ILK distribution would further our understanding of ILK function with regards to cell-ECM adhesion and signal transduction.

Transcription of the ILK gene was confirmed by the amplification of the full ILK transcript (RT-PCR), which revealed an ILK product of approximately 1360 bp (see Figure 5b). This result is consistent with a previous study by Janji *et al*., (2000) who showed that ILK mRNA was expressed at a size of 1359 bp in human melanoma and fibrosarcoma cell lines. The present study also demonstrated that ILK mRNA is translated to ILK protein (59 kDa), where ILK protein expression (Western blotting) was demonstrated in all oesophageal SCC cell lines tested (see Figure 7a). It is important to note that ILK protein was identified from the supernatant protein fractions of a Triton X-100-based extraction (enriching both membrane and cytosolic proteins and shown to preserve integrin function), suggesting that ILK is membrane-associated in oesophageal carcinoma cells and indicates a role for ILK in integrin signalling and ECM adhesion processes.

The result of an expressed 59 kDa ILK protein concurs with previous studies, which have shown ILK to be of equivalent size (Hannigan *et al*., 1996; Dedhar *et al*., 1999; Li *et al*., 1999; Wu, 1999; Friedrich *et al*., 2002). Nikolopoulos & Turner, (2001) however, have reported the molecular mass of ILK as being 50 kDa. These authors suggested that this discrepancy in molecular size might signify different gene products of ILK, although the reason for these differing results is uncertain at present. This study revealed that, under standard culture conditions, ILK expression for three of the five cell lines is relatively similar (WHCO3, WHCO5 and SNO cell lines) whereas the WHCO1 cell line exhibited higher ILK expression levels, while WHCO6 showed a lower ILK expression (densitometry–see Figure 7b). This may indicate dysregulated ILK expression in the latter cell lines although a comparison to normal epithelial oesophageal keratinocytes would be needed to draw further conclusions. The reader is reminded that all oesophageal lines are derived from tumours with similar pathological grading. Unfortunately, due to the difficulties in obtaining, and culturing, normal epithelial oesophageal keratinocytes, this comparison could not be made.

The inconsistencies in ILK protein expression levels (even though samples were normalised for loading variations) lead us away from the possibility of ILK acting as a molecular marker for this pathological grade of oesophageal SCCs. However, it should be remembered that the laser densitometric technique utilised in determining expression levels is a semi-quantitative technique and it would perhaps be prudent for future work to verify these data utilising a more sensitive protocol such as a radio immunoassay (RIA). Furthermore, protein ILK expression do not necessarily reflect ILK mRNA expression levels, which would explain the differences in the intensities of the ILK mRNA and ILK protein bands observed in the WHCO1 and WHCO3 cell line (see Figures 5c and 7a). Real time PCR would have been necessary to precisely determine the levels of cellular ILK mRNA.

While not being a marker, these differences in ILK expression within this single class of pathological grade may signify that the current system for pathologically grading SCC tumours is inconsistent. Although the data regarding ILK protein expression is disappointing, from a pathological grading perspective, this study still provides unique evidence that ILK mRNA and protein are expressed in oesophageal SCC cell lines. However, comparisons to other gastro intestinal cancers cannot be made since there is

currently no data available on the semi-/quantitative expression of ILK for any other cell line.

The two known isoforms of ILK, ILK1 and ILK2, differ only in that they possess unique restriction sites, namely *Bam*HI and *Hinc*II respectively. ILK2 has been found to be expressed in two metastatic cell lines but not in normal adult tissues (Janji *et al*., 2000). ILK2, although only recently discovered, is known to have antigenic cross-reactivity and functional attributes similar to ILK1 (Janji *et al*., 2000). Taken together, this evidence necessitated the determination of the isoform present. In these oesophageal SCCs, the ILK1 isoform was identified (following *Bam*HI restriction), (see Figure 8a, 836 bp product-red arrow and 523 bp product-black arrow), corroborating evidence of ILK1 being the most abundant isoform expressed in metastatic tumours (Janji *et al*., 2000).

Following the attachment of cells to ECM, allows for the localisation of ILK to focal adhesions (Wu & Dedhar, 2000; Nikolopoulos & Turner, 2001). This distribution pattern of ILK suggests roles for ILK in cell adhesion events; as overexpression of ILK results in inhibited adhesion to integrin substrates (Hannigan *et al*., 1996). Furthermore, this localisation controls cell migration processes; since inhibition of the PINCH-ILK interactions impairs coordinated changes in cell shape, which are required for cell migration to occur (Wu *et al*., 1998; Tu *et al*., 2001; Zhang *et al*., 2002). Thus ILK localisation regulated by PINCH (Velyvis *et al*., 2001) is responsible for placing ILK in the most suitable position for mediating specific adhesion and signalling functions. Furthermore, increased ILK expression has been shown to correlate with altered ILK localisation and possible altered function (Somasiri *et al*., 2000). In mesenchymal cells that overexpress ILK, Somasiri *et al*. (2000) demonstrated that ILK is localised to elongated "streaks" at the cell-substratum interface. Although these streaks resemble focal adhesions they differed considerably from control populations.

Localisation of β_3 integrin subunits requires regulation by ILK and therefore association between these molecules (Hannigan *et al*., 1996; Velyvis *et al*., 2001; Zervas *et al*., 2001). The association shown between ILK and β_3 integrins, most likely at focal adhesions, are indicative of an interaction between these two molecules. The physical interaction (direct/indirect) between ILK and the β_3 integrin subunit was verified (by co-

immunoprecipitation) for oesophageal SCCs, where a band of 88 kDa for the β_3 integrin and a 59 kDa band for ILK were obtained (see Figure 9). Similar to other mammalian cell types, oesophageal SCC cell lines show that ILK is directly associated with the β integrin subunit, supporting evidence that ILK controls the expression and activity of the integrin receptors.

As a result of the importance of ILK in signalling processes, and since the WHCO1 cell line seems to express ILK at higher levels in comparison to the other cell lines, it became necessary to investigate the localisation of ILK in these cell lines. Reports demonstrate clearly that localisation of ILK to focal adhesion plaques requires the LIM-containing adaptor protein PINCH and ILKBP (Velyvis *et al*., 2001; Zhang *et al*., 2002), as well as a functional interaction between paxillin and ILK (Nikolopoulos and Turner, 2001). In this thesis, immunofluorescence microscopy showed a prominent cytoplasmic/nuclear and membranal distribution for ILK (Figure 8a), implying that PINCH-ILK and ILK-paxillin interactions are stable and intact. A pericellular membranal distribution for the β_3 integrin (see Figure 10f) for all cell lines was visualised. Miller and Veale, (2001) showed that the β_1 integrin subunit also follows a pericellular distribution in these OSCC cell lines. Hence, it can be speculated that along with interacting with the β_3 integrin subunit, an association may be occurring between ILK and the β_1 subunit in human oesophageal SCCs.

Localisation studies revealed that ILK was detected primarily in the cytoplasmic/nuclear region in the WHCO1, WHCO3, WHCO5, WHCO6 and SNO oesophageal carcinoma cell lines, with a much smaller proportion occurring at the membrane and focal adhesion contacts (see Figure 10). This nuclear localisation of ILK did not agree with the majority of previous studies, which report ILK as primarily having a focal adhesion distribution (Attwell *et al*., 2003; Chun *et al*., 2003). However, Western blotting confirmed our confocal microscopy results, identifying ILK localisation in the nucleus (see Figure 11a). Densitometric analysis further revealed that the nuclear levels of ILK expression were considerably higher in comparison to membrane/cytoplasmic expression levels of ILK (see Figure 11b). With the finding that the β_3 integrin subunit localised exclusively at the membrane (ILK also exhibited membrane localisation) supports the notion of an association between ILK and the β_3 integrin subunit (see Figure 10f).

Epithelial tumours seem to exhibit a downregulation of integrin expression, which is thought to facilitate unregulated growth and invasion (Ziober *et al*., 1996; Meyer and Hart, 1998; Miller and Veale, 2001). Specifically, Miller and Veale, (2001) have shown that expression of the β_1 integrin subunit is drastically reduced or lost in these particular oesophageal SCCs and since the distribution patterns of β_1 and β_3 have been shown to be similar, it is possible that ILK is the causative agent in β_1, and possibly β_3, reduction in carcinoma of the oesophagus. It will thus be of interest for future work to ascertain accurately β_3 integrin subunit expression levels in these cell lines.

We have thus far shown ILK protein and RNA expression in moderately differentiated oesophageal carcinoma cell lines, as well as showing localisation of ILK. The next logical step in developing an understanding of ILK in oesophageal SCCs was to explore the possibility of ILK as a mediator between growth factor expression and integrin signalling pathways. For this, the various cell lines were treated, with EGF and TGFβ1 and the effects on ILK expression examined.

Normal squamous epithelial cells express approximately 2×10^5 EGFR, with certain SCCs overexpressing EGFR up to 50 times higher in comparison to their normal counterparts (Veale and Thornley, 1989). This applies to a variety of epithelial malignancies (see introduction to this chapter and Mayer *et al*., 2000). Additionally, it has been reported that increased numbers of EGFR are required in the early steps of cervical carcinogenesis (Mayer *et al*., 2000) and also during the development and progression of colon cancer (Pouliot *et al*., 2000). Specifically, the oesophageal carcinoma cell lines used in the present study are known to overexpress the EGF receptors significantly higher than normal oesophageal keratinocytes. This applies particularly to the WHCO3 cell line (Veale and Thornley, 1989). While demonstrating increased EGFR expression, no relationship exists between the number of EGF receptors and biological response to EGF in these cell lines. For example, EGF inhibits the colony forming efficiency of SNO cells whereas the WHCO cell lines all differ in their responses to EGF (Veale and Thornley, 1989), highlighting the importance of EGF in growth and differentiation in SCCs. It was therefore of interest to assess EGF effects on ILK protein expression in oesophageal SCCs. Not only would this examination provide us with information on the responses of ILK expression to EGF, but would also

indicate a potential role for ILK as a mediator between the integrin and growth factor signalling pathways in oesophageal carcinoma.

Exogenous TGFβ1 has been found to inhibit epithelial cell growth (Fukai *et al*., 2003; Kim *et al*., 2004). Loss of TGFβ1 growth inhibition is known to occur in oesophageal carcinoma since, emphasising the importance of understanding the role of TGFβ1 in the present study (Osawa *et al*., 2004; Edmiston *et al*., 2005). Understanding the role of TGFβ1 is clearly highlighted by the fact that the expression profiles of integrins are susceptible to regulation by TGFβ1, and integrin-mediated signalling also regulates TGFβ1 expression levels (Kumar *et al*., 1995 cited in Kim *et al*., 2004). Following the relationship identified between ILK and the β integrins in these HOSCCs, we reasoned that TGFβ1 may well impinge upon ILK signalling events.

In HOSCCs examined here, the general response of ILK to both EGF and TGFβ1 treatment was an increase in ILK expression levels over the duration of growth factor exposure (see Figures 12b and 13b). It is likely that this stimulation occurs via activation of the PI3K pathway, which has been shown to be activated in response to the application of these growth factors (Krasilnikov *et al*., 2000; Qui *et al*., 2004). The only other report of EGF and TGFβ1 influencing ILK expression, showed stimulation of ILK expression in a human melanoma cell line following exposure to EGF and TGFβ1 for 24 and 48 hours (Janji *et al*., 2000). However, since several cell lines have been shown to respond rapidly to exogenous EGF and TGFβ1 (Mineo *et al*., 1999; Kim *et al*., 2000) the present study concentrated on shorter exposure times.

Cell lines treated with 10 ng/ml EGF over a 0.5, 1, 3, 6 and 24 hours time course dramatically increased the expression of ILK *in vitro* between 1.5 to 3.3 fold for the WHCO1, WHCO5, WHCO6 and SNO cell lines (see Figure 12b). We expected a similar substantial increase in ILK expression in the WHCO3 cell line, since this line in particular expresses approximately 10.4×10^6 receptors per cell, up to 4 times more than the SNO cell line and 2.5 fold higher than the WHCO1 cell line (Veale and Thornley, 1989). Indeed, the levels of ILK in this cell line were increased to the greatest extent up to 3.4 fold (6 hours EGF). Thus, it appears that the EGF signalling pathway dissociates

from ILK signalling, possibly through a substantial increase in PI3K levels. EGF feeds into the Ras-MAP kinase signalling pathway, controlling cellular growth and differentiation (Juliano and Haskill, 1993; Moro *et al*., 1998; Giancotti and Ruoslahti, 1999; Mariotti *et al*., 2001; Andl *et al*., 2003). Furthermore, since Ras is able to stimulate PI3K, which is a positive regulator of ILK activity (Delcommenne *et al*., 1998; Giancotti and Ruoslahti, 1999; Troussard *et al*., 2000; Mariotti *et al*., 2001), the activation of Ras via EGF may have further implications in the activation of ILK kinase activity.

The noted variations in ILK expression among the cell lines suggest that a mechanism for recycling these growth factors occurs in response to exogenous application depending on the exposure time. In the instances where ILK expression decreased abruptly, the decrease could be attributable to a reduction in the number of receptors at the membrane as a result of receptor internalisation (Doré *et al*., 2001). It is widely accepted that, although EGFR localises primarily at the cell surface, they constantly shuttle and recycle through the cell (Wiley *et al*., 2003). Furthermore, rates in recycling can be accelerated as a consequence of EGF ligand-binding (Mineo *et al*., 1999; Wiley *et al*., 2003). EGFR are internalised from the cell surface in a number of ways, including clathrin-coated pits (Wiley *et al*., 2003; Gao *et al*., 2005; Puri *et al*., 2005; Sigismund *et al*., 2005). Once internalised, EGFR are delivered to endosomes (Baulida *et al*., 1996; Wiley *et al*., 2003), are sorted, and are either recycled back to the plasma membrane or directed to the lysosome for destruction (Mellman *et al*., 1996; Opreska *et al*., 1996; Burke *et al*., 1999). It is highly probable that the fluctuations noted in ILK expression in the WHCO1, WHCO5 and SNO cell lines are attributed to this receptor internalisation/recycling process where lowered EGF signalling occurs as a result of EGFR reduction at the plasma membrane. Since EGF has been shown to stimulate PI3K activity (Normanno *et al*., 2006), lowered EGF signalling results in a concommitant decrease in PI3K activity. As a consequence ILK concentration levels are decreased.

When the OSCC cell lines were treated with TGFβ1 for identical time periods, increased ILK expression was noted in all of the five cell lines compared to untreated cell lysates. The TGFβ1-induced increases in ILK expression were similar to that of EGF. However, the response of ILK to TGFβ1 exposure did not produce the standard

increase in ILK expression as was the case when cells were treated with EGF. Instead, fluctuations in ILK expression levels were observed. This was especially apparent in the WHCO1 and SNO cell lines (see Figure 13b).

Studies addressing the recycling of TGFβ1 receptors have been left largely unexamined. Although scarce, early reports do suggest that internalised receptors are either rapidly replaced or recycled back to the cell membrane (Sathre *et al*., 1991; Mitchell *et al*. 2004). More recently, Mitchell *et al*. (2004) showed that TGFβ1 receptors do indeed recycle and degrade in a clathrin-dependent manner, but that this process is not dependent on ligand stimulation. The Mitchell *et al*. (2004) study was conducted on lung epithelial cells and it is possible that ligand stimulation by exposure to TGFβ1 is required in the case of HOSCCs. Thus, with regard to the HOSCCs in question, the fluctuations observed following TGFβ1 exposure may well be explained by receptor recycling.

While the cellular expression of ILK in HOSCCs is comparable to that of other cell types, particularly noteworthy is that ILK expression in HOSCCs appears to be dependent on the receptor recycling of both the EGF and TGFβ1 receptors. EGF/TGFβ1 has been shown to result in a rapid, marked increase in ILK expression. Since it is known that EGFR overexpression occurs in these HOSCCs, it is plausible that a constitutive increase in ILK expression exists in these HOSCCs due to stimulation of PI3K. A direct consequence of EGF/TGFβ1 stimulation of the ILK pathways could thus be decreased cellular adhesion due to impaired integrin-mediated signalling, or a stimulation of mitogenic pathways such as MAPK thereby promoting transformation.

These results show that ILK expression is regulated, not only by stimuli derived from integrin dependent cell adhesion (Delcommenne *et al*., 1998), but also in a growth factor dependent manner in all the cell lines under investigation. This provides support of a link between integrins and growth factor mediated signalling. In contrast to the data presented here, EGF has been shown to have no effect on ILK expression in HT-144 melanoma cells (Janji *et al*., 2000). However, the melanoma study considered the ILK2 isoform and no mention is made of the effect of EGF on ILK expression in these cells.

While informative, this data does not allow for any comparisons to be made to the metastatic oesophageal cell lines in question. Furthermore, the noted differences between the melanoma cell line and the oesophageal carcinoma cell line in question, in response to EGF, could be accounted for by the differences in their developmental origin. From these sets of data it could be argued that EGF does not affect ILK pre- or posttranscriptional expression levels similarly. It will be interesting to examine EGF effects on ILK mRNA expression in oesophageal SCCs to understand perhaps if EGF similarly affects ILK mRNA and protein.

The identification of ILK expression in oesophageal SCCs in the present study provides a recent addition to the growing list of malignancies in which ILK has been shown to be present. Furthermore, this is the first evidence of ILK expression in oesophageal SCC cell lines. The data obtained has provided novel evidence concerning ILK expression levels and distribution within this class of moderately differentiated oesophageal SCC, and has indicated a role for ILK in cell-ECM adhesion and integrin signalling.

This chapter has addressed the distribution of ILK as well as the growth factors effects on ILK expression. As the noted effects of EGF on ILK expression are almost certainly PI3K dependent perhaps more insight into this particular aspect of ILK could be attained by considering the effects of growth factors on the kinase activity of the catalytic domain of ILK1. The kinase domain is responsible for the regulation of cell-ECM adhesion by being capable of directly phosphorylating the β integrin subunit. Accordingly, the ILK kinase domain forms the focus of chapter 3.

Chapter 3

Growth factor modulation of ILK kinase activity in oesophageal SCCs

3.1 Introduction

The catalytic kinase domain of ILK regulates the β integrin subunits. In order to gain insight into the physiological roles of ILK during cell-ECM adhesion events, and its possible role in invasion and metastasis an understanding is required of the kinase domain of ILK (Troussard *et al*., 2000; Yoganathan *et al*., 2000; Ishii *et al*., 2001; Zhang *et al*., 2002; Cordes, 2004; Stevens *et al*., 2004). The ILK kinase domain will be examined from two aspects. First, and of immense interest to this study, is the effect of growth factors on the kinase activity of ILK. This is of particular importance with respect to EGFR since EGFR is upregulated in oesophageal SCCs (Normanno *et al*., 2006) and in this study (see chapter 2) EGF was shown to upregulate ILK expression levels. Second, an understanding of the alterations of ILK kinase activity upon cell attachment to ECM substrates is provided. For the sake of clarity, these facets will be considered separately and while this chapter will place emphasis on the former, examination of altered ILK activity effects on cell-ECM adhesion will form the basis to chapter 6.

The kinase domain of ILK is serine/threonine directed (Troussard *et al*., 2000; Persad and Dedhar, 2003; Ito *et al*., 2003; Grashoff *et al*., 2004; Chun *et al*., 2005) with ILK acknowledged as having a low basal kinase activity (Wu & Dedhar, 2001). ILK kinase activity is modulated by the interaction of cells with components of the extracellular matrix and subsequent integrin clustering (Janji *et al*., 2000; Yoganathan *et al*., 2000; Wu & Dedhar, 2001; Dai *et al*., 2003). ILK activity is stimulated in a phosphotidylinositol (PI) 3-kinase (PI3K)-dependent manner, which involves binding of the phosphoinositide phospholipid product of PI3K, PIP_3, to the PH-like domain of ILK (Dedhar, 2000; Wu, 2001; Cordes and van Beuningen, 2003; Grashoff *et al*., 2004). Activated ILK in turn, is able to influence changes in cell survival, proliferation and differentiation by modulating integrin-mediated cell adhesion (Dedhar *et al*., 1999; Janji *et al*., 2000; Kaneko *et al*., 2004).

ILK has been demonstrated to be an upstream effector of the PI3K-dependent regulation of two important signalling proteins; namely GSK3β and PKB (see introduction and Persad *et al*., 2000; Yoganathan *et al*., 2000; Wu and Dedhar, 2001; Cordes and van Beuningen, 2003; Quélo *et al*., 2004; Pinkse *et al*., 2005). ILK inhibits GSK3β, which result*s* in β-catenin translocation to the nucleus, causing a subsequent downregulation of E-cadherin expression and cell-cell adhesion respectively (see introduction and Novak *et al*., 1998; Tan *et al*., 2001; Oloumi *et al*., 2004). The regulation of cell survival is also influenced by ILK. ILK achieves this by phosphorylating PKB thus stimulating its activity (Attwell *et al*., 2000; Wu and Dedhar, 2001; Attwell *et al*., 2003; Stevens *et al*., 2004), which results in inactivation of the proapoptotic factor Bad and consequently leads to inactivation of caspase-9 and protection from apoptosis (Wang *et al*., 2001; Cordes and van Beuningen, 2003). This was demonstrated in human prostate carcinoma cells where both ILK and PKB were constitutively activated, and subsequent inhibition of ILK resulted in apoptosis (Persad *et al*., 2000).

EGF and TGFβ1 growth factors are of paramount importance in physiological functions such as proliferation, differentiation and cell adhesion (Ozanne *et al*., 1986; Janji *et al*., 2000; Mayer *et al*., 2000; Fujimoto *et al*., 2001). Furthermore, growth factors such as these have been shown to regulate ILK activity (Delcommenne *et al*., 1998; Dedhar *et al*., 1999). TGFβ1 is an interesting growth factor to consider in regulation of ILK activity, since it is capable of stimulating cellular proliferation, in addition to being largely regarded as a growth inhibitor of epithelial cells (Sporn and Roberts, 1986; Fujimoto *et al*., 2001; Vogelmann *et al*., 2005). Although different cell types have been shown to respond differently to TGFβ1, varying responses within the same cell type may be exhibited under different experimental conditions (Moses *et al*., 1990). For example, TGFβ1 is able to regulate the growth of keratinocytes in cell culture by acting as a potent inhibitor of proliferation, even in the presence of mitogenic concentrations of EGF (Ozanne *et al*., 1986), but induces angiogenesis *in vivo*. Since the mechanism/s that direct TGFβ1 dependent processes are to a large degree still unknown (Janji *et al*., 2000), it is these perplexing actions of TGFβ1 that make it an interesting growth factor to consider in the study of ILK activity in oesophageal SCCs.

EGF has been shown to influence ILK expression in oesophageal SCCs, and TGFβ1 has been shown to modulate the expression of β_3 and β_1 containing integrins (Lai *et al*., 2000), which associate with ILK (Hannigan *et al*., 1996; Attwell *et al*., 2000). This investigation attempted to establish whether regulation of ILK expression occurs in a similar fashion to that of ILK activity, by growth factors as well as to extrapolate these results to the relevance of regulated ILK activity in cell-ECM adhesion events.

As mentioned above positive regulation of ILK activity occurs through phosphorylation provided by PI3K. To ensure appropriate ILK levels, negative regulation is supplied by the PTEN tumour suppressor (Das *et al*., 2003; Leslie and Downes, 2004). While originally identified as a dual specificity phosphatase, it has been revealed by biochemical analysis that PTEN is not a capable protein phosphatase but rather a competent phosphoinositide D3-phosphatase (Al-Khouri *et al*., 2005). PTEN functions by specifically cleaving the D3 phosphate of this second messenger lipid produced by the activity of PI3K (Cristofano and Pandolfi, 2000). The removal of this phosphate group inhibits PI3K function.

PTEN has particular relevance to the present study since it is known that ILK activation requires binding to PIP_3 and subsequent PI3K-dependent activation (Boulter *et al*., 2006). While this leads to the stimulation of ILK activity, the inhibition of ILK occurs through PTEN activity. PTEN negatively regulates ILK activity via the dephosphorylation of PIP_3 (Zheng *et al*., 2003; Leslie *et al*., 2003). This has been demonstrated in PTEN-null prostate cancer cells in which PIP_3 levels are high, ILK is constitutively active (Persad *et al*., 2000).

Since there are no widely expressed PTEN homologues, PTEN activity strongly influences both basal and stimulated PI3K, signifying the importance of this phosphatase in a wide variety of tumour types (Backman *et al*., 2002). PTEN is mutated in a variety of sporadic tumours including glioblastomas and endometrial carcinomas (Ali *et al*., 1999; Bonneau and Longy, 2000; Leslie *et al*., 2001; Leslie and Downes, 2004; Walker *et al*., 2004; Kerr *et al*., 2006). Furthermore, mutations have been observed in brain, breast, prostate and other advanced malignancies (Gu *et al*., 1998; Whang *et al*., 1998; Li *et al*., 2002; Sulis *et al*., 2003; Yang *et al*., 2003). Although inactivation of the PTEN gene occurs frequently there are instances in which tumours

appear to develop mechanisms that reduce the concentration levels of PTEN without mutation of the PTEN gene (Whang *et al*., 1998). Methylation of the PTEN promoter region, associated with reduced PTEN expression, occurs in non-small cell lung cancer (Soria *et al*., 2002). Loss of one allele of PTEN with the preservation of the remaining wild-type allele is also very common (Sulis *et al*., 2003). However, it has been demonstrated that $PTEN^{+/-}$ mice develop colonic adenomas and lymph node hyperplasia at high frequency due to haploinsufficiency (Podsypanina *et al*., 1999). Furthermore, it has been shown that haploinsufficiency results in tumour progression in a mouse SV40T antigen prostate-tumour model (Kwabi-Addo *et al*., 2001). However, PTEN mutations are not frequent in oesophageal carcinoma (Hu *et al*., 1999). Nevertheless, its likely involvement in ILK activity emphasises the importance of PTEN to the present study.

PTEN is involved in many growth factor-mediated signalling pathways (Ebert *et al*., 2002; Vogelmann *et al*., 2005). It seems likely that if indeed growth factors do impinge upon ILK kinase activity in these oesophageal carcinoma cells, it is highly probable that PTEN would mediate these growth factor effects and it is therefore imperative that PTEN be examined in these cell lines. Support of this notion is derived from evidence that demonstrates that TGFβ1-induced effects on cells involve PTEN activity. To corroborate this idea, Vogelmann *et al*. (2005) demonstrated that TGFβ1 treatment results in a reduction in phosphorylated PTEN and causes dissociation from the E-cadherin/β-catenin complex. Furthermore, Ebert *et al*. (2002) demonstrated that TGFβ1 dramatically reduces PTEN in human pancreatic cancer.

ILK kinase activity was examined using myelin basic protein (MBP)-based kinase assays. MBP was used as an analogue, since it contains the β_1 integrin cytoplasmic sequence (Wu, 1999), and phosphorylation of this substrate reveals ILK kinase functionality. Although previous studies involving ILK kinase assays refer to ILK activity as its ability to phosphorylate a myelin basic protein (MBP) substrate (Hannigan *et al*., 1996; Janji *et al*., 1998; Persad *et al*., 2000; Somasiri *et al*., 2000; Kiss *et al*., 2002), it is acknowledged that this is an activity assay and not a strict enzymological definition of enzyme activity. However, for consistency this study will use the published terminology when discussing ILK kinase capabilities (Hannigan *et al*., 1996; Delcommenne *et al*., 1998; Tan *et al*., 2002). Data presented in this chapter

suggest that the EGF and TGFβ1 growth factors are able to modulate the kinase activity of ILK in oesophageal SCCs, and furthermore, that PTEN is directly involved in mediating the growth factor-induced effects of the ILK kinase activity in oesophageal SCC cell lines.

3.2 Methods and Materials

3.2.1 Isolation of ILK Utilising Immunoprecipitation

Cells seeded into Nunc™ culture dishes were treated with EGF (10 ng/ml) and TGFβ1 (1 ng/ml) for a period of 24 hours. 10 ng/ml EGF has been shown to be sufficient for inducing changes in cellular morphology, which promotes dissociation and increases the invasive ability of poorly differentiated human oesophageal SCCs (Shiozaki *et al*., 1996). TGFβ1 is able to either stimulate or inhibit cellular growth and at a concentration of 1 ng/ml has been shown to upregulate ILK expression (Sporn and Roberts, 1992; Janji *et al*., 2000).

Triton X-100 extractions (see Chapter 2, Section 2.2.5) were performed on a dish of either, untreated cells, EGF-treated (10 ng/ml) and TGFβ1-treated (1 ng/ml) cells for the respective oesophageal cell lines. Protein concentrations were determined using the Bradford assay (see Chapter 2, Section 2.2.6). Immunoprecipitation was performed following a modified protocol from Zhang *et al*. (2002). Briefly, cell lysates were incubated with a polyclonal rabbit anti-ILK primary antibody (10 µl) overnight at 4 °C. Traditionally used *S.aureus* cells and currently used protein G sepharose beads (Sigma, USA) were washed 4 times with a 10 mM Tris buffer pH 8 (Appendix 1.7.1). Thereafter, 75 µl of the washed *S.aureus* cells and sepharose beads were added to the supernatant and allowed to incubate for 4 hours at 4 °C. The subsequent immune complexes were centrifuged at 3000 rpm in a MSE bench-top centrifuge for 3 minutes. The supernatant was decanted and 200 µl of kinase buffer (Appendix 1.7.2) was added to the pellet and the tubes gently agitated. Samples were once again centrifuged at 3000 rpm and the supernatant decanted. Pellet samples were stored at −70 °C.

3.2.2 Densitometric Evaluation of ILK Activity Levels When Exposed to EGF and TGFβ1

The immunoprecipitated ILK pellet had the following added to it: 35 µl kinase buffer (see Appendix 1.7.2), 10 µCi [γ-^{32}P] ATP (Amersham, UK) and 10 µg myelin basic protein (MBP) from Sigma, USA. These kinase reaction mixtures were incubated for 2

hours (single time point analysis) at 4 °C with gentle agitation. (An equal volume of double lysis buffer (see Appendix 1.6.4) was added to the samples for two reasons. Firstly, to denature the ILK enzyme thus stopping the kinase reactions, and secondly, to bring the salt concentration of the liquid samples to the same concentration as that of single lysis buffer, which is the ionic concentration required in SDS-PAGE separation. Thereafter, samples were heated in a boiling water bath for 5 minutes. Eppendorf tubes were briefly centrifuged at 10000 rpm in a Sorvall® MC 12 V centrifuge for 10 minutes. Thereafter, the supernatant was transferred to a sterile eppendorf tube. These supernatant samples constituted kinase reaction products of phosphorylated MBP.

Kinase reaction products of phosphorylated MBP were resolved on 8 % SDS-PAGE (see Appendix 1.5.4). Gels were immersed in Coomassie blue stain (see Appendix 1.3.6) for 1 hour and destained, and rinsed in dH_2O and 10 % ethanol for 20 minutes respectively. Gels were dried for 2 hours on a SE1160 slab gel drier. The dried gels were exposed to hyperfilm™ MP autoradiography film overnight at −70 °C. The exposed film was developed in D19B developer (see Appendix 1.4.3) for 5 minutes, briefly rinsed in H_2O and fixed (see Appendix 1.4.4) for 5 minutes.

Kinase assay x-ray plates were semi-quantitatively analysed by laser densitometry using a LKB 2202 Ultrascan Laser Densitometer in order to ascertain ILK activity levels between oesophageal carcinoma cell lines when exposed to growth factors. The area under the peak for each sample was used as a basis for comparison between samples. Activity levels were represented as a percentage of the maximum per 35 μg of protein. Experiments were repeated three times.

3.2.3 Triton X-100 Extraction

As previously documented see Chapter 2, Section 2.2.5

3.2.4 Protein Estimation

As previously documented see Chapter 2, Section 2.2.6

3.2.5 Western Blot Analysis

Triton X-100 extracted lysates from the HOSCCs were resolved on 12 % SDS-PAGE, and transferred to Nitrobind nitrocellulose transfer membrane (MSI, USA), in a BioRad Trans-Blot™ Cell at 400 mA for 3 hours (4 °C) in Western Blot Transfer Buffer (Appendix 1.4.5). After transfer was complete, the nitrocellulose membranes were rinsed twice with PBS (see Appendix 1.1.1), and stored overnight at 4 °C.

Each membrane was blocked in BLOTTO (see Appendix 1.4.1) for 5 minutes to prevent non-specific antibody binding and washed 6 times in PBS. The blots were incubated in a rabbit anti-PTEN primary antibody (1:10000) for 1 hour. Washing was performed 6 times at 5-minute intervals with PBS to remove any residual antibody. When probing for PTEN, membranes were incubated with a HRP-bound secondary anti-rabbit antibody (1:10000) for 1 hour in the dark. Once again membranes were washed 6 times at 5-minute intervals with PBS before being exposed to the Supersignal® West Pico Working Solution (see Appendix 1.4.2) from the West Pico Chemiluminescent Substrate Kit (Pierce, USA) for 5 minutes. Blots were sealed in polyethylene 'saran wrap' and exposed to hyperfilm™ MP autoradiography film (Amersham, UK) for 1.5 minutes. Film was developed in D19B developer (see Appendix 1.4.3) for 5 minutes, rinsed briefly in H_2O before fixing (see Appendix 1.4.4) for 5 minutes. The experiment was repeated three times.

3.2.6 Co-Immunoprecipitation Analysis of ILK and PTEN

Cell lysates were preabsorbed with protein G sepharose beads overnight at 4 °C, centrifuged at 3000 rpm and the supernatant transferred to fresh eppendorf tubes. Cell lysates were either incubated with 5 µl polyclonal rabbit anti-PTEN (Sigma, USA) or polyclonal rabbit anti-ILK antibody overnight at 4 °C. The following day protein G sepharose beads (100 µl) (Amersham, SA) were added to the immune complex for 4 hours. The protein G-ILK1-PTEN complex was centrifuged at 3000 rpm in a Sorvall® MC 12 V centrifuge for 2 minutes. The supernatant was decanted, and 800 µl Tris.HCl buffer pH 8.0 added. Centrifugation was performed once again at 3000 rpm. This washing step was repeated twice more. The supernatant was decanted and an equal volume of double lysis buffer (Appendix 1.6.4) was added to the pellet. The suspension was boiled for 5 minutes and centrifuged for 10 minutes at 12000 rpm in a Sorvall®

MC 12 V centrifuge. The product was resolved on a 12 % SDS-PAGE gel (see Appendix 1.5.4) and transferred to nitrocellulose for 3 hours as in Methods and Material Section 2.2.8. The blot was probed for ILK and the PTEN integrin subunit using standard antibody concentrations. Blots were then exposed to the Supersignal® West Pico Working Solution (see Appendix 1.4.2) for 5 minutes, sealed in polyethylene 'glad wrap' and then exposed to hyperfilm™ MP autoradiography film (Amersham, UK) for 1.5 minutes. Film was developed in D19B developer (see Appendix 1.4.3) for 5 minutes, rinsed briefly in H_2O, before fixing (see Appendix 1.4.4) for 5 minutes.

3.2.7 Indirect Immunofluorescence

The localisation of PTEN was performed utilising indirect immunofluorescence microscopy. All cell lines were grown to 80 % confluency and seeded onto sterile glass coverslips. Coverslips were washed five times in cold PBS, fixed with 4 % paraformaldehyde (Appendix 1.6.1) for 30 minutes and washed again with PBS. Coverslips were immersed in 0.25 % Triton X-100 (Appendix 1.6.2) for 10 minutes to further permeabilise the cells, washed twice with PBS, dipped into dH_2O, and allowed to dry partially. This allowed the DAKO® pen (Denmark) to adhere properly to the glass coverslips. Two circles or wells per coverslip were drawn with the DAKO® pen to create a fluid barrier. One well per coverslip was incubated with a polyclonal rabbit anti-PTEN primary antibody (1:250) for one hour. The other well was incubated in PBS (control). This was followed by a thorough washing in PBS as before. All circled areas were incubated with an anti-rabbit Fluorescine Isothiocyanate (FITC)-conjugated anti-rabbit antibody (1:250). Both wells were incubated for one hour in the dark. The coverslips were washed six times with sterile PBS and then mounted with Elvanol mounting agent (Appendix 1.6.3) onto sterilised glass slides. All incubations were carried out in a humidity chamber to prevent desiccation. Slides were viewed under a Zeiss LSM 410 confocal microscope (FITC excitation 490, emission 525).

3.2.8 Image Capturing

As previously documented see Chapter 2, Section 2.2.13.

3.3 Results

3.3.1 EGF and TGFβ1 Treatment Modulates ILK Kinase Activity

ILK was isolated from total cell lysates by means of immunoprecipitation using protein G sepharose beads and killed *S.aureus* cells. Indeed, the ILK that was immunoprecipitated was utilised to ascertain whether ILK was capable of phosphorylating the MBP substrate during the course of the kinase reaction. It was revealed that both the protein G sepharose beads and *S.aureus* cells precipitated ILK (59 kDa) from all cell lines (Figure 14a). It was further noted that the ILK band immunoprecipitated by *S.aureus* cells was more apparent than ILK immunoprecipitated using the protein G sepharose beads, suggesting the *S.aureus* cells to be binding with greater affinity to the immune complex of ILK. Even though *S.aureus* cells were shown to be more efficient in precipitating ILK, protein G sepharose beads were used in conducting the MBP-based kinase assays, as protein G sepharose beads show increased specificity towards IgG molecules and are at present the method of choice.

ILK kinase assays showed that the ILK kinase catalytic domain was active and capable of phosphorylating the MBP substrate (Figures 14b). Since MBP was a synthetic peptide it lacked kinase activity and not able to undergo autophosphorylation. Therefore, a protein G sepharose only negative control was not included in the study. ILK activity gel separations were densitometrically analysed, which revealed the ILK basal kinase activity to be dissimilar across the cell lines. It must be remembered that per μg of total protein equivalent protein concentrations were loaded for the HOSCCs. A uniform pattern of activity was noted in the WHCO1, WHCO6 and SNO cell lines. The WHCO5 line was 5 fold higher in activity in comparison to the WHCO3 cell line and exhibited a 2.5 fold difference over the WHCO1 and SNO cell lines. The ILK kinase activity of WHCO5 was also 2 fold higher than the WHCO6 cell line (Figure 15a). Lowest ILK activity was noted in the WHCO3 cell line. These data demonstrate that ILK activity levels are inconsistent across the five moderately differentiated oesophageal SCC cell lines examined.

Interestingly, it was observed that treatment of cell cultures with EGF (10 ng/ml) and TGFβ1 (1 ng/ml) caused a decline in the ILK kinase activity in cell lines WHCO1,

WHCO6 and SNO between 1 to 3 fold (established by laser densitometry, Figure 15b). However, paradoxical results were obtained in the case of the WHCO5 cell line, where treatment with TGFβ1 reduced ILK activity, whereas EGF augmented ILK activity in comparison to standard untreated WHCO5. The addition of growth factor had the greatest effects on the WHCO3 line, which showed a dramatic increase in activity to both EGF (3 fold) and, TGFβ1 (3.5 fold). Although TGFβ1 treatment produced a similar result to that of EGF, its effect on ILK activity was more profound. Delcommenne *et al*. (1998) have previously shown that growth factors are able to upregulate ILK activity. While the present data indicate a modulation of ILK activity by the EGF and TGFβ1, a time course analysis would be necessary to fully understand the effects of EGF and TGFβ1 on ILK activity.

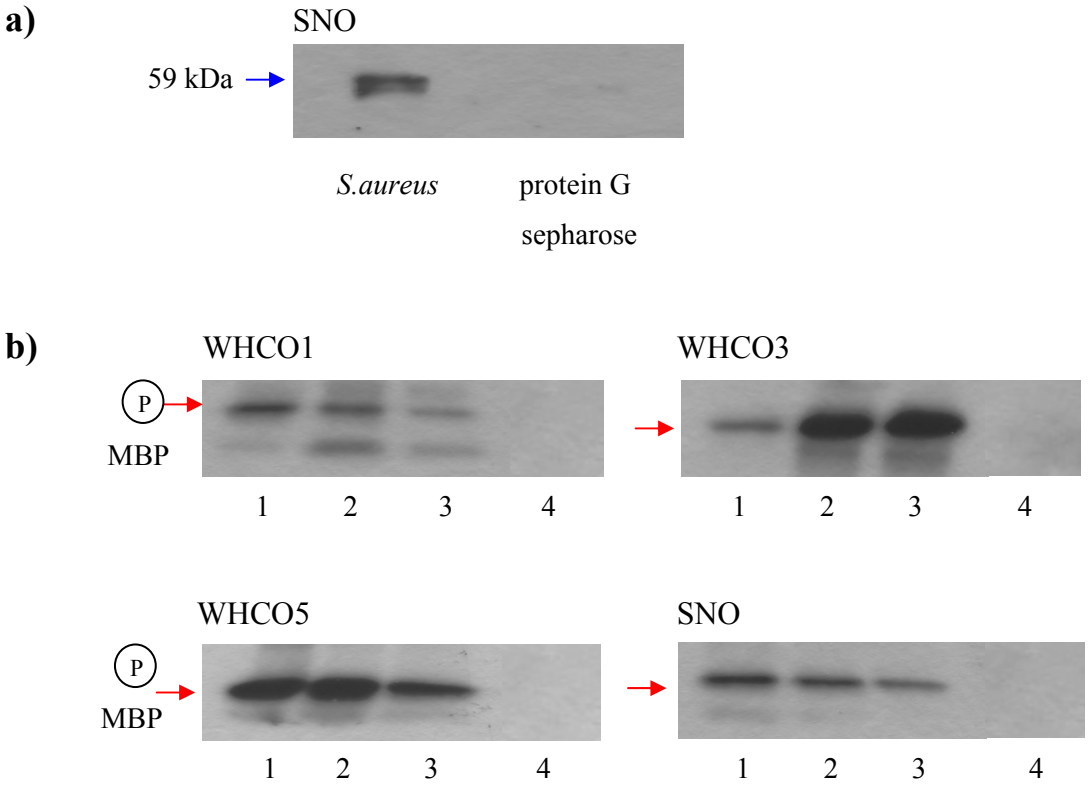


Figure 14: ILK Kinase Assays Characteristic of the Various Cell Lines.
a) SNO immunoprecipitated ILK band using killed *S.aureus* cells and protein G sepharose beads characteristic of all cell lines (blue arrow). Lane 1 – *S.aureus* immunoprecipitated ILK, lane 2 – protein G sepharose immunoprecipitated ILK. b) ILK kinase activity of WHCO1, WHCO3, WHCO6 and SNO cell lines indicating MBP phosphorylation (red arrow). Cell line WHCO3 shows reverse effect of growth factor treatment not characteristic of other cell lines. Cell lysates were preabsorbed with protein G sepharose to prevent non-specific protein G associated products. Lane 1 – phosphorylated MBP (untreated), lane 2 – MBP phosphorylation following EGF treatment, lane 3 – MBP phosphorylation (TGFβ1 treated) and lane 4 – Negative substrate control (MBP excluded). Experiments were repeated three times.

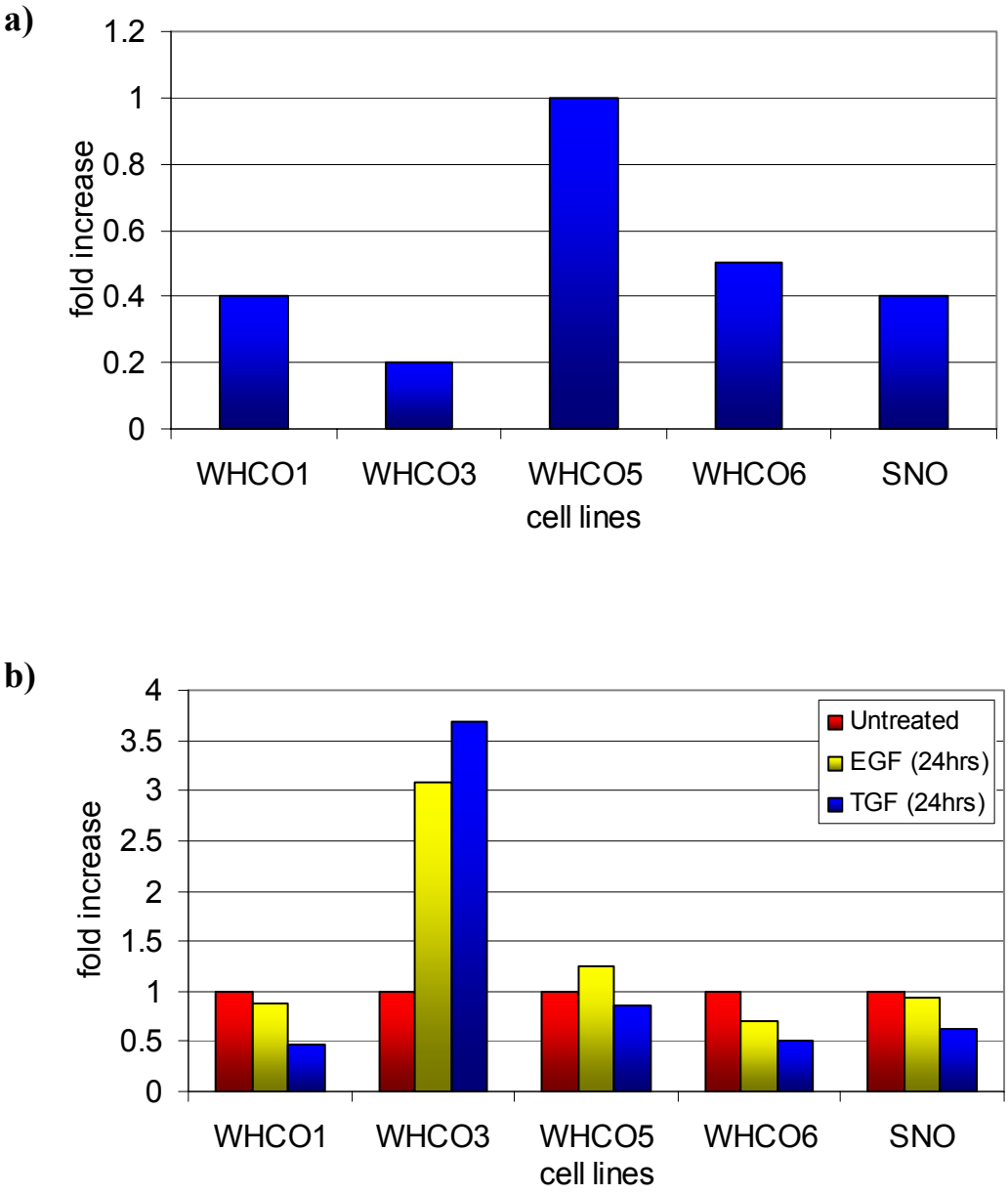


Figure 15: Densitometric Analysis of ILK Activity Levels.

a) Standard ILK activity levels following protein G sepharose immunoprecipitation of ILK and a MBP-based kinase assay. WHCO5 cell line exhibited highest levels of ILK activity, whereas the WHCO3 showed lowest levels of ILK activity. b) Comparative levels of ILK activity are shown between standard untreated cell lines, EGF treated and TGFβ1 treated cell lines. While the WHCO1, WHCO6 and SNO lines all exhibited diminished activity following growth factor addition, the WHCO3 line showed a marked increase in ILK activity. The WHCO5 cell line demonstrated conflicting results with an increase in activity by EGF (10 ng/ml) and a decrease in activity by TGFβ1 (1 ng/ml). Experiments were repeated three times.

3.3.2 Growth Factors Influence PTEN Expression Levels

As described earlier, the activity of ILK is sensitive to PTEN levels which negatively regulates ILK via the dephosphorylation of PIP_3, the product of PI3K, which in turn prevents the activation of ILK (Persad *et al*., 2000; Backman *et al*., 2002; Leslie and Downes, 2004; Walker *et al*., 2004). Thus in normal cellular conditions ILK activity levels are maintained under stringent control by the activities of PI3K and PTEN (Kaneko *et al*., 2003; Miller *et al*., 2003). It is expected that the regulation of PTEN levels via EGF and TGFβ1 would influence ILK kinase activity in these HOSCC cell lines.

The presence of a 56 kDa PTEN band was confirmed via Western blotting analysis in all five HOSCCs (Figure 16a). Densitometric analysis revealed that levels of PTEN expression demonstrated great variation between the various cell lines. PTEN expression was highest in the WHCO1 line, on average 4 fold higher than PTEN expression in the WHCO6 and SNO cell lines. Furthermore, PTEN expression in WHCO1 cells was 1.3 and 2 fold higher than the WHCO3 and WHCO5 cell lines respectively. In contrast, the WHCO6 exhibited lowest PTEN expression, 4 and 2.5 fold lower than WHCO3 and WHCO5 cells respectively (Figure 16b).

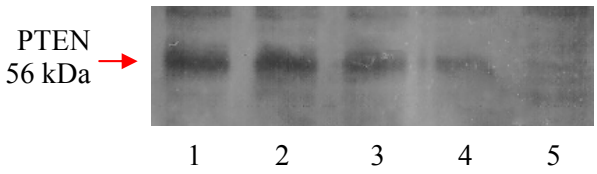
When the respective HOSCCs were treated with EGF (10 ng/ml) for a period of 24 hours, the WHCO1 cell line exhibited a vast increase in PTEN expression of 6.4 fold in comparison to the untreated cell lysate. Similarly, the WHCO5 and SNO cell lines also displayed substantial increases in the expression of PTEN of 6.2 and 3.4 fold respectively. Surprisingly, in the WHCO3 and WHCO6 cell lines noticeable decreases in PTEN expression were observed of 3 and 2.3 fold respectively (Figure 17a and b).

EGF exposure resulted in both an increase and decrease of the protein expression of PTEN. TGFβ1 on the other hand, increased PTEN expression in all five HOSCCs (Figure 18a). Although this increase was modest in the WHCO5 cell line (1.5 fold), more noteworthy increases were shown in the remaining cell lines. In the WHCO3, WHCO6 and SNO cell lines, an average increase of 2 fold was observed, while WHCO1 cells demonstrated the highest increase of 3.9 fold (Figure 18b). A lower

molecular weight band was observed below the PTEN band (Figure 17a and 18a, black arrows). This is attributed to degradation products that are present in the cell lysates.

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a)



b)

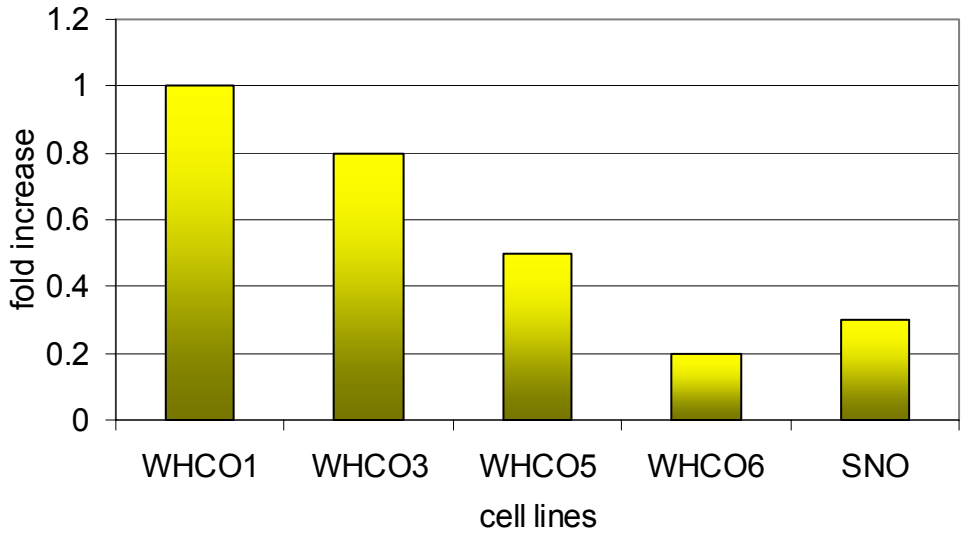


Figure 16: Western Blot and Densitometric Analysis of PTEN Expression Levels. a) Western blots performed utilising a rabbit anti-PTEN antibody (1:10000) on the soluble supernatants of the membrane/cytoplasmic cell lysates revealed PTEN expression at a molecular weight of approximately 56 kDa across all cell lines (indicated by red arrow). Lane 1 – WHCO1, lane – WHCO3, lane 3 – WHCO5, lane 4 – WHCO6, and lane 5 – SNO. b) Densitometric analysis revealed PTEN expression levels were not uniform across all human oesophageal SCC lines under standard culture conditions. The WHCO1 displayed highest PTEN expression 5 fold higher than the WHCO6 cell line (lowest PTEN expression), 1.3 fold higher than WHCO3 PTEN expression, 2 fold higher than WHCO5 PTEN expression, and 3.3 fold higher than SNO PTEN expression. Note: the expression levels are representative as a percentage of maximum per 10 μg of protein from concentrated fractions (included to normalise for loading variation). Experiments were repeated three times.

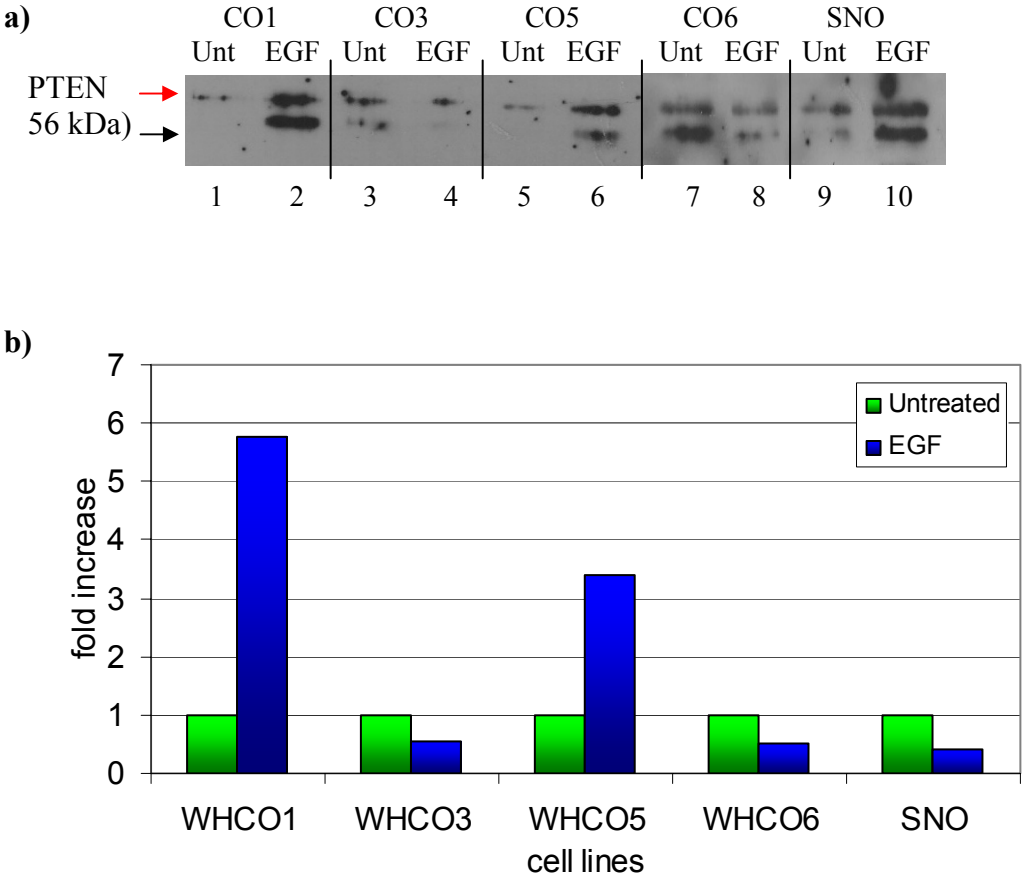


Figure 17: The Effect of EGF on PTEN Expression Levels.

a) Western blot analysis of PTEN expression (red arrow) using a rabbit anti-PTEN antibody (1:10000) following exposure to EGF (10 ng/ml) for 24 hours in the five HOSCCs. Degradation products are indicated by the black arrow. PTEN expression was increased in the WHCO1, WHCO5 and SNO cell lines. A decrease in PTEN expression was in the WHCO3 and WHCO6 cell lines. Lane 1 – untreated WHCO1, lane 2 - EGF-treated WHCO1, lane 3 – untreated WHCO3, lane 4 - EGF-treated WHCO3, lane 5 – untreated WHCO5, lane 6 - EGF-treated WHCO5, lane 7 – untreated WHCO6, lane 8 - EGF-treated WHCO6, lane 9 – untreated SNO and lane 10 - EGF-treated SNO. b) Densitometric analysis of PTEN protein expression levels revealed that PTEN expression was increased substantially in the WHCO1, WHCO5 and SNO cell lines by 6.4, 6.2 and 3.4 fold respectively. In the WHCO3 and WHCO6 cell lines, PTEN expression was decreased following EGF exposure by 3 and 2.3 fold respectively. Note: the expression levels are representative as a percentage of maximum per 10 µg of protein from concentrated fractions (included to normalise for loading variation). Experiments were repeated three times.

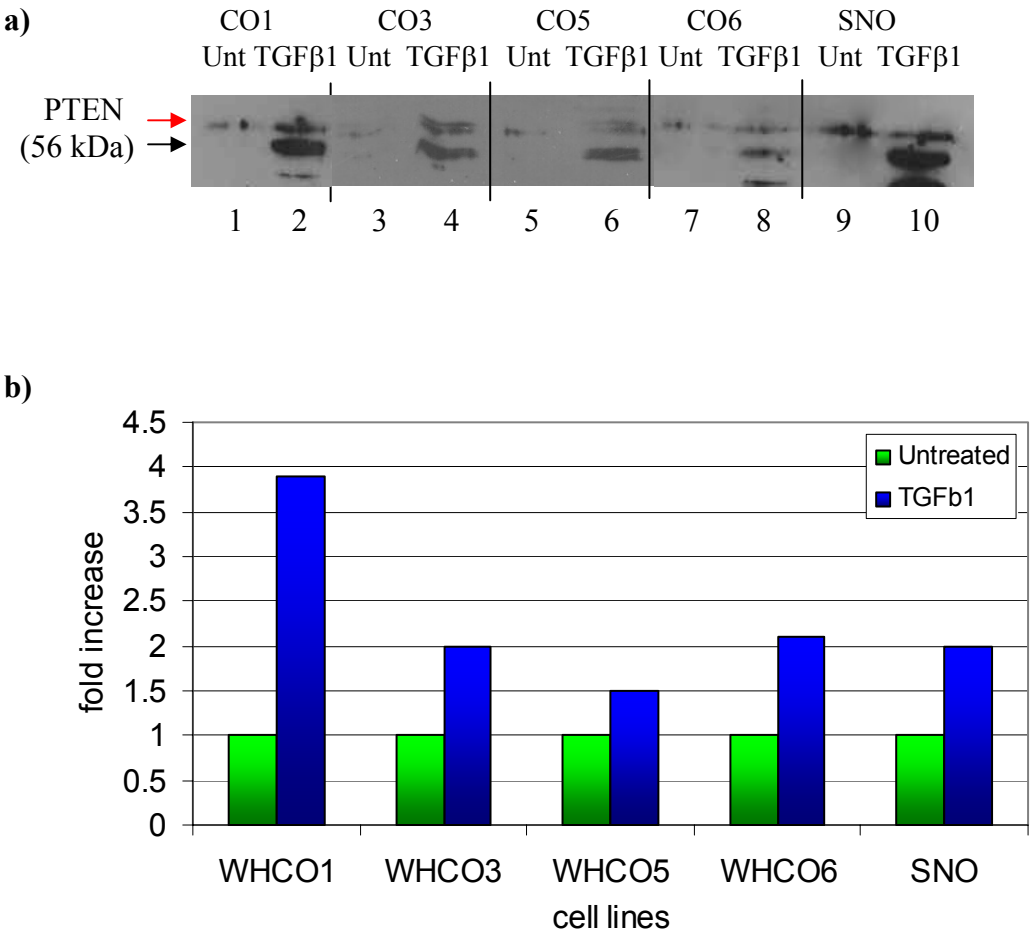


Figure 18: The Effect of TGFβ1 on PTEN Expression Levels.

a) Western blot analysis of PTEN expression (red arrow) utilising a rabbit anti-PTEN antibody (1:10000) following exposure to TGFβ1 (1 ng/ml) for 24 hours in the five HOSCCs. Degradation prodcuts are indicated by the black arrow. Lane 1 – untreated WHCO1, lane 2 – TGFβ1-treated WHCO1, lane 3 – untreated WHCO3, lane 4 - TGFβ1-treated WHCO3, lane 5 – untreated WHCO5, lane 6 - TGFβ1-treated WHCO5, lane 7 – untreated WHCO6, lane 8 – TGFβ1-treated WHCO6, lane 9 – untreated SNO and lane 10 – TGFβ1-treated SNO. b) Densitometric analysis of PTEN protein expression levels. PTEN showed a marked increase in the presence of TGFβ1 in the WHCO1, WHCO3, WHCO5, WHCO6 and SNO cell lines with the WHCO5 cell line exhibiting the highest PTEN expression of 7.1 fold. Note: the expression levels are representative as a percentage of maximum per 10 μg of protein from concentrated fractions (included to normalise for loading variation). Experiments were repeated three times.

3.3.3 ILK and PTEN are Associated in HOSCCs

Due to the regulatory role PTEN plays with regards to ILK function, it stands to reason that these protein moieties should be in close proximity to one another. The approach taken to confirm this idea was co-immunoprecipitation analysis. PTEN was immunoprecipitated from protein cell lysates by precipitating PTEN utilising an antibody directed against PTEN. This immunocomplex was allowed to bind to protein G sepharose and was subsequently probed for ILK by Western blotting. A ILK band was detected in PTEN-immunoprecipitates at the correct molecular weight of 59 kDa in all five cells examined (Figure 19a**,** purple arrow). To ensure that the identified bands were of the appropriate molecular weight corresponding to ILK, a WHCO1 cell lysate was included as a positive control. The positive control was separated in conjunction with the immunoprecipitated samples. It must be mentioned that in all the immunoprecipitated fractions, nonspecific bands presumed to be IgG of both the heavy and light chain (see also Kim *et al*., 2000) were also nonspecifically identified (Figure 19**,** blue and green arrows).

However, when the reverse experiment was performed (i.e. ILK immunoprecipitated and probed for PTEN), a PTEN band was not observed. A smaller molecular weight protein product was present, which was identified as the small chain of IgG at an approximate molecular weight of 30 kDa (Figure 19b**,** blue arrow). In spite of PTEN not being present in ILK immunoprecipitates, the identification of ILK in PTEN immunoprecipitates suggests a novel interaction between these two moieties. This supports the above data that a protein interaction does indeed occur between ILK and PTEN in HOSCCs. In both co-immunoprecipitation experiments, cell lysates were preabsorbed with protein G sepharose to prevent non-specific associated products (see Methods and Materials).

3.3.4 PTEN and ILK Are Similarly Distributed

Having identified a physical relationship between ILK and PTEN, it is reasonable to presume that these signalling moieties move to similar cellular distributions. Since we considered the influence of PTEN on ILK in particular, immunofluorescence studies were conducted, which confirmed PTEN localisation to be similar to that of ILK. In chapter 2 Figure 10 it was revealed that although ILK was shown to be largely

distributed in the cytoplasmic/nuclear region, with variable amounts observed at the membrane (Figure 20, red arrows). PTEN closely followed this distribution pattern, with the majority of PTEN occurring throughout the nuclear region (but not at FAs) (Figure 20**,** blue arrows). A smaller proportion of PTEN was also noted at a cytoplasmic level and is particularly apparent in the WHCO1 cell lines (Figure 20, red arros). The localisation pattern of PTEN in these HOSCCs is similar to that of previous studies, which have shown PTEN to be largely cytosolic and even nuclear in some cells (Walker *et al*., 2004).

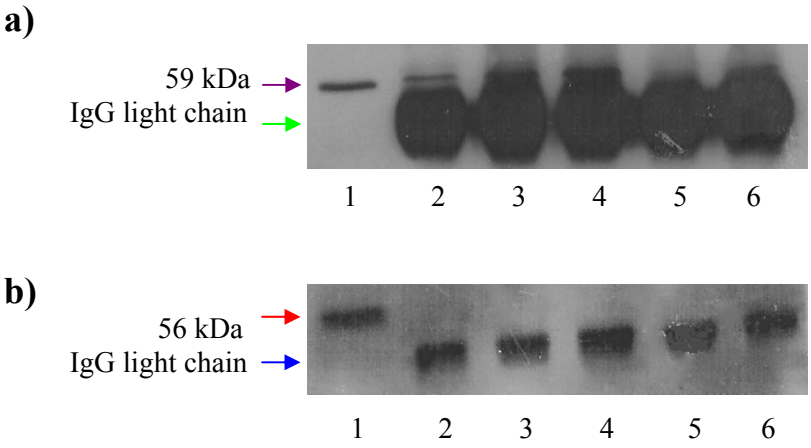


Figure 19: The Association between PTEN and ILK.

a) An ILK band of approximately 59 kDa (indicated by purple arrow) was identified following protein G sepharose immunoprecipitation of PTEN and Western blotting with either a rabbit anti-ILK (1:1500) or a rabbit anti-PTEN antibody (1:10000). The light chain of IgG was also identified (indicated by green arrow). Lane 1 – Control WHCO1, lane 2 – WHCO1, lane 3 – WHCO3, lane 4 – WHCO5, lane 5 – WHCO6 and lane 6 SNO. b) PTEN was not identified when ILK was immunoprecipitated utilising protein G sepharose and subsequently probed for PTEN. PTEN was however observed in the control WHCO1 protein extract (indicated by red arrow). Lane 1 – control WHCO1, lane 2 – WHCO1, lane 3 – WHCO3, lane 4 – WHCO5, lane 5 – WHCO6, lane 6 – SNO.

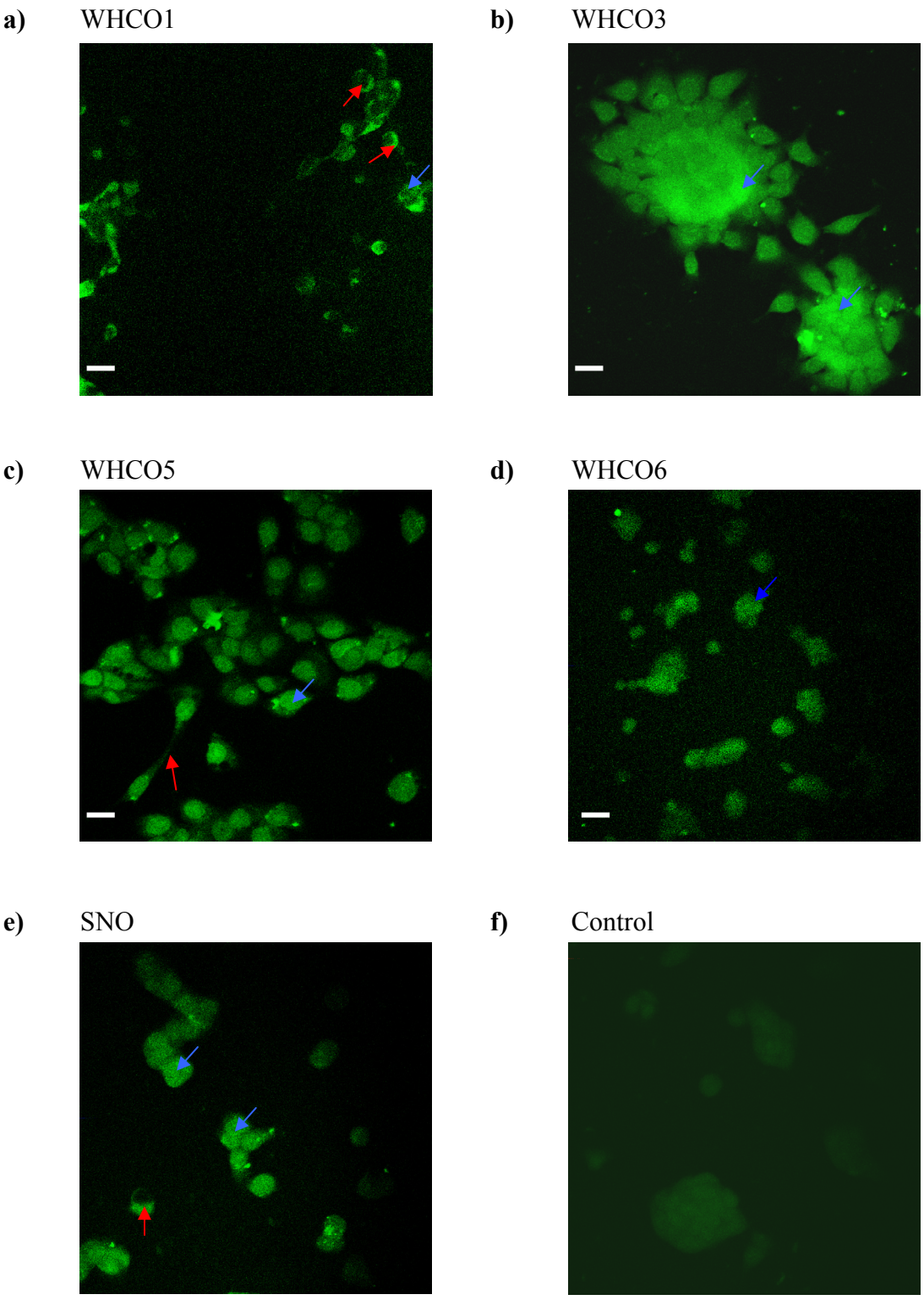


Figure 20: Indirect Immunofluorescence of PTEN in HOSCCs.

a-e) PTEN expressed by HOSCCs showing variable amounts of PTEN distribution with majority of PTEN being located at a nuclear region (blue arrows). A proportion of cytoplasmic localisation was also observed and is particularly visible in the WHCO1 cell line (red arrows). f) Negative control (excluding FITC-conjugated antibody) indicating diffuse staining. Bar represents 10 μm.

3.4 Discussion

Signal transduction events provide a communication network between intracellular and extracellular compartments of cells. Protein phosphorylation is a critically important posttranslational modification, which lies at the heart of the regulatory mechanism controlling cellular activities (Yoganathan *et al*., 2000; Cordes and van Beuningen, 2003; Wu, 2005). These events are regulated by protein kinases and protein phosphatases. Protein kinases are particularly pertinent to tumourigenesis since many cancers are known to express dysregulated kinase activities, examples to date being, colon cancer, breast cancer, lung cancer, and leukaemia's (Yoganathan *et al*., 2000; Cornillon *et al*., 2003; Khyrul *et al*., 2004; Tsuboi *et al*., 2004; Fang and Richardson, 2005).

This study has focused on a key protein kinase, ILK, which is known to be essential not only during normal cellular processes but in addition, critical in the development of metastatic spread of malignant cells (Persad *et al*., 2000; Attwell *et al*., 2003; Khyrul *et al*., 2004; Aoyagi *et al*., 2005). ILK regulates numerous signalling molecules, including GSK3β and PKB (Hannigan *et al*., 1996; Troussard *et al*., 2000; Gary *et al*., 2003), which elicit a wide variety of cellular responses including growth of epithelial cells, apoptosis and cell differentiation (Hannigan *et al*., 1996; Huang *et al*., 2000; Attwell *et al*., 2003; Chun *et al*., 2005). ILK is able to decrease apoptotic tendencies in cells via PKB stimulation and increase invasion and proliferation processes through GSK3β inhibition, thus implicating ILK in tumourigenesis. The final two data chapters of this study are concerned with growth factor effects on ILK kinase activity, the expression of ILK in the nucleus and the role of ILK activity during cell-ECM adhesion, since these oesophageal SCC cell lines in question are known to be highly metastatic most likely through their ability to breach cell-ECM contacts.

ILK isolation accomplished via immunoprecipitation revealed an ILK band of 59 kDa (see Figure 14a). Immunoprecipitated ILK was apparent for both protein G sepharose beads and *S.aureus*, although the formalin fixed intact *S.aureus* cells were more effective in immunoprecipitating the ILK immune complex. In most strains of *S.aureus*, a covalent link attaches protein G to the cell wall and is able to bind immunoglobulin molecules with high affinity (Goding, 1978). IgG is the principal molecule that binds although IgM and IgA are also capable of binding in certain species (Goding, 1978). An

explanation for this strong reaction may be that per volume, *S.aureus* cells contain a larger concentration of protein G in comparison to purified protein G sepharose beads, resulting in relatively more ILK binding to the *S.aureus* cells.

Although *S.aureus* cells seemed to be the obvious choice when proceeding with the MBP-based kinase assays, we avoided their use since the possibility exists that other protein molecules on their cell surfaces could potentially phosphorylate MBP and consequently produce false positives. Protein G is the active component of *S.aureus* in terms of antibody interactions and therefore purified protein G sepharose beads seemed the obvious choice for the ILK kinase assays (Hannigan *et al*., 1996; Attwell *et al*., 2003). Protein G sepharose beads only possess epitopes to bind γ-globulins and do not contain any other protein constituents which may be able to phosphorylate the MBP substrate.

In Chapter 2 it was shown that ILK is able to associate with both the integrin and growth factor receptors suggesting that ILK provides a link between these two classes of receptors. As has been outlined above, the kinase activity of ILK plays a major role in signal transduction pathways that influence many cellular processes and is directly involved in directing the attachment of cells to the ECM by regulating β integrins (Li *et al*., 1999; Zervas *et al*., 2001; Oloumi *et al*., 2004). However, conflicting results have been published with respect to the activity of ILK, as it is not known for certain whether ILK possesses full kinase capabilities (Wu and Dedhar, 2001)**.** This follows a study by Lynch *et al*. (1999), where ILK kinase activity was undetected in ILK immunoprecipitates. Recent evidence indicates that ILK acts as a kinase in certain circumstances where it is capable of phosphorylating substrates such as PKB and GSK3β (Obara *et al*., 2004; Quélo *et al*., 2004; Grashoff *et al*., 2004).

Since the growth factor EGF was shown to increase ILK expression in oesophageal SCCs, it was of interest to examine the kinase activity of ILK and to ascertain the effects of growth factor on its activity. This would perhaps provide a basic understanding of the mechanism/s by which growth factors regulate ILK in carcinoma of the oesophagus. Furthermore, discernment of ILK activity would indicate the propensity of these oesophageal carcinoma cells to metastasise since it has been

established that increased ILK activity correlates with increased invasiveness (Novak *et al*., 1998; Wu *et al*., 1998; Troussard *et al*., 2000).

The activation or inhibition of ILK activity is cell-type dependent and can be modified by growth factors (Delcommenne *et al*., 1998; Novak *et al*., 1998). The effect/s of growth factors on ILK kinase activity has been previously demonstrated where it was shown that rapid stimulation of ILK activity by insulin and PDGF occurred in intestinal epithelial cells (Delcommenne *et al*., 1998). In addition, these authors showed that inhibition of PI3K inhibited insulin-induced ILK activation and that overexpression of a subunit of PI3K activated ILK *in vivo* demonstrating that the PH motif of ILK participates in regulation of ILK kinase activity in response to insulin (Delcommenne *et al*., 1998). Thus, the link that exists between integrins and growth factor mediated signalling is well established (Dedhar *et al*., 1999; Janji *et al*., 2000; Grashoff *et al*., 2004; Kaneko *et al*., 2004). ILK is a crucial mediator in this relationship, as it physically interacts with the β integrins through its kinase domain and links to the growth factor receptors via PINCH and Nck-2 (Nikolopoulos and Turner, 2002).

The mechanisms by which growth factors regulate ILK activity are almost certainly PI3K-dependent since PI3K has been shown on numerous occasions to activate ILK (Delcommenne *et al*., 1998; Dedhar *et al*., 1999; Somasiri *et al*., 2000). Furthermore, ILK is activated in a response to a variety of extracellular stimuli, including cytokines, growth factors, as well as adhesion to extracellular matrices (Delcommenne *et al*., 1998) and inhibition of the lipid phosphatase PTEN, which is known to be a negative regulator of ILK expression (Persad *et al*., 2000; Attwell *et al*., 2003; Grashoff *et al*., 2004).

In this study, growth factors increased ILK protein expression, but caused both increases and decreases in ILK activity in the oesophageal SCC cell lines examined (see Figure 15b). While these HOSCCs exhibit increased EGFR, not all cell lines responded in the same way to EGF exposure. The WHCO3 cell line, in response to EGF and TGFβ1, showed an increase in ILK activity (increased ILK protein expression), whereas the WHCO1, WHCO6 and SNO cell lines all exhibited decreased ILK activity (protein expression increased). The increase in ILK protein expression noted in cell lines WHCO1, WHCO6 and SNO (see Chapter 2, Figure 7), with a decrease in ILK activity in the same cell lines, suggests that PI3K concentrations are not similarly increased by

growth factors. The consequence of this may well be insufficient PI3K to activate the elevated ILK concentrations. Assuming that PI3K levels have remained unchanged, the substantial increase in ILK activity in the WHCO3 cell line was attributed to decreased PTEN since TGFβ1 has been shown to downregulate PTEN mRNA in keratinocytes (Janji *et al*., 2000).

Indeed EGF was shown to reduce PTEN expression levels in the WHCO3 cell line by 3 fold confirming the notion that the ILK-induced kinase activity by EGF is a direct result of reduced PTEN expression (see Figure 17b). In contrast it was thought that EGF and TGFβ1 were stimulating PTEN activity in the WHCO1 and SNO cell lines. Indeed, EGF was shown to increase PTEN expression levels in these cell lines, corroborating our results that EGF regulates ILK activity via PTEN. In the case of WHCO5 and WHCO6 however, PTEN expression levels in the presence of EGF did not correspond to EGF-treated ILK activity levels. While EGF caused ILK activity to increase in WHCO5 cells, this cell line also showed increased PTEN expression when exposed to EGF. Similarly, in the WHCO6 cell line, EGF caused a decrease in ILK activity with a concomitant decrease in PTEN expression levels (see Figure 17b). This implies that another mechanism is in place for the EGF-mediated effects on the WHCO5 and WHCO6 cell lines.

In response to the increased EGFR that have been reported with regards to the oesophageal SCC lines (Veale and Thornley, 1989), one could argue that the WHCO6 cell line is at an upper limit of growth factor response. Thus, growth factor treatment of these cell lines would not elicit any further cellular responses, a consequence being that ILK would not be activated, accounting for the low levels of ILK kinase activity observed in this cell line. This speculation is supported by the suggestion that in fibroblasts higher TGFβ1 concentration results in inhibition of both cell migration and proliferation (Moses *et al*., 1990).

Unlike EGF, TGFβ1 reduced ILK activity in the WHCO5 cell line and produced a similar substantial increase in ILK activity in the WHCO3 cell line. TGFβ1 has previously been shown to elicit a dual effect on cells, either inhibiting cell growth in cancers such as colon, breast and squamous cell carcinoma (Zugmaier *et al*., 1989; Wu *et al*., 1992; Fujimoto *et al*., 2001), or stimulating cell proliferation in malignancies of

squamous carcinoma cell lines and prostate carcinomas (Ozanne *et al*., 1986; Steiner and Barrak, 1992; Fujimoto *et al*., 2001).

A vast increase in ILK activity occurred in the presence of TGFβ1 in the WHCO3 cell line. One would then expect TGFβ1-treated WHCO3 cells to demonstrate a decrease in PTEN expression levels. However, TGFβ1 resulted in an increase in PTEN expression levels in this cell line, suggesting that PTEN is not involved in TGFβ1-mediated regulation of ILK activity in this cell line. On the other hand the results obtained in the WHCO1, WHCO5, WHCO6 and SNO cell lines could be due to the effect of TGFβ1 stimulating PTEN expression in these cells as TGFβ1 increased PTEN expression by as much as 7.1 fold (see Figure 18b).

From these data it is apparent that PTEN does not play a role in the EGF-mediated effects on ILK kinase activity in the case of the WHCO5 and WHCO6 cell lines. Furthermore, when exposed to TGFβ1, WHCO3 cells do not respond in a fashion which would suggest an involvement of PTEN. Another phosphatase capable of inhibiting ILK function which is known as ILK-alkaline phosphatase (ILKAP) may be influenced by the action of growth factors. ILKAP is a protein phosphatase, known to directly associate with ILK, thereby allowing for the inhibition of ILK activity (Leung-Hagesteijn *et al*., 2001; Kumar *et al*., 2004). While ILKAP is able to downregulate integrin- and growth factor-stimulated ILK activity (Leung-Hagesteijn *et al*., 2001), no mention is made concerning the effect of growth factors upon the activity of ILKAP. It is conceivable that EGF and TGFβ1 may be exerting their effects on ILK kinase activity via ILKAP. Thus, for WHCO3 and WHCO5 cells, ILKAP may be downregulated by TGFβ1 (WHCO3) and EGF (WHCO5) accounting for the increases in ILK activity. In the WHCO6 cell line, EGF may also stimulate ILKAP, thus decreasing ILK activity.

The discrepancies that were observed with respect to growth factor modulation of ILK activity within these cell lines may be of particular relevance to oesophageal squamous cell carcinoma. A reasonable assumption may be that cell adhesion is decreased through a disruption of ILK activation by PI3K in the WHCO1, WHCO5, WHCO6 and SNO cell lines. The assumption made here of PI3K levels being unaffected by growth factors would be in disagreement to previous reports. Indeed, PI3K has been shown to be

increased via EGF in malignancies of head and neck squamous cell carcinomas (HNSCC), ovarian tumour cell lines and prostate carcinomas (Ellerbroek *et al*., 2001; Bancroft *et al*., 2002) with a similar interaction occurring between TGFβ1 and PI3K (Gotzmann *et al*., 2002). Another possible explanation could be due to the single time point analysis that was utilised. A time course analysis would perhaps clarify the discrepancies that were observed with regards to growth factor effects on ILK acitivity.

It was clear that in the majority of the cell lines examined, PTEN played a major role in the regulation of growth factor-mediated changes in ILK activity. This implied that an association between ILK and PTEN existed in these cell lines. This was confirmed by co-immunoprecipitation analysis where a physical relationship between ILK and PTEN was identified (see Figure 19a**)**. Surprisingly, when ILK was immunoprecipitated and probed for the presence of PTEN, no PTEN band was observed (see Figure 19b). There are various explanations for PTEN not being identified in ILK-precipitated lysates. ILK may not be associating with PTEN in a 1:1 molar ratio. In other words, when ILK was immunoprecipitated from cell lysates, the concentration of bound PTEN may have been very low, which would account for PTEN not being identified in ILK lysates. In addition, the binding epitopes of PTEN may have been masked, preventing antibody recognition and binding. Subsequently no PTEN product would be observed. However, further support of the notion that ILK and PTEN are associated in these HOSCCs was demonstrated by the similar localisation patterns being observed between PTEN and ILK (see Figure 20).

Although not examined, the role of this interaction may include phosphorylation-based regulation of PTEN activity. Indeed, posttranslational regulation of PTEN is known to occur via serine/threonine phosphorylation (Al-Khouri *et al*., 2005). Studies propose that the phosphorylation of PTEN serves to maintain PTEN in a conformation that blocks membrane association, which could regulate its biological activity (Walker *et al*., 2004). For a while now, Casein kinase II (CKII) has been thought to be the predominant molecule capable of phosphorylating PTEN (Torres and Pulido, 2001; Al-Khouri *et al*., 2005; Gericke *et al*., 2006). However, CKII is not capable of phosphorylating all sites on PTEN and GSK3β has also been shown to be capable of phosphorylating PTEN (Al-Khouri *et al*., 2005). This phosphorylation of PTEN by CKII has important consequences with regards to the biological activity of PTEN, as well as modulating its

stability to proteasome-mediated degradation (Vazquez *et al*., 2000; Torres and Pulido, 2001.

Since ILK is a serine/threonine kinase phosphorylating substrates such as PKB and GSK3β (Cordes and van Beuningen, 2003), the physical association between PTEN and ILK could serve to allow for ILK-mediated PTEN serine phosphorylation. If this were indeed the case, this would suggest that a feedback mechanism would be in place where PTEN could regulate ILK activity via PI3K and ILK in turn could directly regulate PTEN activity (see Figure 21 below). The determination of PTEN expression levels in

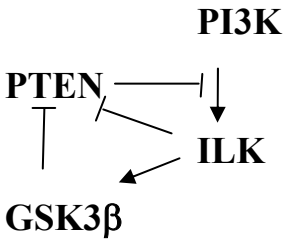


Figure 21: Proposed Feedback Mechanism of ILK Mediated Regulation of PTEN. ILK activation occurs via PI3K phosphorylation. ILK in turn is capable of regulating PTEN phosphorylation. This occurs either indirectly via GSK3β or directly whereby ILK associates with PTEN.

these cell lines has clearly shown that in the WHCO1, WHCO5 and SNO cell lines growth-factor mediated changes in ILK activity depends on the ability of EGF and TGFβ1 to influence PTEN expression. It seems that the WHCO3 cell line has lost its sensitivity to the regulatory mechanisms of EGF and TGFβ1 and if this is indeed the case, elucidating TGFβ1 function in this cell line becomes of paramount importance. Furthermore, it is suggested here that an inverse relationship exists between ILK protein expression and activity in response to growth factors.

The data presented here support evidence that HOSCC ILK is an active kinase (Wu and Dedhar, 2001) and indicates a distinct mechanism of regulation of ILK activity by growth factors within this class of SCCs (Delcommenne *et al*., 1998). These effects may have further consequences in altering cell-ECM adhesion events as an outcome of altered ILK activity. Although not tested, the ability of ILK to phosphorylate MBP in all

the oesophageal SCC cell lines suggests that in addition to associating with the β_3 integrin, ILK may be regulating the β_1 integrin subunit since MBP contains a β_1 integrin sequence. The focus of the chapter 5 will examine the role played by the activity of ILK in cell-ECM adhesion in these cell lines.

The proceeding chapter however will examine the nuclear localisation of ILK that was displayed in chapter 2. In this regard, the caveolae protein, caveolin-1 is important to ILK-mediated nuclear localisation and thus forms the focus of chapter 4.

Chapter 4

ILK Subcellular Localisation is mediated by Caveolin-1

4.1 Introduction

Caveolae ('little caves') were first described morphologically in the 1950's as non-coated vesicles (Razani *et al*., 2000). Caveolae exist as distinct plasma membrane invaginations, which allow for the organisation and recruitment of signal transduction machinery, the transport of cholesterol, as well as the uptake of vitamins and toxins (Pol *et al*., 2000; Park *et al*., 2000; Wiechen *et al*., 2001; Hnasko and Lisanti, 2003). They are vesicular organelles that are especially prevalent in terminally differentiated cell types including adipocytes, endothelial cells and myocytes, but have been identified in the majority of cell types (Couet *et al*., 1997; Razani *et al*., 2000; Epand *et al*., 2005).

Traditionally, caveolae were implicated in transcytosis and potocytosis (Razani *et al*., 2000). Recently though, it has been noted that caveolae are enriched in cell surface receptors such as those for EGF and PDGF and contain intracellular signalling molecules, including Ras and Src (Couet *et al*., 1997; Anderson *et al*., 1998; Kim *et al*., 2002; Tagawa *et al*., 2005). The identification of these signalling molecules now suggest that caveolae further participate in signal transduction events (Kim *et al*., 2000; Williams *et al*., 2004; Lee *et al*., 2005). It has been proposed that caveolae serve as crucial mediators of transport processes where they store inactive signalling molecules for regulated activation and also to facilitate cross-talk between distinct signalling cascades (Couet *et al*., 1997; Williams *et al*., 2004; Epand *et al*., 2005).

The major structural component of caveolae membrane domains are the caveolin proteins, a family of 21-24kDa integral membrane proteins (Liu *et al*., 2001; Williams *et al*., 2004; Epand *et al*., 2005; Orlichenko *et al*., 2005). Three caveolin genes exist (CAV-1, CAV-2 and CAV-3), but it is CAV-1, encoding for caveolin-1 which is of particular interest, since it is able to associate with numerous cell surface growth factor receptors (Couet *et al*., 1997; Lin *et al*., 2005) and cell adhesion receptors (Wary *et al*., 1998; Kim *et al*., 2002; Liu *et al*., 2001). It has been noted, that the binding of caveolin-

1 to these signalling moieties results in an inhibition of their *in vitro* kinase activity, suggesting that caveolin-1 serves as a negative regulator of many signalling proteins (Chun *et al*., 2005).

The interaction of caveolin-1 with EGFR is particularly interesting, since EGF stimulates the recruitment of multiple signalling molecules to caveolae (Kim *et al*., 2000; Epand *et al*., 2004). Furthermore, EGF is known to tyrosine phosphorylate caveolin-1, but surprisingly, the role of tyrosine-phosphorylated caveolin-1 remains largely unknown (Labrecque *et al*., 2004). Studies have suggested however, that tyrosine-phosphorylated caveolin-1 is specifically associated with Grb7, a signalling protein involved in growth factor-induced cell migration (Labrecque *et al*., 2004).

Although caveolin-1 is expressed in the majority of cell types, its expression is downregulated, or absent in oncogenically transformed NIH/3T3 cells, as well as in human breast, colon carcinoma cell lines, and human mammary and lung carcinoma cells (Kato *et al*., 2004; Koleske *et al*., 1995; Engelman *et al*., 1997; Lee *et al*., 1998 cited in Chun *et al*., 2005; Racine *et al*., 1999; Liu *et al*., 2001). However, tissue studies of human breast and prostate cancers have supported a positive association of caveolin-1 expression with tumour initiation and progression, suggesting a tumour promoting function. These reciprocal functions suggest that caveolin-1 may have a variable role depending on the stage of carcinogenesis (Kato *et al*., 2004). Caveolin-1has been previously identified in carcinoma of the oesophagus, where immunoreactivity was shown to correlate with a poor survival rate (Kato *et al*., 2004).

While caveolin-1 possesses the qualities of a scaffolding protein, more interesting is its role as a modulator of cell signalling (Razanai *et al*., 2000). Recently it has been shown that a bona fide caveolin-binding motif exists within the catalytic domain of ILK, which is conserved in the ILK family (Chun *et al*., 2005). This has obvious relevance to this study as caveolin-1 may be directing the signalling of ILK in HOSCCs. The cytosolic retention of ILK could also be attributed to the binding of caveolin-1 since a putative nuclear localization signal (nls), shown to exist within ILK, blocked upon binding to caveolin-1 (Chun *et al*., 2005). Furthermore, in this same study, caveolin-1 binding also resulted in downregulation of the autophosphorylation activity of ILK (Chun *et al*., 2005).

The preceding data chapter dealt largely with how ILK serves as a link between integrin, and growth factor signalling pathways. Due to the cytoplasmic/nuclear distribution of ILK, demonstrated in Chapter 2, we postulated that the above association allows for the internalisation of ILK and subsequent shuttling to the nucleus in oesophageal carcinoma.

Interactions between caveolin-1 and EGFR have been clearly demonstrated in epidermoid carcinoma, human glioblastoma and squamous carcinoma (Kim *et al*., 2006), where EGF has been shown to cause a decrease in caveolin-1 protein expression (Lu *et al*., 2003). This led us to believe that EGF induced a downregulation of caveolin-1 expression in HOSCCs thus providing the necessary stimulus for ILK nuclear localisation. Moreover, we suggest that tyrosine phosphorylation of caveolin-1 provides the mechanism for the EGF-dependent downregulation of caveolin-1.

4.2 Materials and Methods

4.2.1 Cell Lines

As previously documented – see Chapter 2, Section 2.2.1

4.2.2 Antibodies

Caveolin-1 was specifically detected with a polyclonal antibody (Sigma, USA). A polyclonal rabbit anti-ILK antibody and a rabbit anti-caveolin-1 antibody were used in co-immunoprecipitation experiments. Polyclonal horseradish peroxidase (HRP)-bound anti-rabbit secondary antibody was used in Western blot experiments (Separations, SA). Anti-phosphotyrosine (Sigma, USA) was utilised in the detection of phosphorylated caveolin-1. Fluoroscine isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody from Chappel, USA were used in the immunofluorescence experiments.

4.2.3 Nuclear Extraction

As previously documented – see Chapter 2, Section 2.2.11

4.2.4 Protein Estimation

As previously documented – see Chapter 2, Section 2.2.6

4.2.5 Western Blotting Analysis

Samples from cell lines WHCO1, WHCO3, WHCO5, WHCO6 and SNO were resolved on 15 % SDS-PAGE gels. The molecular weight was ascertained utilising a PageRuler™ Prestained Protein Ladder (Fermentas, Canada), which was electrophoresed in conjunction with cell lysates. Samples were transferred to Nitrobind nitrocellulose transfer membrane (MSI, USA), in a BioRad Trans-Blot™ Cell at 400 mA for 3 hours (4 °C) in Western Blot Transfer Buffer (Appendix 1.4.5). After transfer was complete, the nitrocellulose membranes were rinsed twice with PBS (see Appendix 1.1.1), and stored overnight at 4 °C.

Each membrane was blocked in BLOTTO (see Appendix 1.4.1) for 1 hour and washed 6 times in PBS. The blots were incubated in either rabbit anti-caveolin-1 primary antibody (1:5000), anti-ILK antibody (1:1500) or anti-phosphotyrosine antibody (1:500) for 1 hour where the experiment required. Washing was performed 6 times at 5-minute intervals with PBS to remove any residual antibody. Membranes were incubated with a HRP-bound secondary anti-rabbit antibody (1:1500) for 1 hour in the dark. Once again membranes were washed 6 times at 5-minute intervals with PBS before being exposed to the Supersignal® West Pico Working Solution (see Appendix 1.4.2) from the West Pico Chemiluminescent Substrate Kit (Pierce, USA) for 5 minutes. Blots were sealed in polyethylene 'saran wrap' and exposed to hyperfilm™ MP autoradiography film (Amersham, UK) for 1.5 minutes. The film was developed in D19B developer (see Appendix 1.4.3) for 5 minutes, rinsed briefly in H_2O before fixing (see Appendix 1.4.4) for 5 minutes. The experiment was repeated three times.

4.2.6 Indirect Immunofluorescence Microscopy

Similar to ILK localisation, caveolin-1 distribution was analysed utilising indirect immunofluorescence microscopy. Briefly, WHCO1, WHCO3, WHCO5, WHCO6 and SNO cell lines grown to 80 % confluency were seeded onto sterile glass coverslips. The coverslips were washed five times in cold PBS, fixed with 4 % paraformaldehyde (Appendix 1.6.1) for 30 minutes and washed again. Coverslips were immersed in 0.25 % Triton X-100 (Appendix 1.6.2) for 10 minutes, washed twice with PBS, dipped into dH_2O, and allowed to dry partially. Two circles or wells per coverslip were drawn with the DAKO® pen. One well per coverslip was incubated with a polyclonal rabbit anti-caveolin-1 primary antibody (1:500) for one hour. The other well was incubated in PBS (control). This was followed by a thorough washing in PBS as before. All circled areas were incubated with an anti-rabbit Fluoroscine Isothiocyanate (FITC)-conjugated anti-rabbit antibody (1:1000). Both wells were incubated for one hour in the dark. The coverslips were washed six times with sterile PBS and then mounted with Elvanol mounting agent (Appendix 1.6.3) onto sterilised glass slides. All incubations were carried out in a humidity chamber to prevent desiccation. Slides were viewed under a Zeiss LSM 410 confocal microscope (FITC excitation 490, emission 525).

4.2.7 Co-immunoprecipitation Analysis of ILK and Caveolin-1

Cell lysates were precleared with protein G sepharose overnight at 4 °C, centrifuged at 3000 rpm and the supernatants transferred to fresh eppendorf tubes. Co-immunoprecipitation analysis was performed utilising a protein G immunoprecipitation kit (Sigma, USA). Nuclear cell lysates were preabsorbed with protein G sepharose overnight at 4 °C. Cell lysates were centrifuged at 3000 rpm and the supernatant transferred to fresh eppendorf tubes. Nuclear cell lysates (100 μg) were incubated with undiluted polyclonal anti-rabbit caveolin-1 antibody (2 μl) overnight at 4 °C. The following day protein G sepharose beads were washed in 1 ml ice cold PBS. The slurry was centrifuged for 30 seconds at 12000 rpm in a Sorvall® MC 12 V centrifuge and the supernatant decanted. This washing procedure was repeated twice. Thereafter, washed protein G beads in PBS were transferred to the protein-antibody complex and incubated overnight at 4 °C. Immunoprecipitation buffer (500 μl) was then added to the immune complexes containing protein G sepharose and centrifuged for 30 seconds at 12000 rpm. The supernatant was decanted and the washing procedure repeated twice more. After the final wash, 70 μl 2× lysis buffer was added and the immune complexes were boiled for 5 minutes. Samples were then centrifuged for 30 seconds at 12000 rpm and immunoprecipitated caveolin-1 supernatants were transferred to fresh eppendorf tubes. Samples were stored at –20 °C.

4.2.8 Densitometric Analysis

Labworks ™ Image Acquisition and Analysis software (Labworks version 4.5) was used for densitometric analysis to determine quantitatively the concentration level of caveolin-1 in the Western blots. **Note:** the densitometric analysis results are an average of 3 repeated experiments.

4.3 Results

4.3.1 Membrane/Cytoplasmic and Nuclear Caveolin-1 Protein Levels Following EGF Exposure

A caveolin-1 band of 21 kDa was specifically identified in all the HOSCC cell lines by Western blotting utilising an anti-caveolin-1 polyclonal antibody **(**Figure 22a**)**. Densitometric analysis per μg of total protein Triton X-100 extracts loaded revealed that expression levels of caveolin-1 protein were similar across the WHCO1, WHCO3 and WHCO6 cell lines. Expression levels of caveolin-1 were greatest in the SNO cell line, 1.3 fold above the average of the WHCO1, WHCO3 and WHCO6 cell lines. Lowest expression levels were noted in the WHCO5 cell line, which showed a disparately low concentration of caveolin-1. Caveolin-1 expression in the WHCO5 cell line was approximately 8 fold lower than the average of the other four HOSCC cell lines (Figure 22b).

Treating the HOSCC cells with EGF (10 ng/ml) tended to cause a reduction in caveolin-1 protein expression over time in the WHCO3, WHCO5, WHCO6 and SNO cell lines (Figure 23a). WHCO1 cells initially also exhibited decreased caveolin-1 expression. However, prolonged exposure to EGF however, caused caveolin-1 expression levels to increase. The greatest reduction in caveolin-1 expression was noted in the WHCO6 and SNO cell lines, which showed a steady decrease in caveolin-1 expression throughout the EGF exposure period. Although the WHCO6 cell line demonstrated a 1.3 fold increase in caveolin-1 expression following EGF exposure for 1 hour, in contrast to the 1.7 fold reduction observed at 0.5 hours, an overall reduction was observed during the course of EGF treatment. Indeed, a reduction in caveolin-1 expression of 7 fold was noted at 6 hours EGF treatment. EGF exposure for 0.5, 1 and 3 hours respectively in SNO cells, caused caveolin-1 expression to decrease substantially, on average 3.3 fold lower than the untreated membrane/cytoplasmic cell lysate. SNO caveolin-1 expression continued to decrease in that a further reduction of 10 fold was observed after 6 hours EGF treatment (Figure 23b).

With regard to the WHCO5 cell line, an initial decrease, after 0.5 hours EGF exposure, was noticed followed by a subsequent increase in caveolin-1 expression as the cells

were exposed to EGF for greater periods of time. However, throughout the time course, caveolin-1 expression levels were at all times lower than untreated cell lysates. In WHCO5 cells, an initial reduction of 1.4 fold in caveolin-1 expression was noted, which was maintained through to 1 hour EGF exposure. Thereafter, caveolin-1 membrane/cytoplasmic expression levels increased by as much as 1.3 fold at the 3 hour treatment period in comparison to the 0.5 hour period (Figure 23b). WHCO1 cells showed a 1.7 fold reduction in caveolin-1 expression following 0.5 hours EGF in comparison to the untreated control. Following prolonged exposure to EGF however caused caveolin-1 expression levels to increase, where by 6 hours EGF treatment, caveolin-1 had reached expression levels similar to that of the untreated cell lysate (Figure 23b).

The results above support the claim by Lu *et al*., (2003) that a constitutive downregulation of caveolin-1 occurs in response to EGF in human epidermoid carcinoma cells. Furthermore, due to the noted decrease in caveolin-1 expression, it seemed reasonable to assume that caveolin-1 was redistributed to the nucleus following EGF treatment. Examination of this possibility utilised Western blotting of nuclear fractions exposed to EGF for 0.5 hours. Nuclear caveolin-1 concentration levels increased following exposure to EGF in the majority of cell lines examined (Figure 24a). The 0.5 hour EGF time period was chosen since this time period seemed to elicit the greatest reduction in terms of caveolin-1 expression at the membrane/cytoplasm. The largest increases in nuclear concentrations were noted in the WHCO1 and SNO cell lines, producing an increase of 1.2 and 1.4 fold respectively, the WHCO5 cell line only showed a small increase of 1.1 fold in nuclear caveolin-1. The WHCO6 cell line on the other hand, showed no increase in nuclear caveolin-1 concentration, whereas the WHCO3 cell line showed a 1.3 fold decrease in expression (Figure 24b). From these data it could be inferred that EGF treatment results in substantial changes in the cellular distribution of caveolin-1 in the WHCO5 and SNO cell lines, where nuclear concentrations of caveolin-1 are increased with a concomitant decrease in membrane/cytoplasm-associated caveolin-1. Since WHCO1 showed increases in caveolin-1 expression, both at the membrane/cytoplasm and nuclear regions in response to EGF treatment, it can be assumed that no redistribution occurs as a result of EGF. To substantiate these data, we subsequently analysed caveolin-1 distribution following EGF exposure utilising indirect immunofluorescence (Figure 27).

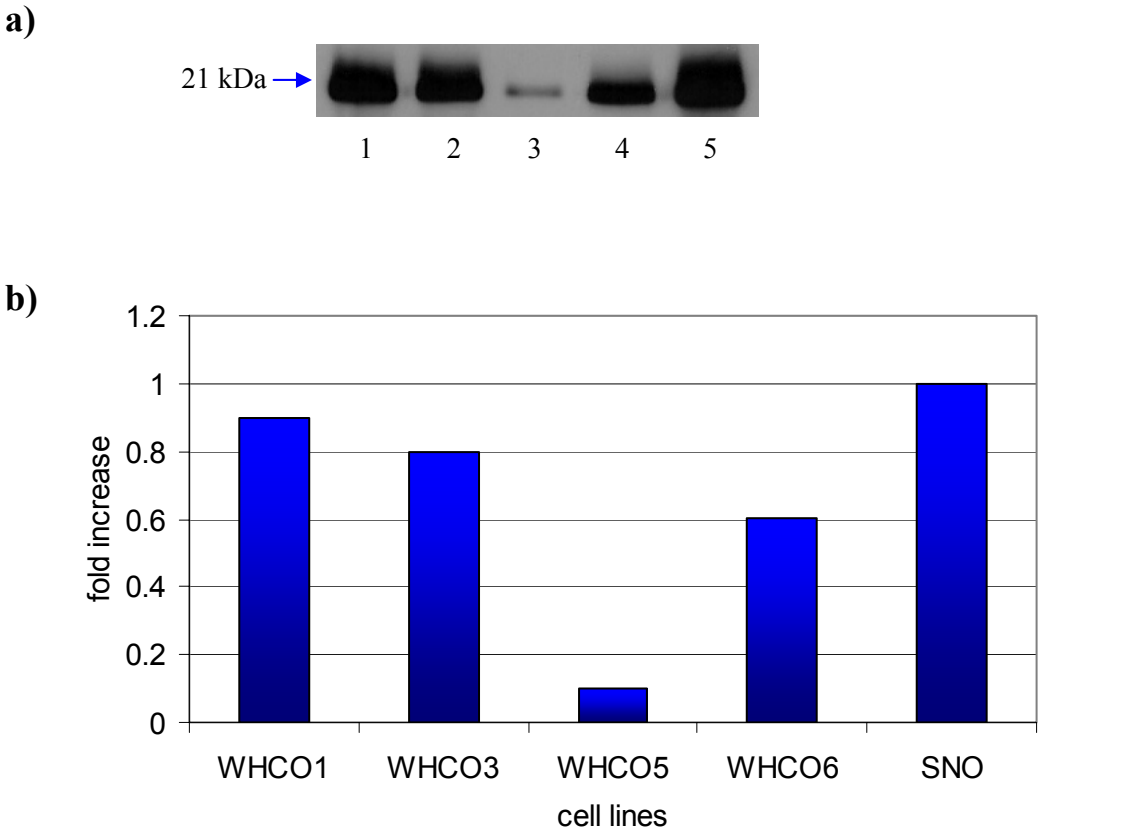
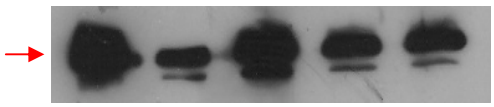


Figure 22: Membrane/Cytoplasmic Expression Levels of Caveolin-1 in HOSCCs. a) Western blot analysis of caveolin-1 expression utilising a rabbit anti-caveolin-1 antibody (1:5000). Caveolin-1 was identified at a molecular weight of 21 kDa in all HOSCCs (indicated by blue arrow). Lane 1 – WHCO1, lane 2 – WHCO3, lane 3 – WHCO5, lane 4 – WHCO6 and lane 5 – SNO. b) Expression levels of caveolin-1, following densitometric analysis revealed highest caveolin-1 expression in the SNO cell line and lowest caveolin-1 expression in the WHCO5 cell line. Note: the expression levels are representative as a percentage of maximum per 10 μg of protein from concentrated fractions (included to normalise for loading variation). Experiments were repeated three times.

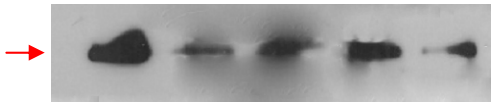
a)

WHCO1



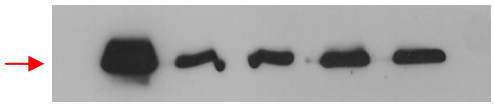
1 2 3 4 5

WHCO3



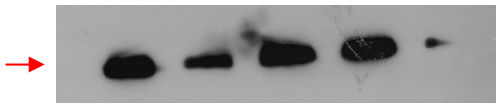
1 2 3 4 5

WHCO5



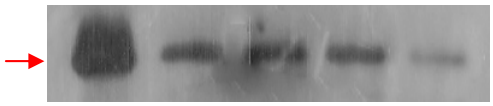
1 2 3 4 5

WHCO6



1 2 3 4 5

SNO



1 2 3 4 5

Please see next page for legend

b)

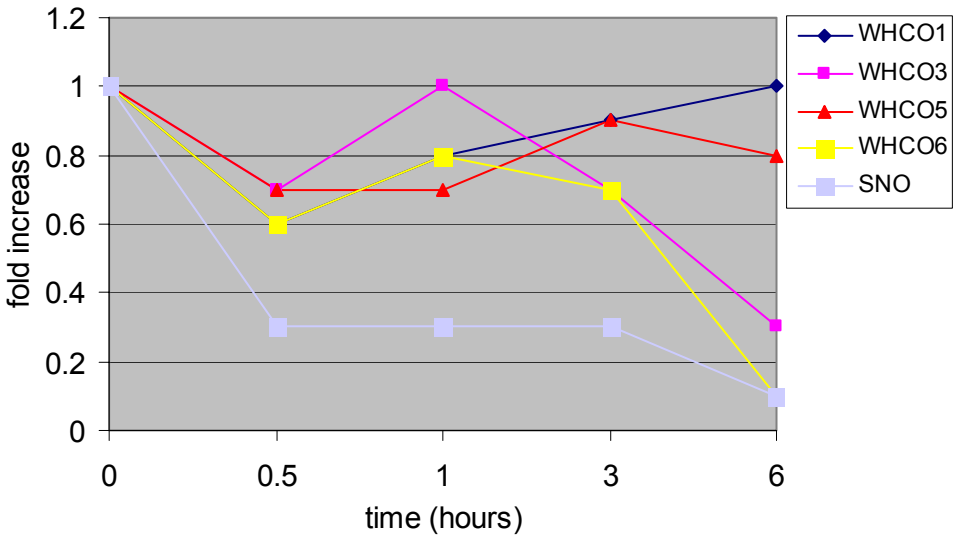


Figure 23: Caveolin-1 Expression Levels Following EGF Exposure.

a) Western blot of caveolin-1 expression utilising a rabbit anti-caveolin-1 antibody (1:5000) following exposure to EGF for 0, 0.5, 1, 3 and 6 hours respectively in the five HOSCCs (red arrows). Lane 1 – 0 hours EGF exposure, lane 2 – 0.5 hours EGF exposure, lane 3 – 1 hour EGF exposure, lane 4 – 3 hours EGF exposure, lane 5 – 6 hours EGF exposure. b) Expression levels of caveolin-1 following EGF exposure as analysed by densitometry. Throughout the EGF exposure period, caveolin-1 expression was reduced in all five HOSCCs Note: the expression levels are representative as a percentage of maximum per 10 μg of protein from concentrated fractions (included to normalise for loading variation). Experiments were repeated three times.

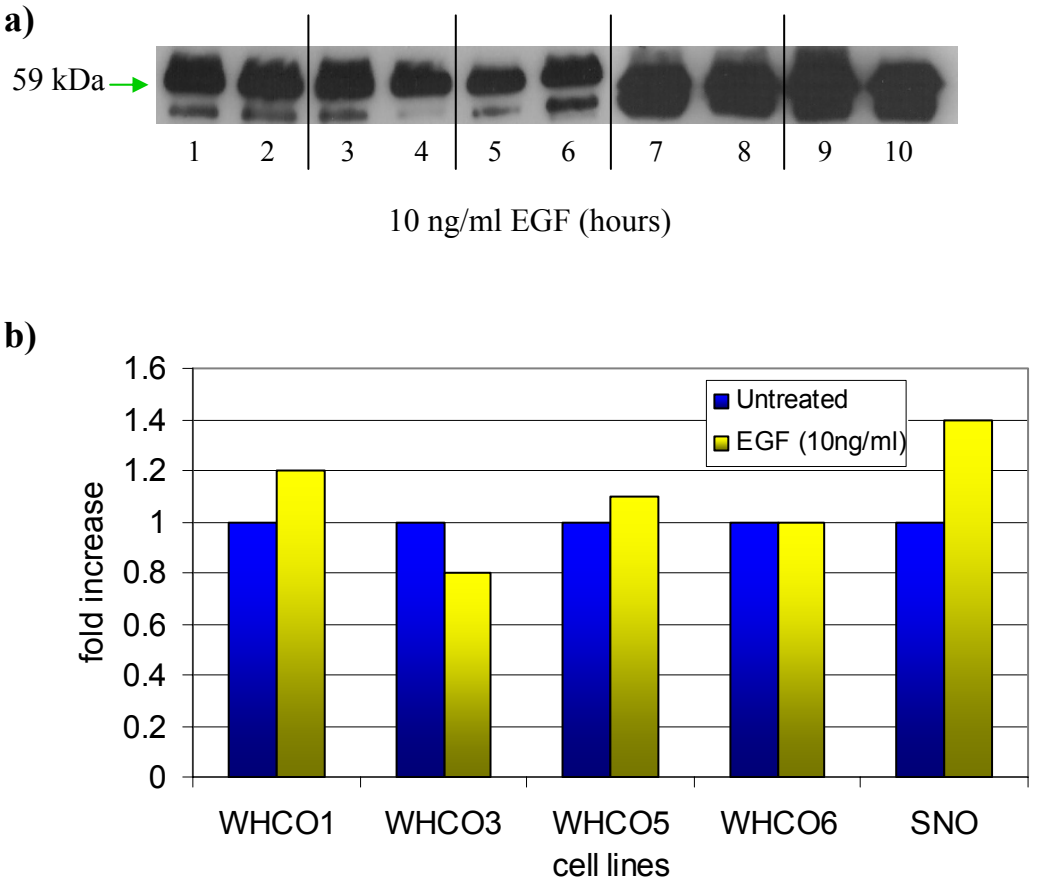


Figure 24: Nuclear Caveolin-1 Protein Levels Following EGF Treatment.

a) Western blot analysis of caveolin-1 utilising a rabbit anti-caveolin-1 antibody (1:5000) in nuclear extracts following EGF (10 ng/ml) treatment for 0.5 hours across all HOSCCs. Lane 1 – untreated WHCO1, lane 2 – EGF-treated WHCO1, lane 3 – untreated WHCO3, lane 4 – EGF-treated WHCO3, lane 5 – untreated WHCO5, lane 6 – EGF-treated WHCO5, lane 7 – untreated WHCO6, lane 8 – EGF-treated WHCO6, lane 9 – untreated SNO and lane 10 – EGF-treated SNO. b) Nuclear caveolin-1 protein levels following densitometric analysis revealed an increase in nuclear caveolin-1 in the WHCO1, WHCO3 and SNO cell lines with no subsequent change observed in the WHCO6 cell line. Surprisingly, the WHCO3 cell line demonstrated reduced caveolin-1 expression following EGF exposure. Note: the expression levels are representative as a percentage of maximum per 10 μg of protein from concentrated fractions (included to normalise for loading variation). Experiments were repeated three times.

4.3.2 Nuclear ILK Concentration Following Exposure to EGF

Due to the observed effects of EGF on the expression of caveolin-1, both at the membrane/cytoplasm and the nucleus, EGF was thought to be involved in nuclear translocation of ILK. HOSCCs were exposed to EGF for 0.5 hours since at this time the most substantial changes in ILK membrane/cytoplasm expression and ILK nuclear levels were noted (Chapter 2, Figure 12).

The response of nuclear ILK to EGF exposure was very similar to that of caveolin-1, where an increased level of ILK was demonstrated. Treatment of HOSCCs with 10 ng/ml EGF for 0.5 hours produced an average increase of 1.1 fold in ILK protein levels in the nuclear extract of the WHCO6 cell line. The WHCO5 cell line produced a much larger increase in ILK expression of 1.6 fold in comparison to the untreated nuclear fraction. Surprisingly, in the WHCO3 cell line a decrease in ILK expression of 1.3 fold was observed following 0.5 hours EGF exposure, while the WHCO1 and SNO cell lines showed no change in ILK expression (Figures 25a and b).

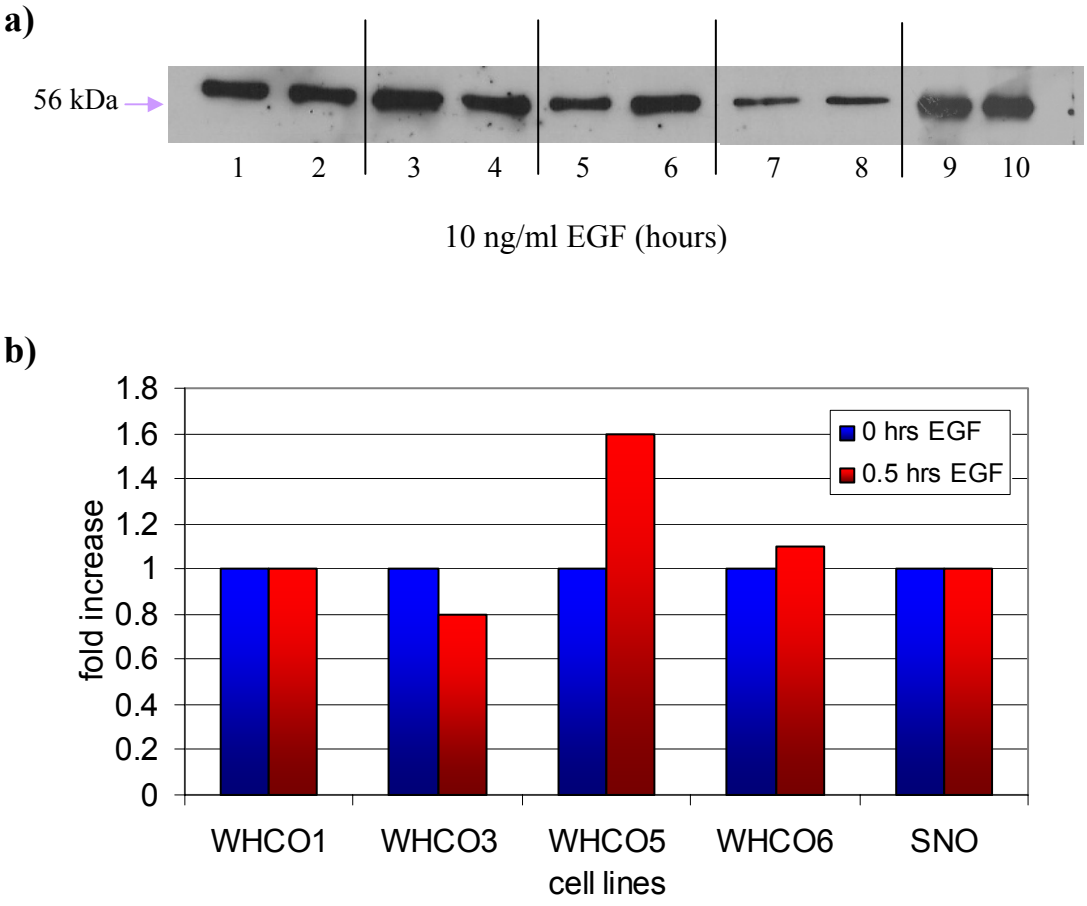


Figure 25: Nuclear ILK Protein Levels Following EGF Treatment.

a) Western blot analysis of nuclear ILK following exposure to 10 ng/ml EGF for 0.5 hours. Lane 1 – untreated WHCO1, lane 2 – EGF-treated WHCO1, lane 3 – untreated WHCO3, lane 4 – EGF-treated WHCO3, lane 5 – untreated WHCO5, lane 6 – EGF-treated WHCO5, lane 7 – untreated WHCO6, lane 8 – EGF-treated WHCO6, lane 9 – untreated SNO and lane 10 – EGF-treated SNO. b) Densitometric analysis revealed that nuclear ILK concentration was increased in the WHCO5, WHCO6 and SNO cell lines. Surprisingly, ILK expression was reduced in the WHCO3 cell line with no change observed in the WHCO1 cell line. Note: the expression levels are representative as a percentage of maximum per 10 μg of protein from concentrated fractions (included to normalise for loading variation). Experiments were repeated three times.

4.3.3 Caveolin-1 is Tyrosine Phosphorylated in HOSCCs

The tyrosine phosphorylation of caveolin-1 has been clearly demonstrated in response to treatment with growth factors such as VEGF, PDGF, as well as EGF where it is thought that the Src family tyrosine kinases are critically involved in this process (Kim *et al*., 2000; Labrecque *et al*., 2004). Surprisingly however, the states of tyrosine phosphorylated caveolin-1 remain largely unknown (Lee *et al*., 2005). A suggestion has been made that phosphorylated caveolin-1 serves as a docking site for other tyrosine phosphorylated molecules (Lee *et al*., 2005).

In terms of caveolin-1 distribution there are indications that tyrosine phosphorylation of caveolin-1 alters its cellular distribution (Pol *et al*., 2000). In order to understand how EGF impacts on caveolin-1 function in oesophageal carcinoma requires investigating the effect of EGF on caveolin-1 tyrosine phosphorylation is required. Since there is evidence that EGF tyrosine phosphorylates caveolin-1 (Kim *et al*., 2000), we examined the tyrosine phosphorylation status of caveolin-1 in the relevant HOSCCs in question, either in the presence or absence of EGF.

Immunoprecipitation analysis revealed that under untreated conditions, a 25 kDa tyrosine phosphorylated caveolin-1 band was identified in the WHCO1 and WHCO5 cell lines when utilising an anti-phosphotyrosine antibody **(**Figure 26**,** orange arrow**)**. Nonphosphorylated caveolin-1 normally migrates at approximately 22-24 kDa (Liu *et al*., 2001; Lee *et al*., 2005). No caveolin-1 tyrosine phosphorylation was noted in the WHCO3, WHCO6 and SNO cell lines. The heavy chain of IgG was observed at an approximate molecular weight of 56 kDa in all cell lines (Figure 26, blue arrow).

While investigating the effect of EGF upon caveolin-1 tyrosine phosphorylation, densitometric analysis could not be performed as a result of inconsistent background (data not shown). Treatment with EGF, and subsequent immunoprecipitation of caveolin-1, revealed that caveolin-1 was not tyrosine phosphorylated. This occurred despite ranging antibody concentrations and reaction conditions being utilised during optimisation. A successful result was achieved with high antibody concentration. However, the background increased concomitantly making it difficult to discern if phosphorylated caveolin-1 was indeed present. It was not possible to say with any

certainty whether EGF resulted in a notable increase in the tyrosine phosphorylation of caveolin-1.

Although the effects of EGF upon the tyrosine phosphorylation of caveolin-1 could not be satisfactorily resolved, it still does not detract from the demonstration that caveolin-1 is tyrosine phosphorylated in HOSCCs and EGF cannot be ruled out as having an effect.

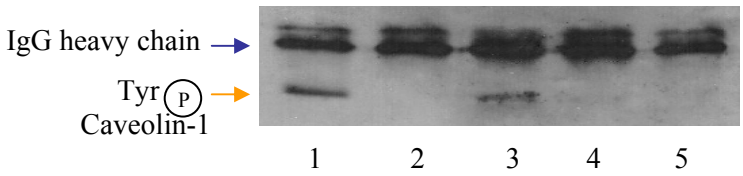


Figure 26: Caveolin-1 Tyrosine Phosphorylation.

Protein G sepharose immunoprecipitated caveolin-1 utilising rabbit anti-caveolin-1 antibody (1:5000) was subjected to Western blot analysis. A phospho-specific tyrosine antibody (1:500) was used to detect tyrosine phosphorylated caveolin-1. Tyrosine phosphorylated caveolin-1 is shown in the WHCO1 and WHCO5 cell lines. Negligible tyrosine phosphorylation was noted in the WHCO6 cell line**.** Tyrosine phosphorylation of caveolin-1 was not observed in the WHCO3 and SNO cell lines and may be due to an undetectable level of phosphorylation in these cell lines. Lane – WHCO1, lane 2 – WHCO3, lane 3 – WHCO5, lane 4 – WHCO6 and lane 5 – SNO cell lines.

4.3.4 Caveolin-1 Distribution Following EGF Treatment

In an attempt to visualise the change in caveolin-1 distribution, indirect immunofluorescence was utilised. This followed from the determination that EGF lowers caveolin-1 expression at the membrane/cytoplasm together with a subsequent increase in expression in the nucleus. Furthermore, Pol *et al*. (2000) demonstrated that caveolae internalise into endocytic compartments, triggered in particular by EGF tyrosine phosphorylation of caveolin-1; the distribution of caveolin-1 was ascertained in response to 0.5 hours EGF exposure.

Previous studies have shown that a short exposure period to EGF is sufficient to elicit caveolin-1 redistribution (Kim *et al*., 2000; Pol *et al*., 2000). No obvious redistribution of caveolin-1 was revealed in the presence of EGF for 0.5 hours; this was apparent in the five cell lines tested. The majority of caveolin-1 seemed to be largely localised at the cell membrane. This was especially apparent for the WHCO1, WHCO3 and WHCO5 cell lines, which showed prominent membrane caveolin-1, both in the presence and absence of EGF (Figure 27**,** yellow arrows). In the WHCO6 and SNO cell lines however, caveolin-1 exhibited a greater cytoplasmic localisation, with membrane localisation occurring to a lesser degree. Furthermore, there appeared to be no substantial change in the distribution of caveolin-1 following EGF treatment in these two cell lines (Figure 27, red arrows).

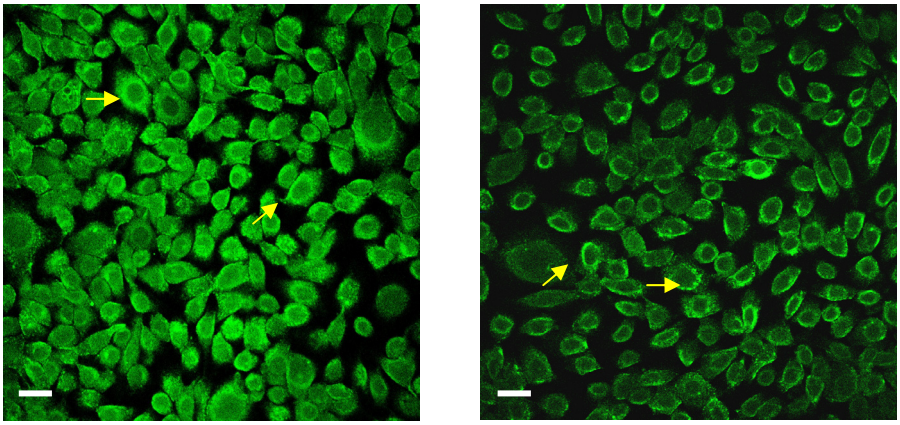
4.3.5 The Association of Caveolin-1/ILK at the Nucleus

Reports have previously documented the association between caveolin-1 and ILK in neuroblastoma (Chun *et al*., 2005). The obvious course of action to follow next was to confirm whether a direct physical interaction existed between caveolin-1 and ILK. Prior to this study it was not known whether a similar protein interaction existed in carcinoma of the oesophagus. In order to establish whether an interaction does indeed occur between caveolin-1 and ILK in HOSCCs, co-immunoprecipitation analysis was performed on nuclear cell lysates, which revealed that caveolin-1 and ILK do indeed associate at nuclear regions of the cell across all cell lines tested. When caveolin-1 was protein G immunoprecipitated from nuclear cell lysates utilising an anti-caveolin-1 antibody, ILK was identified in caveolin-1 fractions following subsequent Western blotting with an anti-ILK antibody (Figure 28**,** red arrow). The heavy chain of IgG was

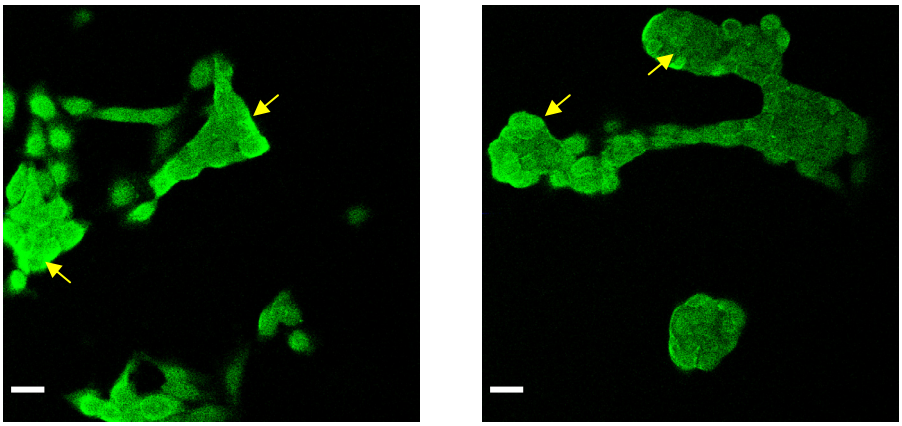
present and is seen just below the 59 kDa of ILK, at an approximate molecular weight of 56 kDa (Figure 28**,** purple arrow). Once again the reader is reminded that cell lysates were preabsorbed with protein G so as to reduce non-specific protein G associated products (see Methods and Materials).

Assuming that membrane/cytoplasmic localisation of ILK requires the interaction with caveolin-1, it stands to reason that for the nuclear entry of ILK to occur, either i) disruption of the caveolin-1/ILK association must take place thereby unblocking the nls of ILK, or ii) transport must occur in conjunction with caveolin-1. Since ILK was also found to be associated with caveolin-1 in the nucleus, the latter option was considered the more feasible. To test this assumption, cell lines were treated with a known inhibitor of caveolin-1 function, namely methyl-β-cyclodextrin.

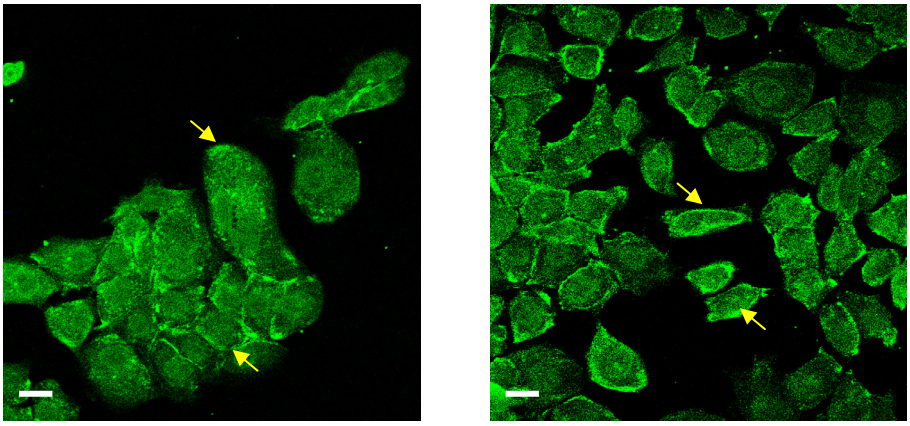
a) WHCO1 (untreated) WHCO1 (EGF)



b) WHCO3 (untreated) WHCO3 (EGF)



c) WHCO5 (untreated) WHCO5 (EGF)



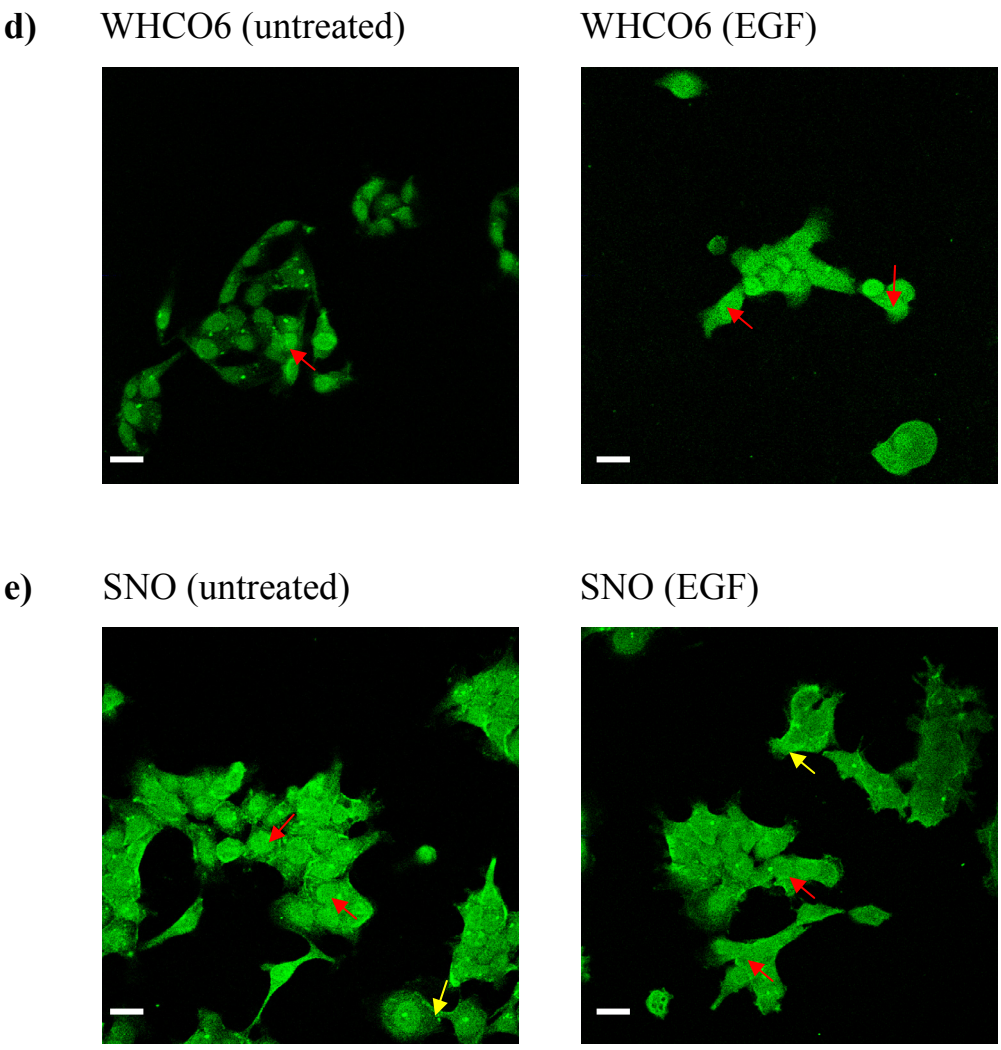


Figure 27: Distribution of Caveolin-1 Following EGF Exposure in HOSCCs.
a-e) Indirect immunofluorescence of caveolin-1 utilising a rabbit anti-caveolin-1 antibody (1:500) and a FITC-conjugated anti-rabbit antibody (1:1000) in HOSCCs, either in the absence or presence of 0.5 hours EGF (10 ng/ml). A significant proportion of caveolin-1 localisation occurred at the plasma membrane in the absence of EGF in the WHCO1, WHCO3 and WHCO5 cell lines (yellow arrows), while WHCO6 and SNO cells showed a greater degree of cytoplasmic caveolin-1 (red arrows). Following EGF exposure no detectable variation in caveolin-1 distribution was apparent in the five cell lines and the majority of caveolin-1 remained localised to the membrane. Bar represent 10 μm.

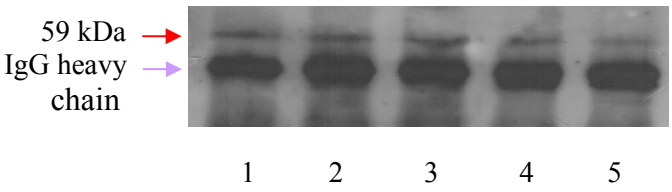


Figure 28: Nuclear Association between Caveolin-1 and ILK.

a) The association between caveolin-1 and ILK in the nucleus was determined by protein G sepharose immunoprecipitated caveolin-1 utilising an anti-caveolin-1 antibody. Western blots of the immunoprecipitated lysates was performed to detect for the presence of ILK with an anti-ILK antibody (1:1500) in all five HOSCCs. ILK was identified at a molecular weight of 59 kDa (orange arrow) with IgG present at a slightly smaller molecular weight (purple arrow). Lane 1 – WHCO1, lane 2 – WHCO3, lane 3 – WHCO5, lane 4 – WHCO6 and lane 5 – SNO.

4.3.6 Inhibition of Caveolin-1 via Methyl-β-Cyclodextrin Prevents ILK Nuclear Localisation

To determine whether ILK requires an association with caveolin-1 for nuclear translocation, cells were exposed to an inhibitor of caveolin-1 function, namely methyl-β-cyclodextrin (10 mM). Methyl-β-cyclodextrin has been shown to sequester cholesterol, disrupt caveolae and causes the mis-localisation of caveolin-1 (Lin *et al*., 2005). Examination of the membrane/cytoplasmic protein levels of ILK expression revealed a reduction in ILK levels upon exposure to methyl-β-cyclodextrin (Figure 29a). This was apparent in all five HOSCCs. In the WHCO1, WHCO3 and WHCO6 cell lines there was an average decrease of 1.7 fold in ILK cytoplasmic protein expression. There were substantial decreases demonstrated in ILK protein expression in the WHCO5 and SNO cell lines of 4 and 2.5 fold respectively (Figure 29b).

When considering the effect of methyl-β-cyclodextrin on nuclear ILK concentration, it was confirmed that similar reductions in ILK protein expression occurred (Figure 30a). On average ILK expression decreased by 1.7 fold in the WHCO1, WHCO3 and SNO cell lines when compared to the untreated cell lysate. Much larger decreases were apparent in the WHCO5 and WHCO6 cell lines with fold decreases of 4 and 2.5 respectively (Figure 30b).

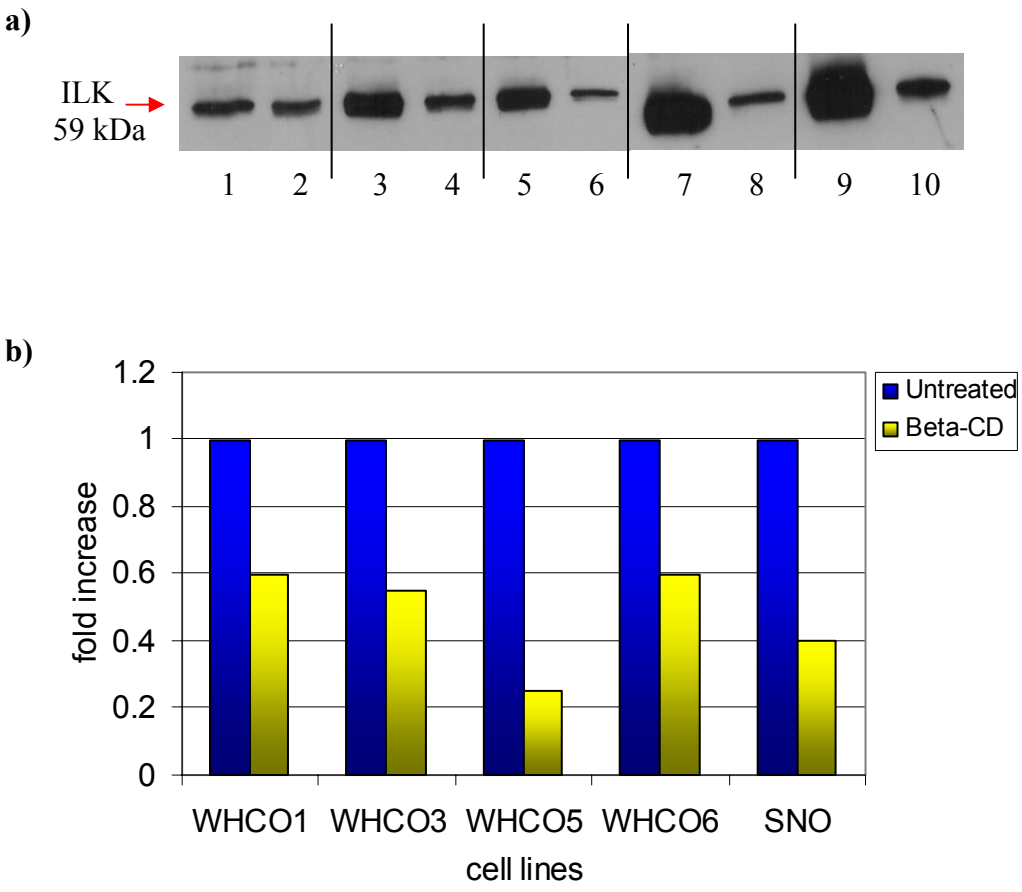


Figure 29: Methyl-β-Cyclodextrin Inhibits Cytoplasmic ILK Protein Expression. a) Western blot analysis of cytoplasmic ILK utilising a rabbit anti-ILK antibody (1:1500) following exposure to the caveolin-1 inhibitor, methyl-β-cyclodextrin (2 ng/ml). Lane 1- untreated WHCO1, lane 2 – cyclodextrin-treated WHCO1, lane 3 – untreated WHCO3, lane 4 – cyclodextrin-treated WHCO3, lane 5 – untreated WHCO5, lane 6 – cyclodextrin-treated WHCO5, lane 7 – untreated WHCO6, lane 8 – cyclodextrin-treated WHCO6, lane 9 – untreated SNO and lane 10 – cyclodextrin-treated SNO. b) Densitometric analysis revealed that a decrease in cytoplasmic levels of ILK occurred to the greatest extent in the WHCO5 and SNO cell lines, i.e. 4 and 2.5 fold respectively. Note: the expression levels are representative as a percentage of maximum per 10 μg of protein from concentrated fractions (included to normalise for loading variation). Experiments were repeated three times.

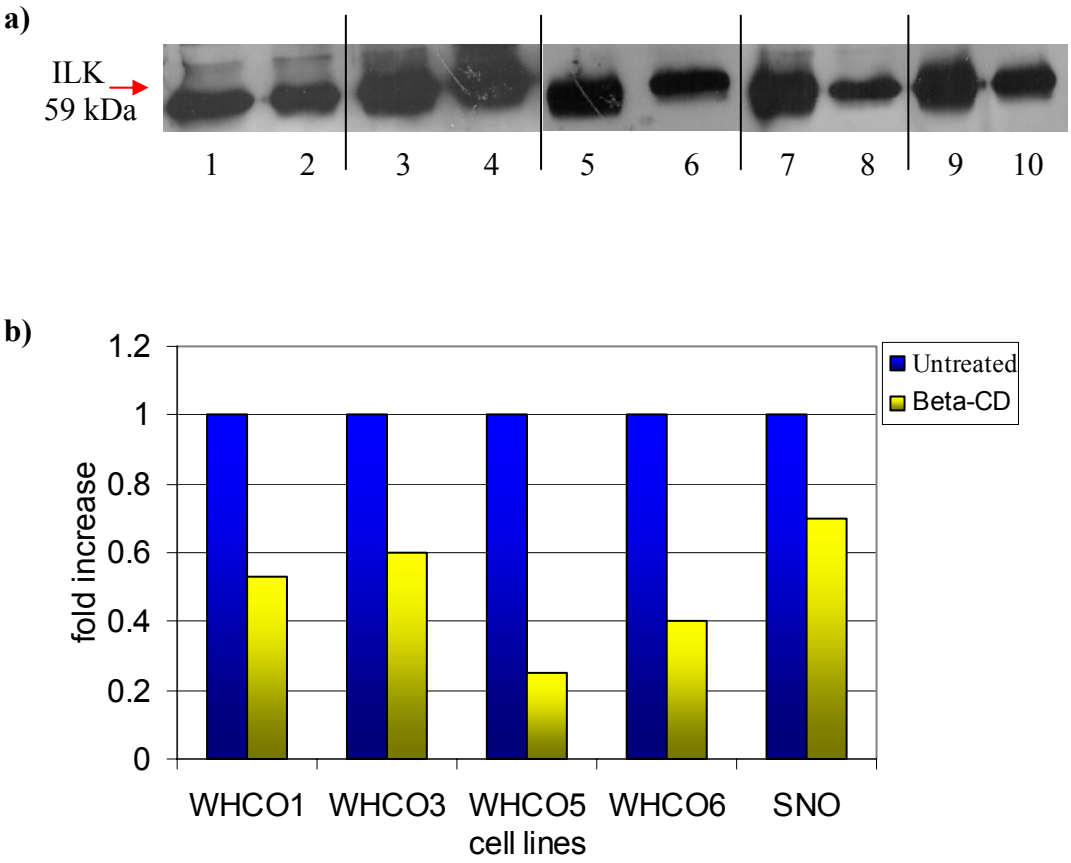


Figure 30: Methyl-β-Cyclodextrin Inhibits Nuclear ILK Protein Expression.

a) Western blot analysis of nuclear ILK utilising a rabbit anti-ILK antibody (1:1500) following exposure to the caveolin-1 inhibitor, methyl-β-cyclodextrin (2 ng/ml). Lane 1- untreated WHCO1, lane 2 – cyclodextrin-treated WHCO1, lane 3 – untreated WHCO3, lane 4 – cyclodextrin-treated WHCO3, lane 5 – untreated WHCO5, lane 6 – cyclodextrin-treated WHCO5, lane 7 – untreated WHCO6, lane 8 – cyclodextrin-treated WHCO6, lane 9 – untreated SNO and lane 10 – cyclodextrin-treated SNO. b) Densitometric analysis revealed that nuclear levels of ILK were decreased to the greatest extent in the WHCO5 and WHCO6 cell lines, i.e. 4 and 2.5 fold respectively. Note: the expression levels are representative as a percentage of maximum per 10 μg of protein from concentrated fractions (included to normalise for loading variation). Experiments were repeated three times.

4.4 Discussion

The retention of ILK at the membrane has been suggested to occur through caveolin-1 binding (Chun *et al*., 2005). This has vital cellular consequences, suitably placing ILK to maintain the biological activities of crucial signalling molecules such as GSK3β and PKB. Ultimately crucial cellular events, such as cell proliferation, migration and differentiation are influenced (Li *et al*., 1999; Cordes and van Beuningen, 2003; Khyrul *et al*., 2004; Graness *et al*., 2005; Vespa *et al*., 2005). The role of growth factors, in particular EGF, on the functioning of ILK formed the subject of previous chapters. EGF was shown not only to directly increase membrane/cytoplasmic ILK expression levels, but also to modulate the kinase activity of ILK. Data presented here lead to the suggestion that EGF has far reaching effects in the nuclear localisation of ILK by downregulating a known membrane-associated protein, caveolin-1.

Lu *et al*., (2003) have shown that EGF exposure for a maximum period of five days elicited a noticeable reduction in caveolin-1 expression; this occurred from approximately three days onwards. However the present study showed that EGF reduced caveolin-1 at a much quicker rate, a considerable decrease was seen as early as 0.5 hours. In terms of caveolin-1, we considered EGF to be important with regards to the distribution and expression levels of caveolin-1. EGF treatment (10 ng/ml) for 0.5, 1, 3 and 6 hours produced a vast decrease in caveolin-1 expression levels in the WHCO1 cell lines of membrane/cytoplasmic cell lysates (see Figure 23). Although expression levels of membrane/cytoplasmic caveolin-1 varied, depending on the time exposure to EGF, the trend suggested a decrease in caveolin-1 expression levels at the membrane/cytoplasm. This is in agreement with previous reports demonstrating a similar reduction in caveolin-1 protein, following the addition of EGF in human epidermoid carcinoma cells, prostate cells and human non-small cell lung cancer cells, as well as rat bladder carcinoma cells (Lu *et al*., 2003).

Examination of the nuclear levels of caveolin-1 revealed an increase in nuclear caveolin-1 concentration (see Figure 24). While this held true for the WHCO1, WHCO5 and SNO cell lines, nuclear caveolin-1 concentration in WHCO3 cells was reduced. It appears that EGF results in caveolin-1 downregulation in this cell line. Furthermore, since it is known that WHCO3 cells particularly overexpress the EGF receptor, it could

be implied that a constitutive downregulation of caveolin-1 exists in this cell line. While the WHCO6 cell line showed a large decrease in membrane/cytoplasmic caveolin-1 associated cell lysates, surprisingly, no change in nuclear caveolin-1 levels was observed. The strong band intensity observed for both untreated and EGF-treated lysates is indicative of an extremely large concentration of caveolin-1 protein. It is possible that of the 10 μg total protein electrophoresed, a proportion may not have transferred across to the nitrocellulose membrane during the Western blotting procedure. This would then suggest that the densitometrically determined caveolin-1 levels are not a true reflection of nuclear caveolin-1 concentration in WHCO6 cells. In the WHCO1, WHCO5 and SNO cell lines however, it was demonstrated that EGF directly increases caveolin-1 transport into the nuclear region and decreases expression at the membrane/cytoplasm. To support this data EGF has been shown previously to cause caveolin-1 redistribution to an intracellular localisation in NRK cells (Pol *et al*., 2000).

An increase was apparent for the expression levels of nuclear caveolin-1 when exposed to EGF for this short time period (on average 1.2 fold higher than untreated cell lysates in the WHCO1, WHCO5 and SNO cell lines). Although this increase in expression was not nearly as substantial in comparison to the decrease noted in the membrane/cytoplasmic fractions, suggests that EGF causes a transfer of caveolin-1 expression from the membrane/cytoplasmic region to the nucleus. Unfortunately, this could not be corroborated by immunofluorescence. An explanation may be that caveolin-1 redistribution only occurs when these HOSCCs are exposed to EGF for a different time period, which would necessitate a time course analysis.

This study was concerned with explaining the nuclear ILK concentration demonstrated in chapter 2. The membrane retention of ILK requires an association with caveolin-1, which blocks a putative nls of ILK located near the caveolin-1 binding site (Chun *et al*., 2005). While these authors showed that disruption of the ILK/caveolin-1 association resulted in increased nuclear ILK, we suggested that translocation of ILK to the nucleus can also occur in conjunction with caveolin-1. Since EGF incurred a decrease in the membrane/cytoplasmic expression of caveolin-1, followed by an increase in nuclear caveolin-1 protein levels (see Figures 23 and 24), it was proposed that EGF would elicit a similar response in nuclear ILK protein levels. Cells were exposed to EGF for 0.5

hours since during this time period EGF seemed to elicit the most noticeable response in membrane/cytoplasmic levels of ILK across all cell lines. In the WHCO5 and WHCO6 cell lines, an increase in nuclear ILK concentration was observed, while no change in expression was noticed in WHCO1 and SNO cells. Surprisingly, the WHCO3 cell line experienced a reduction in nuclear ILK following exposure to EGF for 0.5 hours (see Figure 25).

It is well established that a close relationship exists between EGF and caveolin-1. For instance, caveolin-1 recruitment to the endocytic pathway requires EGF in NRK cells. Interactions between caveolin-1 and EGF have also been demonstrated in numerous other cell types, including rat bladder carcinoma cells and human epidermoid carcinoma cells (Kim *et al*., 2000; Park *et al*., 2000; Kim *et al*., 2002; Lu *et al*., 2003; Lee *et al*., 2005). It is interesting to note that Lee *et al*., (2005) showed in the human epidermoid carcinoma cell line that tyrosine phosphorylated caveolin-1 results in the cell surface localisation of caveolin-1. This suggests that tyrosine phosphorylation of caveolin-1 directly effects its distribution and allows it to act as a signalling molecule (Lee *et al*., 2005). At present however, the complete functional significance of caveolin-1 tyrosine phosphorylation is not yet known. The present study was focused on better understanding the relationship between caveolin-1 and EGF in HOSCCs.

Analysis of caveolin-1 tyrosine phosphorylation revealed that a 25 kDa caveolin-1 tyrosine phosphorylated product is shown in the WHCO1 and WHCO5 cell lines. This molecular weight is supported by a study conducted by Lee *et al*., (2005) who demonstrated an identical molecular weight for phosphorylated caveolin-1. Nonphosphorylated caveolin-1 migrates at approximately 22-24 kDa (Lee *et al*., 2005). In order to confer such a dramatic increase in molecular weight suggests that caveolin-1 undergoes a vast degree of tyrosine phosphorylation.

Although we were able to show that caveolin-1 was tyrosine phosphorylated under standard tissue culture conditions (see Figure 26), the effect of growth factors on the tyrosine phosphorylation status of caveolin-1 could not be discerned. This was due to difficulties in optimising conditions when utilising the phosphospecific tyrosine antibody.

The observed nuclear ILK concentration in these HOSCCs, suggested an interaction between caveolin-1 and ILK. Therefore, the remainder of this chapter was concerned with investigating whether a physical relationship existed between caveolin-1 and ILK, and if so, whether ILK nuclear entry required caveolin-1 expression.

Caveolin-1 is largely regarded as a membrane-associated protein, but is also thought to play a role in cellular signalling and is involved in the shuttling of signalling moieties (Park *et al*., 2000; Wiechen *et al*., 2001; Upla *et al*., 2004; Epand *et al*., 2005). In agreement with this, caveolin-1 was identified at a membrane/cytoplasmic level as well as being expressed in the nucleus (confirmed via Western blotting). With the addition of EGF a dramatic decrease in caveolin-1 membrane/cytoplasmic expression was noted with a concomitant increase in nuclear caveolin-1 in the WHCO5 and SNO cell lines, which implied a cellular redistribution of caveolin-1. Although WHCO1 cells also demonstrated an increase in caveolin-1 nuclear levels (WHCO6 cells demonstrated no change in nuclear expression), a similar increase occurred in the expression of caveolin-1 at the membrane/cytoplasm for both WHCO1 and WHCO6. This implies that in WHCO1 and WHCO6 cells, total protein levels of caveolin-1 are increased by EGF as opposed to a shuttling of caveolin-1 between cellular compartments.

Not surprisingly, confocal microscopy revealed that caveolin-1 was distributed primarily at the membrane in the WHCO1, WHCO3 and WHCO5 cell lines. However, variable caveolin-1 staining was also observed in the cytoplasmic and nuclear region of the WHCO6 and SNO cell lines (see Figure 27). Since caveolae exist either as invaginations in the plasma membrane proper or as vesicles occurring close to the membrane (Razani *et al*., 2005), the cytoplasmic distribution of caveolin-1 in the oesophageal cell lines was not totally unexpected. In the presence of EGF however, no redistribution of caveolin-1 was visible in any of the cell lines, which is surprising considering that caveolin-1 has been reported to internalise into endocytic compartments in response to growth factors such as EGF (Pol *et al*., 2000). Although immunofluorescence microscopy revealed no apparent redistribution of caveolin-1 in the presence of EGF, the fact that EGF affected caveolin-1 expression at the membrane/cytoplasm and nucleus, suggests that EGF is able influence caveolin-1 distribution, which was not observed utilising indirect immunofluorescence. A possible explanation for not being able to demonstrate a noticeable visual change occurring in

the distribution of caveolin-1 in the presence of EGF, could be that the indirect immunofluorescence could not detect the small changes in caveolin-1 redistribution (see Figure 27).

To investigate\ the association between caveolin-1 and ILK, co-immunoprecipitation analysis was performed, which established the association between ILK and caveolin-1 at both membrane/cytoplasm and nuclear levels in the oesophageal carcinoma cell lines in question (see Figure 28). Thus it appears from this data that the transport of ILK to the nuclear region requires an association with caveolin-1. To investigate this possibility, cell lines were treated with a known inhibitor of caveolin-1 function, namely methyl-β-cyclodextrin, and nuclear levels of ILK expression examined.

Exposure of the oesophageal carcinoma cell lines to methyl-β-cyclodextrin resulted in a vast reduction in the expression levels of ILK; up to 4 fold at both a nuclear and cytoplasmic level (see Figures 29 and 30). It is clear that the disruption of caveolin-1 via methyl-β-cyclodextrin impairs the expression of nuclear ILK. Thus, it could be said that not only is a direct/indirect interaction with caveolin-1 required for the retention of ILK at the plasma membrane, but the caveolin-1/ILK association is also necessary for the transport of ILK into the nuclear region. While the present data has not addressed the functional significance of nuclear ILK, it is likely that nuclear ILK plays a role in the phosphorylation of nuclear GSK3β, which would in turn effect the stabilisation of nuclear β-catenin. Indeed, it has been shown previously that nuclear β-catenin is phosphorylated in the nucleus (He, 2006; Oloumi *et al*., 2006). This may have important consequences with regard to transcription processes that ultimately impinge upon cellular processes such as the cell cycle and proliferation.

Since disruption of the ILK/caveolin-1 interaction through methyl-β-cyclodextrin impairs nuclear ILK protein levels, it is apparent that an intact ILK/caveolin-1 interaction is required for ILK nuclear transport. EGF is thought to provide the stimulus for directing the changes in the expression patterns of both ILK and caveolin-1, which subsequently results in a shift from a membranous to a cytoplasmic/nuclear localisation. In the case of caveolin-1, we suggest that EGF achieves this via tyrosine phosphorylation, although this is yet to be verified.

While this potentially explains how ILK is translocated to the nucleus, it does not provide any insight with regard to the functional significance of nuclear ILK. It would be interesting for future studies to examine the role of nuclear ILK in carcinoma of the oesophagus, as it is possible that ILK is acting as a potential nuclear transcription factor, although this is speculative at present.

Chapter 5

Role of ILK in Substrate Adhesion of Oesophageal SCC Cells

5.1 Introduction

Cell-ECM interactions are pivotal in the control of cellular proliferation, migration, survival, differentiation and tissue formation, thereby directly impacting upon processes such as embryogenesis, wound healing, inflammation, and cancer (Jones and Walker, 1999; Li *et al*., 1999; Tu *et al*., 2001; Aplin *et al*., 2002; Wu, 2004; Takanami, 2005; Boulter *et al*., 2006). Cell-ECM adhesion is especially important during malignant processes, since oncogenic transformation routinely induces anchorage independent growth *in vitro*, which is a specific correlate of tumour growth *in vivo* (Ruoslahti and Reed, 1994; Radeva *et al*., 1997). The integrins are the primary cellular receptors that mediate adhesion of cells to the substrate. In this regard, the $\alpha\beta$ associations determine the ligand binding specificities of the receptor for various ECM proteins such as collagen, fibronectin and laminin (Juliano and Haskill, 1993; Ruoslahti, 1996; Kumar, 1998; Katz *et al*., 2000; Aplin *et al*., 2002; Pinkse *et al*., 2005).

The importance of the integrin receptors in metastatic processes has been widely acknowledged. For example, the classic fibronectin receptor, $\alpha_5\beta_1$ is reduced in many human tumours, including pancreatic and skin carcinomas; and a reduction of the collagen receptor, $\alpha_2\beta_1$ is seen in adenocarcinoma of the breast (Weinel *et al*., 1992; Zutter *et al*., 1995; Taverna *et al*., 1998). The importance of integrins is further established by the $\alpha_v\beta_6$ integrin, which is found in almost all squamous cell carcinoma (SCC) tumours and plays a critical role in tumour cell migration and invasion (Koivisto *et al*., 2000). Yet another example is the $\alpha_v\beta_5$ integrin, which is reduced in poorly differentiated SCC lesions (Koivisto *et al*., 2000). Interestingly the β_1 integrin subunit is reduced in oesophageal SCCs (Miller and Veale, 2001). The consequences of aberrant expression of integrins in malignancy are therefore considered as components central to metastatic events.

Over the past few years an understanding of the involvement of ILK in adhesion-dependent processes of metastatic tumours has slowly emerged. Cell adhesion events

play a significant part during metastasis since the invasion of carcinoma cells through basement membranes requires tumour cell adhesion to ECM, proteolytic degradation of the basement membrane and tumour cell motility (Flug and Kopf-Maier, 1995). Perhaps more pertinent is the fact that the development of SCC requires tumour cell interaction with the surrounding ECM (Koivisto *et al*., 2000). The regulatory control that ILK exerts over the β integrin subunits suggests that ILK is certainly involved in mediating cell-ECM processes (Hannigan *et al*., 1996; Mulrooney *et al*., 2000; Stevens *et al*., 2004; Pinkse *et al*., 2005), as well as being involved in ECM assembly (Tu *et al*., 2001). During metastatic events the regulation of β integrin subunits by ILK is almost certainly a vital regulatory step (Mulrooney *et al*., 2000; Ishii *et al*., 2001; Friedrich *et al*., 2002; Chun *et al*., 2005), since neoplastic cells possess the ability to overcome the adhesion constraints that exist in normal cells (Frisch and Francis, 1994; Novak *et al*., 1998; Attwell *et al*., 2000). Therefore, changes in the regulatory control that ILK exerts over integrins, and hence cell-ECM adhesion is pivotal in the development of the metastatic spread of tumours (Novak *et al*., 1998; Takanami, 2005; Yau *et al*., 2005).

The present study attempts to elucidate the effect of reduced ILK on cell adhesion to collagen and fibronectin. Previously, Miller and Veale (2001) showed that the majority of the oesophageal SCC cell lines in question are dependent on collagen and fibronectin for cell adhesion. However, the role of ILK was not taken into consideration when this study was performed. In Chapters 2 and 4, it was demonstrated that ILK is involved in integrin regulation since ILK was capable of phosphorylating an analogue of the β_1 peptide MBP and, that ILK and the β_3 integrin subunit interact. The role of ILK activity on cell-ECM adhesion was examined from two perspectives. Firstly, a direct approach was taken where cells were treated with a specific inhibitor of ILK function, KP-392 (a kind gift from S.Dedhar). Secondly, to discern whether PI3K was involved as an upstream promoter of ILK-mediated cell adhesion events, which was accomplished by treating cells with a potent inhibitor of PI3K function, wortmannin (Powis *et al*., 1994; Schultz *et al*., 1995; Delcommenne *et al*., 1998).

It was hoped that an understanding could be provided of the functional role for the biochemical link demonstrated earlier.

5.2 Methods and Materials

5.2.1 Tissue culture

Cells were cultured under standard conditions as previously documented in Chapter 2, Section 2.2.1.

5.2.2 Cell harvesting and preparation for adhesion assays

Medium was removed and cells washed twice with pre-warmed PBS (37 °C) (see Appendix 1.1.1). Cells were harvested using 0.05 % (w/v) trypsin and 0.01 % (w/v) EDTA (see Appendix 1.1.2), which is a concentration that has been shown to preserve integrin activity (Gui *et al*., 1995). The cell suspension was added to DMEM supplemented with 0.01 % trypsin inhibitor (Appendix 1.8.1), to inactivate TE activity and centrifuged gently (2000 rpm). Cells were resuspended in serum free DMEM containing 0.1 % filter sterilised BSA (see Appendix 1.3.4) and counted using a haemacytometer.

5.2.3 Extracellular matrix cell adhesion assays

Nunc™ 24 multiwell tissue culture dishes were precoated with filter sterilised 50 μg/ml collagen or 50 μg/ml fibronectin (Sigma, USA) (Appendix 1.8.2) for a period of 24 hours at 4 °C modified from Sugiyama *et al*., (1993). Control wells were coated with filter sterilised BSA (Merck AR, SA) under the same conditions. Thereafter, cells were harvested and prepared for plating as above. 250 000 cells/well in 500 μl serum free DMEM were plated in triplicate, per cell line, on ECM coated wells, supplemented with or without 150 nm wortmannin (Sigma, USA) (Appendix 1.8.3) or 100 μM KP-392 (Appendix 1.8.4). Control BSA coated wells were similarly seeded with cells and supplemented with or without wortmannin/KP-392. Cells were cultured under standard temperature, CO_2, and humidity for two hours, which has been shown to be the shortest time to maximise adhesion for the five oesophageal SCC cell lines (Miller, 2001). The supernatant containing nonadhesive cells was removed. Thereafter, each well was rinsed twice in 500 μl PBS and added to the cell suspension containing nonadherent cells. The resultant cell suspension (in total 1500 μl) for each cell line and controls was

counted. The number of adherent cells per well was ascertained by subtracting the number of floating cells from 250 000. The wortmannin cell adhesion assays were repeated on three separate occasions. KP-392 adhesion assays were repeated twice due to insufficient availability of KP-392. However, it was concluded that since the differences between the means of the two repeats were not large, the experiment did not warrant a third repeat.

5.2.4 Statistical analysis

All the data obtained was analysed with Statistica (version 5.5). A one-way analysis of variance (ANOVA) was performed since these data were nonparametric and the sample size (N) was small. Thereafter a Tukey HSD test was performed. All results are represented in Appendix 3.

5.3 Results

5.3.1 Cell-Extracellular Matrix Adhesion Assays

To ensure that cell adhesion was dependent on an ECM substrate, a suitable *in vitro* control substrate was required for this assay. Since cell adhesion has the potential to be artificially benefited when using a polystyrene tissue culture dish as a substrate, this option could not be considered as a relevant control. Instead, a proteinaceous substrate was necessary, in particular one that was not a specific substrate for epithelial cells. BSA is one such commonly used protein (Delcommenne *et al*., 1998), and hence was used to precoat control wells. Since the behaviour of neoplastic cells is strongly affected by their associations with the surrounding ECM (Parise *et al*., 2000), experimental wells were precoated with the two ECM proteins, collagen and fibronectin, that are especially important integrin ligands for epithelial cells. Fibronectin was the obvious choice for substrate-dependent epithelial cells such as these. Furthermore, collagen, which is also an abundant ECM protein, was considered to be useful for comparative purposes.

The data gathered from these cell binding assays generated large and complex matrices that were relatively difficult to analyse statistically (see Appendix 3). The permutations were many thus requiring one way analyses of variance (ANOVA) to interpret the data. To make these statistics easier to comprehend, comparisons will firstly be made of differences in cell adhesion of untreated and treated cells within each cell line, followed by comparisons between the five cell lines. Furthermore, it must be noted that, following trypsinisation, visually these cells attached with no noticeable difference to that observed during typical culturing procedures.

5.3.1.1 Cell Adhesion to Fibronectin Following KP-392 Exposure

Cell Attachment Compared within HOSCCs (BSA Control)

To elucidate the effect of ILK on cell-ECM adhesion, cell attachment to fibronectin was examined in the presence of a specific ILK inhibitor, KP-392, which is known to inhibit the catalytic function of ILK (Persad *et al*., 2000; Troussard *et al*., 2000; Tan *et al*., 2002). Unfortunately, insufficient KP-392 was provided to enable the examination of

ILK inhibition on cell adhesion to both fibronectin and collagen. Importantly, ILK has been shown to be involved in the regulation of fibronectin matrix assembly. A study by Wu *et al*., (1998) demonstrated that ILK overexpression results in a dramatic increase in fibronectin matrix assembly in IEC-18 rat intestinal epithelial cells. Thus, it is for this reason that fibronectin was utilised as a substrate when examining the effect of KP-392 on cell adhesion. Standardly across all the cell adhesion assays, BSA was utilised as a control substrate.

A reduction in cell adhesion as a trend was apparent when cells either not exposed to KP-392 (untreated), or ILK-inhibited (KP-392-treated), were plated on a BSA substrate. However, the difference in cell adhesion between untreated cells and ILK-inhibited cells was only significant in the SNO cell line ($p \leq 0.05$). Not surprisingly, cell adhesion to coated BSA culture dishes was reduced to the greatest extent in the SNO cells line (33 %) following KP-392 exposure. KP-392-treated WHCO3 cells demonstrated a substantial but nonsignificant reduction of 27 % in cell adhesion to BSA compared to untreated WHCO3 cells. Smaller differences were observed in cell lines WHCO1, WHCO5 and WHCO6 (up to 15 %) (Figure 31). A comparison of cell attachment will now be made between the five cell lines

Cell Attachment Compared <u>between</u> HOSCCs (BSA Control)

The SNO cell line exhibited the highest number of untreated cells attaching to a BSA substrate compared to the other cell lines. Significant differences were noted in comparison to the WHCO3 and WHCO5 cell lines, where cell adhesion to BSA of untreated SNO cells was 64 and 47 % higher respectively ($p \leq 0.05$). Cell attachment to BSA in untreated WHCO1 and WHCO6 cells both showed very similar reductions in cell adhesion, on average 13 % lower in comparison to untreated SNO cells ($p \geq 0.05$) (Figure 31).

Across the five HOSCCs, the greatest decrease in cell attachment to BSA was in KP-392-treated WHCO3 cells. Cell adhesion in this cell line was significantly lower than cell adhesion to BSA in ILK-inhibited WHCO1, WHCO5, WHCO6 and SNO cells ($p \leq 0.05$). KP-392 treatment resulted in a 69 % reduction in cell adhesion to BSA in WHCO3 cells compared to cell adhesion in WHCO1 cells. Further large reductions were observed between cell adhesion to BSA in the WHCO3 cell line and cell adhesion

to BSA in the WHCO5, WHCO6 and SNO of 46, 65, and 61 % respectively ($p \leq 0.05$). The WHCO5 cell line showed a considerable reduction in KP-392-treated cell binding to BSA compared to WHCO1 and WHCO6 cells of 42 and 35 % respectively ($p \leq 0.05$). Although cell adhesion to BSA in KP-392-treated WHCO5 cells was 23 % lower than cell attachment in KP-392-treated SNO cells, this difference was not statistically significant ($p \geq 0.05$). Furthermore, nonsignificant differences in cell adhesion of KP-392-treated cells to coated BSA dishes were noted between the WHCO1, WHCO6 and SNO cell lines (up to 21 %) ($p \geq 0.05$).

Cell Attachment to Fibronectin Compared <u>within</u> HOSCCs

With regard to their ability to attach to fibronectin, only WHCO3 cells demonstrated a significant increase in attachment when compared to cell adhesion of untreated WHCO3 cell attachment to BSA ($p \leq 0.05$). In these untreated cells a 44 % increase in cell attachment to fibronectin was observed in comparison to cell attachment to BSA. In untreated WHCO5 cells a 25 % increase was apparent in the binding of cells to fibronectin compared to binding to BSA. Both the WHCO1 and WHCO6 cell lines displayed similar increases in adhesion of 14 %, whereas attachment of cells to fibronectin in the SNO cell line was 11 % higher compared to adhesion to BSA of untreated cells ($p \geq 0.05$) (Figure 31).

When considering the effect of KP-392 on cell attachment to fibronectin a significant difference was only noted in the WHCO5 cell line when compared to fibronectin adhesion of untreated WHCO5 cells ($p \leq 0.05$). The trend for the remaining cell lines however, did suggest a decrease in cell adhesion to fibronectin following the addition of KP-392. A 30 % decrease in cell attachment to fibronectin was apparent in the WHCO5 cell line. The SNO cell line demonstrated a 17 % decrease in the presence of KP-392, while the WHCO3, WHCO6 and WHCO1 cell lines showed modest decreases of 15, 11 and 6 % respectively ($p \geq 0.05$) (Figure 31).

Cell Attachment to Fibronectin Compared <u>between</u> HOSCCs

Comparisons between the five cell lines revealed the SNO cells attached to fibronectin to the greatest extent. A significant difference in cell adhesion to fibronectin was observed between the SNO and WHCO3 cell lines, as well as between the SNO and WHCO5 cell lines ($p \leq 0.05$). Indeed, cell attachment of untreated SNO cells was 42

and 36 % higher than attachment to fibronectin of untreated WHCO3 and WHCO5 cells respectively. Cell adhesion to fibronectin of untreated WHCO1, WHCO6 and SNO cells demonstrated comparable attachment (less than 10 %) ($p \geq 0.05$).

When comparing the effects of KP-392 treatment across the five cell lines it was revealed that the WHCO3 and WHCO5 cell lines exhibited the greatest depression in attachment to fibronectin. Conversely, WHCO1 cells exhibited the most stringent attachment to fibronectin when treated with KP-392. A significant difference in cell adhesion to fibronectin was observed between the ILK-inhibited WHCO1 and WHCO3 cells. A similar significant difference was also noted between WHCO1 cells and the WHCO5 cell line ($p \leq 0.05$). KP-392-treated WHCO1, WHCO6 and SNO cells all demonstrated comparable levels of attachment to fibronectin whereas both the WHCO3 and WHCO5 cell lines showed relatively similar levels (10 %) of cell adhesion to fibronectin ($p \geq 0.05$). In WHCO1 cells, respective increases in cell attachment to fibronectin of 42 and 48 % were apparent compared to fibronectin attachment in the WHCO5 and WHCO3 cell lines ($p \leq 0.05$) (Figure 31).

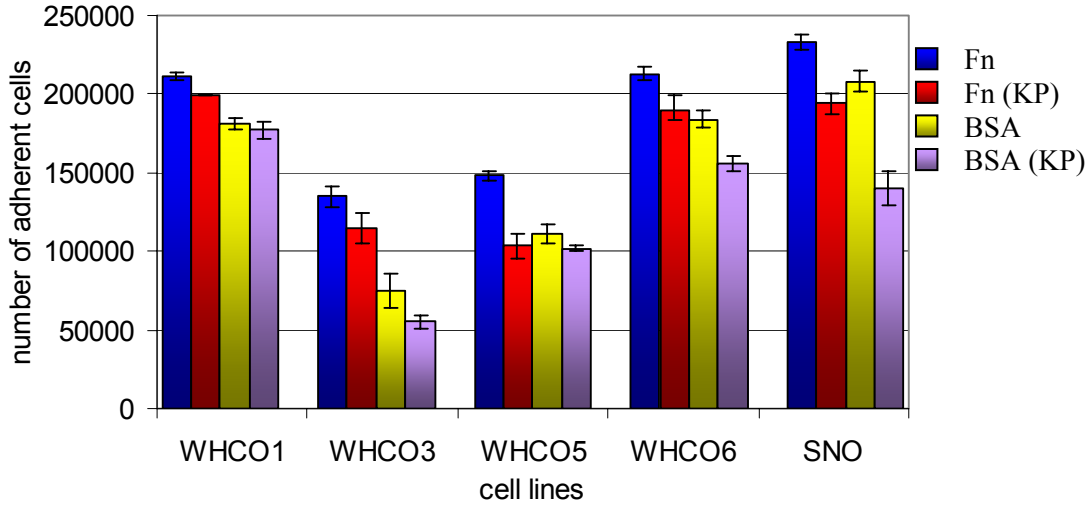


Figure 31: Adhesion to Fibronectin Following KP-392 Exposure in HOSCCs. The WHCO1, WHCO6 and SNO cells demonstrated greatest cell adhesion to both fibronectin and BSA. KP-392 induced a reduction in cell adhesion to fibronectin in all five HOSCCs, which was statistically not significant from untreated cells ($p \geq 0.05$). Blue bars – the number of cells attached to fibronectin, red bars – the number of cells attached to fibronectin in the presence of the ILK inhibitor KP-392, yellow bars – the number of cells attaching to BSA and purple bars– the number of cells attached to BSA in the presence of KP-392. Bars represent standard deviation. Results represent two experiments performed in triplicate.

5.3.1.2 Cell Adhesion to Fibronectin Following Exposure to Wortmannin

Cell Attachment Compared within HOSCCs (BSA Control)

When this data was statistically analysed, a significant difference in cell attachment to coated BSA culture dishes was revealed when comparing untreated WHCO5 cells to wortmannin-treated WHCO5 cells ($p \leq 0.05$). Furthermore, this difference in cell binding to BSA was the greatest in WHCO5 cells (47 %). Both the WHCO6 and SNO cell lines displayed an increase of 17 %, whereas cell adhesion to BSA of untreated WHCO1 and WHCO3 cell lines were similarly increased (by 5 %) compared to cell adhesion to BSA of wortmannin treated cells (Figure 32).

Cell Attachment Compared between HOSCCs (BSA Control)

Examining whether PI3K was involved in ILK-mediated effects on cell attachment to fibronectin, cells were exposed to wortmannin and a fibronectin-based cell adhesion assay was conducted. As was the case with the KP-392 fibronectin assay and the wortmannin collagen adhesion assay, BSA was used as a control protein substrate for the wortmannin fibronectin adhesion assay. Cell adhesion of untreated cells to BSA was highest in the WHCO1 cell line and, in addition, was shown to be significantly different from cell adhesion to BSA in the other untreated cell lines ($p \leq 0.05$). This increase in cell adhesion demonstrated by WHCO1 was 68 % higher in comparison to the SNO cell line and, 53 % and 30 % higher over cell binding to BSA of untreated cells in cell lines WHCO3 and WHCO5 respectively. Furthermore, cell adhesion to BSA of untreated WHCO1 cells was 15 % higher than cell adhesion of the WHCO6 cell line (Figure 32). The data obtained thus showed that wortmannin treatment produced a reduction in cell adhesion to BSA compared to BSA cell adhesion in the absence of wortmannin.

Distinguishing between the levels of cell adhesion to BSA of wortmannin-treated cells across the five cell lines revealed that attachment of the SNO cell line was 67 % lower in comparison to cell adhesion of treated WHCO1 cells. Further, exposure to wortmannin resulted in a 54 % reduction in cell adhesion to BSA in WHCO6 cells compared to treated SNO cells. No significant differences were noted between cell lines WHCO3, WHCO5 and SNO, which all showed similar, cell adhesion to BSA following wortmannin treatment (up to a 23 % difference) ($p \geq 0.05$). Additionally, cell adhesion

to BSA of wortmannin-treated WHCO3, WHCO5 and SNO cells lines was significant from treated WHCO1 and WHCO6 cell lines ($p \leq 0.05$**,** Appendix 3, Figure 32).

Therefore, wortmannin reduced cell adhesion to BSA to the greatest extent in the WHCO3, WHCO5 and SNO cell lines in comparison to the effect of wortmannin treatment in the WHCO1 and WHCO6 cell lines. The differences between the WHCO3, WHCO5 and SNO cell lines were not significant ($p \geq 0.05$). However, cell adhesion of the wortmannin-treated BSA controls in these cell lines was significantly different from the wortmannin treated WHCO1 and WHCO6 BSA controls ($p \leq 0.05$). Furthermore, a significant difference in cell adhesion to BSA, following wortmannin treatment was shown between the WHCO1 and the WHCO6 cell lines ($p \leq 0.05$).

Cell Attachment to Fibronectin Compared <u>within</u> HOSCCs

Similar to the collagen adhesion assay, cell adhesion to fibronectin was considerably higher for the five cell lines in comparison to the attachment of cells to BSA not treated with wortmannin. Of the five SCC cell lines tested, untreated SNO cells demonstrated greatest cell adhesion to fibronectin in comparison to adhesion to BSA (66 %) (Figure 33). This is in concurrence with Miller and Veale, (2001). The WHCO1 cell line showed the lowest number of cells attached to fibronectin compared to WHCO1 cells bound to BSA (31 %). Statistically, all five cell lines showed a significant increase in the number of cells attached to fibronectin compared to untreated cells attaching to BSA ($p \leq 0.05$), implying a preference for fibronectin as a substrate.

Compared to untreated cells, wortmannin treatment reduced the number of cells attached to fibronectin in four of the five cell lines, and although quite noticeable, statistically these differences in cell adherence were not significant ($p \geq 0.05$). As was the case with cell adhesion to collagen, it implies that over the two hour incubation period, wortmannin reduction of ILK did not disrupt the cellular interaction with fibronectin. However, as with the collagen adhesion assay, there is a trend indicating a reduction in cell adhesion to fibronectin following wortmannin treatment. This trend was apparent since significance was obtained at a p-value of 0.1. The SNO cell line demonstrated the greatest reduction in cell adhesion to fibronectin following wortmannin treatment (12 %) in comparison to untreated SNO cells exposed to culture dishes coated with fibronectin. Both treated WHCO3 and WHCO1 cell lines showed

decreased cell adhesion to fibronectin, 9 % and 3 % respectively, in contrast to untreated cells of the same cell lines. The number of untreated cells attached to fibronectin for WHCO5 was comparable to the number of adherent cells following wortmannin treatment (Figure 32).

WHCO6 was the only cell line that did not follow the trend in reduction in cell adhesion. Here cell adhesion to fibronectin was increased following wortmannin treatment by 7 %. Statistics revealed however, that this was not a significant difference ($p \geq 0.05$) (Appendix 3). Even though this difference was shown to be statistically nonsignificant, it suggests that the Wortmannin-induced PI3K/ILK reduction, resulted in stabilisation of the αβ integrin heterodimer-ECM ligand complex, thus increasing cell-ECM adhesion in this cell line.

Cell Attachment to Fibronectin Compared <u>between</u> HOSCCs

Comparisons across the cell lines showed that the WHCO1 cell line had the greatest number of cells attached to fibronectin, just over 4 % higher in comparison to the WHCO3 and WHCO6 cell lines (Figure 32). However, the number of cells attached to fibronectin for these three cell lines were not significantly different from each other ($p \geq 0.05$). In its ability to adhere to fibronectin, untreated WHCO1 cells differed significantly from untreated WHCO5 and SNO cell lines. Untreated WHCO3 and WHCO6 cells showed no significant increase in the number of cells binding to the fibronectin substrate when compared to cell attachment of the WHCO5 cell line. Furthermore, statistically the WHCO5 and SNO lines were not significantly different from one another, indicative of equal levels of binding to fibronectin for these cell lines as well as for the WHCO3 and WHCO6 cell lines ($p \geq 0.05$) (Figure 32).

Wortmannin caused the greatest reduction in cell adhesion to fibronectin in the SNO cell line compared to the other oesophageal SCCs. Analysed statistically, this difference was shown to be significantly different from wortmannin-treated WHCO1, WHCO3, and WHCO6 cells ($p \leq 0.05$), but not from wortmannin-treated WHCO5 cells. The smallest decrease in cell adhesion to fibronectin following wortmannin treatment was in wortmannin-treated WHCO1 and WHCO6 cells, and the numbers of cells adhering in these cell lines were similar. Furthermore, cell adhesion to fibronectin of treated

WHCO3 cells was not significantly different from attachment of wortmannin-treated WHCO5 cells ($p \geq 0.05$) (Figure 32).

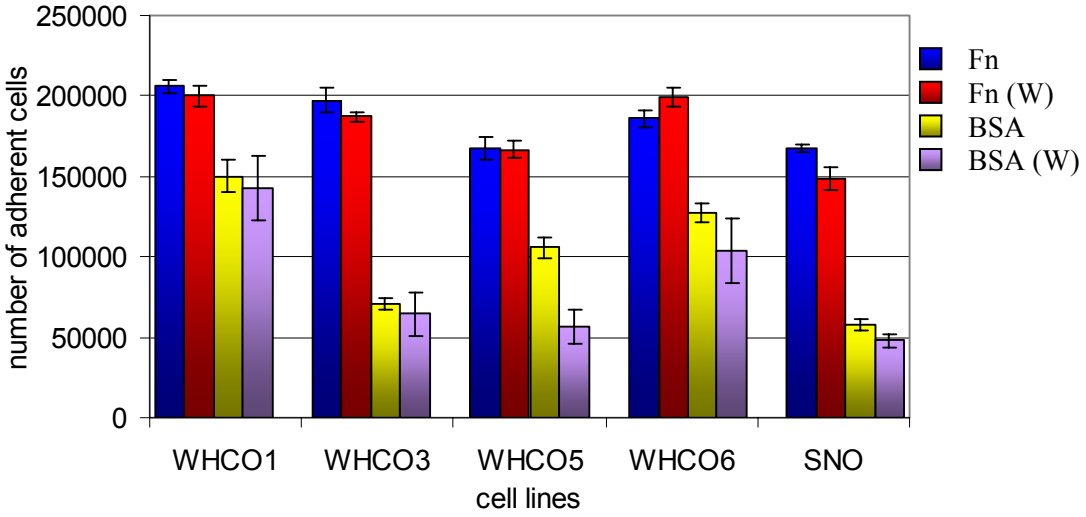


Figure 32: Adhesion to Fibronectin Following Wortmannin Exposure in HOSCCs.

The WHCO1 demonstrated greatest cell adhesion to fibronectin followed closely by WHCO3. Wortmannin (W) induced a reduction in cell adhesion in WHCO1, WHCO3, WHCO5 and SNO but not WHCO6. The differences in cell attachment between wortmannin-treated and untreated cells were not significant ($p \geq 0.05$). Blue bars – the number of cells attached to fibronectin, red bars – the number of cells attached to fibronectin in the presence of the PI3K inhibitor wortmannin, yellow bars – the number of cells attaching to BSA and purple bars – the number of cells attached to BSA in the presence of wortmannin. Bars represent standard deviation. Results represent two experiments performed in triplicate.

5.3.1.3 Cell Adhesion to Collagen Following Exposure to Wortmannin

BSA Cell Attachment Compared within HOSCCs

When considering cell adhesion to the control BSA substrate a slight reduction in adhesion in response to wortmannin treatment was noted, in contrast to cell adhesion to BSA of untreated cells in all human oesophageal SCC cell lines tested. However, this difference was statistically nonsignificant ($p \geq 0.05$, Appendix 3). The trend, however, suggests a reduction in cell adhesion in the wortmannin-treated BSA control in comparison to cell adhesion of untreated cells to BSA (Figure 32). Wortmannin treatment reduced cell adhesion to BSA to the greatest extent in the WHCO3 and SNO cell lines (37 %) and decreased cell adhesion in the WHCO6 and WHCO5 cell lines by 28 % and 21 % respectively. The smallest reduction in cell adhesion to BSA following wortmannin treatment was by the WHCO1 cell line (12 %).

Cell Attachment Compared between HOSCCs (BSA Control)

WHCO1 cells that were not wortmannin-treated exhibited increased cell adhesion to BSA in contrast to that shown by the other four untreated cell lines to the same substrate. This difference was shown to be significant from the WHCO3, WHCO6 and SNO cell lines ($p \leq 0.05$), but not from WHCO5 ($p \geq 0.05$). The difference in cell adhesion between WHCO1 and WHCO5 was only 21 %. Furthermore, cell adhesion to BSA in untreated WHCO1 was 41 % higher compared to the SNO cell line and, 45 % and 42 % higher than untreated WHCO3 and WHCO6 cell lines respectively (Figure 32).

Cell adhesion to BSA by wortmannin-treated WHCO3 and SNO cells was reduced 60 % and 58 % respectively, in comparison to WHCO1, which demonstrated the highest adhesion to BSA following wortmannin treatment. Furthermore, adhesion to BSA of wortmannin-treated WHCO6 cells was 53 % less than treated WHCO1 cells, whereas the WHCO5 cell line was reduced 28 % in comparison. Statistically, cell adhesion of the wortmannin-treated WHCO3, WHCO5, WHCO6 and SNO cell lines to BSA was not significantly different from one another ($p \geq 0.05$). Interestingly, a significant difference was apparent between BSA adhesion of wortmannin-treated WHCO1 cells compared to BSA adhesion of wortmannin-treated WHCO3, WHCO6 and SNO cell

lines ($p \leq 0.05$). No significant difference was apparent between wortmannin-treated WHCO1 and WHCO5 ($p \geq 0.05$) (Figure 32, Appendix 3).

Cell Attachment to Collagen Compared <u>within</u> HOSCCs

Not surprisingly, the number of untreated cells that attached to the collagen substrate was greater than attachment to BSA for all human oesophageal SCC cell lines. In addition, statistical analysis (Appendix 3) showed that the number of untreated cells attached to collagen in comparison to cell attachment to coated BSA culture dishes was significantly different ($p \leq 0.05$). The untreated WHCO3 and SNO cell lines showed the greatest increase in cell adhesion to collagen compared to BSA (up to 62 %). Similarly, the WHCO6 cell line demonstrated a 58 % increase, whereas the lowest adhesion to collagen in comparison to binding to the BSA substrate, were by the WHCO1 and WHCO5 cell lines (Figure 33).

The number of wortmannin-treated cells that attached to collagen increased in comparison to the number of wortmannin treated cells that attached to BSA in all oesophageal SCCs. However, when this data was analysed statistically, it was revealed that four of the cell lines (WHCO1, WHCO3, WHCO6 and SNO) were significantly different from cell adhesion to BSA of wortmannin-treated cells ($p \leq 0.05$). The wortmannin-treated SNO and WHCO3 cell lines exhibited greatest cell adhesion to collagen, up to 71 % higher than wortmannin-treated cell attachment to BSA. Cell adhesion to collagen was reduced to the lowest extent in the WHCO1 and WHCO5 cell lines (27 %), following wortmannin treatment, while cell adhesion to collagen of wortmannin-treated cells in the WHCO6 cell line was 56 % higher than treated cells plated on a BSA substrate (Figure 33).

A comparison of the differences is now required between cell adhesion of untreated cells to that of wortmannin-treated cells to collagen. Not surprisingly, wortmannin reduced cell adhesion to collagen in the five oesophageal SCC cell lines. However, the effect of wortmannin treatment on cell adhesion to collagen was not significantly different compared to collagen cell adhesion of untreated cells ($p \geq 0.05$) (Figure 33). The WHCO5 cell line exhibited the greatest reduction in cell adhesion following wortmannin treatment of 21 %, followed by WHCO1, WHCO3, WHCO6 and SNO,

which were all reduced by just over 1 %. This trend of reduced adhesion will be elaborated upon in the discussion.

Cell Attachment to Collagen Compared between HOSCCs

In the ability to bind collagen, it was untreated WHCO1 cells, which displayed the most rigorous attachment to collagen compared to the other four cell lines. This difference was 26 % higher than collagen adhesion of untreated WHCO6 cells and 19 % higher than the WHCO5 cell line. Cell attachment of untreated WHCO1 cells to collagen was only slightly higher than cell adhesion of untreated WHCO3 and SNO cells to collagen (just over 1 %). Statistically, a significant difference was noted only between the WHCO1 and WHCO6 cell lines ($p \leq 0.05$) (Figure 33).

The number of wortmannin-treated WHCO5 and WHCO6 cells attached to collagen was notably reduced in comparison to treated WHCO1, WHCO3 and SNO cells. Moreover, these differences were shown to be significant ($p \leq 0.05$). The WHCO5 cell line showed a 36 % reduction compared to WHCO1 and was decreased by 25 % and 32 % in comparison to cell adhesion of treated WHCO3 and SNO cells to collagen respectively. The levels of cell adhesion in treated WHCO6 cells were similar to that of the WHCO5 cell line (differing by just over 1 %) (Figure 33).

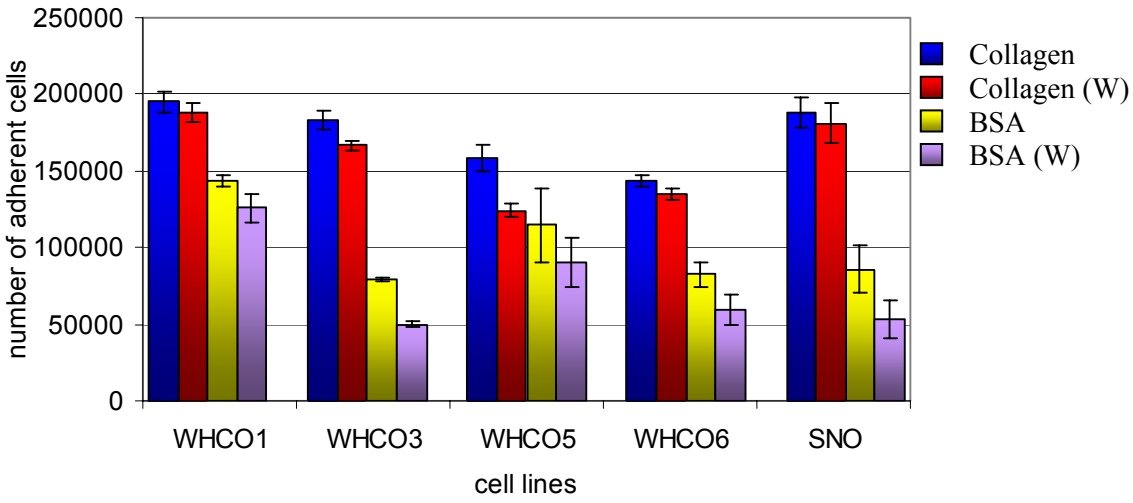


Figure 33: Adhesion to Collagen Following Wortmannin Exposure in HOSCCs. The WHCO1, WHCO3 and SNO cell lines attached more readily to collagen. Attachment to BSA was highest in the WHCO1 and WHCO5 cell lines. Wortmannin treatment reduced adhesion of cells to collagen, but this was statistically not significant ($p \geq 0.05$) from untreated cells. Blue bars – the number of cells attached to fibronectin, red bars – the number of cells attached to fibronectin in the presence of the PI3K inhibitor wortmannin, yellow bars – the number of cells attaching to BSA and purple bars – the number of cells attached to BSA in the presence of wortmannin. Bars represent standard deviation. Results represent two experiments performed in triplicate.

5.3.1.4 A Comparison between Wortmannin and KP-392 Exposure on Cell Adhesion

Comparisons will now be made between cell adhesion of wortmannin-treated cells and cell adhesion of KP-392-treated cells. It is important to note that in both wortmannin and KP-392 fibronectin assays the differences in attachment of untreated cells to fibronectin were nonsignificant in the majority of cell lines examined ($p \geq 0.05$). In untreated WHCO3 cells however, a significant difference was observed between fibronectin cell binding of the respective fibronectin assays ($p \leq 0.05$). A large reduction in cell adhesion of 32 % was shown in WHCO3 cells between the fibronectin assays. SNO cells also showed a large difference of 28 % while in the WHCO1, WHCO5 and WHCO6 cell lines these differences were considerably less (up to 13 %).

When comparing the differences between wortmannin and KP-392 on the attachment of cells to fibronectin the following was apparent. The trend following treatment of cells with both wortmannin and KP-392 was a reduction in cell adhesion across all five HOSCCs. However, unlike the wortmannin treatment, which exhibited no significant differences between cell attachments of untreated cells to that of treated cells, a significant difference was noted in KP-392-treated WHCO5 cell line compared to untreated WHCO5 cells ($p \leq 0.05$) (Figures 31 and 32).

5.3.1.5 A Comparison between Collagen and Fibronectin as Substrates for Cell Adhesion Following Wortmannin Exposure

Finally, when comparing the effect of wortmannin on collagen adhesion to that of fibronectin adhesion (at the $p \leq 0.05$ level), the following similarities and distinctions were apparent. Wortmannin caused a reduction in cell adhesion, although nonsignificant, to collagen compared to cells that plated in the absence of wortmannin in all the cell lines. Similarly, in the fibronectin adhesion assay, wortmannin caused a reduction in cell adhesion but again not significant ($p \geq 0.05$). However, this was the case for only four of the cell lines. When plated on fibronectin, the WHCO6 cell line demonstrated an increase in cell adhesion following wortmannin treatment. Cell adhesion to collagen of wortmannin-treated WHCO6 cells did not demonstrate this increased attachment. This further corroborates the notion that the WHCO6 cell line

expresses different integrin receptors when binding to either collagen or fibronectin. Thus the wortmannin-mediated PI3K/ILK reduction may affect distinct signalling pathways in the WHCO6 cell line in the presence of differing ECM ligands. Although this increase in WHCO6 cell adhesion to fibronectin following wortmannin treatment was not significant from cell adhesion in the absence of wortmannin, it was the only cell line that exhibited increased cell attachment subsequent to wortmannin treatment.

It appears that these oesophageal SCC cell lines adhere to fibronectin more readily than collagen. Cell adhesion between collagen and fibronectin was statistically shown as not significant in the WHCO1, WHCO3 and SNO cell lines ($p \geq 0.05$). Nevertheless, a trend exists that suggests an increase in cell adhesion to fibronectin in these cell lines when compared to collagen adhesion. A significant difference was noted between collagen and fibronectin cell adhesion for untreated/wortmannin treated WHCO6 cells as well as for wortmannin treated WHCO5 cells ($p \leq 0.05$) (Figures 32 and 33).

5.4 Discussion

Integrins have long been recognised as the dominant family of cell adhesion receptors that mediate attachment to the ECM (Puddefoot *et al*., 1997; Koivisto *et al*., 2000; Reyes-Reyes *et al*., 2002). Integrin ligation to the ECM activates the PI3K signalling pathway, which leads to the activation of an integrin-mediated moiety, namely ILK (Somasiri *et al*., 2000). ILK is thought to play an essential role during the assembly and functioning of the cell-matrix adhesion structures (Zhang *et al*., 2002). Two lines of evidence support this notion. Firstly, ILK activity is stimulated by extracellular matrix attachment (Delcommenne *et al*., 1998; Duxbury *et al*., 2005) and secondly, ILK is capable influencing the process of cell adhesion to the ECM (Ishii *et al*., 2001). In light of this role of ILK, it was of interest to elucidate the effect that inhibiting ILK has on cell-ECM adhesion in HOSCC.

Inhibition of ILK was exercised through the use of KP-392. KP-392 is a small molecule ATP-analog, which has previously been shown to inhibit ILK activity in a highly selective, dose-dependent manner (D'Amico *et al*., 2000; Persad *et al*., 2001; Yoganathan *et al*., 2002; Attwell *et al*., 2003; Troussard *et al*., 2006). In order to ascertain whether PI3K was mediating the ILK-induced effects on cell-ECM adhesion, cell adhesion assays were also conducted in the presence of an inhibitor of PI3K activity, wortmannin.

The biochemical function of wortmannin on ILK has been demonstrated by Delcommenne *et al*., (1998). These authors showed that in rat intestinal epithelial cells (IEC-18), ILK activity depended on PI3K, and furthermore, that PI3K was functioning upstream of ILK. This experiment was accomplished by measuring ILK activity at various time intervals following plating on fibronectin and wortmannin treatment (Delcommenne *et al*., 1998). ILK activity is stimulated upon attachment to ECM components. However, this stimulation is only transient, peaking at approximately 30-45 minutes and declining rapidly so that at 60 minutes it is almost diminished (Delcommenne *et al*., 1998; Dedhar *et al*., 1999). Since this is the only known study to consider the effects of Wortmannin on ILK it was assumed that, in these oesophageal SCCs, wortmannin would induce a similar effect on the PI3K/ILK signalling sequence in HOSCCs.

To establish the effects of wortmannin and KP-392 exposure, on the ability of the oesophageal carcinoma cells to adhere to fibronectin, it was shown that both wortmannin and KP-392 resulted in a reduction in cell attachment to fibronectin. In the case of wortmannin exposure, these differences were not statistically significant in any of the cell lines. However, KP-392-treated WHCO5 cells exhibited a significant reduction in cell attachment to fibronectin ($p \geq 0.05$). Furthermore, the decreases in cell adhesion to fibronectin that were observed in KP-392-treated cells of the remaining oesophageal lines were more substantial than that of wortmannin-treated cells (12-30 % reduction in adhesion). This implies that ILK is indeed involved in directing cell-ECM adhesion in HOSCCs. Furthermore, it is proposed that the inhibition of cell-fibronectin adhesion is not mediated by PI3K, which is especially apparent in the WHCO5 cell line. It is possible that another protein moiety such as PTEN may be more directly involved during ILK-mediated cell-ECM adhesion events.

In support of the data which showed a trend in reduced cell adhesion in the presence of KP-392, Attwell *et al*., (2003) demonstrated that treatment of PC3 cells with KP-392 resulted in reduction in cell attachment to fibronectin. It was suggested that the decrease in cell adhesion associated with decreased ILK activity could be due to the role of ILK in focal adhesion formation and actin organisation (Attwell *et al*., 2003). In addition to the known substrates of ILK, which include CH-ILKBP, paxillin and vinculin, these authors propose that currently unidentified substrates of ILK are not phosphorylated, which in turn prevents proper focal adhesion formation and integrin malfunction integrin (Attwell *et al*., 2003).

When considering the effects of both wortmannin and KP-392, the cell adhesion assays, performed above, established that all the oesophageal SCCs tested depended on the presence of either collagen or fibronectin for cell adhesion, since significant differences were noted between cell adhesion to collagen/fibronectin and the BSA controls (see Figures 31**,** 32 and 33). Attachment to ECM substrates by stratified epithelial squamous cells *in vivo* requires adhesion to collagen and fibronectin. Since this study demonstrated that cell attachment to either collagen or fibronectin was greater than adhesion to BSA, it implies that the behaviour of cultured cells *in vitro* is similar to that of cells *in vivo*.

In the presence of wortmannin, the collagen-based adhesion assay showed that WHCO5 and WHCO6 cell adhesion to collagen was reduced in comparison to the other cell lines. Interestingly, when cells were exposed to either wortmannin or KP-392 in the fibronectin-based adhesion assay, it was the WHCO5 and SNO cell lines, which showed greatest reduction in adhesion. However, in the instance of wortmannin treatment, the WHCO6 cell line exhibited increased binding to fibronectin, which was not exhibited by KP-392-treated WHCO6 cells (see Figures 31 and 32). Furthermore, fibronectin adhesion of wortmannin-treated WHCO6 cells was much higher compared to collagen adhesion of wortmannin-treated WHCO6, where this difference was shown to be statistically significant ($p \leq 0.05$). A possible explanation for this apparent increase in cell adhesion could be that integrin expression levels are increased in the WHCO6 cell line. Indeed, differential integrin expression could account for the noted differences in cell attachment to the ECM substrates between the five cell lines. However, the process of establishing which integrin is expressed when this cell line attaches to ECM substrates is not trivial and remains of interest for future work.

It has been discovered that the β_1 integrin requires a period in excess of two hours for biological maturation (Heino *et al*., 1989). Thus adhesion of oesophageal SCCs to the various ECM proteins can be considered to be a consequence of the integrins already embedded in the cell membrane and not due to *de novo* integrin synthesis (Miller, 2001), since the adhesion assays were performed over a time period of two hours. Furthermore, the increase in cell adhesion on the ECM proteins in comparison to BSA controls is indicative of integrin engagement via "inside-out signalling". As a reminder to the reader that "inside-out signalling" refers to the ability of intracellular signals to modulate integrin affinity towards ECM proteins (Holly *et al*., 2000). Due to ILK involvement in integrin signalling, ILK may well be mediating this activation process.

The number of cells attaching to either collagen or fibronectin treated with wortmannin or KP-392 exhibited no significant difference in comparison to attachment of untreated cells ($p \geq 0.05$). This cell adhesion assay was modified from an established protocol from Sugiyama *et al*. (1993), and it is feasible that this assay technique lacks sensitivity. However, there was a reduction in the number of cells attaching to both collagen and fibronectin following wortmannin treatment (besides WHCO6) and a decrease in cell attachment to fibronectin following KP-392 treatment (see Figures 31**,** 32 and 33).

Interestingly, WHCO6 was the only cell line to exhibit an increase in cell adhesion to fibronectin following wortmannin treatment. Although the increase was nonsignificant, adhesion in the WHCO6 cell line was different to the rest of the cell lines, which requires some sort of explanation. Speculating on the reason for this result is difficult since this particular outcome was observed in only one cell line. This result does suggest however, that the reduction of PI3K in WHCO6 via wortmannin leads to activation of integrin and increased binding towards ECM ligand. Alternatively, it is possible that deficient β integrin subunit phosphorylation, due to inhibited ILK, prevents the removal of the integrin heterodimer from the cell-matrix adhesion site. In support of this, Mulrooney *et al*. (2000) have shown that, in migrating parietal endoderm cells, ILK is capable of phosphorylating the β_1 integrin subunit, resulting in a subsequent release of β_1 from the cytoskeleton and removal of cell-matrix adhesion.

Besides the WHCO6 cell line, the other four cell lines displayed a reduction in cell adhesion to either collagen or fibronectin after wortmannin treatment, in comparison to untreated cells (see Figures 32 and 33). KP-392 induced a similar reduction on cell attachment, which was apparent for all five HOSCCs. Although this decline in cell adhesion to collagen/fibronectin for wortmannin- and KP-392-treated cells was not significant ($p \leq 0.05$) the trend cannot be ignored. Since ANOVA is a robust statistical test and the sample size (N) of this study was low; it is feasible that choosing another arbitrary p-value ≥ 0.05, would substantially increase the probability of significance for wortmannin and KP-392 effects on cell adhesion. For example, if a p-value of 0.1 was chosen, it was apparent that wortmannin and KP-392 induced a significant reduction in cell adhesion in all the cell lines. This would then imply that ILK inhibition via KP-392 is sufficient to disrupt the structure of the αβ integrin heterodimer-ECM ligand interaction and, is influenced by the upstream effects of PI3K. A consequence of this would be reduced cell adhesion to collagen and fibronectin ECM substrates.

The increased adhesion to fibronectin in the wortmannin treated WHCO6 cell line was unexpected but can be explained by previous studies which have indicated that ILK overexpression, and not inhibition, reduces cell adhesion. For example, overexpression of ILK in IEC-18 induces a less adhesive phenotype (Hannigan *et al*., 1996; Radeva *et al*. 1997). Furthermore, Attwell *et al*. (2000) showed that ILK overexpression in mouse mammary epithelial cells (SCP2) inhibits anoikis, a form of apoptosis, which occurs

when cell-ECM interactions are disrupted. Taking these data into consideration, one would expect ILK inhibition to correlate with stabilisation of the αβ integrin heterodimer, a consequence being increased cell adhesion to ECM.

Another possible explanation for the observed effects of wortmannin on the increased cell adhesion noted in the WHCO6 cell line is due to reduced wortmannin activity over the 2 hour incubation period utilised during the cell adhesion assay. While Delcommenne *et al*., (1998) demonstrated that 20 minutes was sufficient time for wortmannin to reduce PI3K and subsequently ILK activity, the authors make no mention of the feasible possibility that wortmannin activity peaks at 20 minutes, and subsequently becomes reduced over prolonged periods. If this were indeed the case then the 2 hour incubation period in the present study would be indicative of diminished wortmannin activity and hence, elevation of PI3K and subsequent ILK activity levels. This would result in the stabilisation of the αβ integrin heterodimer complex.

To our knowledge this was the first investigation to consider the effects of inhibited ILK on cell-ECM adhesion in oesophageal SCCs. In fact, this is the first study to examine cell adhesion in SCCs in general. Previous studies have examined ILK activity-based adhesion in other cell types, including IEC-18 intestinal epithelial cells and Scp2 mammary epithelial cells (Delcommenne *et al*., 1998; Persad *et al*., 2000). These differences in tissue types may require different integrins for ECM specificity, hence accounting for the differences in cell adhesion to fibronectin.

Interpreted in conjunction with the ILK activity levels elucidated in Chapter 3, there does not seem to be a similarity between the patterns of basal ILK activity and the effect of reduced ILK activity on cell adhesion. Recalling that KP-392 and wortmannin specifically inhibits ILK and PI3K respectively, ascertaining the time period for optimal inhibition of ILK and PI3K, would require a time course experiment for both these inhibitors. Subsequently, the length of time that the cells are exposed to either KP392 or wortmannin during the course of the cell adhesion assay can be altered accordingly. Only then would it be possible to further understand the behaviourial change on cell adhesion as a consequence of KP392-induced ILK inhibition. The surprising high binding capacity to BSA that was observed in the cell adhesion was surprising. It suggested that impurties may have been present in the BSA which allowed for binding

to certain proteins and accounted for the successful binding to BSA in this study (see Figures 31, 32 and 33).

A complex reciprocal interaction exists between ILK and the integrin receptors where, integrins modulate ILK activity and, ILK regulates integrin activity via phosphorylation of the β integrin subunit (Hannigan *et al*., 1996; Mulrooney *et al*., 2000; Janji *et al*., 2000; Somasiri *et al*., 2000). From a statistical standpoint, this investigation implies that ILK alone is not involved in adhesion events in oesophageal carcinoma over the time period analysed but certainly plays a key role. However, due to the obvious trend showing a reduction in cell adhesion in ILK-inhibited cells to both collagen and fibronectin observed in all five HOSCCs and the demonstration of an interaction between ILK and the β integrin subunit (see Chapter 2), the involvement of ILK in cell-ECM processes in HOSCCs cannot be ignored. These results may also have been affected by differences in collagen and fibronectin receptor numbers which would have required different analyses to resolve. Furthermore, in the instances of the WHCO1, WHCO3 and SNO cell lines, ILK regulation of cell attachment is almost certainly mediated by PI3K. However, the WHCO5 and WHCO6 cells did not follow the trend of reduced cell adhesion. PI3K inhibition via wortmannin treatment understandably had less of an affect on cell adhesion since this approach indirectly influenced ILK activity. However, direct ILK inhibition via KP-392 treatment resulted in a more substantial effect on cell attachment. This is evidence for a definite role for ILK in cell adhesion in HOSCCs.

Chapter 6

General Discussion and Conclusion

6.1 The Importance of ILK in Human Oesophageal Squamous Cell Carcinomas

At present, it is widely accepted that the signalling cascades existing within cells are a tightly regulated phenomena (Persad *et al*., 2000; Yoganathan *et al*., 2000; Brakebusch and Fässler, 2003; Mould and Humphries, 2004). This regulation occurs primarily through the ability of numerous protein kinases and phosphatases to either phosphorylate or dephosphorylate protein substrates (Yoganathan *et al*., 2000). ILK is a serine/threonine protein kinase that is capable of influencing cell proliferation, migration, differentiation and survival (Novak *et al*., 1998; Dedhar *et al*., 1999; Yoganathan *et al*., 2000; Wu and Dedhar, 2001; Pasquet *et al*., 2002). ILK influences these signalling processes by regulating intermediate molecules such as GSK3β, PKB, and PINCH, as well as the integrin class of adhesion receptors (Tan *et al*., 2001; Yamaji *et al*., 2002; Cordes and van Beuningen, 2003; Gary *et al*., 2003; Obara *et al*., 2004; Pinkse *et al*., 2005; Yau *et al*., 2005).

The way in which ILK accomplishes this regulation is through its kinase domain, which serves to phosphorylate specific residues present on target proteins. For example, in response to phosphorylation by ILK, PKB elicits a response that promotes cell survival whereas phosphorylation of GSK3β initiates a signalling response that leads to differentiation and proliferation in cells (Dedhar *et al*., 1999; Attwell *et al*., 2000; Yoganathan *et al*., 2000; Dai *et al*., 2003).

From a tumour biology perspective, ILK is vital to the control of these aforementioned processes in order to maintain cellular functioning within normal limits. Disturbances in this system are seen in cancers, good examples being colon, prostate, breast and brain. What makes the present study particularly exciting is that ILK has not previously been described in any SCC and, due to the importance of oesophageal SCCs in South Africa, it has become critical to investigate the role of ILK in oesophageal carcinoma cells.

It is well established that alterations in ILK impinge upon essential signalling pathways that ultimately impact upon cell behaviourial traits (Walsh and Dedhar, 2002; Dai *et al*., 2003; Stevens *et al*., 2004; Vouret-Craviari *et al*., 2004; Wu, 2004; Watanabe *et al*., 2005; Boulter *et al*., 2006). For example, overexpressed ILK alters the phenotype of cells by inducing an epithelial to mesenchymal transformation (Novak *et al*., 1998; Somasiri *et al*., 2000), as well as resulting in increased anchorage-independent growth, cell cycle progression and invasiveness (Radeva *et al*., 1997; Dedhar, 2000; Dai *et al*., 2003; Ito *et al*., 2003). Conversely, inhibition of ILK has been shown to reduce tumour growth in pancreatic cancer (Yau *et al*., 2005). Current literature suggests that ILK is directly associated with another aspect of cell behaviour, that is, cell-ECM adhesion (Hannigan *et al*., 1996; Mulrooney *et al*., 2000; Attwell *et al*., 2003). Cell adhesion is important as it is directly linked to the ability of tumour cells to leave their original site and metastasise. Due to the fact that ILK has not been previously considered in carcinoma of the oesophagus, a biochemical description of ILK was first required, before an understanding of the significance of ILK in cell adhesion events could be achieved.

6.2 ILK and Integrin Signalling Pathways are Closely Associated

Typically, ILK distribution has been shown to occur along focal adhesion plaques in the majority of cell types examined, implicating this protein kinase in integrin-mediated adhesion events (Zervas *et al*., 2001; Dai *et al*., 2003; Aoyagi *et al*., 2005; Cho *et al*., 2005). ILK localisation requires the adaptor protein, PINCH, which binds, through its LIM domain to the ankyrin (ANK)-repeat domain of ILK (Wu, 2004). The ANK repeats are necessary but not sufficient for ILK localisation to focal adhesions as demonstrated by Li *et al*. (1999). Briefly, these authors showed that an ILK mutant lacking the C-terminal binding site, but containing the ANK repeats, failed to localise to focal adhesions in Chinese hamster ovary cells (CHO). Furthermore, they showed that ILK was not recruited to epithelial cell-cell adhesion sites (Li *et al*., 1999). This interaction has been found in both mammalian cells as well as *in vitro* (Wu, 1999). The observation that ILK is a focal adhesion constituent, strongly suggests that ILK could function as one of the major kinases responsible for phosphorylation in focal adhesions during integrin-mediated signal transduction (Li *et al*., 1999). Examination of the localisation

of ILK would hence allow for the functional role/s of ILK in carcinoma of the oesophagus to be gauged, by identifying the site/s of ILK functioning in the cell.

It was shown that ILK and the β_3 integrin were interacting, as demonstrated by co-immunoprecipitation analysis (see Figure 9). Miller and Veale (2001) previously showed that the β_1 integrin subunit followed a membranal distribution, which suggests similar distributions between ILK and this subunit. Co-localisation between ILK and the β_3 integrin was demonstrated in these oesophageal carcinoma cell lines. ILK is able to associate with the majority of β integrin subunits including β_1, β_2 and β_3 (Hannigan *et al*., 1996; Dedhar *et al*., 1999; Somasiri *et al*., 2000).

Indirect immunofluorescence showed that ILK displayed prominent and distinctive cytoplasmic localisation. Unexpectedly prominent nuclear localisation was also demonstrated, with variable amounts of ILK distributed at the plasma membrane (see Figure 10a-e). The demonstration of ILK nuclear localisation was somewhat surprising, especially since ILK is largely regarded as a focal adhesion protein. Subsequent Western analysis however confirmed that ILK was prominently present in nuclear extracts of all five HOSCCs (see Figure 11). Interestingly, Chun *et al*. (2005) demonstrated recently that for cytoplasmic retention of ILK an association with a cholesterol-dependent molecule, caveolin-1 is required. When this interaction was disrupted it resulted in subsequent ILK nuclear localisation. Since it is likely that ILK nuclear localisation could be explained by mechanism/s involving caveolin-1, this molecule was considered important with regards to ILK distribution in HOSCCs. ILK nuclear localisation and caveolin-1 will be discussed at a later stage in this chapter.

At present, it is generally thought that the complete elucidation of the functional significance of ILK activity would aide in providing novel research approaches to the development of specific treatments in malignancies (Yoganathan *et al*., 2000). The activity of ILK is PI3K-dependent and is stimulated by numerous signalling molecules including growth factors and ECM (Dedhar *et al*., 1999; Troussard *et al*., 2000; Dai *et al*., 2003; Barker *et al*., 2005). In support of this, it has been shown that the interaction of ILK with the cytoplasmic tail of the β_1 integrin subunit activates ILK when epithelial cells adhere to the ECM in a β_1 integrin-dependent manner (Hannigan *et al*., 1996; Somasiri *et al*., 2000).

Integrin activation is also directly affected by the kinase activity of ILK. The interaction of integrin receptors to specific ECM ligands results in the activation of PI3K signalling, which in turn activates ILK (Somasiri *et al*., 2000; Attwell *et al*., 2003; Grashoff *et al*., 2004; Khyrul *et al*., 2004). It has long been known that ILK interacts with the integrin class of receptors and, in doing so, is able to phosphorylate the cytoplasmic domain of the β integrin subunit (Hannigan *et al*., 1996; Janji *et al*., 2000; Mulrooney *et al*., 2000). Since the β integrin subunits do not possess any catalytic activity, this phosphorylation by ILK is thought to regulate integrin activity (Hannigan *et al*., 1996; Attwell *et al*., 2000; Mulrooney *et al*., 2000). Furthermore, this interaction is reciprocal since the integrins are also able to regulate ILK activity (Pinkse *et al*., 2005). In confirmation of this, it has been shown that the interaction of ILK with the cytoplasmic tail of β_1 integrin subunit activates ILK when epithelial cells adhere to the ECM in a β_1 integrin-dependent manner (Hannigan *et al*., 1996; Delcommenne *et al*., 1998; Somasiri *et al*., 2000).

From the identification of an integrin β_3-ILK association in it would appear that integrin-mediated signalling events are regulated by ILK in oesophageal carcinoma. Since this interaction is not compromised in HOSCCs suggests that integrin signalling pathways depend on ILK functionality. In this particular instance, it implies that HOSCCs behave similarly to other tissue types, in which integrin activation relies heavily on ILK activity (Hannigan *et al*., 1996; Mulrooney *et al*., 2000; Boulter *et al*., 2006). Thus, it appears that ILK and the integrin receptors are closely intertwined. Integrins however, are not the only molecules responsible for ILK activation. Stimulation of ILK activity is also mediated by signalling molecules, including growth factors and the ECM (Hannigan *et al*., 1996; Dedhar *et al*., 1999).

Perhaps it is important at this point to clarify that while PINCH is necessary for focal adhesion localisation of ILK to occur, caveolin-1 is required for cytoplasmic retention. Thus, the association currently shown between ILK and β_3 integrin further indicates that a stable complex exists between the ANK repeats of ILK and the LIM domains of PINCH in oesophageal SCCs, although this has not been validated in these HOSCCs. Nonetheless, the present investigation still corroborates previous evidence of ILK functioning in integrin-mediated signalling pathways.

The growth factor receptor link to the integrin receptors is well established (Miyamoto *et al*., 1996; Lai *et al*., 2000; Pouliot *et al*., 2000; Yu *et al*., 2000; Mariotti *et al*., 2001), and the above data point towards an involvement of ILK in integrin receptor signalling. The following section elaborates upon the findings which clearly demonstrate that ILK is intricately involved in growth factor signalling pathways.

6.3 Functional ILK Activity in Relation to ILK Protein Expression

The first step in understanding ILK in carcinoma of the oesophagus required the confirmation that the ILK gene in oesophageal SCCs was undergoing active transcriptional and translational processes. This was verified by the expression of a 59kDa ILK protein band, and the RT-PCR amplification of a 1360bp ILK fragment. ILK has been identified in a variety of tissues that include those of the pancreas, stomach, liver, thymus, brain, prostate, and intestine and is found to be abundantly expressed in skeletal and heart muscle (Hannigan *et al*., 1996; Janji *et al*., 2000; Friedrich *et al*., 2002). Furthermore, its overexpression has been demonstrated in certain malignancies that include prostate and colon carcinoma, medullablastoma, melanoma and gastric carcinoma (Chung *et al*., 1998; Persad *et al*., 2000; Dai *et al*., 2003; Ito *et al*., 2003). The current study is the first demonstration of ILK expression in not only SCC tumour cell lines but furthermore, the first identification in oesophagus.

The signalling pathways of ILK are known to interdigitate with both the integrin and growth factor receptors, allowing for crosstalk between these two classes of receptors (Delcommenne *et al*., 1998; Janji *et al*., 2000; Mulrooney *et al*., 2000; Nikolopoulos and Turner, 2002; Barker *et al*., 2005; Boulter *et al*., 2006; Shafiei and Rockey, 2006). To establish if growth factor receptor signalling and ILK were associated, the effect of growth factors on ILK expression and activity was examined. Analysis revealed that the EGF and TGFβ1 growth factor upregulated ILK protein expression in all five oesophageal carcinoma cell lines, implying that within a certain class of oesophageal SCC cell lines, similar regulatory mechanisms for ILK signalling exist. With regards to EGF the increased expression of ILK following EGF exposure in these cell lines was not unexpected, considering that these HOSCCs overexpress EGFR (Veale and Thornley, 1989). Thus it is apparent that EGF-stimulated ILK could have further consequences with regards to the tumourigenicity of oesophageal carcinoma. In

addition, it suggests that growth factor and ILK signalling pathways are closely associated and that crossmodulation exists between these signalling molecules (see Chapter 2, Figures 12 and 13).

The only other data considering growth factor effects on ILK clearly demonstrated an increase in ILK regulation following growth factor exposure (Delcommenne *et al*., 1998; Janji *et al*., 2000; Cho *et al*., 2005). These authors similarly showed that ILK2 expression was increased following EGF- and TGFβ1-stimulation in human melanoma cell lines, while a separate study revealed that insulin was capable of stimulating ILK activity in intestinal epithelial cells (Delcommenne *et al*., 1998; Janji *et al*., 2000). The current data provide the first evidence that ILK is expressed and stimulated in response to growth factor treatment in SCC tumour cell lines of oesophagus. These data corroborated the above evidence from Janji *et al*. (2000) which was the only basis of comparison for the effects of the EGF and TGFβ1 growth factors on ILK expression.

Lynch *et al*. (1999) have expressed their doubt as to whether ILK behaves as a bona fide protein kinase. These authors suggested that ILK regulates PKB phosphorylation indirectly rather than directly. This speculation is based on their observation that a mutation of a specific serine residue present in PKB, a recognised autophosphorylation site, rendered PKB active. They created an ILK dominant negative mutant and showed that this mutant could still phosphorylate PKB (Lynch *et al*., 1999 cited in Yoganathan *et al*., 2000), indicating that ILK activity is not required for PKB phosphorylation. However, more recently it was shown that, in a dominant negative form of ILK, ILK activity is severely decreased and inhibits PI3K-dependent phosphorylation of PKB in a dominant negative manner. This suggests that ILK activity is required for PKB phosphorylation (Yoganathan *et al*., 2000; Walsh and Dedhar, 2001). There is thus conflicting evidence regarding the kinase activity of ILK. The kinase activity of ILK is the primary way in which ILK regulates cell survival, but as mentioned at the beginning of this discussion, there are numerous cellular processes that are regulated by ILK. One such behaviourial event is that of cell-ECM adhesion. Thus it was necessary, and of extreme importance to ascertain the functionality of the ILK kinase domain.

The kinase domain of ILK was shown to be active following MBP-based kinase assays. Since MBP is a β integrin subunit analogue, this result highlighted the possibility of an

interaction between ILK and integrin mediated pathways in oesophageal SCCs. This association will be considered further on but presently, the effect of growth factors on ILK activity will be discussed, to ascertain if growth factors affect ILK activity in a similar fashion to ILK protein expression. Due to the noted increase in ILK protein expression following growth factor treatment, a similar increase in ILK kinase activity was expected in the presence of growth factors in these oesophageal SCC cell lines. Furthermore, our suggestion is supported by Delcommenne *et al*. (1998) who demonstrated increased ILK activity in response to insulin and PDGF growth factors in IEC-18 intestinal epithelial cells.

Growth factors are tightly linked to cell proliferation in epithelial cells (Ozanne *et al*., 1986). Since ILK is involved in cell cycle progression via PKB-independent pathways involving GSK3β, β-catenin, or AP-1 transcription factor (Radeva *et al*., 1997; Persad *et al*., 2000; Troussard *et al*., 2000; Pinkse *et al*., 2005), it was thought that growth factors would stimulate ILK activity. While EGF increased ILK protein expression across all cell lines it is noteworthy that EGF and TGFβ1 resulted in both increases/decreases in ILK activity in the WHCO3 and WHCO5 with a decrease in ILK activity in the remaining cell lines. Thus subsequent to growth factor treatment, there was an inclination towards a reduction in ILK activity, opposed to the increase noted in ILK expression. This is linked to the capacity of TGFβ1 to be either stimulatory at one concentration or inhibitory at another concentration (Fujimoto *et al*., 2001; Hu and Zuckerman, 2001). The growth factor-induced upregulation of ILK protein expression in these HOSCCs implicated ILK in the progression of oesophageal carcinoma. The demonstration that growth factors both stimulated and inhibited ILK activity further implied that ILK is more intimately involved in oesophageal carcinoma development than originally suggested. Indeed, growth factor-mediated changes in ILK activity could possibly lead to the dysregulation of both integrin-mediated cell adhesion and integrin signalling cascades, resulting in aberrant expression of critical protein kinases such as FAK and MAPK.

This reduction in ILK activity was unexpected considering that these oesophageal carcinoma cell lines overexpress EGFR (Veale and Thornley, 1989). In squamous carcinoma cells, EGFR has been found to be responsible for the activation of the Ras/Raf/ERK signalling pathway as well as the PI3K signalling pathway as a direct

result of increased EGF signalling (Mariotti *et al*., 2001). This identifies a route by which cellular events such as cell adhesion may be regulated, since PI3K activation may affect ILK/integrin-mediated cell attachment to ECM substrates. This route may occur through PI3K-mediated regulation of FAK. However, since HOSCCs demonstrated increased ILK protein expression following both EGF and TGFβ1 treatment, and decreased ILK activity following treatment with the same growth factors, it suggests that PI3K levels are not similarly stimulated by growth factors.

An alternate signalling pathway may be activated by EGF in HOSCCs instead of PI3K. This may include stimulation of an ILK inhibitor, PTEN. Therefore, PTEN expression levels were monitored in the presence of growth factors. To ensure proper tissue homeostasis, epithelial cell growth and survival need to be tightly regulated. Thus ILK, which is positively regulated by PI3K, requires a negative regulator. From the above hypothesis of unchanged activity levels of PI3K following growth factor treatment, the PTEN lipid, which is a protein phosphatase, serves as such a negative regulator of ILK. PTEN was the obvious protein phosphatase to consider when explaining the effects of growth factors on ILK. Since it has been estimated that 60 % of all solid tumours contain inactivating mutations of PTEN (Persad *et al*., 2000), it is evident that this protein phosphatase is crucial during the maintenance of ILK activity. Persad *et al*. (2000) demonstrated that ILK activity is anchorage independent in PTEN-null prostate carcinoma cells and, anchorage dependent in cells expressing PTEN.

The EGF-induced effects on ILK activity are mediated by PTEN in the WHCO1, WHCO3 and WHCO5 cell lines, since PTEN expression in these cells correlated with ILK activity levels (see Figure 17b). In the case of WHCO6 and SNO cells, PTEN could not be considered to be regulating the growth factor-mediated responses in ILK activity. Instead, it is proposed that another growth factor signalling moiety such as ILKAP is regulated by EGF in WHCO6 and SNO cells subsequently resulting in a reduction of ILK activity (see Figure 17b). Evidence has suggested that TGFβ1 causes a reduction in PTEN in keratinocytes (Janji *et al*., 2000). Thus, ILKAP may also play a role in mediating ILK activity in TGFβ1-treated WHCO3 cells (see Figure 18a and b). In support of this notion, ILK was shown to be negatively regulated when physically associated with ILKAP in transformed human embryonic kidney (HEK293) cells (Leung-Hagesteijn *et al*., 2001).

The effect of growth factors on PTEN expression implies that PTEN has a greater role to play in growth factor-mediated effects of ILK activity. Furthermore, although interactions between growth factor receptors and PI3K signalling have been shown in other malignancies, including ovarian and prostate carcinomas (Ellerbroek *et al*., 2001; Bancroft *et al*., 2002), this interaction has not been demonstrated in carcinoma of the oesophagus. Determining this interaction thus remains an area of focus for future studies. However, the demonstration of PTEN levels in response to growth factors in these cell lines has provided insight into the mechanism of growth-factor modulation of ILK activity in carcinoma of the oesophagus.

From the above data it seems likely that ILK is intricately involved in growth factor and integrin signalling processes of oesophageal carcinoma cell lines. To our knowledge this is the first investigation regarding ILK in oesophageal SCCs. These differences in ILK activity in response to growth factor exposure imply that ILK regulation within these HOSCCs is highly complex. Furthermore, these noted differences in ILK activity were somewhat surprising since these cell lines are similarly pathologically graded.

Although these data provide a basis for the kinase activity of ILK in HOSCCs the adaptor-type role of ILK cannot be discounted. Complexes between ILK, PINCH and parvin would also modulate some of the ILK activity described (Nikolopoulos and Turner, 2001). Besides these complexes, ILK binding to paxillin and affixin can also significantly regulate ILK acitivty (Wu and Dedhar, 2001).

6.4 Significance of the ILK and PTEN Interaction

It is well established that ILK is an effector of PI3K signalling, which upon dephosphorylation of PI3K via PTEN, leads to inhibition of ILK activity (Attwell *et al*., 2003; Grashoff *et al*., 2004; Obara *et al*., 2004). It stands to reason that in order for these regulatory processes to be performed efficiently, PI3K, PTEN and ILK need to be in close proximity. The above data clearly suggested that PTEN mediates the growth factor effects of ILK activity. Thus, it is obvious that PTEN plays an important role with regard to the regulation of ILK signalling in HOSCCs.

It was surprising that initial co-immunoprecipitation analysis did not reveal PTEN in ILK-precipitated lysates (see Figure 19a). However, when co-immunoprecipitation was performed on PTEN-immunoprecipitated lysates, ILK was identified at a molecular weight of 59 kDa (see Figure 19b). The surprising result of no PTEN band being present in ILK immunoprecipitated lysates was possibly due to ILK not associating with PTEN in a 1:1 molar ratio. Another explanation was that the binding epitopes of PTEN were masked, preventing antibody recognition and binding. However, subsequent analysis of PTEN localisation revealed a very similar pattern of distribution to that noted for ILK (see Figure 20), which corroborates the data from the successful co-immunoprecipitation analysis.

It is proposed that the ILK/PTEN interaction serves an important regulatory function. Since our data confirmed an interaction between ILK and PTEN in HOSCCs, it is suggested that ILK may also be capable of modulating PTEN activity through phosphorylation (see Figure 21). PTEN phosphorylation is speculated to serve a regulatory role in PTEN function, although evidence to confirm this has remained elusive (Vazquez *et al*., 2000). Recent evidence however, suggests that PTEN phosphorylation serves to maintain PTEN in a conformation that prevents membrane association (Das *et al*., 2003; Leslie and Downes, 2004). Although originally thought to involve CKII, it has now come to light that GSK3β is also capable of directly phosphorylating PTEN (Al-Khouri *et al*., 2005; Gericke *et al*., 2006). Although not demonstrated, it is possible that the identified interaction between ILK and PTEN serves to provide a similar regulatory function for PTEN.

6.5 Caveolin-1 Regulates ILK Subcellular Localisation

Experiments reported on in chapter 2 clearly demonstrated the prominent nuclear localisation of ILK in HOSCCs. While this distribution of ILK is not unheard of, it is not the typical distribution pattern normally exhibited by ILK. Obviously, it was therefore of interest to ascertain the mechanism in which ILK achieved this nuclear distribution. Previously it was demonstrated in COS-1 cells that ILK dissociation from caveolin-1 unblocks a nuclear localisation signal within ILK, thus allowing ILK to translocate to the nucleus (Chun *et al*., 2005). It is therefore apparent that caveolin-1 had to be considered when examining the nuclear localisation of ILK in HOSCCs.

Throughout this study, growth factors have been shown to influence various cellular aspects of ILK function (see Chapters 2 and 3). EGF is particularly relevant to the HOSCCs in question due to the noted upregulation of EGFR. Previous studies investigating caveolin-1 signalling have also implicated EGF as playing a pivotal role in caveolin-1 regulation (Pol *et al*., 2000; Kim *et al*., 2002). This is highlighted by the fact that in cell types including human epidermoid carcinoma cells, prostate cells and non-small lung cancer cells, EGF was shown to downregulate caveolin-1 expression (Lu *et al*., 2003). Other studies have shown that the relationship between caveolin-1 and EGF is reciprocal where the upregulation of caveolin-1 attenuated EGF signalling in rat brain, spleen and lung cells (Park *et al*., 2000). Furthermore, EGFR has been found to interact directly with caveolin-1 (Couet *et al*., 1997). In this study it was revealed that the binding of caveolin-1 to EGFR resulted in a downregulation of the autophosphorylation activity of EGFR in Chinese hamster ovary cells. Therefore, it is highly probable that EGF is almost certainly a key player in ILK and caveolin-1 interactions in HOSCCs.

Due to caveolin-1 displaying dissimilar expression across different malignancies, it was necessary to determine caveolin-1 expression in HOSCCs. Caveolin-1 expression has been shown to be reduced in human mammary carcinoma cells as well as ovarian carcinoma (Wiechen *et al*., 2001). Paradoxically however, caveolin-1 expression has previously been demonstrated to be increased in prostate cancer and oesophageal cancer, which suggests a positive role of tumour development (Li *et al*., 2001; Kato *et al*., 2002; Mouraviev *et al*., 2002). Furthermore, re-expression of caveolin-1 in human breast cancer cells inhibits cell growth, which directly implicates caveolin-1 in the regulation of cell proliferation (Lee *et al*., 1998; Kim *et al*., 2000). In the majority of HOSCC cell lines examined here relatively uniform caveolin-1 expression levels were demonstrated (see Figure 22). It is important to realise that the comparative changes in caveolin-1 expression in the malignancies discussed above were made with respect to normal tissue. Even though in the present study comparisons could not be drawn to normal oesophagus, it still suggests that within this class of moderately differentiated HOSCC, caveolin-1 does not display altered regulation and is more than likely not involved in the tumourigenicity of HOSCCs.

Analysis of EGF exposure over a 6 hour time course revealed that caveolin-1 was reduced in all five cell lines, implying a redistribution of caveolin-1 in the presence of EGF (see Figure 23). Indeed, examination of nuclear caveolin-1 levels in the presence of EGF revealed an increased trend in expression levels in the majority of cell lines (see Figure 24a and b). This supports previous data which demonstrate an internalisation of caveolin-1 when treated with the EGF growth factor (Pol *et al*., 2000). WHCO3 cells proved the exception as a reduction in nuclear caveolin-1 was observed in this cell line following EGF treatment. Thus, in the WHCO1, WHCO5, WHCO6 and SNO cell lines these results confirmed that EGF induced a redistribution of caveolin-1 to a nuclear region. In the instance of WHCO3 cells, it implied a constitutive downregulation of caveolin-1 expression at both a membrane/cytoplasmic and nuclear level.

In addition to a cytoplasmic ILK distribution, ILK also displayed prominent nuclear localisation (see Figures 9 and 10 in Chapter 2). Since EGF increased nuclear caveolin-1 concentration, we next tested whether EGF induced a similar response in nuclear ILK concentration. Indeed, Western analysis showed that nuclear ILK protein levels were increased following EGF treatment in WHCO1, WHCO5, WHCO6 and SNO cells. As was noticed for caveolin-1 expression, EGF-treated WHCO3 cells exhibited a reduction in nuclear ILK concentration. However, unlike membrane/cytoplasmic caveolin-1 expression, an increased level of ILK expression was displayed in EGF-treated WHCO3 cells (Figure 25). It is apparent that in the WHCO3 cell line, different mechanisms exist for the regulatory control of both caveolin-1 and ILK in comparison to the remaining HOSCCs.

Upon ligand binding the activated EGF receptor mediates a number of crucial biological responses, including the stimulation of cell proliferation, migration and differentiation (Ullrich and Schlessinger, 1990; Boonstra *et al*., 1995, Wells *et al*., 1998; Andl *et al*., 2003; Normanno *et al*., 2006). These processes usually involve receptor tyrosine kinases, which when bound to their cognate ligand activates their intrinsic kinase activity. What ensues is the recruitment and activation of signal transduction machinery which ultimately leads to a cascade of events (Puri *et al*., 2005). An example of this includes the ErbB receptor tyrosine kinases of which EGFR is a member. This signalling pathway has the potential to signal very potently through tyrosine phosphorylation of either the ras/raf/MAPK pathway for cell proliferation, and through

the PI3K/Akt pathway for cell survival (Normanno *et al*., 2006). Tyrosine phosphorylated signalling cascades are thus central to the coordination of the biological functioning of cells.

With this in mind, it has recently been revealed that EGF is capable of tyrosine phosphorylating caveolin-1, suggesting that caveolin-1 may act as a signalling molecule (Kim *et al*., 2000; Labrecque *et al*., 2004; Lee *et al*., 2005). Although this tyrosine phosphorylation is thought to have important consequences with regards to caveolin-1, at present the complete functional significance is not known. In the present study it is proposed that caveolin-1 tyrosine phosphorylation via EGF induced the noted redistribution of caveolin-1. Utilising a phospho-specific tyrosine antibody, it was confirmed that caveolin-1 was tyrosine phosphorylated in the WHCO1; WHCO5 and WHCO6 cell lines (see Figure 26). The inability to demonstrate tyrosine phosphorylation in the WHCO3 and SNO cell lines is most likely as a result of a low level of tyrosine phosphorylated levels, which remained undetected. Unfortunately, levels of caveolin-1 tyrosine phosphorylation could not be detected following EGF treatment. Although inconsistent, these data suggest that caveoloin-1 tyrosine phosphorylation in HOSCCs is mediated by EGF, which has crucial consequences with regards to caveolin-1 function, specifically localisation.

Having established that EGF altered caveolin-1 expression levels with a subsequent increase in nuclear caveolin-1 concentration, confirmation of this redistribution was sought. Surprisingly, no visual detection of this redistribution was observed in the distribution patterns of caveolin-1 when treated with EGF (see Figure 27). Even though 0.5 hours was more than sufficient to elicit changes in both membrane/cytoplasmic and nuclear caveolin-1 concentration levels, it could be that this period of time does not result in a noticeable visual redistribution or that there is no significant relocalisation.

Due to the similarities noted for expression of both caveolin-1 and ILK in the WHCO1, WHCO5, WHCO6 and SNO cell lines when exposed to EGF, this suggested that ILK translocation to the nucleus involved an association with caveolin-1. The finding that ILK is associated with caveolin-1 in nuclear lysates is indicative of ILK translocation requiring an interaction with caveolin-1 (see Figure 28). Although this is in disagreement with the study by Chun *et al*. (2005), who demonstrated that the

disruption of the ILK/caveolin-1 interaction allows for ILK nuclear localisation, the present data cannot be ignored. It appears that both these mechanisms are plausible. Perhaps these differences can be explained by differences in cell type. As mentioned earlier, the noted overexpression of EGFR in HOSCCs may have an influence. It has been clearly demonstrated that EGF exposure results in a more intracellular distribution of caveolin-1 (Pol *et al*., 2000). This strengthens the suggestion made here that ILK nuclear translocation occurs when bound to caveolin-1 in HOSCCs. It is further proposed that this redistribution is mediated by EGF tyrosine phosphorylation of caveolin-1.

Methyl-β-cyclodextrin sequesters cholesterol, which is required for caveolae function (Ilangumaran *et al*., 1998). To confirm the data from the present study that ILK does indeed require an interaction with caveolin-1 for nuclear translocation, then downregulating caveolin-1 with methyl-β-cyclodextrin should produce a concomitant reduction in nuclear ILK protein levels. The decrease in ILK expression at both cytoplasmic and nuclear levels in cells treated with methyl-β-cyclodextrin substantiates the above claim (see Figures 29 and 30). To our knowledge this is the first demonstration that an association between ILK and caveolin-1 is necessary in order to allow for ILK translocation to a nuclear region. Unfortunately, the role of nuclear ILK is uncertain at present but it is possible that it is directly involved in the phosphorylation of nuclear β-catenin as well as GSK3β. This would have consequences with regards to transcription of genes such as cyclin D1, which is involved in cell cycle control.

For ultimate proof that ILK and caveolin-1 do indeed associate in these cell lines, double label immunofluorescence could be performed which would indicate visually the co-localisation between ILK and caveolin-1. Pull down assays would indicate biochemically that this association does indeed occur.

Thus far, this study has provided groundbreaking evidence in that it has identified ILK in oesophageal SCCs for the first time and, implicated ILK in integrin and growth factor receptor signalling pathways. Furthermore, it has established that a unique ILK nuclear localisation mechanism exists in HOSCCs that involves caveolin-1. Even though the biochemistry of ILK was an important aspect, it primarily served to complete the

understanding of the significance of ILK in cell-ECM adhesion events, which formed the main thrust of this study.

6.6 ILK Function in Cell-ECM Interactions

The ability of malignant cells to metastasise entails the detachment of cells from the primary tumour mass, migration on ECM and, adhesion in the new environment once the cell has circulated to a secondary site (von Schlippe *et al*., 2000; Stewart *et al*., 2004). The majority of cancer deaths are a result of the invasion of cells into surrounding tissues and widespread metastasis to vital organs (Qian *et al*., 1994; Cooper *et al*., 2003). This metastatic phenotype is dependent on an altered behaviour at various cellular levels (Kurschat and Mauch, 2000). An example of such a behaviourial change is differences in expression of certain adhesion molecules (Zutter *et al*., 1998; Christofori, 2003).

In recent years the understanding of cell-cell and cell-matrix interactions that are involved in tumour invasion and metastasis on a molecular and cellular level has improved greatly. The best characterized of the molecules that mediate these associations are the integrin class of receptors, which exhibit altered expression in certain malignancies. For instance, altered integrin expression is noted in a host of malignancies, including breast, lung, prostate, ovary and oesophagus (Giancotti and Ruoslahti, 1990; Zutter *et al*., 1995; Taverna *et al*., 1998; Koivisto *et al*., 2000; Felding-Habermann *et al*., 2001; Miller and Veale, 2001). Furthermore, the integrins are of relevance because they play a central role in anchorage dependent growth, apoptosis, differentiation, migration processes and it is these processes that are characteristically dysregulated in malignancy (Humphries *et al*., 2004; Stewart *et al*., 2004; Brockbank *et al*., 2005; Ginsberg *et al*., 2005; Reddig and Juliano, 2005).

A possible way in which integrins induce a metastatic phenotype is through interactions with other adhesion pathways (Jones and Walker, 1999; Kurschat and Mauch, 2000; Tan *et al*., 2001; Vespa *et al*., 2005). An example of this includes FAK and paxillin which regulate integrin-mediated adhesion and migration and positively modulate cell-cell adhesion mediated by N-cadherin in HeLa cells (Yano *et al*., 2004). ILK binds to the cytoplasmic domains of β_1 and β_3 integrins, and is activated by integrin-ligand

interactions (Wu and Dedhar, 2001; Vouret-Craviari *et al*., 2004). ILK is also capable of mediating cell-cell interactions. For example, ILK overexpression results in reduced expression of the invasive suppressor, E-cadherin, and the acquisition of an invasive phenotype (Novak *et al*., 1998; Jones and Walker, 1999). Conversely, inhibition of ILK has been shown to result in increased E-cadherin expression (Tan *et al*., 2001). Thus it is obvious that ILK is able to mediate both cell-cell and cell-ECM processes by regulating either cadherin or integrin molecules.

The fact that ILK follows a pattern of distribution similar to that of β integrins and was able to associate with β integrins was indicative of a role for ILK in cell-ECM adhesion events in carcinoma of the oesophagus. While previous studies placed more emphasis on the effects of ILK overexpression on ECM attachment, this study took a different approach and examined the effect of reducing ILK on cell-ECM adhesion. Since it has been shown that selective inhibition of ILK activity, with KP-392 treatment results in decreased adhesiveness to ECM in cell types that include intestinal epithelial cells (Attwell *et al*., 2003), a similar occurrence was expected in the HOSCCs under investigation. Following KP-392 treatment a decrease (albeit nonsignificant) in cell adhesion to fibronectin was apparent (see Figure 31). Since this trend of reduction was observed in all the cell lines, it indicated that ILK inhibition resulted in destabilisation of the αβ integrin heterodimer complex and subsequent reduced cell adhesion to the ECM.

Examination of cell adhesion to collagen and fibronectin in the presence of the PI3K inhibitor, wortmannin revealed a trend of reduced cell attachment to these ECM substrates. The exception was the WHCO6 cell line, which exhibited increased attachment to fibronectin when treated with wortmannin. A possible explanation for this is that the inhibition of PI3K in wortmannin- treated WHCO6 cells results in stimulation of integrin receptors such as $\alpha_5\beta_1$, and $\alpha_v\beta_6$**,** which are specific for fibronectin (Watt, 2002). Interestingly, the $\alpha_v\beta_6$ integrin is often expressed in squamous cell carcinomas (Jones *et al*., 1997) and for this reason may have further relevance to these HOSCCs. However, the trend across the cell lines suggests that PI3K is an upstream mediator of the ILK-induced effects on cell-ECM in HOSCCs

It would appear from the present study that, had ILK not been forcefully inhibited, αβ integrin heterodimer stabilisation would have occurred with a subsequent increase in the adhesion of cells to ECM proteins. This is contrary to Mulrooney *et al*. (2000), who showed that ILK expression results in β integrin subunit removal from focal adhesions, with a subsequent loss in adhesion. Furthermore, with the knowledge that ILK activity is transiently increased when cells bind to fibronectin (Hannigan *et al*., 1996; Delcommenne *et al*., 1998), these recent results suggest that reduced cell adhesion as a consequence of reduced ILK may be specific for oesophageal carcinoma. However, until the details of these interactions are unraveled in other SCCs, further comparisons cannot be made.

6.7 Conclusion

Understandably to date the literature surrounding ILK has been primarily biochemical. As a consequence of these biochemical signals elicited by ILK, various critical behaviourial aspects of cellular functions are regulated. ILK accomplishes this role by mediating a multitude of integrin and growth factor signalling pathways. Ultimately, cellular events such as cell proliferation, cell migration and cell spreading are affected, which all play an essential role in the metastatic spread of tumours. Cell adhesion is yet another important cellular event, which involves ILK. Surprisingly, although the influences of ILK on cell adhesion have been examined to a large extent in many other tumours and tissue types, the role of ILK on cell adhesion in oesophageal SCCs has been largely overlooked. Oesophageal carcinoma is an aggressive metastatic malignancy, which relies on the dysregulation of signalling pathways that allow for tumour establishment at distant tissue sites. The present study has clearly demonstrated the requirement of ILK in both integrin and growth factor signalling cascades in HOSCCs. Therefore, ILK may be a prime candidate for the regulation of the signalling cascades involved in the metastatic spread of HOSCC.

Interestingly, throughout this discourse, variation in results has been shown between cell lines in certain instances. This is surprising, since histopathogically all five cell lines have been graded in the moderately differentiated class of HOSCCs. This implies that the current system for the pathological grading of oesophageal tumours is inconsistent and that it requires re-evaluation. As biochemical techniques become more

sophisticated, molecular descriptors need to be developed for more accurate and consistent grading of oesophageal malignancies.

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Appendix 1

1.1 Tissue culture

1.1.1 Phosphate Buffered Saline (PBS)

136.9 mM Sodium Chloride

2.63 mM Potassium Chloride

10.1 mM Disodium hydrogen orthophosphate (anhydrous)

1.76 mM Potassium dihydrogen orthophosphate

Make up with distilled water (dH2O)

Adjusted pH to 7.2-7.3

Sterilise and store at 4 °C

1.1.2 TE – Trypsin in Ethylenediamine-tretraacetic acid (EDTA)

0.02 % EDTA in 500 ml PBS

0.1 % Trypsin in 1 litre PBS

Mix EDTA:Trypsin (1:1)

Final: 0.01 % EDTA/0.05 % Trypsin

1.1.3 Trypan Blue (counting viable cells)

0.4 % Trypan Blue

Make up in PBS

1.2 RNA Extractions

1.2.1 Phenol/Chloroform solution

25:24:1 mixture of phenol:chloroform:isopropyl alcohol

Store at 4 °C

1.2.2 Sodium Acetate

3 M NaAc

pH adjusted to 5.2 using glacial acetic acid

Make up with dH_2O

1.3 Triton X-100 Extraction

1.3.1 Phenyl-methyl-sulphonyl fluoride (PMSF) stock solution

20 mM PMSF powder (Merck, SA)

Make up with Methanol

Store at 4 °C

1.3.2 PMSF/Aprotinin stock solution

0.5 % PMSF stock solution (see Appendix 1.3.1)

1 % Aprotinin (Trasylol® Bayer, SA)

Make up in PBS

Store at 4 °C

1.3.3 Triton X-100 Extraction Buffer

0.5 % Triton X-100

50 mM Tris

150 mM NaCl

1 mN $CaCl_2$

1 mM $MgCl_2$

0.01 % Aprotinin

Make up to volume with dH_2O

1.3.4 Bovine Serum Albumin (BSA) solution

0.1 % BSA (Merck, SA)

In a 0.1 % Sodium dodecyl sulphate (SDS) in 20 mM Tris.HCl (pH 7.4) solution

Store at 4 °C

1.3.5 Trichloroacetic Acid

7.5 % TCA

Make up to volume with dH_2O

1.3.6 Coomassie Blue Stain

0.25 % Coomassie blue powder

50 % Methanol

10 % Acetic acid

Make up to volume with dH_2O

Store at room temperature

1.3.7 Elution Solution

67 % Methanol

32 % dH_2O

1 % concentrated Ammonia

Store at room temperature

Made up to 50 ml with dH_2O

1.4 Western Blotting

1.4.1 Blocking Buffer (BLOTTO)

0.606 % Tris.HCL (pH 7.8)

0.0294 % Calcium Chloride (dihydrate)

5 % Non-fat milk powder

0.01 % Antifoam (1 drop)

0.05 % Triton X-100

Make up with dH_2O

Store at 4 °C

1.4.2 Working Solution

Mix equal volumes of:

Luminol (Supersignal® West Pico Chemiluminescent kit, Pierce, USA)

Peroxide (Supersignal® West Pico Chemiluminescent kit, Pierce, USA)

Note: the total volume is dependent on the size of the nitrocellulose being treated

1.4.3 Developer: D19B

2.2 g Metol

72 g Sodium sulphite

8.8 g Hydroquinone (quinol)

48 g Sodium Carbonate

4 g Potassium Bromide

Make up to 1000 ml with warm dH_2O

1.4.4 Fixer

200 g Sodium thiosulphate

40 g Sodium or potassium metasulphite

Make up to 1000 ml with warm dH_2O

1.4.5 Transfer Buffer

0.3 % Tris

1.41 % Glycine

20 % Methanol

Dissolve in dH_2O

Adjust to pH 8.3 with HCl

Store at 4 °C

1.5 Electrophoresis

1.5.1 Agarose Gel (2 %)

0.6 g Agarose

Make up to 30 ml with TAE buffer (1×)

Heat until agarose dissolves

Pour gel and add

1.5 µl EtBr

Allow gel to set for approximately 40 minutes

1.5.2 TAE Buffer (20×)

1.6 M Tris

0.8 M $NaAc.3H_2O$

40 M EDTA $Na_2.2H_2O$

Adjust pH to 7.2.

Make up to 1000 ml with dH_2O

1.5.3 TAE Buffer (1×)

To 950 ml of dH_2O

Add 50 ml TAE buffer (20×)

1.5.4 SDS Polyacrylamide gel

Separating gel:

8 % Acrylamide

25 % of a 1.5 M Tris.HCl (pH 8.8) separating buffer

4 % of a 0.17 M SDS solution

4 % of a 0.16 M NN'-Methylenebisacrylamide solution

Make up to 10 ml with dH_2O

Just prior to pouring add:

1.8 % of a 0.05 M ammonium persulphate (APS) solution

0.25 %l N,N,N',N'-tetramethylene-diamine (TEMED)

Allow to set for approximately 20-30 minutes

Stacking gel:

5 % Acrylamide

25 % of a 0.5 M Tris.HCl (pH 6.8) stacking buffer

4 % of a 0.17 M SDS solution

4 % of a 0.16 M NN'-Methylenebisacrylamide solution

Make up to 10 ml with dH_2O

Just prior to pouring add:

1.8 % of a 0.05 M APS

0.2 % TEMED

Allow to set for approximately 15 minutes

1.5.5 Electrophoresis Running Buffer for SDS-PAGE

3.5 M SDS (Dissolve into dH_2O)

0.2 M Glycine

25 mM Tris

Make up Glycine and Tris solutions separately in dH_2O

Mix SDS and Glycine/Tris solutions together

Adjust pH to 8.3 with HCl

Store at room temperature

1.5.6 Sample buffer (single lysis)

1.5 % Tris.HCl (pH 6.8)

2 % SDS

10 % Glycerol

5 % β-mercaptoethanol

Make up to 100 ml with dH_2O

Store at 4 °C

1.6 Indirect Immunofluorescence

1.6.1 Paraformaldehyde Solution (4 %)

Solution 1:

166 ml of a 0.14 M sodium dihydrogen orthophosphate (anhydrous)

34 ml of a 0.63 M sodium hydroxide solution

Solution 2:

4 % Paraformaldehyde

Dissolve in Solution 1

Heat solution to 80 °C and stir until solution is clear

Allow solution to cool and adjust pH to between 7.2-7.4

Filter and store solution at 2 °C

1.6.2 Triton X-100 (0.25 %)

In 10 ml 1× PBS add:

0.025 ml Triton X100

1.6.3 Elvanol mounting agent

7.5 g Glycerol

With continual but slow stirring add:

3 g Polyvinyl Alcohol (grade 51-05)

Mix well

Add slowly down the side of the tube to avoid clumping of the polyvinyl alcohol

7.5 ml dH_2O

Stir for 24 hours at room temperature

Add:

15 ml 0.1 M Tris.HCl (pH 8.5)

Continue stirring in a 50 °C water bath for 48 hours

Clear by centrifugation (3000 rpm) for 30 minutes at room temperature

Separate supernatant into aliquots, seal and store at 4 °C

1.6.4 Double lysis buffer

Use same weights/volumes as for single lysis buffer (Appendix 1.5.6)

Make up to 50 ml with dH_2O

Store at 4 °C

1.7 Kinase Assay

1.7.1 Tris buffer

10 mM Tris

Adjust pH 8.8

Store at room temperature

1.7.2 Kinase buffer

50 mM Hepes

10 mM $MnCl_2$

10 mM $MgCl_2$

Adjust pH 7

Add 10 μCi of [γ-^{32}P]ATP

Store at 4 °C

1.8 Cell Adhesion Assay

1.8.1 Trypsin Inhibitor

0.01 % Aprotinin

Make up to 100 ml with serum free DMEM

Store at 4 °C

1.8.2 Extracellular matrix proteins

Collagen: 50 μg/ml

Make up in 10 nM acetic acid

Fibronectin: 50 μg/ml

Make up in sterile PBS

1.8.3 Wortmannin Stock

150 nM Wortmannin

Make up with serum free DMEM

Store in the dark at 4 °C

1.8.4 KP-392 Stock

100 μM KP-392

Make up with DMSO

Store at 4 °C

Appendix 2

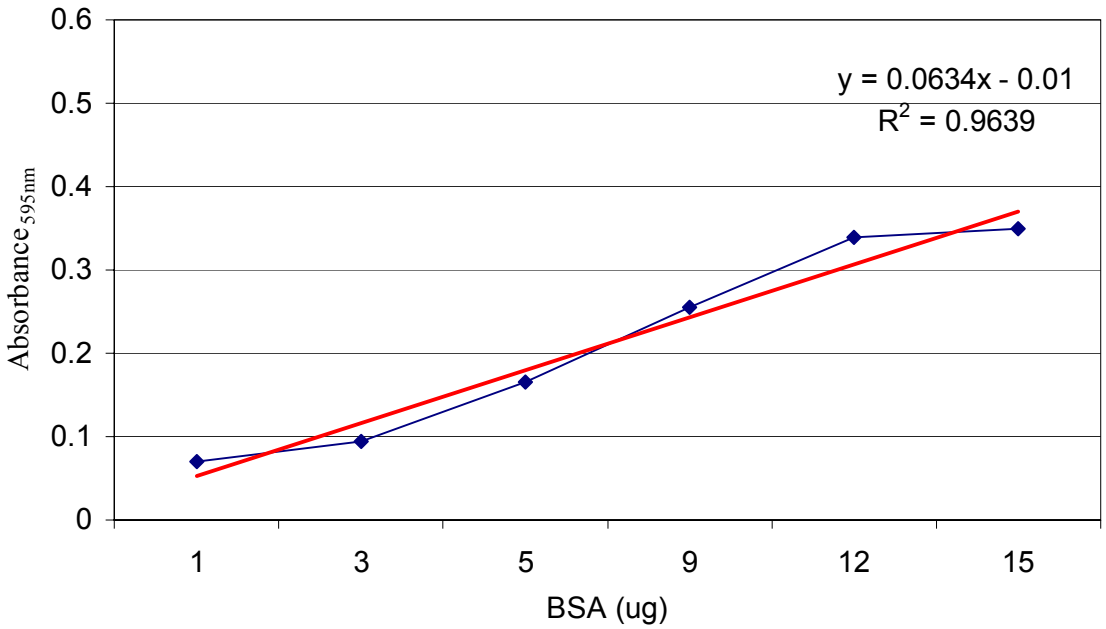


Figure A: BSA standard curve for protein estimation.

The absorbencies (595 nm) of 1, 3, 6, 9, 12, and 15 µg BSA were used to construct a standard curve for protein estimation of the Triton X-100 extracts. The concentrations of the total protein of the extracts were calculated from this BSA standard curve ($y=0.0635x–0.01$. $R^2=0.9639$).

Appendix 3

3.1 Statistical Results of Fibronectin Cell Adhesion Assay

Table 1: Analysis of Variance of the Number of Cells Attached to Fibronectin (Fn).

Indicates significant difference ($p \leq 0.05$).

Effect	df effect	MS effect	df error	MS error	F	P
Cell Line	4	25882500E3	40	161620832	160.1433	0.000000
Treatment	3	10614493E3	40	161620832	65.6753	0.000000
Cell line & Treatment	12	687027776	40	161620832	4.2509	0.000259

Table 2: Tukey HSD Test for Fibronectin (Fn) Adhesion Assay Following KP-392 Exposure.

Marked effects are significant at $p \leq 0.05$

	WHCO1 Fn	WHCO1 Fn (KP)	WHCO1 BSA	WHCO1 BSA (KP)	WHCO3 Fn	WHCO3 Fn (KP)	WHCO3 BSA	WHCO3 BSA (KP)	WHCO5 Fn	WHCO5 Fn (KP)
WHCO1 Fn		0.999646	0.321162	0.163602	0.000180	0.000179	0.000179	0.000179	0.002759	0.000179
WHCO1 Fn (KP)	0.999646		0.956846	0.828039	0.000217	0.000179	0.000179	0.000179	0.002759	0.000179
WHCO1 BSA	0.321162	0.956846		1	0.010759	0.000179	0.000179	0.000179	0.002759	0.000179
WHCO1 BSA(KP)	0.163602	0.828039	1		0.028060	0.000245	0.000179	0.000179	0.085652	0.000180
WHCO3 Fn	0.000180	0.000217	0.010759	0.028060		0.900075	0.000313	0.000179	0.630994	0.247690
WHCO3 Fn(KP)	0.000179	0.000179	0.000198	0.000245	0.900075		0.046107	0.000313	0.009840	0.999802
WHCO3 BSA	0.000179	0.000179	0.000179	0.000179	0.000313	0.046107		0.900075	0.000179	0.446797
WHCO3 BSA(KP)	0.000179	0.000179	0.000179	0.000179	0.000179	0.000313	0.900075		0.000179	0.004551
WHCO5 Fn	0.002759	0.063379	0.911775	0.985652	0.630994	0.009840	0.000179	0.000179		0.000513
WHCO5 Fn(KP)	0.000179	0.000179	0.000180	0.000180	0.247690	0.999802	0.446797	0.004551	0.000513	
WHCO5 BSA	0.000179	0.000179	0.000184	0.000198	0.715862	1	0.107550	0.000608	0.003624	0.999999
WHCO5 BSA(KP)	0.000179	0.000179	0.000179	0.000180	0.199276	0.999275	0.521567	0.006269	0.000404	1
WHCO6 Fn	1	0.996605	0.212335	0.099980	0.000179	0.000179	0.000179	0.000179	0.001479	0.000179
WHCO6 Fn (KP)	0.873928	0.999995	0.999991	0.998987	0.000893	0.000180	0.000179	0.000179	0.376545	0.000179
WHCO6 BSA	0.521567	0.994261	1	1	0.004348	0.000185	0.000179	0.000179	0.755796	0.000179

WHCO6 BSA (KP)	0.000825	0.019933	0.674070	0.873928	0.887463	0.033204	0.000179	0.000179	1	0.001544
SNO Fn	0.844196	0.174869	0.001765	0.000709	0.000179	0.000179	0.000179	0.000179	0.000181	0.000179
SNO Fn (KP)	0.978581	1	0.998596	0.978581	0.000382	0.000179	0.000179	0.000179	0.186727	0.000179
SNO BSA	1	0.999995	0.499940	0.286913	0.000181	0.000179	0.000179	0.000179	0.006269	0.000179
SNO BSA (KP)	0.000182	0.000362	0.036076	0.086123	1	0.652653	0.000206	0.000179	0.887463	0.096367

	WHCO5 BSA	**WHCO5 BSA (KP)**	**WHCO6 Fn**	**WHCO6 Fn (KP)**	**WHCO6 BSA**	**WHCO6 BSA (KP)**	**SNO Fn**	**SNO Fn (KP)**	**SNO BSA**	**SNO BSA (KP)**
WHCO1 Fn	0.000179	0.000179	1	0.873928	0.521567	0.000825	0.844196	0.978581	1	0.000182
WHCO1 Fn (KP)	0.000179	0.000179	0.996605	0.999995	0.994261	0.019933	0.174869	1	0.999995	0.000362
WHCO1 BSA	0.000179	0.000179	0.212335	0.999991	1	0.674070	0.001765	0.998596	0.499940	0.036076
WHCO1 BSA (KP)	0.000198	0.000180	0.099980	0.998987	1	0.873928	0.000709	0.978581	0.286913	0.086123
WHCO3 Fn	0.715862	0.199276	0.000179	0.000893	0.004348	0.887463	0.000179	0.000382	0.000181	1
WHCO3 Fn (KP)	0.999999	1	0.000179	0.000180	0.000185	0.033204	0.000179	0.000179	0.000179	0.652653
WHCO3 BSA	0.107550	0.521567	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000206
WHCO3 BSA (KP)	0.000608	0.006269	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179
WHCO5 Fn	0.003624	0.000404	0.001479	0.376545	0.755796	1	0.000181	0.186727	0.006269	0.887463
WHCO5 Fn (KP)	0.999999	1	0.000179	0.000179	0.000179	0.001544	0.000179	0.000179	0.000179	0.096367
WHCO5 BSA		0.999995	0.000179	0.000179	0.000181	0.012852	0.000179	0.000179	0.000179	0.416036

WHCO5 BSA (KP)	0.999995		0.000179	0.000179	0.000179	0.001147	0.000179	0.000179	0.000179	0.073994
WHCO6 Fn	0.000179	0.000179		0.755796	0.376545	0.000497	0.932432	0.932432	1	0.000181
WHCO6 Fn (KP)	0.000179	0.000179	0.755796		1	0.163602	0.021726	1	0.963358	0.003020
WHCO6 BSA	0.000181	0.000179	0.376545	1		0.457240	0.004348	0.999974	0.715862	0.015333
WHCO6 BSA (KP)	0.012852	0.001147	0.000497	0.163602	0.457240		0.000180	0.068507	0.001765	0.988414
SNO Fn	0.000179	0.000179	0.932432	0.021726	0.004348	0.000180		0.058591	0.674070	0.000179
SNO Fn (KP)	0.000179	0.000179	0.932432	1	0.999974	0.068507	0.058591		0.997437	0.001056
SNO BSA	0.000179	0.000179	1	0.963358	0.715862	0.001765	0.674070	0.997437		0.000187
SNO BSA (KP)	0.416036	0.073994	0.000181	0.003020	0.015333	0.988414	0.000179	0.001056	0.000187	

3.2 Statistical Results of Collagen Cell Adhesion Assay

Table 3: Analysis of Variance of the Number of Cells Attached to Collagen.

Indicates significant difference ($p \leq 0.05$).

Effect	df effect	MS effect	df error	MS error	F	P
Cell line	4	966730E4	100	405195E3	23.8584	0.000000
Treatment	3	631596E5	100	405195E3	155.8744	0.000000
Cell line & Treatment	12	259739E4	100	405195E3	6.4102	0.000000

Table 4: Tukey HSD Test for Collagen Adhesion Assay Following Wortmannin Exposure.

Marked effects are significant at $p \leq 0.05$.

	WHCO1 Coll	WHCO1 Coll (W)	WHCO1 BSA	WHCO1 BSA (W)	WHCO3 Coll	WHCO3 Coll (W)	WHCO3 BSA	WHCO3 BSA (W)	WHCO5 Coll	WHCO5 Coll (W)
WHCO1 Coll		0.913879	0.003765	0.000169	0.99996	0.590239	0.000164	0.000164	0.157006	0.000166
WHCO1 Coll (W)	0.913879		0.600847	0.014932	0.999994	1	0.000164	0.000164	0.157006	0.000166
WHCO1 BSA	0.003765	0.600847		0.990895	0.098184	0.918936	0.000196	0.000164	0.9993	0.008787
WHCO1 BSA (W)	0.000169	0.014932	0.990895		0.000655	0.077916	0.01565	0.000164	0.999563	0.974425
WHCO3 Coll	0.99996	0.999994	0.098184	0.000655		0.995312	0.000164	0.000165	0.393406	1
WHCO3 Coll (W)	0.590239	1	0.918936	0.077916	0.995312		0.000164	0.000164	0.805288	0.000424
WHCO3 BSA	0.000164	0.000164	0.000196	0.01565	0.000164	0.000164		0.000164	1	0.049818
WHCO3 BSA (W)	0.000164	0.000164	0.000164	0.000165	0.000164	0.000164	0.558323		0.000164	0.02596
WHCO5 Collagen	0.157006	0.9993	0.999563	0.393406	0.805288	1	0.000164	0.558323		0.000165
WHCO5 Coll (W)	0.000166	0.008787	0.974425	1	0.000424	0.049818	0.02596	0.000164	0.000164	
WHCO5 BSA	0.000164	0.000534	0.558323	0.999979	0.00017	0.003237	0.239288	0.000165	0.294009	0.294009
WHCO5 BSA (W)	0.000164	0.000164	0.002046	0.224997	0.000164	0.000165	0.999987	0.000196	0.03539	0.999999
WHCO6 Coll	0.003765	0.600847	1	0.990895	0.098184	0.918936	0.000196	0.074905	0.000174	0.310913
WHCO6 Coll (W)	0.000323	0.136589	0.999999	1	0.008787	0.42304	0.001131	0.000164	0.999563	0.974425
WHCO6 BSA	0.000164	0.000164	0.000298	0.040294	0.000164	0.000164	1	0.000164	0.891587	0.999994

WHCO6 BSA (W)	0.000164	0.000164	0.000164	0.000179	0.000164	0.000164	0.974425	0.346338	0.000165	0.063831
SNO Coll	1	0.998314	0.028393	0.000238	1	0.932912	0.000164	0.999999	0.000164	0.000196
SNO Coll (W)	0.9994	1	0.168059	0.001308	1	0.9994	0.000164	0.000164	0.50533	0.0002
SNO BSA	0.000164	0.000164	0.000534	0.081027	0.000164	0.000164	1	0.000164	0.908615	0.00078
SNO BSA (W)	0.000164	0.000164	0.000164	0.000166	0.000164	0.000164	0.77057	1	0.000164	0.000167

	WHCO5 BSA	**WHCO5 BSA (W)**	**WHCO6 Coll**	**WHCO6 Coll (W)**	**WHCO6 BSA**	**WHCO6 BSA (W)**	**SNO Coll**	**SNO Coll (W)**	**SNO BSA**	**SNO BSA (W)**
WHCO1 Coll	0.000164	0.000164	0.003765	0.000323	0.000164	0.000164	1	0.9994	0.000164	0.000164
WHCO1 Coll (W)	0.000164	0.000164	0.003765	0.000323	0.000164	0.000164	0.998314	1	0.000164	0.000164
WHCO1 BSA	0.000534	0.000164	0.600847	0.136589	0.000298	0.000164	0.028393	0.168059	0.000534	0.000164
WHCO1 BSA (W)	0.558323	0.002046	1	0.999999	0.040294	0.000179	0.000238	0.001308	0.081027	0.000166
WHCO3 Coll	0.999979	0.224997	0.990895	1	0.000164	0.000164	1	1	0.000164	0.000164
WHCO3 Coll (W)	0.00017	0.000164	0.098184	0.008787	0.000164	0.000164	0.932912	0.9994	0.000164	0.000164
WHCO3 BSA	0.003237	0.000165	0.918936	0.42304	1	0.974425	0.000164	0.000164	1	0.77057
WHCO3 BSA (W)	0.239288	0.999987	0.000196	0.001131	0.346338	0.999999	0.000164	0.000164	0.211306	1
WHCO5 Collagen	0.000196	0.074905	0.000164	0.000164	0.000165	0.000164	0.50533	0.908615	0.000166	0.000164
WHCO5 Coll (W)	0.03539	0.000174	0.999563	0.891587	0.063831	0.000196	0.0002	0.00078	0.122609	0.000167
WHCO5 BSA		0.310913	0.974425	0.999994	0.42304	0.001308	0.000165	0.000179	0.600847	0.000298

WHCO5 BSA (W)	0.999999		0.558323	0.967325	1	0.463639	0.000164	0.000164	1	0.162465
WHCO6 Coll	0.866006	0.866006		0.029683	0.000298	0.000164	0.028393	0.168059	0.000534	0.000164
WHCO6 Coll (W)	0.558323	0.002046	0.002046		0.003237	0.000165	0.001946	0.018004	0.007584	0.000164
WHCO6 BSA	0.967325	0.029683	0.999999	0.999999		0.891587	0.000164	0.000164	1	0.558323
WHCO6 BSA (W)	0.42304	1	0.000298	0.003237	0.891587		0.000164	0.000164	0.761492	1
SNO Collagen	0.001308	0.463639	0.000164	0.000165	0.000164	0.000164		1	0.000164	0.000164
SNO Coll (W)	0.000165	0.000164	0.028393	0.001946	0.000164	0.000164	1		0.000164	0.000164
SNO BSA	0.000179	0.000164	0.168059	0.018004	1	0.761492	0.000164	0.000164		0.38376
SNO BSA (W)	0.000298	0.162465	0.000164	0.000164	0.558323	1	0.000164	0.000164	0.38376	

3.3 Statistical Results of Fibronectin Cell Adhesion Assay

Table 5: Analysis of Variance of the Number of Cells Attached to Fibronectin (Fn).

Indicates significant difference ($p \leq 0.05$).

Effect	df effect	MS effect	df error	MS error	F	P
Cell Line	4	179171E5	100	325847E3	54.9864	0.000000
Treatment	3	838100E5	100	325847E3	257.2068	0.000000
Cell line & Treatment	12	175173E4	100	325847E3	5.3759	0.000000

Table 6: Tukey HSD Test for Fibronectin (Fn) Adhesion Assay Following Wortmannin Exposure.

Marked effects are significant at $p \leq 0.05$.

	WHCO1 Fn	WHCO1 Fn (W)	WHCO1 BSA	WHCO1 BSA (W)	WHCO3 Fn	WHCO3 Fn (W)	WHCO3 BSA	WHCO3 BSA (W)	WHCO5 Fn	WHCO5 Fn (W)
WHCO1 Fn		0.00024	0.000167	0.999999	0.951909	0.000164		0.000164	0.042791	0.000164
WHCO1 Fn (W)	1		0.001051	0.00021	1	0.999402	0.000164	0.000164	0.195346	0.162321
WHCO1 BSA	0.00024	0.001051		1	0.002403	0.064978	0.000164	0.000164	0.978289	0.98702
WHCO1 BSA (W)	0.000167	0.00021	1		0.000307	0.007396	0.000164	0.000164	0.683677	0.738503
WHCO3 Fn	0.999999	1	0.002403	0.000307		0.999972	0.000164	0.000164	0.321992	0.275281
WHCO3 Fn (W)		0.999402	0.064978	0.007396	0.999972		0.000164	0.000164	0.939362	0.913643
WHCO3 BSA	0.000164	0.000164	0.000164	0.000164	0.000164	0.000164		1	0.000164	0.000164
WHCO3 BSA (W)	0.000164	0.000164	0.000164	0.000164	0.000164	0.000164	1		0.000164	0.000164
WHCO5 Fn	0.042791	0.195346	0.978289	0.683677	0.321992	0.939362	0.000164	0.000164		1
WHCO5 Fn (W)	0.033625	0.162321	0.98702	0.738503	0.275281	0.913643	0.000164	0.000164	1	
WHCO5 BSA	0.000164	0.000164	0.007396	0.064978	0.000164	0.000164	0.113719	0.018381	0.00017	0.000173
WHCO5 BSA (W)	0.000164	0.000164	0.000164	0.000164	0.000164	0.000164	0.997479	1	0.000164	0.000164
WHCO6 Fn	0.998619	1	0.014159	0.001303	1	1	0.000164	0.000164	0.683677	0.626029
WHCO6 Fn (W)	1	1	0.001234	0.000221	1	0.99965	0.000164	0.000164	0.217359	0.181593
WHCO6 BSA	0.000164	0.000164	0.808135	0.994292	0.000164	0.000179	0.000229	0.000168	0.026257	0.033625

WHCO6 BSA (W)	0.000164	0.000164	0.004008	0.038888	0.000164	0.000164	0.17499	0.032019	0.000167	0.000168
SNO Fn	0.042791	0.195346	0.978289	0.683677	0.321992	0.939362	0.000164	0.000164	1	1
SNO Fn (W)	0.000198	0.000607	1	1	0.001303	0.038888	0.000164	0.000164	0.943774	0.962471
SNO BSA	0.000164	0.000164	0.000164	0.000164	0.000164	0.000164	0.99929	1	0.000164	0.000164
SNO BSA (W)	0.000164	0.000164	0.000164	0.000164	0.000164	0.000164	0.779466	0.98702	0.000164	0.000164

	WHCO5 BSA	**WHCO5 BSA (W)**	**WHCO6 Fn**	**WHCO6 Fn (W)**	**WHCO6 BSA**	**WHCO6 BSA (W)**	**SNO Fn**	**SNO Fn (W)**	**SNO BSA**	**SNO BSA (W)**
WHCO1 Fn	0.000164	0.000164	0.998619	1	0.000164	0.000164	0.042791	0.000198	0.000164	0.000164
WHCO1 Fn (W)	0.000164	0.000164	1	1	0.000164	0.000164	0.195346	0.000607	0.000164	0.000164
WHCO1 BSA	0.007396	0.000164	0.014159	0.001234	0.808135	0.004008	0.978289	1	0.000164	0.000164
WHCO1 BSA (W)	0.064978	0.000164	0.001303	0.000221	0.994292	0.038888	0.683677	1	0.000164	0.000164
WHCO3 Fn	0.000164	0.000164	1	1	0.000164	0.000164	0.321992	0.001303	0.000164	0.000164
WHCO3 Fn (W)	0.000164	0.000164	1	0.99965	0.000179	0.000164	0.939362	0.038888	0.000164	0.000164
WHCO3 BSA	0.113719	0.997479	0.000164	0.000164	0.000164	0.000164	0.321992	0.001303	0.000164	0.000164
WHCO3 BSA (W)	0.018381	1	0.000164	0.000164	0.000179	0.000164	0.939362	0.038888	0.000164	0.000164
WHCO5 Fn	0.00017	0.000164	0.683677	0.217359	0.000229	0.17499	0.000164	0.000164	0.99929	0.779466
WHCO5 Fn (W)	0.000173	0.000164	0.626029	0.181593	0.000168	0.032019	0.000164	0.000164	1	0.98702
WHCO5 BSA		0.001303	0.000164	0.000164	0.026257	0.000167	1	0.943774	0.000164	0.000164

WHCO5 BSA (W)	0.001303		0.000164	0.000164	0.033625	0.000168	1	0.962471	0.000164	0.000164
WHCO6 Fn	0.000164	0.000164		1	0.867013	1	0.00017	0.013433	0.00203	0.000196
WHCO6 Fn (W)	0.000164	0.000164	1		0.000164	0.002403	0.000164	0.000164	1	0.999999
WHCO6 BSA	0.867013	0.000164	0.000166	0.000164		0.000164	0.683677	0.007816	0.000164	0.000164
WHCO6 BSA (W)	1	0.002403	0.000164	0.000164	0.000166		0.217359	0.000699	0.000164	0.000164
SNO Fn	0.00017	0.000164	0.683677	0.217359	0.000164	0.000164		0.895194	0.000164	0.000164
SNO Fn (W)	0.013433	0.000164	0.007816	0.000699	0.769505	0.769505	0.026257		0.003785	0.000234
SNO BSA	0.00203	1	0.000164	0.000164	0.026257	0.000167	0.000167	0.007396		0.000164
SNO BSA (W)	0.000196	0.999999	0.000164	0.000164	0.895194	0.007396	0.943774	0.943774	0.000164	

3.4 Number of Cells Attaching to Fibronectin and Collagen

Table 7: Cell Counts of Untreated and Wortmannin-Treated Cells Attaching to Fibronectin (Fn) and BSA.

	fibronectin	fibronectin (W)	fibronectin	fibronectin (W)	BSA	BSA (W)	BSA	BSA (W)
WHCO1	205000	205000	199000	193000	169000	145000	142000	108000
	217000	220000	193000	196000	175000	103000	154000	205000
	211000	202000	211000	184000	121000	121000	139000	175000
WHCO3	178000	181000	211000	190000	61000	46000	80000	109000
	190000	190000	208000	196000	67000	52000	73000	70000
	187000	181000	211000	184000	75000	34000	70000	76000
WHCO5	160000	170000	184000	166000	106000	82000	90000	64000
	178000	160000	172000	169000	100000	28000	130000	70000
	163000	184000	148000	151000	103000	61000	106000	34000
WHCO6	199000	217000	190000	202000	127000	118000	135000	118000
	175000	202000	148000	187000	133000	163000	124000	94000
	205000	184000	199000	205000	106000	88000	139000	43000
SNO	175000	155000	172000	163000	63000	49000	60000	55000
	169000	157000	163000	142000	55000	37000	66000	45000
	166000	148000	160000	124000	46000	58000	57000	43000

Table 8: Cell Counts of Untreated and Wortmannin-Treated Cells Attaching to Collagen and BSA.

	Collagen	collagen (W)	collagen	collagen (W)	BSA	BSA (W)	BSA	BSA (W)
	205000	166000	199000	190000	145000	139000	142000	145000
WHCO1	187000	181000	172000	157000	151000	142000	154000	103000
	208000	178000	202000	165000	133000	106000	139000	121000
	175000	166000	199000	166000	76000	46000	83000	50000
WHCO3	163000	169000	187000	175000	79000	55000	76000	53000
	187000	160000	190000	163000	82000	49000	80000	46000
	151000	124000	187000	133000	85000	52000	169000	120000
WHCO5	169000	112000	151000	128000	67000	74000	175000	130000
	155000	130000	136000	118000	70000	61000	121000	106000
	133000	135000	142000	127000	82000	97000	86000	60000
WHCO6	154000	145000	139000	130000	58000	40000	95000	50000
	151000	139000	145000	133000	103000	58000	73000	52000
	199000	208000	196000	172000	91000	88000	127000	55000
SNO	184000	202000	181000	139000	43000	22000	82000	67000
	214000	196000	157000	169000	61000	28000	110000	60000

Table 9: Cell Counts of Untreated and KP-392-Treated Cells Attaching to Fibronectin and BSA.

	Fibronectin	fibronectin (KP-392)	fibronectin	fibronectin (KP-392)	BSA	BSA (KP-392)	BSA	BSA (KP-392)
WHCO1	217000	199000	210000	204000	174000	178000	180000	185000
	208000	199000	214000	197000	188000	187000	183000	176000
	208000	199000	210000	193000	180000	166000	180000	179000
WHCO3	148000	136000	132000	120000	98000	46000	77000	60000
	136000	97000	137000	125000	56000	61000	73000	63000
	122000	112000	136000	101000	72000	58000	75000	49000
WHCO5	154000	85000	160000	107000	98000	99000	105000	110000
	168000	110500	157000	102000	124000	102000	100000	99000
	160000	115000	164000	100000	112000	106000	109000	106000
WHCO6	222000	210000	217000	188000	190000	145000	180000	195000
	214000	174000	210000	195000	172000	163000	193000	177000
	204000	186000	212000	187000	190000	160000	178000	182000
SNO	238000	208000	233000	200000	217000	121000	213000	211000
	238000	190000	225000	190000	214000	163000	205000	210000
	222000	184000	231000	192000	193000	136000	207000	206000