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The influence of p90RSK on FAK-dependent signalling in human oesophageal squamous carcinoma cells.

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28th May 2017

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28th May 2017

“Man cannot discover new oceans unless he has the courage to lose sight of the shore”

Andre Gide

Abstract

The focal adhesion kinase or FAK plays an important role in detecting and transducing signals that are generated by cell-substrate attachment (Focal adhesions). When these pathways are activated under atypical conditions they may promote metastasis, uncontrolled proliferation and a chemo-resistant phenotype. However the mechanisms by which this protein is activated ectopically in human oesophageal squamous cell carcinomas cell lines (HOSCC) is unknown. In the current study it was hypothesised that the p90 ribosomal S6 kinase, a key member of multiple pro-survival pathways (activator of the Y-box binding protein-1), activates FAK. RSK may promote FAK activation directly, from its location at the plasma membrane, or it may modulate FAK activation indirectly via the regulation of one of its substrates. RSK inhibits the activation of the glycogen synthase kinase 3β (GSK3β) by phosphorylation at Ser9. GSK3β also localises at focal adhesions and may therefore play a role in mediating FAK activity. To ascertain the role RSK plays in FAK activation, 3 inhibition studies were performed. In the first assay, RSK was specifically inhibited within HOSCC and the levels of active FAK monitored (two different environmental conditions). FAK activation was monitored by detecting the auto-phosphorylation of FAK at Tyr397. A GSK3β inhibition assay was then performed in which GSK3β was specifically inhibited and the levels of active FAK monitored. Lastly, a dual inhibition assay was performed where both RSK and GSK3β were inhibited simultaneously and the levels of active FAK monitored. A 10% SDS-PAGE analysis when coupled with western immunoblotting, semi-quantitatively determined the relative abundance of phospho-FAK (Tyr397), phospho- GSK3β (Ser9) and phospho-β-catenin (Ser33, Ser37 & Thr41) within each cell line. The overall net changes in the phospho-protein profile indicated that all of the HOSCC cells had distinct cellular responses to the three inhibitor combinations. However RSK did not appear to activate/inhibit FAK activity directly, in most of the HOSCC cells, but rather modulated FAK activation through the inhibition of GSK3β. The effects the RSK/GSK3β pathway had on FAK activation was partially dependent on the HOSCC cells containing active levels of PTEN. Interestingly, the inhibition of both GSK3β and RSK reduced the levels of active FAK in 3 of the 5 HOSCC cell lines, indicating that this might be a good anti-cancer therapeutic. RSK appeared to play a more context specific role in FAK activation within the HOSCC cells suggesting that the grading system for moderately differentiated carcinomas needs to be improved. This paper also highlights the importance of studying the effects the microenvironment has on neoplasmic transformation as varied environmental conditions, during the RSK inhibition studies, drastically impacted the effects the RSK inhibitor had on FAK activation.

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List of Abbreviations

A431	Human epidermoid carcinoma cell line
Akt/PKB	Protein kinase B
APC	Adenomatous polyposis coli
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CBB	0.25 % Coomassie brilliant blue G-250
CK1	Casein kinase 1 (CK1)
CK2	Casein kinase 2
CTKD	C-terminal kinase domain
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulfoxide
ECM	Extra cellular matrix
EDTA	Ethylenediamineterta-acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
elF4E	Eukaryotic initiation factor 4E
EMT	Epithelial mesenchymal transition
ERK	Extracellular signal-regulated kinase
FA	Focal adhesions
FAK/PTK2	Focal adhesion kinase
FAT	Focal adhesion targeting domain of FAK
FCS	Foetal calf serum
FERM	N-terminal band 4.1, ezrin, radixin, moesin Homology domain
FRNK	FAK related non-kinase
GSK3β	Glycogen synthase kinase 3β

HNSCC	Head and neck squamous cell carcinomas
HOSCC	Human oesophageal squamous cell carcinomas
HRP	Horseradish peroxidase
HT29	Human colon adenocarcinoma cell line
IgG	Immunoglobulins
IOD	Integrated optical density
JAK	Janus kinase
JKAP	JNK pathway-associated phosphatase
JNK	c-Jun N-terminal kinases
LEF	Lymphoid enhancer factor
LKB1	Liver kinase B1
MAPK	Mitogen activated protein kinase
MAPKAP-K1/RSK/p90RSK	MAPK-activated protein kinase-1
MDM2	Mouse double minute 2 homolog
MMP7	Matrix metalloproteinase7
MMP9	Matrix metallopeptidase 9
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-Diphenyltetrazolium bromide
MWM	Molecular weight marker
Na_3VO_4	Sodium orthovanadate
NaF	Sodium fluoride
NTKD	N-terminal kinase domain
PBS	Phosphate buffered saline
PDK1	Phosphoinositide-dependent kinase 1
PDK2	3-phosphoinositide-dependent kinase
p-FAK (Tyr 397)	phospho-FAK (Tyr397)

p-GSK3β (Ser 9)	phospho-GSK3β (Ser9)
PI3K	Phosphatidylinositol-3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKD1	Phosphoinositide-dependent kinase 1
PMSF	Phenyl-methyl-sulphonyl fluoride
PTEN	Phosphatase and tensin homolog deleted in chromosome 10
PVDF	Polyvinylidene fluoride
Pyk2/CAKβ	Proline-rich tyrosine kinase 2/cell adhesion kinase β
p-β-catenin (Ser33, Ser37 & Thr41)	phospho-β-catenin (Ser33, Ser37 & Thr41)
RSK/p90RSK	p90 ribosomal S6 kinase
RSK1	p90 ribosomal S6 kinase 1
RSK2	p90 ribosomal S6 kinase 2
RSK3	p90 ribosomal S6 kinase 3
RSK4	p90 ribosomal S6 kinase 4
S6K	p70 Ribosomal S6 kinases
SCC	Human oral squamous cell carcinomas cells
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	Serine
STAT	Signal transducer and activator of transcription protein
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-Tween
TCA	Trichloroacetic acid
TCF	T-cell factor

TGF-β1	Transforming growth factor-β1
Thr	Threonine
TSC	Tuberous sclerosis complex
Tyr	Tyrosine
YB-1	Y-box binding protein-1
OSCC	Oral squamous cell carcinoma

Chapter 1

1. Introduction

1.1. Cellular adhesion and substrate dependent cells: links to proliferation and the transformed state

Multicellular organisms are the products of cellular collaboration. For cellular collaboration to be effective an extensive network of regulatory queues and an elaborate communication network are needed in order to maintain tissue homeostasis (Yamada and Geiger, 1997; Fu *et al.*, 2012). Cells, which originate from multicellular organisms, are naturally dependent on external stimuli for survival and die in its absence (Yamada and Geiger, 1997). The type of external stimulation each cell is exposed to depends largely on the microenvironment in which the cells are found (Wang *et al*., 1998). External stimulation, a chemically or mechanically derived signal, triggers a variety of intracellular signal transduction cascades that ultimately modulate cellular activity and morphology (Yamada and Geiger, 1997).

The external environment modulates cellular activity, outside-in signalling in a variety of ways. Soluble ligands (such as the epidermal growth factor (EGF)), hormones, cytokines, neighbouring cells and components of the extracellular matrix (ECM) all act as a form of external stimuli to a cell (Yamada and Geiger, 1997; Carpenter and Cohen, 1990). Cellular receptors (such as integrins, E-cadherins and epidermal growth factor receptors) detect these forms of external stimulation and trigger specific intracellular signal transduction cascades in response to the stimuli (Yamada and Geiger, 1997). The signal transduction cascades can trigger a variety of cellular responses depending on the origin of the signal. There are several signal transduction cascades that relay externally derived signals intracellularly. These cascades may alter cell differentiation patterns, trigger cell motility, promote cell proliferation or cause cell senescence (Ohashi *et al*., 2010; Carragher and Frame, 2004).

Cells are bombarded with signals that are generated by multiple forms of external stimulation (Wang *et al*., 1998; Kholodenko *et al*., 1997; Nakakuki *et al*., 2010). This provides cells with copious amounts of information about the microenvironment in which they are found, making signal interpretation and signal integration a large concern as this may play a role in modulating disease (Kholodenko *et al*., 1997; Nakakuki *et al*., 2010). In essence cells are exposed to several forms of external stimulation simultaneously triggering a wide range of internal signal transduction pathways, which the cell must be able to interpret accurately in

order to respond to its environment appropriately (Kholodenko *et al*., 1997; Nakakuki *et al*., 2010).

There are two hypothesised reasons why multiple forms of stimulation may be necessary to trigger a specific cellular response (whether it is cellular senescence, migration or proliferation). The first being, that several forms of external stimuli may be necessary to obtain a strong enough signal to generate a specific cellular response (signal amplification). In this case multiple externally triggered signal transduction cascades have a single intermediate in common and several pathways need to be activated in order to amplify the signal appropriately (Kholodenko *et al*., 1997). This phenomenon is known as cross talk (Logue and Morrison, 2012). Cross talk occurs when different external stimuli activate similar pathways via different mechanisms or different pathways converge at a specific intermediate (Logue and Morrison, 2012). Cell signalling intermediates may therefore have different cellular roles when acting outside of their canonical pathways and these roles may underpin disease (Logue and Morrison, 2012). With the knowledge that intracellular signalling pathways may no longer be considered isolated, how these pathways converge may be imperative for our understanding of how diseased phenotypes are maintained and propagated (Logue and Morrison, 2012). Points of cross talk are particularly important to elucidate when they play a vital role in cell survival regulation (Logue and Morrison, 2012).

Lastly, several externally triggered signal transduction pathways may interact/feed into each other, either through positive or negative feedback loops, generating spatiotemporal response patterns (context dependent cellular responses). This suggests that there is an interplay between signal transduction pathways, triggered by different forms of external stimulation, and that the resulting cellular response could be dependent on this interplay (Nakakuki *et al*., 2010). So intracellular signalling pathways, triggered simultaneously by different forms of external stimuli, may either amplify/modify or nullify a resulting cellular response that would have otherwise been generated had only one of the forms external stimulation been present (Nakakuki *et al*., 2010). This knowledge challenges scientists that have generally been studying signal transduction pathways separately/in isolation (Aksamitiene *et al*., 2012). Deciphering how signal transduction pathways, generated by more than one form of external stimuli, integrate to generate a specific cellular response is of paramount importance. The understanding that spatiotemporal response patterns are possible and in fact a probable makes

the treatment of complex disorders such as cancer more challenging (Aksamitiene *et al*., 2012).

For stationary cells, cell survival and proliferation is dependent on the pro-survival intracellular signalling cascades that are triggered by cell-substrate adhesion (Frisch and Francis, 1994). These cells, that are heavily reliant on their extracellular matrix (ECM) for survival, will often undergo a form of caspase mediated cell death in its absence (when found in suspension or under conditions of anchorage independence) (Frankel *et al.*, 2001; Frisch and Francis, 1994). This form of cell death, that is triggered when substrate dependent cells become detached from their ECM, is known as anoikis. For certain cell types, cell-ECM interactions are imperative not only for their ability to divide, but also for their ability to maintain the correct cellular morphology (Frisch and Francis, 1994; Hill and Mackenzie, 1984; Vachon, 2011). This indicates that the signal transduction cascades, that are propagated via ECM attachment, are not solely regulating proliferation but also have roles in cellular differentiation, survival and cell polarity (Frisch and Francis, 1994; Hill and Mackenzie, 1984; Vachon, 2011). For example epithelial and oesophageal cells are more dependent on substrate anchorage, needing this interaction for cellular division and the maintenance of the correct histo differentiation patterns (Frisch and Francis, 1994; Hill and Mackenzie, 1984; Vachon, 2011)

This dependency on ECM attachment for pro-survival signalling is a precautionary measure that protects against inept cellular migration and proliferation (Frisch and Francis, 1994). This ensures that cells that may have acquired the ability to proliferate uncontrollably, cancerous cells, are still unable to metastasize (Isohata *et al.*, 2009). An example of how cells are able to overcome this constraint is through an epithelial-mesenchymal transition (EMT) (Isohata *et al.*, 2009; Rees *et al*., 2006). EMT transitions require a cell to have lost its cell-cell interactions allowing the cell to alter its differentiation pattern (Isohata *et al*., 2009; Rees *et al.*, 2006). The cell adopts an embryonic differentiation pattern which is commonly seen in mesenchymal cells (Isohata *et al*., 2009; Rees *et al*., 2006). These cells are characteristically more mobile (Isohata *et al.*, 2009; Rees *et al*., 2006). In altering its differentiation patterns, these cells alter their dependency on certain signal transduction cascades for survival (Isohata *et al*., 2009; Rees *et al*., 2006). Understanding the molecular mechanisms these cells adopt to promote cell survival is imperative in order to obtain a greater understanding of the cancerous

state (Carragher and Frame, 2004; Frisch and Francis, 1994; Hill and Mackenzie, 1984; Ohashi *et al.,* 2010).

1.2. Focal adhesion and its role in cell-ECM interactions

As a signal transduction hub, focal adhesions (FA) are usually responsible for relaying signals to, or from, the ECM to the cell (Carragher and Frame, 2004; Vachon, 2011). These signal relays often involve multiple effectors that activate and repress many different proteins such as transcription factors, protein kinases and protein phosphatases (Carragher and Frame, 2004; Wozniak *et al.*, 2004). These signal transduction pathways that are awakened by different stimuli alter gene expression patterns, cellular morphology, its proliferative ability and even affects cell motility (Carragher and Frame, 2004). These protein complexes (FA) can therefore have far reaching effects on cell survival signalling and proliferation (Fanucchi and Veale, 2009; Ohashi *et al.,* 2010). The multifaceted nature of these complexes ensures that they can activate or repress many cellular pathways that play a role in controlling cell proliferation and differentiation (Carragher and Frame, 2004). Focal adhesions generate signals that activate an array of intracellular signalling pathways including the phosphatidylinositol-3 kinase (PI3K) pathway and the mitogen-activated protein kinase (MAPK) cascade (Vanhaesebroeck and Alessi, 2000; Heavey *et al*., 2014; Karelina *et al*., 2014).

Focal adhesion formation is stimulated by cell-ECM interactions and these interactions are most commonly modulated by integrins (or growth factor receptors) (Petit and Thiery, 2000; Zhong and Rescorla, 2012). Integrins, transmembrane glycoproteins, provide a link between the cytosolic machinery (FA) of the cell and the ECM (Dunty *et al*., 2004; Ly and Corbett, 2005). For focal adhesions to form, two separate integrin monomers (α and β monomers) must cluster together making it possible for the integrins to associate with a specific external stimuli (this is modulated by cytosolic proteins) (Jamali, Jamali and Mofrad, 2013; Miyamoto, Teramoto, and Gutkind, 1996). The type of external stimuli may vary and could be a ligand, growth factor or ECM component (Jamali, Jamali and Mofrad., 2013; Miyamoto, Teramoto, and Gutkind, 1996). Upon binding of a stimulus, the integrin protein`s conformation is altered allowing protein binding sites, on its cytoplasmic tails, to interact with various proteins which will eventually collectively form a focal adhesion (Jamali, Jamali and Mofrad, 2013; Cram and Schwarzbauer, 2004; Margadant *et al.*, 2012). These proteins can be recruited by the integrin membrane clustering process or by the proteins that were

initially affected by the event (Carragher and Frame, 2004). More than 50 individual proteins make up the focal adhesions including protein tyrosine kinases (Petit and Thiery, 2000; Zhong and Rescorla, 2012). These proteins play a crucial role in the signal transduction cascades that alert the cell to changes in its environment (Zhong and Rescorla, 2012).

1. 3. FAK, its activators, repressors and role in focal adhesion based signalling

Focal adhesion kinases (FAK) are one of the first proteins to be recruited by the integrin heterodimer in FAs (Fanucchi and Veale, 2009; Mitra and Schlaepfer, 2006; Parsons, 2003). FAK is a 125 kDa non-receptor tyrosine kinase (Carragher and Frame, 2004; Panetti, 2002; Zachary and Rozengurt, 1992). This protein can be subdivided into 3 domains (Figure 1) (Dunty *et al*., 2004; Jones, *et al.,* 1988; Panetti, 2002; Parsons, 2003). The N-terminal domain contains an N-terminal band 4.1, ezrin, radixin, moesin homology (FERM) domain (Jones *et al*., 1988; Panetti, 2002). The chief kinase is located at the centre of the amino acid sequence and is linked to a focal adhesion targeting (FAT) domain, at C-terminus (Jones *et al*., 1988; Panetti, 2002). The protein also contains SH2 docking sites which allow proteins that contain this domain to bind to FAK (Dunty *et al*., 2004; Stewart *et al*., 2002; Zhong and Rescorla, 2012, Carragher and Frame, 2004). FAK has a built in autophosphorylation site at tyrosine 397, see Figure 1 (Calalb and Polte, 1995; Dunty *et al.*, 2004; Lim *et al.,* 2008; Panetti, 2002; Owen *et al.,* 1999). The autophosphorylation of this site is thought to be inhibited by the FERM domain (which lies within this region) (Calalb and Polte, 1995; Dunty *et al*., 2004; Lim *et al.,* 2008; Panetti, 2002; Owen *et al.,* 1999).

The activation of FAK is complex in that it is not completely understood and appears to be varied within the cell. In previous years the displacement of the FERM domain, by the binding of proteins such as the cytoplasmic tails of β1 integrins, was thought to partially activate FAK resulting in its autophosphorylation at tyrosine 397 (Calalb and Polte, 1995; Zhong and Rescorla, 2012). This in turn would have created a binding site for Src proteins (that phosphorylate FAK at Tyr576 and Tyr577) to fully activate the protein (Figure 1) (Calalb and Polte, 1995; Zhong and Rescorla, 2012).

However, more recently the mechanism by which FAK is partially activated (autophosphorylation Tyr397) has been called into question with some studies suggesting that FAK dimerization is responsible for the partial activation of its kinase activity (Brami-Cherrier *et al*., 2014; Katz *et al*., 2002). These studies suggest that focal adhesions and specifically Paxillin recruits FAK to the membrane creating points of local enrichment that

promote FAK dimerization (Brami-Cherrier *et al*., 2014; Katz *et al.*, 2002). The dimers form via the association of their N-terminal FERM domains and are thought to be stabilised by a FERM: FAT domain interaction (Brami-Cherrier *et al*., 2014). It is therefore suggested that the dimerization event triggers the autophosphorylation of FAK at Tyr397 (Brami-Cherrier *et al.*, 2014).

Regardless of whether FAK is partially activated in monomeric or dimeric form, FAK appears to require the activity of Src proteins to fully activate it (in integrin-ECM based signalling) (Caron-Lormier and Berry, 2005; Calalb and Polte, 1995; Mitra and Schlaepfer, 2006; Zhong and Rescorla, 2012). The activation of this protein is further complicated by the fact that FAK has the ability to act as both a scaffolding protein and a protein kinase (Dunty *et al*., 2004). This means that FAK's activity may not require dimerization when acting in its role as a scaffolding protein (Dunty *et al*., 2004). This adds yet another layer to the complexity to the regulation of FAK activity within the cell.

It is important to note that the FAK/Src complex plays a large role in focal adhesion based cell survival signalling (Mitra and Schlaepfer, 2006; Carragher and Frame, 2004; Zhong and Rescorla, 2012). This complex does this by recruiting a variety of proteins to FA and phosphorylating several of them (Mitra and Schlaepfer, 2006; Carragher and Frame, 2004; Zhong and Rescorla, 2012). For example FAK binds p130Cas, see Figure 1 (Mitra and Schlaepfer, 2006; Carragher and Frame, 2004; Owen *et al*., 1999). p130Cas plays a role in mediating kinases activity by allowing kinases to come into close proximity with their substrates (Mitra and Schlaepfer, 2006; Owen *et al.*, 1999). These adaptor proteins ensure that signal transduction cascades are initiated (Carragher and Frame, 2004). FAK SH2 docking sites, produced by the phosphorylation of FAK at Y925 and Y861 (FAT Domain), enables pro-survival signalling molecules such as Grb2 to bind to FAs (Carragher and Frame, 2004; Owen *et al*., 1999). Grb2 elicits its response by activating the MAPK pathway (Figure 2) (Mitra and Schlaepfer, 2006; Carragher and Frame, 2004). Other proteins that are recruited to the focal adhesion complex are vinucullin, VASP, vinexins, ponsin, Arp2/3, α-actinin, F-actin, PYK2, Abl, PKC, PAK and ILK (Mitra and Schlaepfer, 2006; Bershadsky *et al*., 2006).

FAK activity is also repressed by activity of the JNK pathway-associated phosphatase (JKAP) and phosphatase and tensin homolog (PTEN) (Li *et al*., 2010; Zhang *et al*., 2014). Both of these protein phosphatases modulate focal adhesions by dephosphorylating FAK

thereby inhibiting cell migration (Zhang *et al*., 2014; Li *et al.*, 2010; Gu *et al*., 1999). The FAK related non-kinase (FRNK) (transcribed by an alternative promoter in the intron of the FAK gene) has identical sequence homology to the COOH-terminal domain of FAK and also acts as an inhibitor of FAK activity (Aguirre, 2002; Nolan *et al*., 1999; Xu *et al*., 1998). FRNK is only really ubiquitously expressed during embryonic development making it an unlikely player in regulating FAK activity within adult tissues (Nolan *et al*., 1999). The proline-rich tyrosine kinase 2/cell adhesion kinase β (Pyk2/CAKβ) is the only other isoform of FAK (Sieg *et al*., 1998; Sasaki *et al*., 1995). Pyk2, although a non-receptor tyrosine kinase, is not ubiquitously expressed in all cells, as is the case with FAK (Zhang *et al.*, 2014; Zhao *et al*., 2000; Mitra *et al*., 2005). In fact the expression of Pyk2 has been limited to the central nervous system, hematopoietic and endothelial lineages (Mitra *et al.*, 2005, Zhang *et al*., 2014; Zhao *et al.*, 2000). As a result of this it is unlikely that Pyk2 will play a role in epithelial cells pro-survival signal transduction cascades (Xu *et al*., 1998).

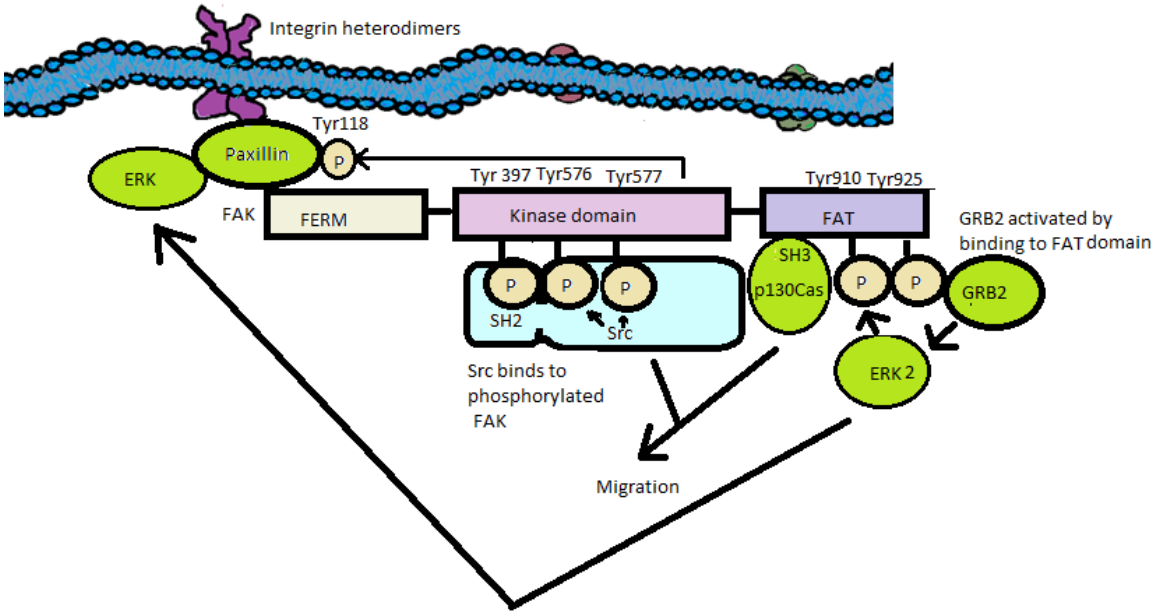


Figure 1. A schematic representation of the structure and function of the principle focal adhesion kinase.
The phosphorylation of FAK, Tyr576 and Tyr577, by Src is necessary for the optimal activity of the protein. FAK activates substrates such as Grb2, Paxillin and p130Cas. The image was modified (Microsoft Paint ©) from the review, Focal adhesion kinase: in command and control of cell motility, written by Mitra *et al.* (2005) (Figure 3; pg 61).

1.4. The pro-survival intracellular signalling mechanisms of FAK

FAK plays a vital role propagating cell survival based signals, by activating the MAPK pathway for example, that its activity is often repressed in cells undergoing a form of programmed cell death (Levkau *et al*., 1998). One of the initial steps following apoptosis induction is actually the caspase 3-mediated cleavage of FAK (Levkau *et al*., 1998). This effectively inhibits the focal adhesions ability to trigger cell survival pathways during apoptosis (Levkau *et al*., 1998). It therefore stands to reason that high levels of the active form of this protein would cause endothelial and epithelial cells to become resistant to anoikis, apoptosis and promote cell-survival based signalling ectopically (Frisch and Jolla, 1996).

The role of FAK within the cell is not limited to the membrane level since it occurs within all subcellular locations including the nucleus (Fanucchi and Veale, 2009). The pro-survival role of FAK within the nucleus is closely linked to the regulation of p53 turnover (Figure 2) (Lim *et al*., 2008; Van Miltenburg *et al.*, 2014; Dunty *et al.*, 2004). It is thought that FAK acts in its capacity as a scaffolding protein to mediate the interaction between p53, a well-known tumour suppressor protein, and MDM2 via its FERM domain (Lim *et al*., 2008). FAK therefore aids in the ubiquitin-mediated degradation of p53 (Lim *et al.*, 2008). This ensures that the pro-apoptotic gene targets of p53 remain transcriptionally silent (Lim *et al*., 2008). This therefore inhibits proteins such as p21, a pro-apoptotic protein, which promotes cell cycle arrest (Graham, *et al*., 2010). The loss of p53 activity ensures that cell survival and proliferation continues unabated (Lim *et al*., 2008).

In a study observing how different cellular lineages perceived and interpreted cell-ECM signals, it was found that active FAK provided cells from different lineages with resistance to anoikis (Zouq *et al*., 2009). The mechanisms by which this resistance was achieved varied from one cell lineage to another (Zouq *et al*., 2009). For example epithelial cells require Paxillin, its SH2 domain, in order to activate the Protein kinase B (Akt) and PI3K Pathway (Zouq *et al*., 2009). While in fibroblasts, FAK mediated anoikis resistance required the activation of p130Cas (Zouq *et al.*, 2009). This in turn activated the Ras-Rac pathway via Crk and Nck (Zouq *et al*., 2009). However possible mechanisms by which FAK, pro-survival based signalling can be activated ectopically has yet to be determined.

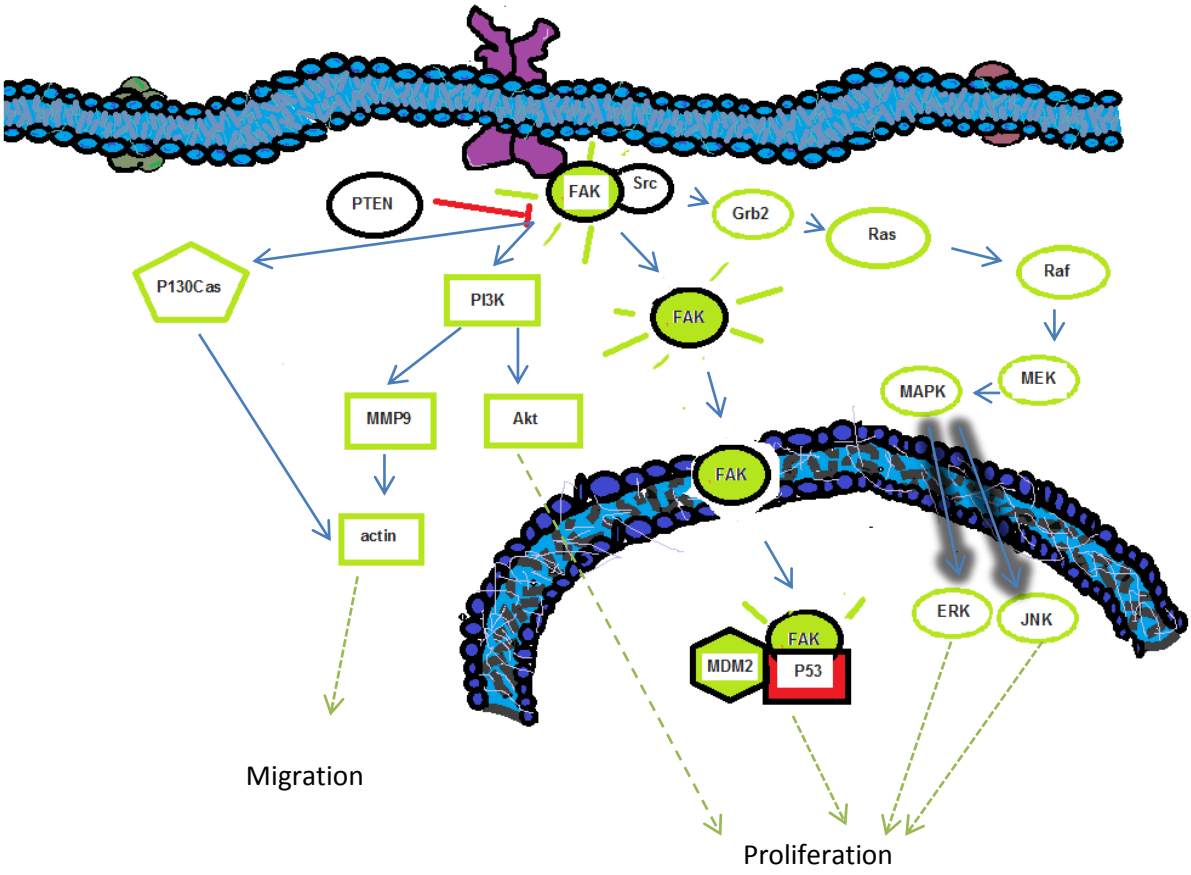


Figure 2. A schematic representation of FAK-regulated signal transduction cascades.
Green FAK is representative of an active FAK. Red regions indicate the inhibition of p53 by FAK and the consequences for this inhibition on the p53-apoptosis pathway. The MAPK pathway is activated by FAK as seen by the uncoloured green circles whilst PTEN inhibits FAK activity. The PI3K pathway is also activated by FAK, indicated by uncoloured green squares, and the consequences for the activation of this pathway are highlighted. Abbreviations: mitogen activated protein kinase kinase (MEK), the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinases (JNK), Mouse double minute 2 homolog (MDM2), matrix metallopeptidase 9 (MMP9), protein kinase B (Akt/PKB) and PTEN. The image was modified (Microsoft Paint ©) from papers published by Fu and colleagues, 2012.

1.5. RSK, its activators, repressors and sublocalization within the cell

The ribosomal S6 kinase (RSK) family is a group of Ser/Thr kinases that can be divided into 2 categories namely the p70 Ribosomal S6 kinases (S6K) and p90 ribosomal S6 kinases (RSK) (Yntema *et al.,* 1999; Frödin and Gammeltoft, 1999; Li *et al.,* 2013). The p90 kDa ribosomal S6 kinases (RSK), also known as the MAPK-activated protein kinase-1 (MAPKAP-K1), play a large role in multiple pathways (Anjum and Blenis, 2008; Gayanilo, *et al*., 2014). RSK is an unusual protein in that it contains two catalytic domains at the N- and C-terminus of the protein (Frödin and Gammeltoft, 1999; Gayanilo *et al*., 2014; Jones *et al*., 1988). These kinase domains are known as NTKD and CTKD respectively (Frödin and Gammeltoft, 1999; Gayanilo *et al*., 2014).

There are four isoforms of the p90 ribosomal S6 kinase (RSK, p90RSK, MAPKAP-K1) in mammals, RSK1 to 4 (Yntema *et al*., 1999; Jacquot *et al.,* 1998). The isoforms, RSK1-3, are expressed in all cells, however, their individual abundance within cell lineages appears to be tissue specific (Yntema *et al*., 1999). The RSK isoforms all share a similar overall structure and appear to have overlapping roles in signal transduction cascades (Leighton *et al*., 1995; Carriere *et al*., 2008). They display a degree of functional redundancy and are activated in a similar way (all key residues are conserved amongst the isoforms) (Carriere *et al.,* 2008). However, it is specifically known that RSK1 and RSK 2 have been implicated in cancer development and maintenance (Carriere *et al.,* 2008). Due to the functional redundancy of these isoforms they will collectively be referred to as RSK in this paper.

RSK activation, canonically, requires the binding of the extracellular signal-regulated kinase (ERK) to its C-terminal docking site (RSK) (Roux *et al*., 2003). The C-terminal domain acts as a negative regulator for the activity of the RSK N-terminal kinase (Richards *et al*., 1999). As a result of this in the first stage of RSK activation ERK phosphorylates RSK at ser-573, in the activation loop of the COOH-terminal kinase (Ballif *et al*., 2005; Kuang *et al*., 2009). This triggers the phosphorylation of 2 other sites on the linker region by ERK (Thr-359/Ser-363) and other kinases (Ballif *et al*., 2005; Kuang *et al*., 2009). The phosphorylation of the linker region is thought to assist in unfolding the protein thereby priming it for full activation (Ballif *et al*., 2005; Kuang *et al*., 2009). Lastly the C-terminal kinase domain creates a binding site for 3-phosphoinositide-dependent kinase, PDK2, by phosphorylating Ser380 (Ballif *et al*., 2005; Richards *et al*., 1999). PDK2 in turn phosphorylates Ser-221 in the N-

terminal kinase domain of RSK, activating its kinase activity (Ballif *et al*., 2005; Kuang *et al*., 2009; Richards *et al*., 1999). The final step of RSK activation involves the separation of the RSK-ERK complex (Ballif *et al*., 2005; Kuang *et al*., 2009). This is triggered by the autophosphorylation of RSK, by the N-terminal kinase domain, at serine 732 (Roux *et al*., 2003; Ballif *et al*., 2005; Kuang *et al*., 2009).

RSK is activated by ERK, a key member in the MAPK pathway, identifying this protein as a downstream signalling molecule that promotes cell survival based signalling (Figure 3) (Gayanilo *et al*., 2014). RSK is also associated with the rapamycin-mTOR pathway and the PI3K pathway, see Figure 3 (Zeng and Kinsella, 2008; Gayanilo *et al*., 2014). As a downstream signalling molecule in a signal transduction cascade, RSK modulates the activity of a variety of proteins in order to elicit a cellular response (Zeng and Kinsella, 2008; Gayanilo *et al*., 2014). The substrates that RSK act on can be found in any sublocation within the cell (Anjum and Blenis, 2008; Frödin and Gammeltoft, 1999; Qi *et al*., 2007). RSK substrates include CREB, Fascin-1, Hsp27, c-FOS, cAMP, Bad, Bim and c-Met (Kang *et al*., 2010; Li *et al*., 2013).

One such substrate of RSK, the glycogen synthase kinase 3β (GSK3β), is known to play a fundamental role in several signal transduction pathways that regulate cell cycle progression, proliferation, differentiation and migration, see Figure 3 (Domoto *et al*., 2016; Jope and Johnson, 2004). GSK3β and its isoform GSK3α are ubiquitously expressed serine-threonine kinases (Hansen *et al.*, 1997). The reported roles GSK3β plays in neoplasmic transformations are exceedingly conflicting. Its been found to be overexpressed and activated, by phosphorylation at tyrosine 216, in several forms of cancer including epithelial ovarian tumours, pancreatic and colorectal cancers (Domoto *et al*., 2016; Rask *et al*., 2003; Shakoori *et al*., 2005; Shakoori *et al*., 2007; Mai *et al*., 2009; Ougolkov *et al*., 2005). The inhibition of GSK3β actually suppressed cancer proliferation and induced apoptosis (Ougolkov *et al*., 2007; Ougolkov *et al*., 2005). GSK3β has also been found to promote migration through the activation of Rac 1, which promotes lamellipodia formation (Koivisto *et al*., 2003; Vaidya *et al*., 2006).

Literature has shown that GSK3β acts as tumour suppressor that negatively regulates the activity of Wnt pathway (Waaler *et al*., 2011). It does this by preventing β-catenin from activating the T-cell factor/lymphoid enhancer factor (TCF/ LEF), which transcribe several

genes that underpin the transformed state (c-Myc, cyclin D1, matrix metalloproteinase 7 (MMP7) etc.) (Cook *et al*., 1996; Manoukian and Woodgett, 2002; Fuchs *et al*., 2005; Jope and Johnson, 2004). GSK3β, adenomatous polyposis coli (APC) and axin collectively bind to/phosphorylates β-catenin at Ser 33, Ser 37 and Thr 41 (Hinoi *et al*., 2000; Rask *et al*., 2003; Waaler *et al*., 2011). This phosphorylation triggers the ubiquitination of β-catenin by β-TrCP which ultimately results in the protein being degraded by the proteasome (Hinoi *et al*., 2000; Fuchs *et al*., 2005). The constant activation of the Wnt-pathway upon the loss of GSK3β activity often leads to cancer and its inhibition is often associated a reduction in cancer cell growth (Kwong *et al*., 2002). GSK3β has also been implicated in the PI3K-Akt pathway (Figure 3) and has several other substrates including c-jun, cyclic AMP response protein-1, heat shockfactor-1 and Myc (Sutherland *et al*., 1993; Grimes *et al*., 2001; Hinoi *et al*., 2000; Rask *et al*., 2003). RSK and Akt both inhibit GSK3β activity, by phosphorylating its inhibitory site (Serine 9), thereby regulating its function within the cell (Jope and Johnson, 2004; Stambolic and Woodgett, 1994; Pap and Cooper, 1998). It is possible that RSK may elicit a specific cellular response via the inhibition of one of its substrates such as GSK3β.

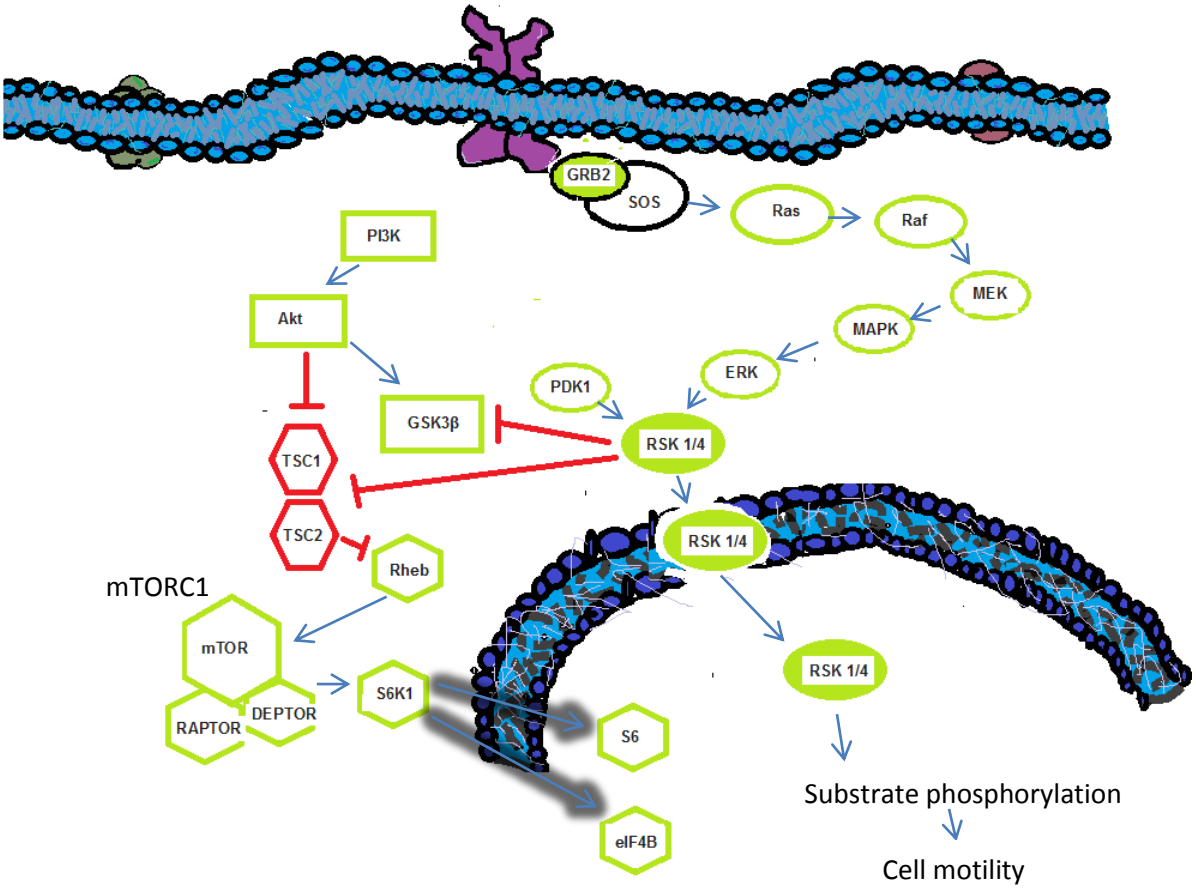


Figure 3. A schematic representation of the canonical pathway of RSK. The MAPK-RSK pathway is commonly activated by growth factors; this pathway is indicated by the green uncoloured spheres. RSK requires the activity of both PDK1 and ERK for its activation. Once activated RSK modulates the mTOR pathway by inhibiting TSC1/TSC2, as seen by the uncoloured hexagon. RSK also acts on other substrates such as GSK3β and promote gene expression. The activity of RSK within the cell often leads to cell motility. Abbreviations: mammalian target of rapamycin (mTOR), mTOR complex 1 (mTORC1), tuberous sclerosis complex (TSC), protein kinase B (Akt/PKB) and eukaryotic initiation factor 4E (eIF4E). The image was produced (Microsoft Paint ©) by making use of the information provided from papers published by Brett and colleagues (2014) and Anjum and Blenis (2008).

1.6. RSK, cell motility and pro-survival based signalling

RSK has a diverse range of functions within the cell and may therefore play a vital role in cell survival based signalling (Xian *et al*., 2009). Recently it was found that RSK activity induced by high levels of fibroblast growth factor receptor 1, resulted in non-transformed mammary cells being able to survive and proliferate in suspension (loss of Cell-ECM interactions) (Xian *et al*., 2009). The role of RSK in anoikis resistance, cell survival and EMT was further solidified by the fact that these cells underwent apoptosis when RSK was inhibited (Xian *et al*., 2009). Other studies corroborated this discovery finding RSK activity necessary for the

weakening of cell-cell contacts and the increased cellular migration of cancer cells (Čáslavský, Klímová, and Vomastek, 2013). This is achieved by altering the cells gene expression profile (Doehn *et al*., 2009). RSK activates the expression of a variety of pro-invasive proteins such as uPAR, whilst inhibiting the expression tumour suppressor proteins (Doehn *et al*., 2009). The response RSK elicits in epithelial cells can be linked to cell scattering, wound healing, cell multilayering and ECM invasion (Doehn *et al*., 2009). This implies that RSK mediates cell motility and invasion via gene regulation (Doehn *et al*., 2009). Ultimately these findings indicate that RSK may well play a role in pro-survival based signalling and that ectopically RSK activation can lead to increased cell invasiveness.

1.7. A link between RSK and the focal adhesion pathway

With the knowledge that RSK plays such a varied role in cell signalling cascades, few studies have focused on the possible interactions RSK might mediate at the membrane level. The sublocalisation of RSK has become increasingly important since the study conducted by Woo *et al*. (2004). In this study it was found that the need for ERK to activate RSK was circumvented in cells exposed to epidermal growth factors, EGF (Woo *et al*., 2004). In this case EGF caused the translocation of RSK to the plasma membrane where it was activated by phosphorylation, before being translocate into the nucleus (Woo *et al*., 2004). Based on its subcellular locations within the cell, RSK may activate multiple proteins including Filamin A, which assists in cell motility by remodelling the actin cytoskeleton (Woo *et al*., 2004).

RSK was in fact found to localise near focal adhesion complexes (Gawecka *et al*., 2012). Gawecka and colleagues found that RSK co-localised with talin, a protein that is thought to play a role in integrin activation. It was also found that RSK, at this position in the focal adhesion complex, may be able to modulate the activation of integrins (as RSK2 located near the cytoplasmic tails) (Gawecka *et al*., 2012). RSK may therefore play a role in initiating several focal adhesion based signalling processes, including those that regulate the cytoskeletal contacts at that point (Gawecka *et al*., 2012). Therefore RSK, outside of its canonical MAPK pathway, may play an active role in focal adhesion signalling by activating FAK, see Figure 4A (Gawecka *et al*., 2012).

1.8. Human oesophageal squamous cell carcinomas (HOSCC)

Cells that show a high degree of dependency on cell-ECM, focal adhesion based signalling, for survival often adopt interesting mechanisms to circumvent these controls in cancer (Ohashi *et al*., 2010). For example oesophageal squamous cells are highly substrate-dependent before transforming into highly invasive cancer cells (Ohashi *et al.*, 2010).

The degree of metastasis seen in human oesophageal squamous cell carcinomas (HOSCC) made this form of cancer the sixth most deadliest form of cancer in the world (Ohashi *et al*., 2010, Ferlay *et al*., 2012). In South Africa 1 in every 6250 individuals will be affected by this disease and only 10 % of all of these patients will survive longer than 5 years after the initial prognosis is given (Hendricks and Parker, 2002; Ohashi *et al*., 2010). Understanding the molecular mechanisms that allow this form of cancer to circumvent anoikis and promote cell survival/proliferation can only aid in the battle against this disease. Because the transition from the original state to the highly metastatic and invasive HOSCC cell lines is so drastic, it may involve multiple alterations to the intermediates, of the signal transduction pathways, that mediate cell anchorage dependent survival (Fanucchi and Veale, 2009).

1.9. The effects of RSK on focal adhesion signalling through its interactions with key players within the focal adhesion pathway in HOSCC

As a prominent player in focal adhesions, FAK plays an important role in detecting/ transducing signals that are generated by cell-ECM interactions (Mitra and Schlaepfer, 2006; Carragher and Frame, 2004; Zhong and Rescorla, 2012). Most of these signals promote cell survival which when, activated under atypical conditions, could explain how cancer cells circumvent anoikis and promote cell survival (Frisch and Jolla, 1996). In reality should FAK be activated non-canonically it would convey cell-ECM based signals regardless of whether it (cell-ECM interactions) were present or absent (Frisch and Jolla, 1996; Zouq *et al*., 2009). For this reason FAK is overexpressed in many different forms of cancer (colon, cervix and breast cancer) and is thought to play a prominent role in maintaining the transformed state (Van Miltenburg *et al*., 2014).

HOSCC have been shown to resist anoikis in suspension through the creation of multicellular aggregates that sustain FAK activity (Zhang *et al*., 2004). FAK promoted cell survival signalling and fibronectin deposition (Zhang *et al.*, 2004). This coupled with the knowledge that FAK is overexpressed in other cancers suggests that the overexpression and activity of

FAK might play a fundamental role in the transformation of epithelial cells (Canel *et al*., 2006; Frisch and Jolla, 1996). In fact FAK activation/overexpression has been associated with cancerous strains that are resistant to chemotherapeutics and the reduction of FAK activation in these cell lines rendered them susceptible to chemotherapeutic agents (Wilson *et al*., 2014; Eke *et al*., 2012). This indicates that FAK signalling plays a vital role in propagating a chemo resistant phenotype in SCC that its loss decreased cancer cell viability and its proliferative capacities (Golubovskaya *et al*., 2012; Eke *et al*., 2012).

Therefore the mechanism by which this protein is activated, non-canonically, may be a good therapeutic target for anti-cancer therapeutics (Frisch and Francis, 1994; Carlson *et al*., 2004; Fanucchi and Veale, 2009; Lim *et al*., 2008; Ohashi *et al*., 2010). Understanding the mechanisms that regulate FAK activation may help to provide a more detailed image of cancer progression in HOSCC cancer cells (Carlson *et al*., 2004; Fanucchi and Veale, 2009; Lim *et al.*, 2008; Ohashi *et al*., 2010).

RSK has been said to play a role in several signal transduction pathways, such as the PI3K pathway and the MAP kinase pathway, however, its role in focal adhesion complex regulation has only recently been suggested by Gawecka and colleagues (2012). Based on the subcellular location of RSK and the pathways that are said to activate this protein, RSK may play a role in activating multiple proteins in focal adhesions, including FAK (Gawecka *et al.*, 2012; Woo *et al*., 2004). In its canonical pathway, RSK promotes cellular motility, EMT and metastasis in epithelial cells. With little information being known about the relationship between RSK and FAK, it is possible that RSK may directly activate this protein.

In previous years a link between RSK activity and the phosphorylation of FAK, Tyr861 was made in HNSCC cell lines (Kang *et al*., 2010). However, this study considered it unlikely that RSK would modulate the activation of FAK directly, instead suggesting that RSK may modulate FAK activity via one of its substrates (Kang *et al*., 2010). In 2014 the effect of RSK inhibition on the proliferation of oral squamous cell carcinomas was investigated. Here loss of RSK resulted in the inhibition of its downstream targets; it caused cell cycle arrest (via its modulation of p21 expression) and apoptosis (Chiu *et al*., 2014). It is possible that the role RSK plays in modulating p21 expression may be through its activation of FAK, see Figure 4A (Chiu *et al.*, 2014).

Therefore this investigation wishes to determine whether or not cross talk exists between the focal adhesion pathway and the MAPK-RSK pathway in HOSCC (Figure 4A). It is predicted that this study will elucidate novel mechanisms by which FAK is regulated ectopically and thereby provide scientists with a better understanding of how HOSCC modulates cell survival based signalling (Figure 4A and B). This knowledge could not only be imperative for ones understanding of the molecular mechanisms that propagate this disease, but may also improve our mechanisms of combating this disease.

1.9.1. The Aim of this investigation:

The role RSK plays in modulating focal adhesion based signalling will be investigated by determining whether RSK activates the principle focal adhesion kinase (FAK) (either directly or indirectly) in HOSCC cell lines (Figure 4A and 4B).

1.9.2. The Objectives

- The basal levels of phospho-β-catenin (Ser33, Ser37 & Thr41) (phosphorylation targets β-catenin for ubiquitination), active FAK (phospho-FAK (Tyr397)) and inactive GSK3β (phospho- GSK3β (Ser9)) will be semi-quantitatively determined for all the HOSCC cell lines including the control cell lines (the HT29 and A431 cell line).
- To putatively determine whether RSK activates FAK directly, RSK will be specifically inhibited (by BI-D1870) within the HOSCC cell lines and the subsequent fluctuations in the level of active FAK monitored (Figure 5C and 5D).
- To determine whether RSK is inhibited effectively within the HOSCC cell lines, RSK will be specifically inhibited and the levels of inactive GSK3β monitored (RSK phosphorylates GSK3β at Serine 9) (control).
- To explore whether FAK activation is indirectly caused by RSK activity via the activation of its substrate the glycogen synthase kinase 3β (GSK3β); both RSK and GSK3β will be specifically inhibited (by BI-D1870 and AR-A014418) simultaneously and the levels of active FAK monitored (Figure 5E).
- To determine the effect of GSK3β inhibition has on the levels of phospho-FAK (Tyr397), GSK3β will be specifically inhibited (AR-A014418) in each of the HOSCC cell lines and the subsequent levels of active FAK will be monitored (control).

- To determine whether GSK3β is effectively inhibited within the HOSCC cell lines, GSK3β will be specifically inhibited and the levels of phospho-β-catenin (Ser33, Ser37 & Thr41) monitored (GSK3β targets β-catenin for ubiquitination) (control).

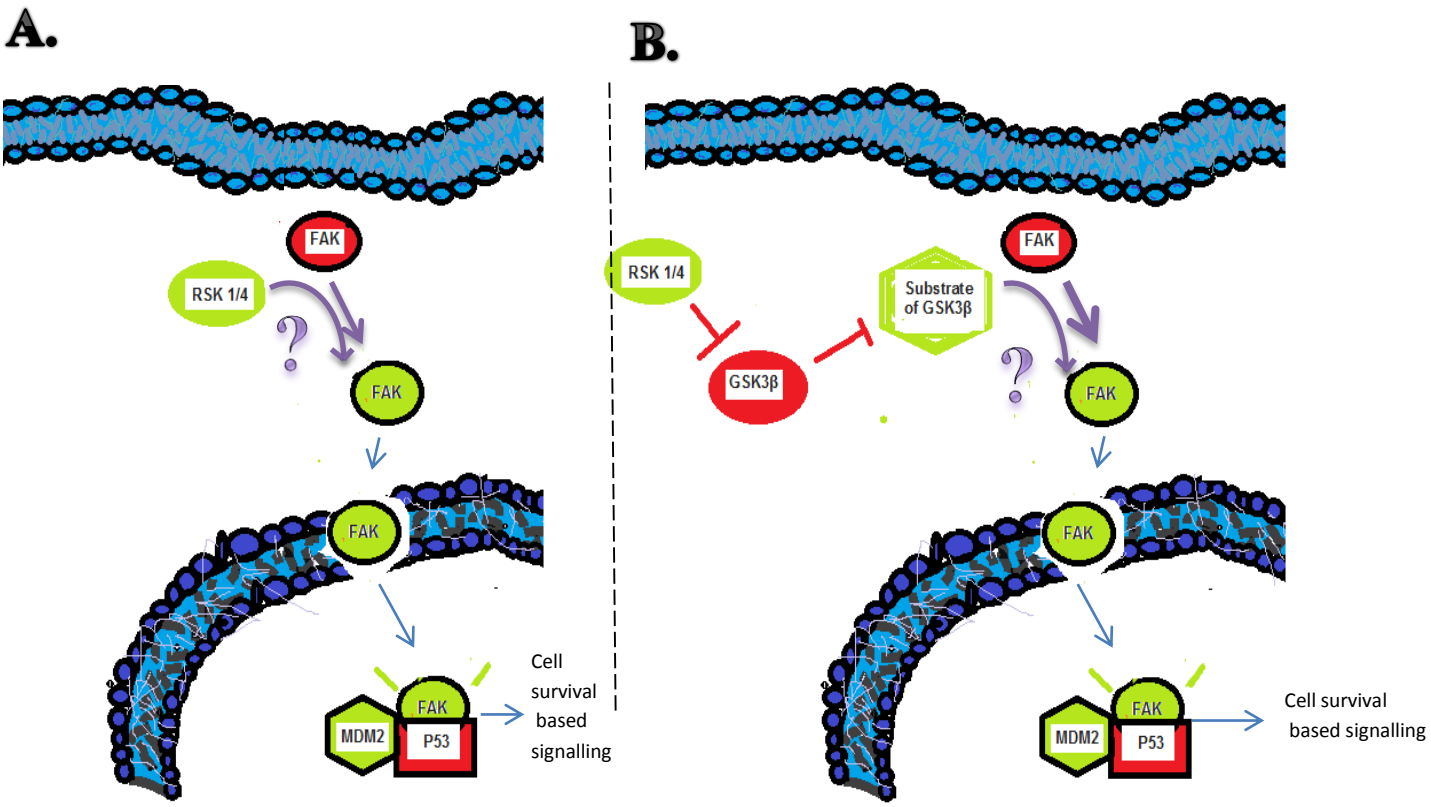


Figure 4 (A and B). A schematic representation of the proposed mechanisms by which focal adhesion-based signalling can be modulated by RSK in HOSCC. Green represents active proteins whilst red represents proteins that are inactive. Red lines represent inhibition. **A.** FAK is suggested to be ectopically activated by RSK, not the result of cell-ECM interactions, and this results in pro-survival signalling as illustrated by the inhibition of the p53-apoptosis pathway. The involvement of RSK in this pathway, as seen in purple, is being proposed in this article and has not yet been proven**. B.** Alternatively FAK may be ectopically activated by a protein that is ordinarily repressed be GSK3β activity, not the result of cell-ECM interactions, and this results in pro-survival signalling. The image was produced (Microsoft Paint ©) by making use of the information provided from papers published from Lim *et al*., 2008.

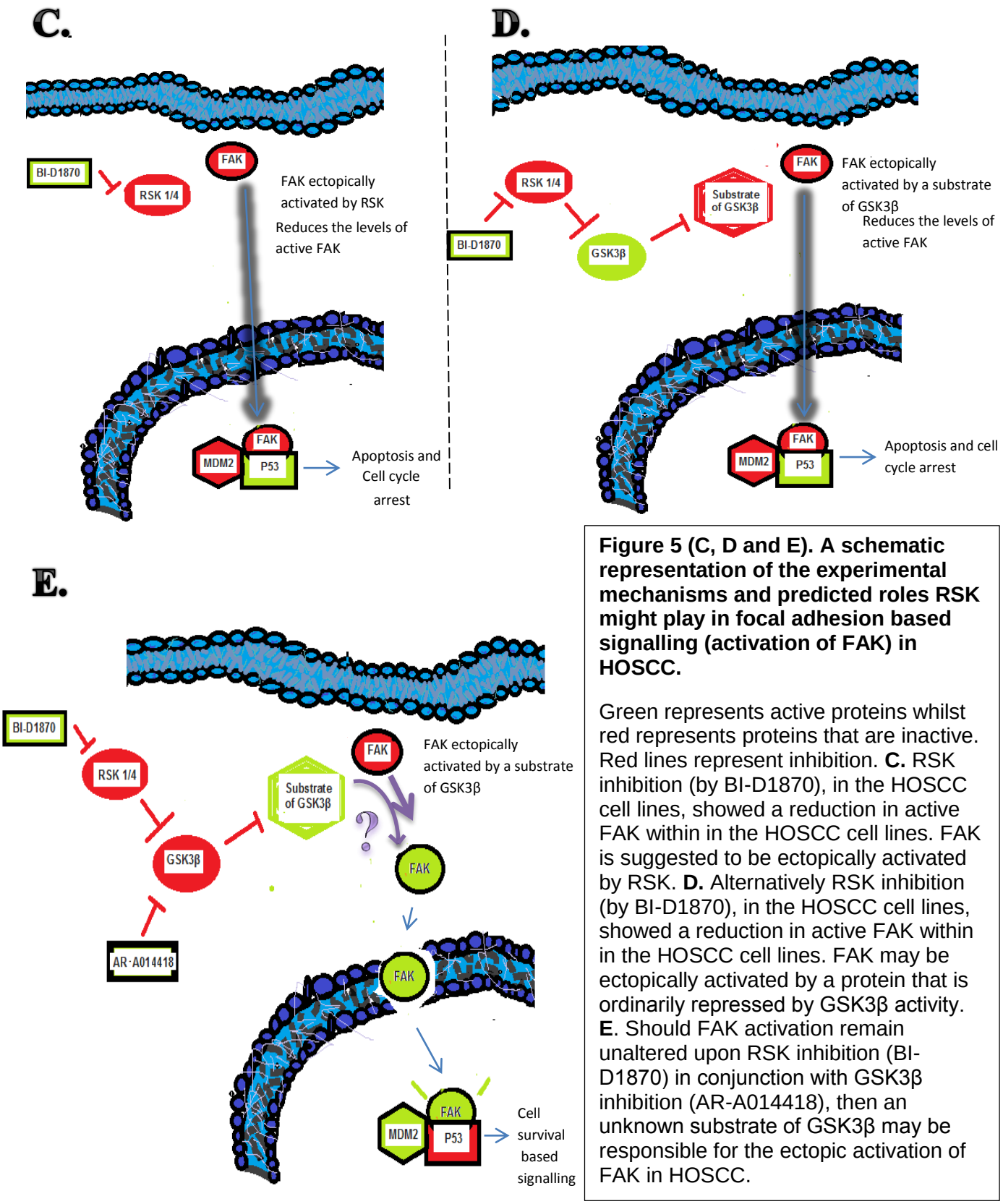


Figure 5 (C, D and E). A schematic representation of the experimental mechanisms and predicted roles RSK might play in focal adhesion based signalling (activation of FAK) in HOSCC.

Green represents active proteins whilst red represents proteins that are inactive. Red lines represent inhibition. **C.** RSK inhibition (by BI-D1870), in the HOSCC cell lines, showed a reduction in active FAK within in the HOSCC cell lines. FAK is suggested to be ectopically activated by RSK. **D.** Alternatively RSK inhibition (by BI-D1870), in the HOSCC cell lines, showed a reduction in active FAK within in the HOSCC cell lines. FAK may be ectopically activated by a protein that is ordinarily repressed by GSK3β activity. **E**. Should FAK activation remain unaltered upon RSK inhibition (BI-D1870) in conjunction with GSK3β inhibition (AR-A014418), then an unknown substrate of GSK3β may be responsible for the ectopic activation of FAK in HOSCC.

Chapter 2

2. The basal levels of key phospho-intermediates involved in the focal adhesion and Wnt pathway within the HOSCC cells

2.1. Introduction

Several studies have focused on the overexpression of FAK in neoplasmic cells. The overexpression of this protein can have multiple effects on the diseased phenotype. Lyngeal squamous cell carcinoma cells that overexpress FAK have a distinctive survival advantage, increased proliferative capacities and a reduced susceptibility to apoptosis induction (Li *et al*., 2012). FAK overexpression also correlated with rectal cancer resurgence following multiple rounds of chemoradiation (del Pulgar *et al*., 2016). Thoracic oesphageal cancer was also found to overexpress FAK (Miyazaki *et al*., 2003). Only 38 % of patients with FAK overexpression survived longer than 5 years, significantly lower than patients that did not overexpress this protein. Characteristics of FAK overexpression were predominantly linked to lymph node metastasis and tumour invasiveness (Miyazaki *et al*., 2003). This would suggest that FAK overexpression is a marker for the transformed state but does this correlate with FAK activation?

The high abundance of active FAK, phospho-FAK (Tyr397), has been associated with particularly invasive cancer strains suggesting that active FAK may promote lymph node metastasis and promote cell survival (Rodrigo *et al*., 2007; Aust *et al*., 2014). FAK activity also appears to be essential for anoikis resistance and promotes EMT in epithelial cells (Bolos *et al*., 2010; Halder *et al*., 2005). Circulating breast cancer cells that expressed activated signaling kinases (FAK and PI-3K) were found to be malignant/metastatic (Kallergi *et al*., 2007). Interestingly, osteosarcoma cells that overexpressed high levels of GKS3β, FAK and β5 integrin were found to be less responsive to neoadjuvant chemotherapeutics (Le Guellec *et al*., 2013). This suggests that high levels of FAK activation could be used as a marker for more virulent forms of cancer. This study therefore wishes to determine whether high levels of active FAK are a hallmark of human oesphageal squamous cell carcinomas. Provided that FAK activation is a common trait in HOSCC cells, the mechanisms by which this protein is activated ectopically will need to be elucidated.

Key signal transduction intermediates that may provide insight into the ectopic mechanisms of FAK activation are proteins that associate with the Wnt pathway, GSK3β and β-catenin.

GSK3β has the capacity to both promote and inhibit cancer (Waaler *et al*., 2011; Ougolkov *et al*., 2007; Ougolkov *et al*., 2005). The role GSK3β plays in the transformed state appears to be dependent on the cell lineage. In oral cancers GSK3β acts as a tumour suppressor protein that regulates transcription, accelerated cell cycle progression and promotes epithelial cell homeostasis (Kim *et al*., 2007; Iamaroon *et al*., 2009).

It does this in part, by inhibiting the Wnt pathway (MacDonald *et al*., 2009). GSK3β triggers the ubiquitination/degradation of β-catenin by phosphorylating it at Ser33, Ser37 and Thr41 (Hinoi *et al*., 2000; Rask *et al*., 2003; Waaler *et al*., 2011). This halts the Wnt pathway thereby preventing inapt cell renewal/proliferation (MacDonald *et al*., 2009). Should GSK3β be inhibited continually it would lead to excessive Wnt signaling and a loss in phospho-β-catenin (Ser33, Ser37 & Thr41) (MacDonald *et al*., 2009). Attaining the relative abundance of phospho-β-catenin within neoplasmic cells would provide scientists with an indicator of GSK3β activity.

The inhibition of GSK3β itself, by phosphorylation at Ser9, is upregulated by the oncogenic activation of either p90 ribosomal S6 kinase (RSK), Protein kinase A(PKA), Akt and protein kinase C (PKC) (Fang *et al*.,2000; Kim *et al*.,2007; Jope and Johnson, 2004; Stambolic and Woodgett, 1994; Pap and Cooper, 1998). The activation of these proteins and subsequent inhibition of GSK3β, underpins the diseased state in oral squamous cell carcinomas (Iamaroon *et al*., 2009; Lim *et al*., 2005). Therefore it stands to reason that cancer cell lines with high to moderate levels of GSK3β inhibition, phospho-GSK3β (Ser9) contain equally high levels of its antagonists (actively inhibiting GSK3β). This study also wishes to determine whether GSK3β inhibition is prominent in HOSCC cells. Not only will this indirectly determine whether GSK3β antagonists (such as the p90 ribosomal S6 kinase) are active, it may also ascertain whether GSK3β acts in the capacity of a tumour suppressor or oncogene within these cells. GSK3β or one of its antagonists may play a large role in cancer cell progression by playing an active role in FAK activation.

Lastly, the levels of phospho-β-catenin (Ser33, Ser37 & Thr41) will also be monitored to ascertain the level of GSK3β activity that underpins the transformed state in HOSCC cells. This will provide some indication as to whether GSK3β or one of its antagonists is more likely to play a role in FAK activation within these cells.

2.2. Methods and Materials

2.2.1. Cell culture

Five South African moderately differentiated human oesophageal squamous cell carcinoma cell lines were obtained from the Cell Biology Research Laboratory, the School of Molecular and Cell Biology, University of the Witwatersrand. The cell lines were denoted as the WHCO1, WHCO3, WHCO5, WHCO6 (Veale and Thornley, 1989) and the SNO (Bey *et al.*, 1976) cell lines. To date, there appears to be no archetypical oesophageal squamous cell lines that accurately represent the correct physiological profile of non-transformed oesophageal squamous cells *in vivo* (Jankowski *et al.*, 1995; Underwood *et al.*, 2010). Even the HET-1A cell line, the most commonly used model for organotypical oesophagus *in vitro*, was found to lack squamous differentiation (Underwood *et al.*, 2010). This cell line failed to express the classic epithelial markers, E-cadherin and casein kinase 5/6, displaying mesenchymal markers such as vimentin and N-cadherin instead (Underwood *et al.*, 2010). This suggests that the HET-1A cell line, or its equivalent, cannot act a representative model for 'normal' oesophageal squamous epithelium (Jankowski *et al.*, 1995; Underwood *et al.*, 2010). This makes the HET-1A cell line unsuitable as a comparative control in this investigation (Underwood *et al.*, 2010).

The HT29 cell line, derived from a human colon adenocarcinoma, and the A431 cell, originating from a human epidermoid carcinoma, were therefore used as comparative controls. The HT29 cell line contains constitutively high quantities of phospho-FAK (Tyr397), a truncated adenomatous polyposis coli (APC) and a mutant p53 (R273H) suggesting it would be an suitable comparative control (Golubovskaya *et al*., 2003; Bossi *et al*., 2006; Chandra *et al*., 2012). This cell line also acted as a negative control for phospho-β-catenin (Ser33, Ser37 & Thr41) as the presence of a truncated APC results in the stabilization of β-catenin (preventing the phosphorylation of β-catenin at Ser 33, Ser37 & Thr41) within this cell line (Morin *et al*., 1996; Chandra *et al*., 2012). The A431 cell line overexpresses epidermal growth factor receptor (EGFR), FAK and integrin β1 (Lu *et al*., 2001; Kao *et al*., 2008; Slack-Davis *et al*., 2007; Brockbank *et al*., 2005). The A431 cell line also contained high levels of phospho-GSK3β (Ser9) making it an appropriate comparative control (Saito *et al*., 1994).

All cells were maintained as monolayer cultures in Dulbecco's modified eagles medium (DMEM/Hams F12 (3:1)) (Life Technologies) (Appendix A, Section 1.7.1., Section 1.7.2. and Section 1.7.3.) supplemented with 10 % foetal calf serum (FCS) in 5 % carbon dioxide, at 37°C (humidified environment). FCS supplemented the media with growth factors, proteins or lipids whilst DMEM/Ham`s F12 contained essential amino acids, glucose and vitamins. It is important to note that the same batch of FCS was used concurrently for each experiment.

2.2.2. Subculture

Cell cultures were propagated in 10cm dishes. Once the cells reached an approximate confluency of 80 %, the media was aspirated and the cell monolayer washed twice with warm (37°C) sterile phosphate buffered saline (PBS), pH 7.3 (Appendix A, Section 1.1.1.). A 2 ml solution containing ethylenediamineterta-acetic acid (EDTA) (BDH Laboratory reagents) and trypsin (Gibco BRL) (Appendix A, Section 1.6.4 and Section 1.6.5) was then added to the tissue culture dish. The dish was then placed in an incubator for 5 minutes at 37°C to facilitate the disruption of cell-cell and cell-ECM based attachment/adhesion. A Leitz Watzlar inverted light microscope was used to verify cell-cell/cell-ECM detachment. A fraction of the detached cells, in the trypsin-EDTA solution (Appendix A, Section 1.6.6.), were seeded into a new tissue culture dish and 10 ml of fresh media (containing 10 % FCS) added. Cells were then maintained as described in Section 2.2.1.

2.2.3. Whole cell protein extraction

Whole cell protein extractions were performed as outlined by Laemmli, 1970. A whole cell protein extraction was opted for, instead of a nuclear or cytoplasmic protein extraction, as the proteins of interest (FAK and β-catenin) are located within all subcellular locations within the cell (Fanucchi and Veale, 2009; Willert and Jones, 2006; Krieghoff *et al*., 2006).

Cells were allowed to proliferate until a confluency of 80 % was reached. The cells were then washed twice with 1 ml of PBS. The cells were then exposed to 1 ml of PBS containing phenyl-methyl-sulphonyl fluoride (PMSF) (1 mM) (Trasylol ®, Bayer S.A.), sodium fluoride (NaF) (5 µl/ml) (Sigma-Aldrich ®) and sodium orthovanadate (Na_3VO_4) (100 µl/ml) (Sigma-Aldrich ®) (Appendix A, Section 1.1.3,Section 1.1.4. and Section 1.1.5). PMSF, an irreversible serine protease inhibitor, was utilised to prevent protein degradation within the whole cell lysates (James, 1978; Powers *et al*., 2002). As the proteins were extracted for

phospho-antibody based experiments, phosphatase inhibitors (NaF and Na_3VO_4) were incorporated in the extraction procedure in order to prevent the loss of certain post translational modifications during the extraction procedure (Gordon, 1991; Di *et al.*, 2013; Posner *et al.*, 1994). The cells were then carefully mechanically harvested into this PMSF/PBS solution using a rubber policeman. The harvested cells were transferred into sterile Eppendorf® Safe-Lock microcentrifuge tubes and centrifuged in a TOMY HF-120 (1145 xg), 1 min, to yield a highly concentrated mass of cells (pellet).

The supernatant was then aspirated and the pellet resuspended in Laemmli double lysis buffer (Appendix A, Section 1.1.2.) (ratio of 1:2). The lysis buffer (Appendix A, Section 1.1.2.) contained β-mercaptoethanol (reduce disulphide bonds) and sodium dodecyl sulphate (SDS) which encouraged protein denaturation preparing the samples for SDS-PAGE analysis. The sample was then boiled for 5 minutes and the lysates spun down in a PRISM™ Refrigerated Microcentrifuge (12000 xg) for 15 minutes. Lastly, the samples, appropriate for SDS-PAGE analysis, were stored at -20°C.

2.2.4. Protein determination

A modified Bradford assay as proposed by Bramhall *et al.* (1969) was utilised to semi-quantitatively determine the concentration of protein within each of the whole cell protein extracts. This ensured that equimolar/equivalent quantities of protein were loaded during SDS-PAGE analyses for each of the HOSCC cell lysates (for comparative purposes). Whatman ® Filter paper was hydrated in distilled water, for 20 minutes, and dehydrated using a series of one, five minute incubations in 95 % ethanol, 100 % ethanol and 100 % acetone (applied sequentially) (Appendix A, Section 1.2.1). The dehydrated filter paper was then placed in a sterile fume hood to air dry. A known concentration of protein (1 µg/µl) was produced by solubilizing bovine serum albumin (BSA) (BDH Laboratory reagents) in Laemmli double lysis buffer (ratio of 1:1). The stock BSA solution (1 µg/µl) was then spotted onto the dry filter paper to generate standard concentrations of BSA (1 µg, 3 µg, 6 µg, 12 µg, 16 µg and 20 µg of protein) which collectively formed a standard curve. Additionally, 2 µl of each of the HOSCC whole cell protein extracts were spotted onto the same filter paper. The filter paper was air-dried and exposed to trichloroacetic acid (TCA) (Appendix A, Section 1.2.2.) for 45 minutes. TCA caused the proteins to precipitate onto the filter paper and removed any residual SDS that may have remained from the lysis buffer (safeguarded against SDS interference with regard to the spectrophotometer readings). The filter paper was placed

in 0.25 % Coomassie brilliant blue G-250 (CBB) (Appendix A, Section 1.2.3.) for 5 minutes (washed off residual TCA). The filter paper was exposed to fresh CBB stain for 1 hour, placed in a destain solution (Appendix A, Section 1.2.4) for 1 hour (to remove all background staining) and then allowed to air dry in a sterile fume hood.

The resulting blue-stained circles, located on the filter paper, represented precipitated protein. Each of these circles were then cut out and individually placed into 5 ml of elution solution (Appendix A, Section 1.2.5.). The stains circles were then left in the dark overnight. The absorbance readings for each of these solutions were obtained using an Abbota SV1100 spectrophotometer (set to a wavelength of 595 nm). Note that elution solution served as a blank for the spectrophotometer. CBB, upon protein dye binding, absorbed light in the 595 nm range and this absorbance was equivalent to the concentration of protein present (Bradford, 1976). A standard curve was then developed using the known protein concentrations of BSA (absorbance at 595 nm over protein concentration). The resulting absorbance readings, for the known BSA standards (eluents of known protein concentration), were then utilised to generate a standard curve of absorbance (at 595 nm) versus protein concentration (µg/µl) (Appendix B, Section 2.1) generated in Microsoft® Excel 2010 (Version 14.0). This standard curve was then used to determine the protein concentration of each of the HOSCC cell lysates (of unknown protein concentration). Only standard curves with an R^2 of greater than 0.98 was utilised (indicative of how well the standard curve fits the data). These concentrations were then used to standardize the amount of protein loaded per SDS-PAGE analysis.

2.2.5. Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE)

A 10 % (w/v) discontinuous SDS-PAGE analysis, as described by Laemmli (1970) was used to obtain high resolution protein separations (on the basis of size). The proteins, present in each of the HOSCC whole cell lysates, were effectively separated using this technique. Ten percent discontinuous SDS-PAGE gels were generated using the Mighty Small™ SE245Dual Gel Caster set (Hoefer Scientific). A 10 % separating gel solution, containing SDS, (Appendix A, Section 1.3.2 and Section 1.3.3) was placed between 2 glass plates located within the Dual Gel Caster. Approximately 200 µl of a 0,2 % SDS overlay (Appendix A, Section 1.3.4) was added to prevent oxygen affecting the polymerization reaction and the gel was allowed to polymerise for approximately 25 minutes. The overlay was discarded before a 5 % stacking gel solution (Appendix A, Section 1.3.3) was poured directly above the

separating gel (between the 2 glass plates). A comb, with the capacity to generate either 10 or 20 µl wells, was then inserted into the stacking gel before it was allowed to polymerise (for approximately 25 minutes). The comb is used to generate wells, each able to accommodate up to10 or 20 µl of sample.

The polymerised gel was then removed from the caster set and inserted into the Mighty Small™ Electrophoresis Unit (Hoefer ®), filled with electrophoresis tank buffer (pH 8.3) (Appendix A, Section 1.3.1.). The comb was removed and 40 µg of protein, from each whole cell protein lysate, was loaded into each of the wells. Approximately 1 µl of PageRuler Plus Pre-stained Protein ladder (molecular weight marker) (Thermo Fisher Scientific ®) was loaded into the last well of each gel. The proteins, collectively forming the Pre-stained protein ladder, represented proteins with a mass of approximately 250, 130, 100, 70, 55, 35 and 25 kDa (used to monitored protein migration in the gel and visualise protein transfer step). The gel was fully resolved using constant current, 21 mA (400 V) per gel (45 minutes). Once the molecular weight marker, 25 kDa, reached a set point on the separating gel (two centimetres from the end), the gel was removed from the Mighty Small™ Electrophoresis Unit. The SDS-PAGE gel could then be utilised for western immunoblotting.

Visualisation of the resolved protein pattern was attained by staining the SDS-PAGE gel with CBB stain (Appendix A, 1.3.5.) for 1 hour. The gel was destain solution (Appendix A, Section 1.3.6.) for 1 hour and left in distilled water overnight. The gel was scanned using a Hewlett Packard ScanJet G3110 scanner.

2.2.6. Western immunoblotting

The western immunoblotting procedure is able to semi-quantitatively determine the abundance of a specific protein (or post translational modification located on a protein) within a whole cell protein lysate. Western immunoblotting does this, by capitalising on monoclonal/polyclonal antibodies ability to bind exclusively to a specific protein. It does this making use of a specific polyclonal/monoclonal antibody-antigen interaction, a secondary antibody system conjugated with a detector molecule and an effective detection system (Towbin, Staehelin, and Gordon, 1992). Western blots were performed as outlined by Towbin, and colleagues (1992).

A 10 % (v/w) discontinuous SDS-PAGE analysis was initially required in order to fully resolve the proteins located in the HOSCC lysates as described in Section 2.2.5. The SDS-PAGE gels were cut, at a range around the molecular weight of the protein of interest as indicated by the PageRuler Plus Pre-stained Protein ladder. The proteins, resolved by SDS-PAGE analysis, were transferred onto an BioTrace™ PVDF nitrocellulose membrane (Pall) using a Bio-Rad Criterion™ Blotter containing transfer buffer (Appendix A, Section 1.4.1.), at constant current of 400 mA (4°C).The protein transfer took approximately 2 to 3 hours to complete (dependent on the molecular weight of the protein of interest). Each monoclonal/polyclonal antibody has specific protocol requirements that were utilised, following this step in order to obtain western blots with little to no non-specific antibody binding.

2.2.6.1 Anti-phospho-FAK (Tyr397)

The protein transfer step, described in Section 2.2.6., took 3 hours to complete. The membrane was washed in Tris-buffered saline (TBS) (Appendix A, Section 1.4.1.) and placed in a casein based-blocking solution (Appendix A, Section 1.4.1.5.) for 1 hour to prevent non-specific antibody binding. The membrane was then washed in Tris-buffered saline-Tween (TBS-T)(Appendix A, Section 1.4.1) and placed in a primary polyclonal anti-phospho-FAK (Tyr397) polyclonal rabbit antibody solution, 1 % BSA in TBS-T (antibody dilution:1:1000), overnight at 4°C (Cell Signalling Technology ®). The membrane was washed in TBS-T several times to remove any unbound antibody (5 minutes per wash). The membrane was then incubated in a goat-anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody solution, blocking solution (Appendix A, Section 1.4.1) (antibody dilution: 1:2000), for 1 hour at room temperature (in the dark) (Sigma-Aldrich ®). Several washes, in TBS-T, were performed to remove residual secondary antibody from the membrane (5 minutes per wash). The membrane then underwent a process outlined in 2.2.7 to 2.2.9.

2.2.6.2. Anti-phospho-GSK3β (Ser9)

The protein transfer step, described in Section 2.2.6., took 2 hours to complete. The membrane was washed in TBS (Appendix A, Section 1.4.1.), to remove any residual transfer buffer. Next the membrane was placed in a casein based-blocking solution (Appendix A, Section 1.4.1.) for 1 hour to prevent non-specific antibody binding. Excess blocking solution was removed by washing the membrane with TBS-T (several 5 minute washes). The membrane was incubated in an anti-phospho-GSK3β (Ser9) polyclonal rabbit antibody

solution, 2.5 % BSA in TBS-T (antibody dilution: 1:1000), overnight at 4°C (Cell signalling technology ®). The membrane was washed in TBS-T several times to remove any unbound antibody (5 minutes per wash). The membrane was then incubated with a goat-anti-rabbit HRP-conjugated secondary antibody solution, 2, 5 % casein based-blocking solution (Appendix A, Section 1.4.1.) (antibody dilution: 1:3000), for 1 hour at room temperature (in the dark) (Sigma-Aldrich ®). Several washes, in TBS-T, were performed to remove residual secondary antibody from the membrane (5 minutes per wash). The membrane then underwent a process outlined in 2.2.7 to 2.2.9.

2.2.6.3. Anti-phospho-β-catenin (Ser33, Ser37 & Thr41)

The protein transfer step, described in Section 2.2.6., took approximately 3 hours to complete. The membrane was then washed in TBS-T (Appendix A, Section 1.4.1.) and placed in a primary polyclonal anti-phospho-β-catenin (Ser33, Ser37 & Thr41) polyclonal rabbit antibody solution, 2 % BSA in TBS-T (antibody dilution:1:1000), overnight at 4°C (Cell signalling technology®). The membrane was washed in TBS-T several times to remove any unbound antibody (5 minutes per wash). The membrane was then incubated in a goat-anti-rabbit HRP-conjugated secondary antibody solution, 2.5 % TBS-for BLOTTO blocking solution (Appendix A, Section 1.4.1.) (antibody dilution: 1:2500), for 1 hour at room temperature (in the dark) (Sigma-Aldrich ®). Several washes in TBS-T were performed to remove excess secondary antibody from the membrane (5 min per wash). The membrane then underwent a process outlined in 2.2.7 to 2.2.9.

2.2.6.4. Anti-β-actin

The protein transfer step described in Section 2.2.6 took 2 hours to complete. The membrane was washed in TBS (Appendix A, Section 1.4.1.) and placed in a casein based-blocking solution (Appendix A, Section 1.4.1.) for 1 hour. The membrane was then washed in TBS-T (Appendix A, Section 1.4.1.) and placed in a primary polyclonal anti-β-actin polyclonal rabbit antibody solution (2.5 % casein-based blocking solution in PBS (antibody dilution: 1:2500)) overnight at 4°C (Cell signalling technology ®). The membrane was washed in TBS-T several times (5 minutes per wash). The membrane was incubated with a goat-anti-rabbit HRP-conjugated secondary antibody solution, blocking solution (Appendix A, Section 1.4.1.) (antibody dilution: 1:10000), for 1 hour (in the dark) (Sigma-Aldrich ®). Several washes, in TBS-T, were performed to remove residual secondary antibody from the

membrane (5 min per wash). The membrane then underwent a process outlined in 2.2.7 to 2.2.9.

2.2.7. Antibody detection

The membrane was incubated in a luminol/hydrogen peroxide solution (1:1 ratio), SuperSignal ® West, for 5 minutes in the dark (Appendix A, Section 1.4.4.). The membrane was placed in Versafilm ® clear cling-wrap and exposed to CL-XPosure X-ray film (Pierce Chemical ©). The X-ray film was exposed to the membrane for 10 minutes (in the dark). A latent image was formed by deposits of free silver ions that are liberated when light photons strike the silver bromide crystals found on the X-ray film. Next, the X-ray film was placed in developer solution (Thermo Fisher Scientific ®) (Appendix A, Section 1.4.2.). The film was washed in water and then placed in fixer solution (Appendix A, Section 1.4.3.). Finally the film was rinsed in water before it was allowed to air dry. A Hewlett Packard ScanJet G3110 scanner was then used to obtain digital images of the X-ray film.

2.2.8. Densitometry

All densitometric analyses were performed using MATLAB® R2013a image acquisition and analysis software. MATLAB semi quantitatively determined the relative abundance of a specific phospho-protein as determined by western blotting. The band intensities/integrated optical densities (IOD) represent the magnitude of the HRP-dependant chemiluminescent signal generated during the western blotting technique. This provided an estimation of the relative abundance of key phospho-proteins within each of the HOSCC cell lines (described in more detail below). All densitometric data generated was compared to the optical density of the A431 or WHCO6 cell lines (Appendix B, Section 2.2.). To generate accurate and comparable data, the computed IOD, determined by MATLAB, were normalised for all western blot replicates as described in Appendix B, Section 2.6 (Degasperi *et al*., 2014)

2.2.9. Data analysis

All experiments were performed in triplicate, unless otherwise stated. Results were represented by the mean plus/minus the standard error of the mean. The statistical significance of the densitometric data obtained by western immunoblot analysis, was determined by performing a standard Student's *t*-test (Appendix B, Section 2.2.) using GraphPad Prism®, Version 7.0 (GraphPad Software, Inc., La Jolla, USA) ($p<0.05$ indicated statistical significance).

2.3. Results section

2.3.1 Efficacy of the whole cell protein extraction and protein determination technique

The separation of proteins into descreet polypeptide bands, by SDS-PAGE analysis indicated that the whole cell protein extraction procedure is an effective mechanism of extracting cellular proteins from HOSCC cell lysates (Figure 6). Upon staining the polypeptides with Coomassie Brilliant Blue stain a reasonably uniform banding pattern, across all of the lanes of the SDS-PAGE gel, was made apparent (Figure 6). This uniform banding pattern, present across all of the HOSCC cell lines, provided proof of the accuracy and reliablilty of the protein estimation technique (approximately 20 µg of protein was loaded into each lane, see Appendix B, Section 2.1., Figure 6). The molecular weight marker (MWM), located in lane 1, was fully resolved. This was noted by the fact that the MWM split into a range of distinctive bands (each a representative of a polypeptide with a set molecular weight) upon SDS-PAGE analysis. This indicates that the blue-stained polypeptides, located in lanes 2 to 8, were fully separated on the basis of size and had goo d resolution at the regions of interest, indicated by arrows on Figure 6. As there are polypeptides present at the molecular weight regions of the proteins of interest, it is likely that these key signal transduction intermediates (FAK, GSK3β and β-catenin) are present and were effectively extracted from the HOSCC cell lysates. This warranted further investigation into the presence of key signal transduction intermediates at set molecular weight marker regions. Western immunoblot analyses were then preformed to confirm that these proteins are present within the HOSCC cell lines and verify that the post translational modifications, made to each of these key intermediates, remained intact (further validating the effectivity of the protein extraction procedure). Lastly the western blot analyses were used to determine the relative abundance of key phospho-proteins (indicative of the activation/inhibition state of each protein) within each of the HOSCC cell lines at a specific point in time.

All of the key signal transduction intermediates, mentioned in Figure 7, were present within the HOSCC cell lines. The western immunoblotting technique physically verified that key post translational modifications located on cellular proteins were retained (through the use of phosphatase inhibitors, Section 2.2.4. of the methods and materials) in the HOSCC cell lysates. As the major phospho-intermediates pertaining to this study were readily detectable by western immunoblotting, the protein extraction procedure was considered successful (Figure 7). This ensured that the activation/ inhibition state of each protein could be studied

by western immunoblotting thus providing the physical surety needed to continue the current study. Lastly, the western immunoblotting procedure yielded little to no non-specific antibody binding indicating that the western blotting technique is capable of producing reliable and repeatable western blots. As western immunoblotting (for phospho-FAK (Tyr397)) is known to produce reliable results (Fanucchi and Veale, 2009), smaller western blots were employed, dimensions that adequately cover a range around the molecular weight of the protein of interest (Figure 7). This was done to conserve reagents.

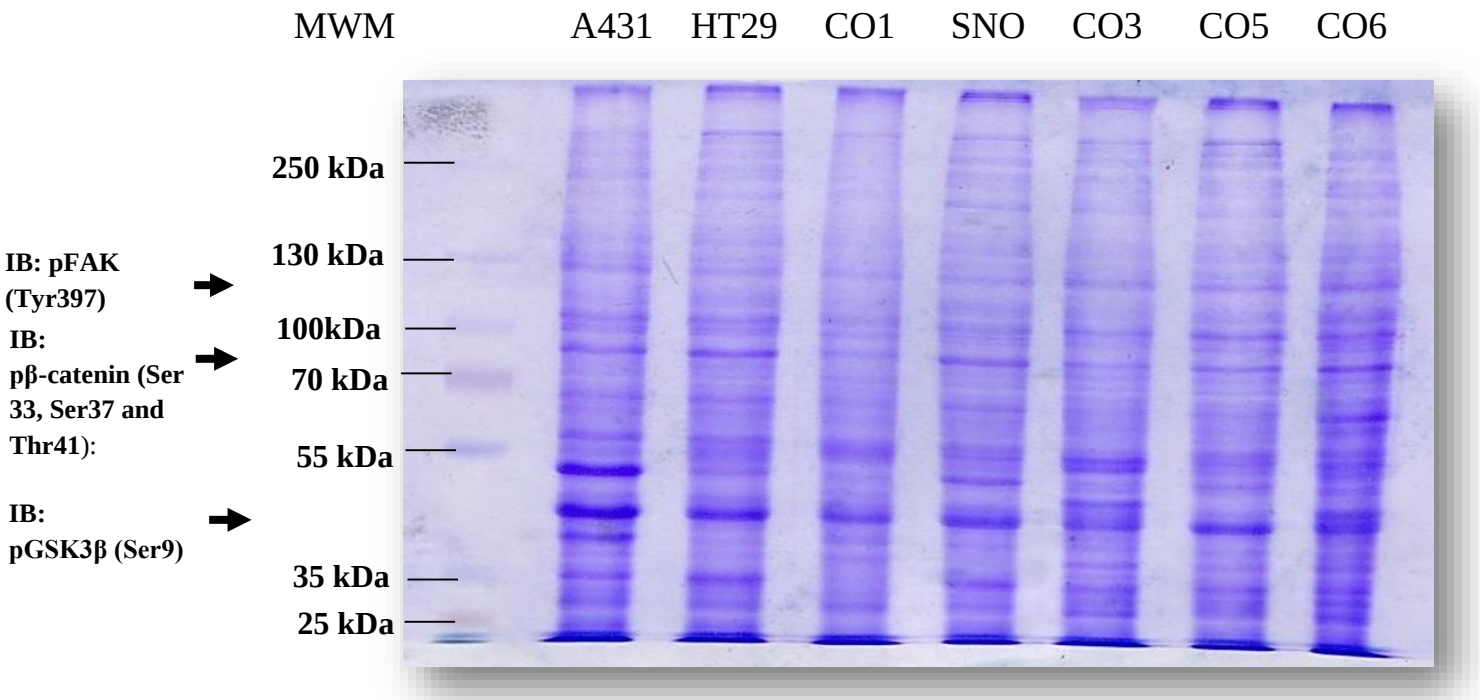


Figure 6. High resolution separation of the cellular proteins that were extracted from the HOSCC cell lines, A431 and HT29 cells.
A 10 % (w/v) discontinuous SDS PAGE analysis revealed that the protein determination and extraction procedure was successful. This can be seen by uniform banding pattern of the blue-stained protein polypeptides noted across all seven of the lanes. A molecular weight marker was fully resolved in the first lane of the gel. The gel was stained, with Coomassie brilliant blue. The suggested positions of phospho-β-catenin (Ser33, Ser37 &Thr41) (92kDa), phospho-FAK (Tyr397) (125kDa) and phospho-GSK3β (Ser9) (46kDa), relative to the molecular weight marker (MWM), are indicated by arrows. (CO1:WHCO1; CO3: WHCO3; CO5: WHCO5 and CO6: WHCO6)

2. 3.2. The relative abundance of key phospho-intermediates differ greatly amongst the HOSCC cell lines

Phospho-FAK (Tyr397) (125kDa), phospho-GSK3β (Ser9) (46kDa) and phospho-β-catenin (Ser33, Ser37 & Thr41) (92kDa) were all detected in the HOSCC cell lines under standard tissue culture conditions (Figure 7). The β-actin western blots provided physical evidence to support the success of the protein estimation technique. Equal protein loading across all of the HOSCC cell lines was confirmed by β-actin immunoblots that sported a uniform banding pattern (Figure 7). As archetypical oesophageal squamous cell lines are currently unavailable (Section 2.2.1, method and materials), the HT29 cell line and A431 cell line were used as comparative controls to determine the relative abundance of phospho-FAK (Tyr397) within the HOSCC cell lines (Section 2.2.1, method and materials). Both of these cell lines are known to have a high abundance of phospho-FAK (Tyr397) and this was confirmed in the current study (Figure 7 and Figure 8) (Golubovskaya *et al*., 2003). With the exception of phospho-β-catenin (Ser33, Ser37 & Thr41), in the HT29 cell line (Section 2.2.1, method and materials), all of the key phospho-proteins were detected within these two comparative controls (HT29 and A431 cell lines) (Figure 7). The WHCO6 cell line acted as an internal control for the western blotting technique ensuring that the protein levels detected on one immunoblot was comparable to another. All densitometric data was made relative to the IOD of the A431/WHCO6 cell line (Section 2.2.8, method and materials)

The relative abundance of phospho-FAK (Tyr397), phospho-GSK3β (Ser9) and phospho-β-catenin (Ser33, Ser37 & Thr41) differed greatly amongst the HOSCC cell lines, relative to the IOD of the A431 cell line (Figure 8). This suggests that the mechanisms by which the transform state is propagated/ maintained within one WHCO cell line may not be universally applicable to all HOSCC cells. It should therefore be noted that the high/low abundance of a specific phospho-protein may or may not be a hallmark of the transformed state of HOSCC cells (a commonality between all HOSCC cells) (Figure 8). This resulted in the WHCO cell lines being segregated, based on the relative abundance of key phospho-intermediates, in order to develop a more holistic view of the phospho-protein profile located within HOSCC cells. The overall trends, with regard to the relative abundance of the key phospho-intermediates, have been summarised Table 1.

Most HOSCC cell lines, excluding the WHCO1 cells, contained an equivalent/greater level of phospho-FAK (Tyr397) than that which was found within the HT29 cell line (Figure 8 and Table 1). This indicates that the majority of HOSCC cell lines contain endogenously high levels of this phospho-protein, as the HT29 cells are known to contain high levels of phospho-FAK (Try397) (Golubovskaya *et al*., 2003). The WHCO6 cell line contained exceedingly high levels of phospho-FAK (Tyr397) and was second only to the A431 cell line (contained greatest relative abundance of phospho-FAK (Try397)). The endogenous levels of phospho-FAK (Tyr397), noted for all HOSCC cell lines, was significantly different to levels of this protein found within the A431 cells (Appendix B, Section 2.2.). Three of the five HOSCC cell lines were found to contain moderate to high levels of phospho-GSK3β (Ser9). This suggests that, in general, HOSCC cells are likely to maintain moderate to high levels of phospho-GSK3β (Ser9). The WHCO3 and WHCO1 cell lines were the exception to the rule, as these cells contained a relatively low abundance of phospho-GSK3β (Ser9). When comparing the levels of phospho-GSK3β (Ser9) found within the A431 cell line to that of the HOSCC cells, only levels of phospho-GSK3β (Ser9) located within the SNO cell line were not considered statistically significant (Appendix B, Section 2.2.). As most of the WHCO cell lines contained moderate to high levels of phospho-β-catenin (Ser33, Ser37 & Thr41) when compared to the A431 cell line, the HOSCC cells generally contain moderate/high levels of this phospho-protein (Figure 8). The levels of phospho-β-catenin (Ser33, Ser37 & Thr41) differed significantly in the WHCO1, WHCO5 and WHCO6 cells when compared to the A431 cell line (Appendix B, Section 2.2.).

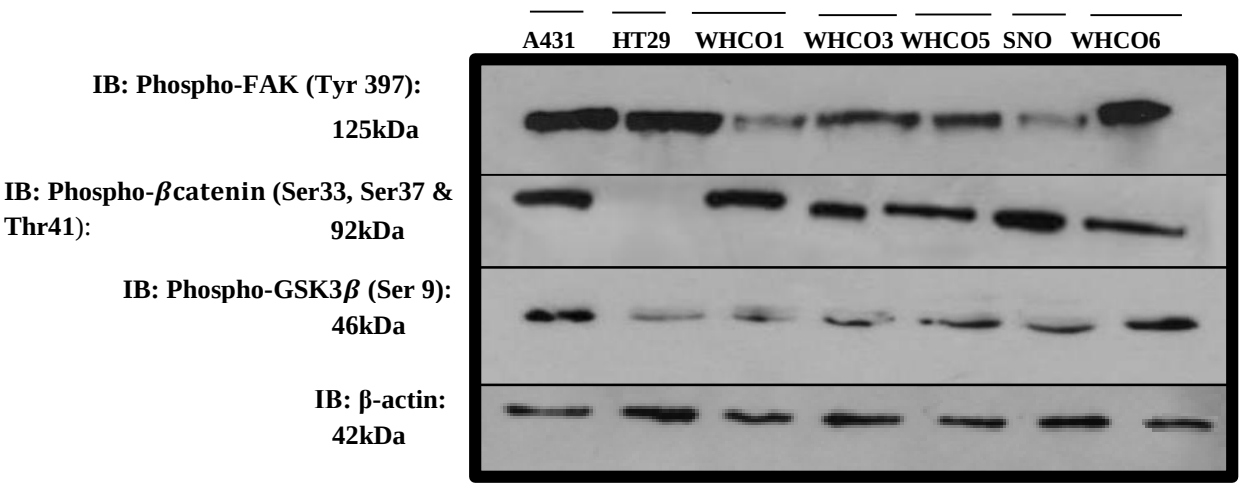


Figure 7. Representative western immunoblots used to detect the basal levels of phospho-GSK3β (Ser9), phospho-FAK (Tyr397) and phospho-β-catenin (Ser33, Ser37 & Thr41) in HOSCC cell lines.

The levels phospho-GSK3β (Ser9), phospho-FAK (Tyr397) and phospho-β-catenin (Ser33, Ser37 & Thr41) were detected in the WHCO cells, as well as the 2 control cell lines, HT29 and A431 cells. The relative abundance of the phospho-proteins were detected under standard tissue culture conditions using polyclonal (anti-phospho-FAK (Tyr397), anti-phospho-GSK3β (Ser9), anti-phospho-β-catenin (Ser33, Ser37 & Thr41) and anti-β actin) antibodies. Three biological replicates were performed. The HOSCC cell lines, in general, appeared to contain high to moderate levels of phospho-FAK (Tyr397) and phospho-GSK3β (Ser9). The HT29 cell line acted as a negative control for phospho-β-catenin (Ser33, Ser37 & Thr41) abundance. The western blot analyses, for the detection of β-actin, acted as a loading control, indicate equal protein loading, for the western blotting technique.

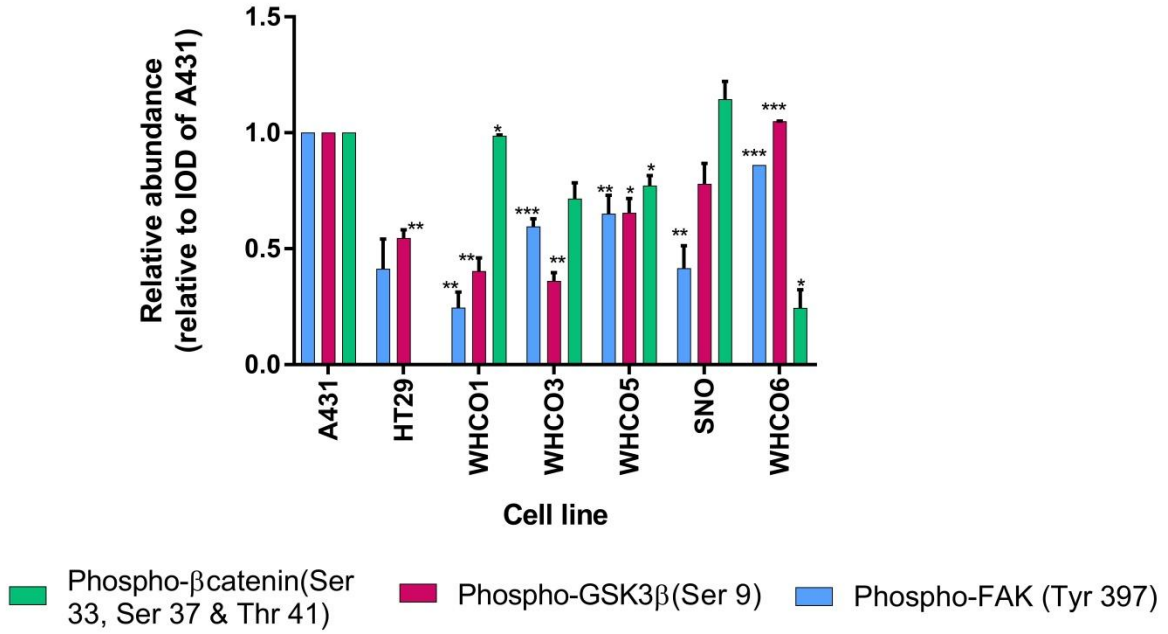


Figure 8. The relative abundance of certain phospho-intermediates appeared to be HOSCC cell line specific under standard tissue culture conditions.
The relative abundance of each phospho-protein was obtained by normalising semi-quantitatively determined densitometric analyses relative to the A431 cell line (Appendix B, Section 2.2.). The level of each phospho-protein appeared to vary greatly from one HOSCC cell line to the next. Phospho-FAK (Tyr397) is highly abundant within most of the HOSCC cell lines (equivalent/greater levels of phospho-FAK (Tyr397) than the HT29 cell line). Secondly, most of the HOSCC cell lines contained high to moderate levels of phospho-GSK3β (Ser9) and phospho-β-catenin (Ser33, Ser37 & Thr41) when compared to the A431 cell line. Only the WHCO3 and WHCO1 cell lines had low levels of phospho-GSK3β (Ser9). A star (*) represents any statistical difference, when compared to the A431 cell line (*p <0 .05, **p < 0.01, ***p < 0.001) (Appendix B, Section 2.2.).

Table 1. The relative abundance of key phospho-intermediates in the HOSCC cells under standard tissue culture conditions and the resulting phospho-protein trends.

The level of each phospho-protein, within the WHCO cells, was characterised as high, medium and low. This was done by comparing the relative abundance of each protein with the endogenous levels of set-protein in the A431 and HT29 cell lines (Section 2.2.1, methods and materials). A star (*) graphically represented any statistical difference, when compared to the A431 cell line using a standard Student's *t*-test (*p <0 .05, **p < 0.01, ***p < 0.001) (Appendix B, Section 2.2.).

Protein	Cell line						
	A431 (mean)	HT29 (mean)	WHCO1 (mean)	WHCO3 (mean)	WHCO5 (mean)	SNO (mean)	WHCO6 (mean)
Phospho-FAK (Tyr397) (Active protein)	Extremely high (1)	High (0.41)	Moderate /Low** (0.25)	Very high** (0.65)	Very high** (0.65)	High** (0.41)	Extremely high**** (0.86)
Phospho-GSK3β (Ser9) (Inactive protein)	High (1)	Moderate ** (0.55)	Low** (0.4)	Low** (0.36)	Moderate* (0.65)	High (0.78)	High**** (1.05)
Phospho-β-catenin (Ser33, Ser37 & Thr41) (Target for ubiquitination)	High (1)	None (0)	High* (0.99)	Moderate (0.72)	Moderate* (0.77)	High* (1.14)	Low* (0.24)

2.4. Discussion:

Human oesophageal squamous cell carcinoma cells are extremely resilient and adaptive. Single HOSCC cells are capable of forming a tumour and appear to be highly resistant to anti-cancer therapeutics (Tripathi, 2016; Van Miltenburg *et al*., 2014; Ge *et al*., 2015; Li *et al*., 2014; Hasan *et al*., 2016). Some have even suggested that there may be regenerative pockets of □stem cell like cells□ that assist in maintaining the diseased state (Ge *et al*., 2015; Xia *et al*.,2016; Zhao *et al*., 2013). Even though HOSCC development has been associated with several risk factors such as the use of tobacco, hot beverages and poor oral hygiene, the disease is still prevalent in our communities (Castellsague *et al*., 2000; Abnet *et al*., 2005; Wei *et al*., 2005). It is imperative that scientists identify accurate biomarkers for this

debilitating disease. Cancer biomarkers identify key molecular pathways that maintain the cancerous state in the hopes that these pathways may be targeted by anti-cancer therapeutics (Li *et al*., 2014; Hasan *et al*., 2016). However, biomarkers need to be accurate in order for the anticancer therapeutics to be effective at treating the disease (Li *et al*., 2014; Hasan *et al*., 2016). Biomarkers should also be found to identify patients that are at risk of cancer resurgence (Li *et al*., 2014; Hasan *et al*., 2016). With the HOSCC cells heterogeneity, identifying universal biomarkers that underpin the diseased state is challenging to identify and harder to capitalise on.

2.4.1. High levels of active FAK may be a biomarker for the transformed state in HOSCC cells

Phospho-FAK (Tyr397), active FAK, was detected in all of the HOSCC cell lines under standard tissue culture conditions. Previous studies could only detect phospho-FAK (Tyr397), via western immunoblot analyses, in SNO and WHCO6 cell lines, at 80 µg of protein (Fanucchi and Veale, 2009). This discrepancy may have been due to the use of different primary antibodies during the western blotting technique. An anti-phospho-FAK (Tyr397) monoclonal mouse antibody was used previously as opposed to an anti-phospho-FAK (Tyr397) polyclonal rabbit antibody that was used in the current study. The level of active FAK within the two control cell lines (the HT29 and A431 cell lines) were also detected by western immunoblotting. The A431 cell line had the highest level of active FAK of all of the cell lines that were used in the current study. The levels of FAK activation within the A431 cell line was found to be statistically different, at a p-value 0.01, when compared to the HOSCC cell lines used in the current study. This suggests that the A431 cell line maintains extremely high levels of active FAK higher than that of the HT29 cell line (a cell line that is known to contain high levels of active FAK) (Golubovskaya *et al*., 2003).

With the exception of the WHCO1 cells, all HOSCC cell lines had greater or equivalent levels of active FAK than that of the HT29 cell line. This suggests that WHCO cell lines generally contain high levels of active FAK, phospho-FAK (Tyr397). It can therefore be said that high levels of this phospho-protein may act as a hallmark/biomarker for moderately differentiated HOSCC cells (Fanucchi and Veale, 2009). This may in part explain why this disease is so virulent. Based on the literature, high levels of this active protein is associated with highly invasive cancers such as ovarian, breast and colon cancers (Golubovskaya *et al*., 2009; Wang *et al*., 2008). The over expression and activity of FAK was associated with

increased cell survival, EMT and migration (within ovarian, breast and colon cancers) (Golubovskaya *et al*., 2009; Wang *et al*., 2008). FAK activation was indispensable for the metastasis and invasion of mesenchymal triple negative breast cancer cells, one of the most invasive and virulent forms of breast cancer (Taliaferro-Smith *et al*., 2015). The WHCO6, WHCO5 and WHCO3 cell lines maintain exceptionally high levels of active FAK suggesting that these cell lines may also be more predisposed to anoikis resistance, EMT and metastasis. However, although FAK activation plays a prominent role in propagating migratory/pro-survival based signalling in differently differentiated cells the mechanisms by which FAK does this may be different. Likewise the mechanisms by which this protein is activated within cancerous cells may also be cell lineage specific.

Nevertheless, based on the role FAK plays in the transformed state, this protein may be a good molecular target for anti-cancer therapeutics (Taliaferro-Smith *et al*., 2015; Tripathi, 2016). However, with FAK playing a role in normal cell functioning, as a key member of the focal adhesion pathway, inhibiting this protein may prove ineffective (Fanucchi and Veale, 2009; Mitra and Schlaepfer, 2006; Parsons, 2003). With the knowledge that high levels of FAK activation is common within the HOSCC cells, the mechanisms by which this protein is activated will be explored further in Chapter 3.

2.4.2. GSK3β inhibition, an indicator of its antagonists activity within the HOSCC cells?

The inhibition of GSK3β by phosphorylation at Ser9 is upregulated by the oncogenic activation of either p90 ribosomal S6 kinase (RSK), Protein kinase A (PKA), Akt or protein kinase C (PKC) (Fang *et al*., 2000; Kim *et al*., 2007; Jope and Johnson, 2004; Stambolic and Woodgett, 1994; Pap and Cooper, 1998). Therefore should any of the above mentioned proteins be active within the HOSCC cell lines, GSK3β will be phosphorylated at serine 9. Anyone of the above mentioned antagonists may play a role in FAK activation. This makes the verification of the activity of these proteins within the HOSCC cells imperative.

The GSK3β inhibition was detected in all of the HOSCC cell lines and the two comparative controls under standard tissue culture conditions. This suggests that all of the WHCO cell lines maintained sufficient levels of phospho-GSK3β (Ser9) for it to be detectable, using the western immunoblotting technique. The level of GSK3β inhibition appeared to be the greatest in the WHCO6, SNO and WHCO5 cell lines. The other two cell lines contained low levels of GSK3β inhibition. This would suggest that there are two subgroups of WHCO cells, containing either low or high/moderate abundance of GSK3β inhibition. This finding was

corroborated by a previous study conducted by Nicolene Shaw, a previous colleague of the Cell Biology laboratory (Shaw, 2012). In the previous study, the levels of GSK3β inhibition were also found to be low within the WHCO3/WHCO1 cell lines, whilst being high to moderate within the other three WHCO cell lines. It is likely, based on the relative abundance of phospho-GSK3β (Ser9), that the GSK3β antagonists are active within the HOSCC cell lines. The degree to which the antagonists are active within the HOSCC cells appears to vary, as the levels of GSK3β targeted for ubiquitination differ from cell line to cell line. Lastly, the exact antagonist or combination of antagonists (p90RSK, Akt, PKC and/or PKA) that are responsible for GSK3β inhibition, by phosphorylation at Serine 9, within the HOSCC cells are currently unknown. Previous knowledge, obtained by Shaw and colleagues, detected high levels of Akt/PKB within the WHCO6/SNO cell lines, suggesting that PKB may, in part, play a role in the inhibition of GSK3β within these cell lines (2012).

Interestingly β-catenin has been found to be overexpressed in HOSCC cell lines in which GSK3β activity is lost (Deng *et al*., 2015). As GSK3β triggers the ubiquitination and eventual degradation of β-catenin, by phosphorylating it at Ser33, Ser37 and Thr41, cancer cells with high levels of GSK3β inhibition should contain higher levels of cytoplasmic β-catenin (Hinoi *et al*., 2000; Rask *et al*., 2003; Waaler *et al*., 2011). A study conducted by Hasan and colleagues found that the Wnt pathway played a key role in HOSCC progression (Hasan *et al*., 2016). A high level of cytoplasmic β-catenin was noted in the early stages of oesphageal dysplasia (Hasan *et al*., 2016). This suggests that GSK3β inhibition also affects other pro-proliferative pathways which may impact FAK activation.

Interestingly, the levels of phospho-β-catenin (Ser33, Ser37 & Thr41) were detected in all of the HOSCC cell lines and the A431 cell line under standard tissue culture conditions. All of the HOSCC cell lines had high to moderate levels of β-catenin, targeted for ubiquitination, except the WHCO6 cells. This suggests that GSK3β may not be the only protein that targets β-catenin for ubiquitination within the HOSCC cells. Cell lines with high levels of GSK3β inhibition, still contained high levels of phospho-β-catenin (Ser33, Ser37 & Thr41). It is possible that PKC, a protein that also phosphorylates β-catenin at Serine33, Serine37 and Threonine41, activity may be responsible for this discrepancy within the SNO cells (Goode *et al*., 1992). Overall, high levels of phospho-β-catenin (Ser33, Ser37 & Thr41) suggest that the Wnt pathway is still effectively inhibited within most of the HOSCC cells. The WHCO6 cells had very low levels of phospho-β-catenin (Ser33, Ser37 & Thr41), and high levels of GSK3β inhibition, suggesting that the Wnt pathway may play a more prominent role in maintaining

the transformed state of within these cells. This is interesting as the WHCO6 cells also contain the highest levels of active FAK when compared to all of the other WHCO cells.

In recent years there is an exceeding large amount of evidence to suggest that both FAK and the Wnt signal transduction pathways play a crucial role in promoting cancer (Fonar and Frank, 2011). The overactivation of either of these pathways is often associated with several forms of cancer. However, the interplay between the 2 pathways is complex and appears to be cell line dependent (Fonar and Frank, 2011; Santos *et al*., 2010; Rangaswami *et al*., 2012). Interestingly, FAK activation seemed to promote the stabilization of β-catenin and the activation of the Wnt pathway during embryonic development (Santos *et al*., 2010; Rangaswami *et al*., 2012; Fonar *et al*., 2011). These to pathways may therefore work synergistically to maintain the transformed state (Santos *et al*., 2010; Rangaswami *et al*., 2012). The link between these two pathways may be more prominent within the WHCO6 cell line. This indicates that FAK activation may involve multiple signalling intermediates from several intrinsic pro-survival/migratory pathways (Chapter 1).

Chapter 3

3. p90RSK, a regulator of FAK activation within the HOSCC cell lines?

3.1. Introduction:

Another key signal transduction intermediate that is known to play a prominent role in neoplastic transformations is the p90 ribosomal S6 kinase (RSK) (Kang *et al*., 2010). The MAPK/RSK signal transduction pathway is an iconic/well studied signal transduction cascade that regulates cell proliferation, differentiation, cell survival, proliferation, metabolism and motility (Mendoza *et al*., 2011; Redman *et al*., 2013; Clement *et al*., 2013). RSK, when activated indirectly by EGFR, efficiently activates the Y-box binding protein-1 (YB-1) (Stratford *et al*., 2008). The YB-1 protein is a notorious oncogenic transcription factor that plays a role in cell proliferation, migration and EMT (Syed *et al*., 2014). The activation of RSK and therefore its substrates, such as YB-1, promoted chemo-resistance, invasion and an EMT phenotype in malignant melanoma cells. The inhibition of RSK sensitised these cells to chemotherapeutics highlighting the role this protein plays in maintaining the cancerous state (Syed *et al*., 2014). Interestingly, when RSK activity is dysregulated within cancerous cells, these cells often display three key features: cell motility, metastasis and increased invasive capacities. For this reason RSK activity is likely to be associated with more virulent forms of cancer (melanomas, ovarian cancer, prostate, head and neck squamous cell carcinomas (HNSCC) etc.) not unlike the HOSCC cells (Kang *et al*., 2010; Syed *et al*., 2014; Torchiaro *et al*.,2016; Chen *et al*., 2013).

A gene-based pathway analysis of prostate cancer found that the MAPK/RSK pathway played a prominent role in the diseased state particularly when combined with gene mutations in PTK2/FAK and EGFR (Chen *et al*., 2013). The MAPK/RSK pathway is known to be activated by FAK (Yurdagul *et al*., 2016). Therefore should RSK ectopically activate FAK it would create a positive feedback loop that promotes sustained RSK signalling (Yurdagul *et al*., 2016). This feedback loop could explain why certain cancer strains are more malignant than others (Torchiaro *et al*., 2016).

FAK activation appears to be highly abundant within the HOSCC cells (Chapter 1), which could in part be due to the activity of RSK. FAK itself is known to play a significant role in tumour cell migration, propagating a chemo resistant phenotype and anchorage independent

proliferation in SCC (Serrels *et al*., 2012; Eke *et al*., 2012). However, the mechanisms by which FAK activation occur, within these cells, is unknown. As FAK function is imperative for normal cellular functioning, it may be difficult to target this protein in the treatment of cancer (Serrels *et al*., 2012). As focal adhesions are dependent on FAK activity for normal cell functioning, the mechanisms by which this protein is activated, ectopically, may provide scientists with a viable alternative for anti-cancer therapeutics (Carlson *et al*., 2004; Fanucchi and Veale, 2009; Lim *et al*., 2008; Ohashi *et al*., 2010).

This study therefore aims to determine whether RSK plays a role in FAK activation within the HOSCC cells. In an attempt to elucidate whether RSK activates FAK, RSK will be specifically inhibited, within the HOSCC cell lines, and fluctuations in the levels of active FAK monitored. Should FAK activation be RSK-dependent (there is a significant decrease in FAK activation post RSK inhibition) it may provide a new target for anti-cancer therapeutics.

3.2. Methods and Materials

3.2.1. Cell culture

Described previously (Chapter 2, Section 2.2.1).

3.2.2. Subculture

Described previously (Chapter 2, Section 2.2.2)

3.2.3. RSK inhibition assay

Cells were seeded into 6 cm dishes and allowed to proliferate, until a confluency of 80 % was reached. The media was discarded and the cell culture washed twice with warm PBS (37°C). Subsequently, three millilitres of fresh culture media, containing 10 % FCS, was dispensed into the tissue culture plate. The media contained a concentration of 10 µM BI-D1870 (stock diluted in dimethyl sulfoxide (DMSO) (Appendix A, Section 1.5.1. and Section 1.5.2. (Millipore) as described by Sapkota *et al.* (2007) . BI-D1870 was used because it is a highly specific ATP competitive inhibitor for RSK function, it inhibits the N-terminal domain kinase (Sapkota *et al.*, 2007). This inhibitor is selective for all isoforms of RSK (Sapkota *et al*., 2007). Simultaneously, controls for each cell line were prepared containing 0.1 % DMSO. The cell monolayers were then incubated, for 30 minutes, at 37°C in a humid incubator with 5 % carbon dioxide.

3.2.4. Whole cell protein extraction

Described previously (Chapter 2, Section 2.2.3.).

3.2.5. Protein determination

Described previously (Chapter 2, Section 2.2.4.).

3.2.6. Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE)

Described previously (Chapter 2, Section 2.2.5.).

3.2.7. Western immunoblotting

Described previously (Chapter 2, Section 2.2.6).

3.2.8. Antibody detection

Described previously (Chapter 2, Section 2.2.7).

3.2.9. Densitometry

MATLAB® R2013a image acquisition and analysis software was used for all densitometric analyses as described previously (Chapter 2, Section 2.2.8). This provided an estimation of the relative abundance of key phospho-proteins within each of the HOSCC cell lines (described in more detail below). All densitometric data generated was compared to the optical density of A431 or WHCO6 cell lines (Appendix B, Section 2.3.). To generate accurate and comparable data, the IOD, determined by MATLAB, were normalised for all western blot replicates as described in Appendix B, Section 2.6. (Degasperi *et al*., 2014)

3.2.10. Data analysis

All experiments were performed in triplicate. Results were represented described previously (Chapter 2, Section 2.2.9). The statistical significance of the densitometric data was determined by performing a standard Student's *t*-test (Appendix B, Section 2.3.) using GraphPad Prism®, Version 7.0 (GraphPad Software, Inc., La Jolla, USA) (where $p<0.05$ indicated statistical significance).

3.3. Results section

3.3.1. The efficacy of the RSK inhibitor, BI-D1870, under standard tissue culture conditions:

Phospho-GSK3β (Ser9) and phospho-FAK (Tyr397) were successfully detected in all of the HOSCC cell lines, post RSK inhibition (Figure 9). Fluctuations in these key phospho-intermediates were visualised via the western immunoblotting technique. All normalised densitometric data was made relative to IOD of the A431/WHCO6 cell line (Methods and materials, Section 3.3.9) and the WHCO6 cell line acted as an internal control. β-actin western blots were obtained to confirm equal protein loading and had a fairly uniform banding pattern (Figure 9). Comparative studies were then performed assessing the difference between the relative abundance of a set phospho-protein in a HOSCC cell lines exposed to BI-D1870 as opposed to the DMSO control. The net change (Appendix B, Section 2.5.) in the cellular levels of these key phospho-intermediates, post RSK inhibition, has been summarised in the Table 2 below.

Decreased levels of cellular phospho-GSK3β (Ser9) post RSK inhibition, verified the activity of the RSK inhibitor (BI-D1870) within the HT29 cell line (Figure 9). RSK phosphorylates GSK3β at Serine 9 (substrate of RSK), therefore a loss of phospho-GSK3β (Ser9) is indicative of a loss in RSK activity (Stambolic *et al*., 1994; Sutherland *et al*., 1993). Effective RSK inhibitors hamper this proteins ability to phosphorylate its substrates hence resulting in a decrease in the phosphorylation of GSK3β at Serine 9. The activity of BI-D1970 was verified within three of the Five HOSCC cell lines, by decreased levels of endogenous phospho-GSK3β (Ser9) (post RSK inhibition) (Figure 9 and Figure 10). The levels of phospho-GSK3β (Ser9) appeared to increase in WHCO5 and WHCO3 cell lines, post RSK inhibition (Figure 10 and Table 2). However the degree to which the levels of phospho-GSK3β (Ser9) fluctuated appeared to vary from one WHCO cell line to the next. The WHCO1 and SNO cells suffered the greatest losses in phospho-GSK3β (Ser9), post RSK inhibition (Table 2). The decreased levels of phospho-GSK3β (Ser9) noted within the SNO cells following RSK inhibition, were considered statistically significant, at a p-value □ 0.01. Therefore, in general, RSK inhibition by BI-D1870 was successfully verified within the HOSCC cell lines. Interestingly, the level of phospho-GSK3β (Ser9) remained relatively unaltered within the A431 cell line regardless of whether the cells were exposed to BI-D1870

or not (Figure 10 and Table 2). This suggests that the RSK inhibitor may or may not be active within this cell line.

3.3.2. The cellular levels of phospho-FAK (Tyr397) within the HOSCC cell lines were variably affected by the loss of RSK

The cellular levels of phospho-FAK (Tyr397) appeared to decrease in the SNO and WHCO6 cells, upon the loss of RSK (Figure 10 and Table 2). The relative abundance of this phospho-protein increased in WHCO3 and WHCO5 cells that were exposed to the RSK inhibitor (Figure 10). This indicates that RSK inhibition within the HOSCC cells variably affected the endogenous levels of phospho-FAK (Tyr397). RSK inhibition decreased the levels of cellular phospho-FAK (Tyr397) within the HT29 cell line (Figure 10). However, the relative abundance of this phospho-protein remained relatively unaltered within the A431 and WHCO1 cell lines regardless of whether the cells were exposed to BI-D1870 or not (Figure 10 and Table 2).

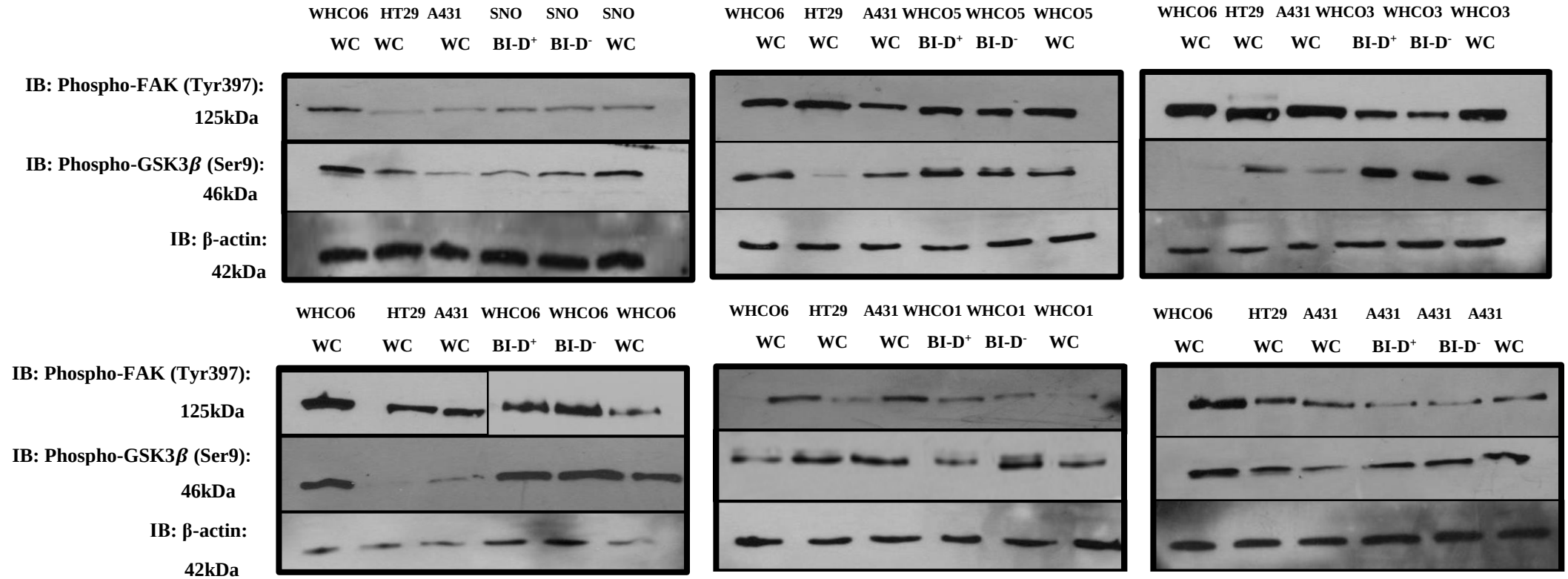


Figure 9. Representative immunoblots for the detection of phospho-FAK (Tyr397) and Phospho-GSK3β (Ser9) within the HOSCC cell lines post RSK inhibition (BI-D1870).

The levels phospho-GSK3β (Ser9) and phospho-FAK (Tyr397) were detected in the WHCO cells as well as the 2 control cell lines, HT29 and A431 cells. The relative abundance of phospho-FAK (Tyr397), within the HOSCC cell lines appeared to be variably affected by the loss of RSK. Cells were exposed to the RSK inhibitor for 30 minutes and control was added to the experiment (cells exposed to 0.1 % DMSO). The western blot analyses for the detection of β-actin acted as a loading control for the western blotting technique (indicating equal protein loading). Three biological replicates were performed. (BI-D$^+$: with 10 µM BI-D1870; BI-D$^-$: without 10 µM BI-D1870/containing 0.1 % DMSO; WC: whole cell protein extract under standard tissue culture conditions)

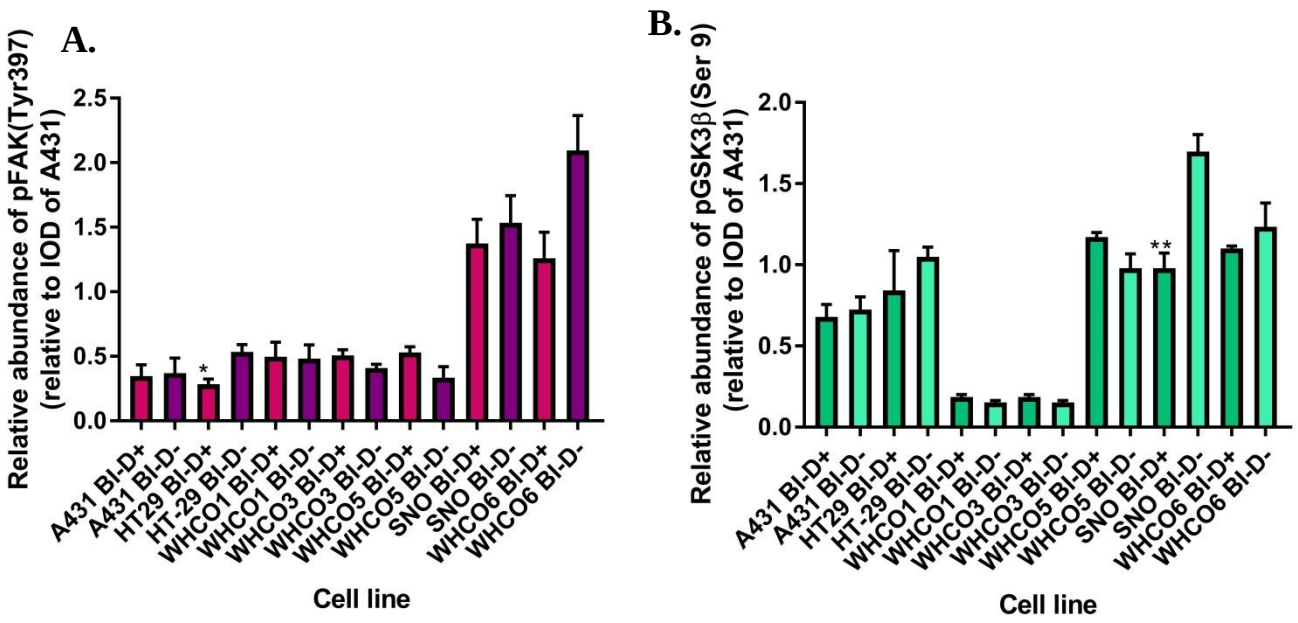


Figure 10. The relative abundance of certain phospho-intermediates within the HOSCC cell lines appears to be variably affected by the loss of RSK activity (10 μM BI-D1870).

The relative abundance of each phospho-protein was obtained by normalising semi-quantitatively determined densitometric analyses relative to the A431 cell line (Appendix B, Section 2.6.). A star (*) represents any statistical difference when compared to the control (exposed to 0.1 % DMSO) for each cell line (*p <0.05) (Appendix B, Section 2.3.). **A**. The specific inhibition of RSK by BI-D1870, decreased the cellular levels of phospho-FAK (Tyr397) within the WHCO6 and SNO cell lines. **B.** The cellular levels of phospho-GSK3β (Serine 9) decreased in most of the HOSCC cell lines post RSK inhibition. As RSK is responsible for the phosphorylation of GSK3β at serine 9, monitoring the loss of this particular phosphorylation verified the activity of the inhibitor within these cell lines. Unfortunately as the net change in phospho-GSK3β was found to be positive in the WHCO3 and WHCO5, BI-D1870 activity could not be verified within these 2 cell lines. **(**BI-D^+: with 10 μM BI-D1870; BI-D^-: without 10 μM BI-D1870/containing 0.1 % DMSO).

Table 2. Net change in the relative abundance of a few key phospho-intermediates within the HOSCC cell lines post RSK inhibition (10 μM BI-D1870).

Net changes in the cellular levels of each phospho-protein, within the WHCO cells post RSK inhibition, were determined as outlined in Appendix B, Section 2.5. A star (*) graphically represented any statistical difference, when compared to the control (exposed to 0.1 % DMSO) for each cell line using a standard Student's *t*-test (*p <0 .05;**p < 0.01) (Appendix B, Section 2.3.)(↑: Net increase in the cellular levels of a phospho-protein; ↓: Net decrease in the cellular levels of a phospho-protein; ≡: Equivalent net change in the cellular levels of a phospho-protein).

	Cell line						
Phospho-Protein	A431 ↑/↓/≡ (% change)	HT29 ↑/↓/≡ (% change)	WHCO1 ↑/↓/= (% change)	WHCO3 ↑/↓/≡ (% change)	WHCO5 ↑/↓/≡ (% change)	SNO ↑/↓/≡ (% change)	WHCO6 ↑/↓ /≡ (% change)
Phospho-FAK (Tyr397) (Active protein)	≡ (-2)	↓* (-47)	≡ (6)	↑ (25)	↑ (88)	↓ (-10)	↓ (-36)
Phospho-GSK3β (Ser9) (Inactive protein)	≡ (-7)	↓ (-21)	↓ (-54)	↑ (22)	↑ (22)	↓** (-43)	↓ (-21)

3.4. Discussion:

Only about 30 % of cellular proteins have the capacity to be phosphorylated (Ubersax and Ferrel, 2007; Tichy *et al*., 2011). Observing the levels of a specific phosphorylated protein involves monitoring infinitesimally small alterations in the phospho-proteome (Ubersax and Ferrel, 2007; Tichy *et al*., 2011). This would suggest that any fluctuations in the levels of a specific phospho-protein, that are detectable, may likely indicate a substantial change in the regulation/activation of this phospho-intermediate within the cells (Ubersax and Ferrel, 2007; Tichy *et al*., 2011). Although not all of the observed changes in the levels of key phospho-proteins post RSK inhibition were found to be statistically significant, the alterations in the phospho-proteome did indicate the general trends HOSCC cells embody under certain cellular conditions.

Cells rely on complex signaling systems to □sense □ changes in the external environment in which they are found (Nakakuki *et al*., 2010; Kholodenko *et al*., 1997). To prevent inappropriate/disproportionate cellular responses from occurring, due to the ectopic activation/repression of individual signal transduction cascades, cells must detect and integrate signals from several sources of external stimulation (Arkun, 2016). Signal transduction cascades therefore regulate one another at points of cross talk (Nakakuki *et al*., 2010; Kholodenko *et al*., 1997). These pathways, when activated in unison can either up or down regulate a signal (Arkun, 2016; Nakakuki *et al*., 2010). Therefore, cross talk between pathways may actually act as an additional layer of cellular control. When one signal transduction pathway is dysregulated, several other cascades (salvage pathways) may be activated in an attempt to salvage the activity of the dysregulated pathway, and maintain a specific cellular response (Nakakuki *et al*., 2010; Kholodenko *et al*., 1997). The salvage pathways may modulate signal transduction intermediates at points of cross talk between the two pathways or alter the expression/activity of regulators of the dysfunctional pathway.

3.4.1. RSK inhibition (BI-D1870) within the human epidermoid carcinoma cell line and the possible salvage pathways that tempered the effects of the inhibitor

The specific inhibition of RSK (BI-D1870) appeared to have little to no effect on the endogenous levels of active FAK and GSK3β inhibition within the A431 cells (Figure 9 and Figure 10). The lack of any significant difference in GSK3β inhibition within these cells post RSK inhibition may suggest that BI-D1870 is not functional within this cell line (Chapter 3, Section 3.3.1). Although this is probable it is more likely that the effects of the inhibitor, BI-D1870, are being offset by a salvage pathway within this cell line. In reality any signal transduction cascade that promotes the activation of either protein kinase A (PKA), PKB/Akt or protein kinase C (PKC) within the A431 cells could act as a possible salvage pathway for the loss of RSK (Fang *et al*., 2000; Kim *et al*., 2007; Jope and Johnson, 2004; Stambolic and Woodgett, 1994; Pap and Cooper, 1998). All three of these proteins when activated, promotes the inhibition of GSK3β by phosphorylation at serine 9, mimicking the activity of RSK within these cells (Fang *et al*., 2000; Kim *et al*., 2007; Jope and Johnson, 2004; Stambolic and Woodgett, 1994; Pap and Cooper, 1998). Therefore should any of these proteins be activated, within the A431 cells upon the loss of RSK it may explain why the cellular levels of GSK3β inhibition remained unaltered (Table 2).

Interestingly, the A431 cells are known to contain high levels of the EGF receptor (EGFR) (Wu *et al*., 2013). Cancer cells which express high levels of EGFR or contain EGF receptors with activating mutations exhibit higher levels of EGF signalling (Faber *et al*., 2009). EGF signalling promotes cell proliferation, migration, adhesion, cell survival and metastasis (Wu *et al*., 2013). EGFR signalling does this by promoting the activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT), PI3K/Akt and MAPK/RSK signal transduction cascades (see Figure 11) (Faber *et al*., 2009; Wu *et al*., 2013; Uribe *et al*., 2011). Interestingly, the loss of both PI3K/Akt and MAPK/RSK signalling was necessary to promote apoptosis in non-small lung cancers with mutant EGF receptors (promote activation) (Faber *et al*., 2009). This suggests that there is synergy between these two pathways and that the loss of one pathway was not sufficient at curbing the aberrant growth of non-small lung cancer (Faber *et al*., 2009). Interestingly, the inhibition of the PI3K/Akt pathway by metformin, inhibited the proliferation of the A431 cells (Liu *et al*., 2015). This indicated that the PI3K/Akt pathway plays a prominent role in maintaining the transformed state within this cell line (Liu *et al*., 2015). It is therefore likely that the activation of Akt/PKB, possibly via an EGFR-dependent signalling pathway, may compensate for the loss of RSK activity (BI-D1870) within the A431 cells. Several growth factors located within FCS were introduced concurrently with BI-D1870 as fresh culture media (containing 10 % FCS and BI-D1870) was exposed to the A431 cells at the beginning of the inhibitory assay (Methods and Materials, Section 3.2.3). Serum stimulation may therefore be responsible in part for the variable nature or noise noted in the experimental data, as the activation of several signal transduction cascades within the HOSCC cell lines may mask the overall effects of the RSK inhibitor, and even this may be context dependent. Therefore the role fresh culture media and FCS plays in promoting spatiotemporal responses, within the A431 cell line post RSK inhibition, was explored further in Chapter 4.

The loss of RSK had little to no effect on FAK activation within these cells, suggesting that RSK may not play a prominent role in FAK activation within the A431 cell line. Salvage pathways may compensate for the loss of RSK; however, it is also probable that RSK may mediate FAK activation via the inhibition of GSK3β. Here the activation of this protein, FAK, may be modulated by a substrate of GSK3β. FAK may be ectopically activated by a protein that is ordinarily repressed by GSK3β activity. The loss of GSK3β activity, by RSK, could promote this substrates ability to activate FAK. Should this be the case FAK activation

would have remained relatively the same within this cell line, due to the activity of Akt (maintaining the inhibition of GSK3β) (Table 2). This was explored further in Chapter 4.

3.4.2. The relative abundance of active FAK within the WHCO cells appears to be variably affected by the inhibition of RSK (10 µM BI-D1870).

The WHCO cell lines can be separated into 2 subgroups, with the exception of the WHCO1 cell line, based on the net change in FAK activation, post RSK inhibition. The levels of FAK activation appeared to increase in one subgroup whilst decreasing in the other, post RSK inhibition. This may indicate that there is variability amongst the carcinomas given the same physiological grading, moderately differentiated carcinomas (Fanucchi and Veale, 2009).

The WHCO1 cell line deviated from the common trends noted for the other four HOSCC cell lines. The levels of FAK activation within the WHCO1 cell line appeared to be unaffected by the specific inhibition of RSK (BI-D1870) (Figure 10 and Table 2). Interestingly, the inhibition of RSK also had little to no impact on FAK activation within the A431 cell line. This may suggest that the signal transduction pathways that maintain and propagate the transformed state within the A431 cells may also play a role in the WHCO1 cells. However, a decrease in GSK3β inhibition, upon the loss of RSK, was noted for the WHCO1 cell line, but not the A431 cells. This verified the activity of the RSK inhibitor within this cell line. Diminished GSK3β inhibition within the WHCO1 cells may suggest that the salvage pathways mentioned previously for the A431 cells may not be as present or effective enough to offset the effects of the loss of RSK (By BI-D1870) within this cell line. Interestingly, Nicolene Shaw and colleagues found that the WHCO1 cell line had extremely low levels of active PKB/Akt (phospho-PKB (Ser473)) (around 10 % of the active PKB found in the WHCO6 cell line) (2012). The low levels of active PKB within the WHCO1 cell line suggests that this cell line is unable to use the PI3K/Akt pathway to compensate for the loss of RSK (as hypothesized for the A431 cells) resulting in the loss of GSK3β phosphorylation. This was further confirmed by the fact that the WHCO1 cell line contains very high levels of Phosphatase and tensin homolog (PTEN), as determined by Glen Driver and Robin Veale (2006) (Appendix B, Section 2.4.). In fact the WHCO1 cell line had the highest levels of the negative regulator, of the PI3K/Akt pathway (PTEN), of all of the HOSCC cells.

Despite the loss of GSK3β inhibition, the active form of GSK3β did not impact the levels of active FAK within this cell line. This may suggest that FAK activation is being propagated by growth factor mediated signal transduction pathways or integrin heterodimers. These

canonical mechanisms of FAK activation may be masking the impact RSK inhibition has on FAK activity within this cell line (Figure 11). Chapter 4 will explore whether the results obtained from the RSK inhibition studies are in fact the result of a spatiotemporal response (a consequence of cells being exposed to multiple forms of external stimulation simultaneously). However, it is important to note that should this not be a spatiotemporal response, it is likely that RSK activity plays no role in FAK activation within the WHCO1 cells, when grown as monolayer (attached to a substrate).

The cellular levels of both active FAK and inactive GSK3β decreased within the HT29, SNO and WHCO6 cells, post RSK inhibition (BI-D1870), (Figure 10 and Table 2). The activity of the RSK inhibitor was verified within these cell lines as the relative abundance of phospho-GSK3β (Ser9), inhibited GSK3β, decreased post RSK inhibition. It is therefore likely that RSK promotes FAK activation decreased within these cell lines post RSK inhibition. Interestingly the net change in the cellular levels of active FAK post RSK inhibition was only found to be statistically significant for the HT29 cell line (Appendix B, Section 2.3.). The other two cell lines showed a clear decrease in the cellular levels of active FAK, upon the loss of RSK (BI-D1870), and this depression may yet be statistically significant provided that more biological replicates be performed.

The net change in the cellular levels of active FAK/inactive GSK3β, post RSK inhibition, within the HT29 and WHCO6 cells is almost identical. This may suggest that the signal transduction pathways that maintain the transformed state within these two cell lines may be similar. It is interesting that the two cell lines that contain mutant p53, hotspot mutation R175H (SNO) and R273H (HT29), were found to have RSK-dependent FAK activation (Fanucchi and Veale, 2009; Bossi *et al*., 2006). In a previous study conducted by Fanucchi and Veale, the mutant p53 located within the SNO cells rendered FAK resistant to caspase 3 mediated cleavage, upon apoptosis induction and staurosporine mediated cell detachment (Fanucchi and Veale, 2009). In this study the induction of staurosporine mediated apoptosis resulted in the activation and translocation of FAK into the nucleus of SNO/WHCO6 cells (Fanucchi and Veale, 2009). In the nucleus, FAK acted in its capacity as a scaffolding protein promoting the degradation of p53 and hence promoting pro-survival based signalling (Fanucchi and Veale, 2009). It is unlikely that RSK caused the activation of FAK under these conditions as RSK is inhibited by staurosporine, a nonspecific ATP-competitive kinase inhibitor (Fischer *et al*., 2010; Nguyen, 2008; Belmokhtar *et al*., 2001). However, it is possible that RSK may play a role in activating FAK, promoting cell survival based

signalling, in WHCO cells that are grown in suspension, inducing anoikis naturally . It is important to note however that RSK-dependent FAK activation may be context dependent (a spatiotemporal response).

The endogenous levels of active FAK and inhibited GSK3β increased in the WHCO3 and WHCO5 cell lines post RSK inhibition. This was surprising finding as it suggests that RSK inhibition inhibits FAK activity within these cell lines. It also suggests that the loss of RSK promoted the phosphorylation of its own substrate, GSK3β at serine 9. It is possible that the WHCO3 and WHCO5 cell lines have alternative salvage pathways that result in the activation of FAK. Serum stimulation may have promoted the activation of FAK and the inhibition of GSK3β, via either integrin or growth factor dependent pathways (Sieg *et al*., 2000). This may have masked the effects of the RSK inhibitor (BI-D1870) within these cell lines. Although the exact components of serum are nebulous, it is known that serum, FCS, contains a vast array of growth factors including the platelet derived growth factor (Childs *et al*., 1982; Brindley *et al*., 2012). As serum stimulation, involving growth factor receptor signalling, occurred concurrently with RSK inhibition, it`s possible that any fluctuations in the relative abundance of key phospho-proteins may be the accumulation/amalgamation of all of these signalling ques combined. This would suggest that the responses noted in reaction to the inhibition of RSK may be context dependent. This was explored further in Chapter 4.

Recent studies have found that the exact role RSK plays in signal transduction pathways appears to be context specific and therefore cannot be generalised (Aronchik *et al*., 2014). This study found that cells may only be reliant on RSK activity for cell survival under conditions of anchorage independence (Aronchik *et al*., 2014). Aronchik and colleagues also found that RSK activity appeared to be cell lineage dependent (Aronchik *et al*., 2014). This suggests that the activity of RSK within the HOSCC cell lines may be variable and context specific. To determine whether this is indeed the case further studies were performed in Chapter 4.

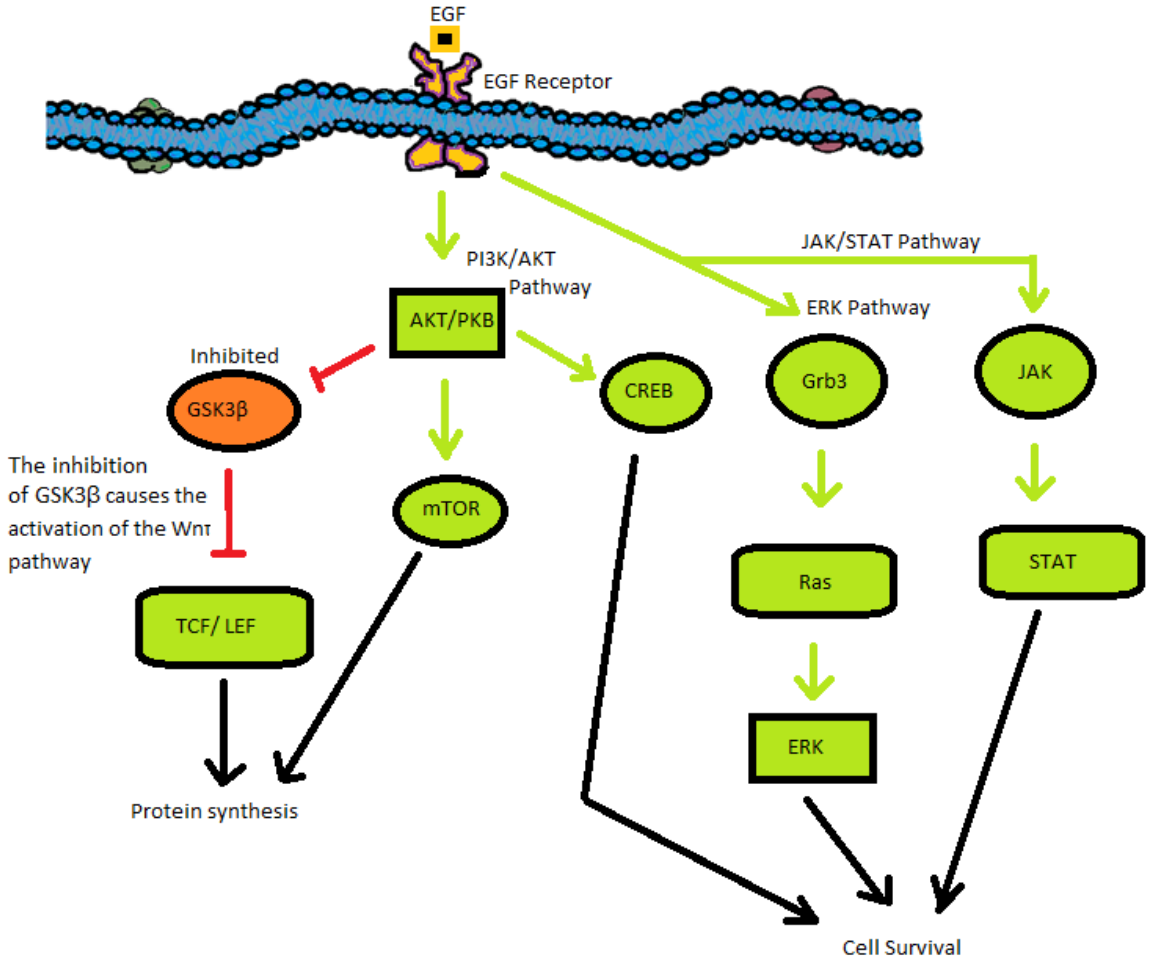


Figure 11. A schematic overview of epidermal growth factor (EGF) mediated signal transduction cascades.
The activation of the EGFR receptor triggers the activation of the PI3K/Akt pathway, the MAPK/ERK pathway and the JAK/STAT pathway. (ERK: Extracellular signal regulated kinase; PI3K: phosphatidylinositol 3-kinase; Janus Kinase: JAK; STAT: Signal transducer and activator; GSK3β: Glycogen synthase kinase 3β; T-cell factor/lymphoid enhancer factor: TCF/ LEF). The image was produced (Microsoft Paint ©) by making use papers published by Jope and Johnson (2004), Zheng *et al*. (2007) and information provided by C. Hooper (Abcam © 2016).

Chapter 4

4. 4. GSK3β, RSK and serum stimulation: the link to FAK activation within HOSCC cells?

4.1. Introduction:

4.1.1. GSK3β, RSK and FAK activation?

GSK3β is distinctive in that it is ubiquitously expressed and perpetually active within the cell (Woodgett, 1990). This protein`s activity is regulated by inhibition (Fang *et al*., 2000; Kim *et al*., 2007; Jope and Johnson, 2004; Stambolic and Woodgett, 1994; Pap and Cooper, 1998). Several proteins including RSK, PKB and PKC play a role in modulating the activity of GSK3β by inhibition (Fang *et al*., 2000; Kim *et al*., 2007; Jope and Johnson, 2004; Stambolic and Woodgett, 1994; Pap and Cooper, 1998). However, GSK3β activity is also interesting in that GSK3β preferentially phosphorylates pre-primed substrates (Fiol *et al*., 1987; Frame *et al*., 2001; Dajani *et al*., 2001; ter Haar *et al*., 2001). Substrates with a consensus sequence of Ser/ThrXXXSer/Thr (pre-primed phosphorylation) are often phosphorylated by GSK3β at the initial Ser/Thr residue 4 amino acid residues (X) from the pre-primed phosphorylation (Fiol *et al*., 1987; Frame *et al*., 2001; Dajani *et al*., 2001; ter Haar *et al*., 2001). The N terminal phosphorylation of GSK3β at Ser9 inhibits GSK3β activity by acting as a pseudo-substrate that outcompetes pre-primed substrates for the phosphate binding site (Frame *et al*., 2001). GSK3β, when activated, plays a role in inflammation, cell survival based signalling, apoptosis and glycogen biosynthesis (Embi *et al*., 1980; Doble and Woodgett, 2003; Sun *et al*., 2009).

GSK3β activity also plays a prominent role in embryonic development, regulating cell polarity and migration and is thought to play a large role in regulating actin cytoskeletal dynamics/cell-ECM interactions spatiotemporally (Sun *et al*., 2009). Embryonic stem cells were found to undergo differentiation when exposed to a particular substrate-specific level of elasticity provided that the MAPK pathway and GSK3β remained active (Shimizu *et al*., 2012). However, when the MAP kinase, GSK3β and Src were inhibited, the embryonic stem cells retained their pluripotency, encouraging self-renewal, independent of substrate elasticity (Shimizu *et al*., 2012). This indicates that Src, GSK3β and the MAPK pathway play a

prominent role in promoting mechanically triggered cell differentiation (Shimizu *et al*., 2012). This suggests that GSK3β activity plays a fundamental role in regulating several signal transduction pathways.

Although the part GSK3β plays in cancer progression is not fully understood (Chapter 1, Section 1.5), it appears as though the role this protein plays in neoplasmic transformations is very much cell lineage dependent. In pancreatic cancer cells GSK3β activity played a fundamental role in promoting cancer cell invasion and chemo-resistance (Kitano *et al*., 2013). The loss of GSK3β activity reduced cell proliferation and rendered the cells susceptible to certain chemotherapeutics (Kitano *et al*., 2013). GSK3β was also found to enhance p53 mediated DNA transcription, thereby repressing cancer cell growth, in some cancer cells whilst promoting the MDM2 mediated degradation of p53 in others (Turenne and Price, 2001; Kulikov *et al*., 2005). Interestingly GSK3β appeared to be localised at focal adhesions within melanoma cells suggesting that GSK3β plays a prominent role in promoting more motile melanoma cells. It's interesting to note that majority of GSK3β was inhibited within these cells (Kitano *et al*., 2013). Only a small subset of GSK3β remained active within these cells (Kitano *et al*., 2013). The complete inhibition of GSK3β resulted in a decrease in FAK activation, increasing the size of the focal adhesions (John *et al*., 2012). This suggests that GSK3β may play a role in FAK activation within the HOSCC cell lines.

Interestingly previous studies have suggested that GSK3β, a substrate of RSK, may inhibit FAK activity by phosphorylating it at serine 722 (Kobayashi *et al*., 2006; Bianchi *et al*., 2005; Sutherland, 2011). This suggests that the role GSK3β plays in FAK activation appears to be inconsistent with some studies suggesting GSK3β may inhibit FAK activation whilst other suggest it may promote FAK activity (Kobayashi *et al*., 2006; Bianchi *et al*.,2005). The role GSK3β played in FAK activation, by triggering the auto phosphorylation of FAK at Tyrosine 397, is highly contested. Whether GSK3β ectopically activates/inhibits FAK within the HOSCC cell lines still remains unclear.

It is also possible that RSK may indirectly effect FAK activation by inhibiting GSK3β (Chapter 3, Section 3.4.1; Chapter 1, Section 1.9.2). GSK3β phosphorylates several substrates causing the inhibition of these proteins activity. Should GSK3β be inactivated, by RSK, it would promote the activity of these substrates. One such substrate of GSK3β is the phosphatase and tensin homolog deleted in chromosome 10 (PTEN). Active GSK3β has been found to de-stabilise PTEN, a cellular inhibitor of FAK activation, by phosphorylating it at

Thr366 (Maccario *et al*., 2007; Sapkota *et al*., 2007). This post translational modification has been known to hinder PTEN activity promoting FAK activation. Therefore RSK may promote FAK inactivation via the inhibition of GSK3β and subsequent stabilisation of PTEN within HOSCC cells.

Interestingly, a study conducted on human placental cells, derived from trophoblasts, found that Kisspeptin inhibited cell growth via an interesting p90RSK- GSK3β pathway. In these cells Kisspeptin-10 promoted RSK activation which in turn phosphorylated GSK3β at serine 9. However this study found that this promoted β-catenin stabilization and FAK activation via a feedback loop. However the exact mechanism by which FAK activation was promoted is unclear (Roseweir *et al*., 2012).

Therefore the role RSK and GSK3β play in FAK activation will be determined by performing dual inhibition studies. It is hoped that these studies will provide more clarity as to whether RSK activates or inhibits FAK activation, either directly or indirectly, within the HOSCC cell lines.

4.1.2. Serum stimulation: a spatiotemporal response?

Cells, originating from multicellular organisms, are continually being exposed to an array of external stimuli (Wang *et al*., 1998; Kholodenko *et al*., 1997; Nakakuki *et al*., 2010). How cells integrate these externally-derived signals, promoting a specific cellular response, is largely context dependent (Kholodenko *et al*., 1997; Nakakuki *et al*., 2010). This makes studying complex diseases challenging. It`s imperative, in the study of complex disorders, not to underestimate the role the micro-environment, in which these cells are found, plays in maintaining the transformed state (Kholodenko *et al*., 1997; Nakakuki *et al*., 2010). Serum stimulation could play a role in promoting specific signal transduction cascades, within established cell lines, particularly because its constituents are unknown (Childs *et al*., 1982; Brindley *et al*., 2012). However FCS is a necessary complication as moderately differentiated human squamous cell carcinoma cells are challenging to maintain *in vitro*. The opposite is also true; serum deprivation is equally unnatural (Yamada and Geiger, 1997; Zhu *et al*., 2006; Li *et al*., 2008; Leong *et al*., 2003). It places cells under extreme stress resulting in several cell survival pathways being favoured in an attempt to inhibit apoptosis and promote cell survival (Zhu *et al*., 2006; Li *et al*., 2008; Leong *et al*., 2003). Serum deprivation also causes morphological changes in established cell lines (Qi *et al*., 1997; Han *et al*., 2006).

Serum stimulation initially flooded the cell monolayer with growth factors, amino acids and other essential nutrients. This initial influx of signaling may have masked the impact of the RSK inhibitor within the HOSCC cells, particularly as the incubation period for BI-D1870 is 30 minutes. The initial serum stimulation, concomitantly with BI-D1870, may have promoted a specific spatiotemporal response within the HOSCC cell lines. However serum deprivation could produce an equally definitive spatiotemporal response. To ascertain the effects RSK inhibition has on the phospho-proteome profile of the HOSCC cells under conditions that most closely mimic the natural environment, these cells will be pre-exposed to serum 23 hours and 30 minutes prior to being exposed to the RSK inhibitor. This will be done in order to determine whether the results obtained in Chapter 3 can be reproduced under slightly different environmental conditions.

4.2. Methods and Materials

4.2.1. Cell culture

Described previously (Chapter 2, Section 2.2.1).

4.2.2. Subculture

Described previously (Chapter 2, Section 2.2.2).

4.2.3. RSK and GSK3β inhibition assay

Due to the vastly different incubation periods of BI-D1870 and AR-A014481 (Appendix A, Section 1.5.2 and Section 1.6.2.) the cell cultures were not exposed to both inhibitors simultaneously. Instead the cell monolayer was exposed to AR-A014481 prior to BI-D1870 in such a way that both inhibitors reach optimal activity within the cells at the same time. This split-inhibition incubation period ensured that both inhibitors reached optimal activity simultaneously within the cells (a likely representation of the inhibition of both RSK and GSK3β).

Cell cultures were propagated in 6 cm plates. Once the cell monolayer reached a confluency of 65 %, the cells were washed twice in warm sterile PBS (37°C). Subsequently, three millilitres of fresh culture media, containing 10 % FCS and a final concentration of 10 µM AR-A014481 (Sigma-Aldrich ®) (Appendix A, Section 1.6.1 and Section 1.6.2), was added to the cell culture as described by Kitano *et al*. (2013) (Bhat *et al*., 2003). AR-A014418 was

selected as it is a highly specific ATP competitive inhibitior for GSK3β which does not inhibit closely related kinases (cdk2 and cdk5) (Bhat *et al*., 2003). Simultaneously, controls for each cell line were prepared containing 0.187 % DMSO. Next, the cells were incubated for 23 hours and 30 minutes at 37°C, in a humid incubator with 5 % carbon dioxide. Following this incubation period, a concentration of 10 µM BI-D1870 was inserted into the tissue culture plate (previously exposed to AR-A011481), as described by Sapkota *et al.* (2007). A further 0.1 % DMSO was added to the controls, previously exposed to 0.187 % DMSO. The cell cultures were then re-incubated for 30 minutes, at 37°C in an incubator with 5 % carbon dioxide.

A second control for GSK3β inhibition was performed as described by Kitano *et al*. (2013). Cell cultures were propagated in 6 cm plates. Once the cell monolayer reached a confluency of 65 % the cells were washed twice in warm sterile PBS (37°C). Subsequently, three millilitres of fresh culture media, containing 10 % FCS and a final concentration of 10 µM AR-A014481 (Sigma-Aldrich ®) (Appendix A, Section 1.6.1 and Section 1.6.2), was added to the cell culture as described by Kitano *et al*. (2013) (Bhat *et al*., 2003). Simultaneously, controls for each cell line were prepared containing 0.187 % DMSO. The cells were incubated for 24 hours at 37°C, in a humid incubator with 5 % carbon dioxide.

A third control was prepared for RSK inhibition as described by Sapkota *et al.* (2007). Cell cultures were propagated in 6 cm plates. Once the cell monolayer reached a confluency of 65 % the cells were washed twice in warm sterile PBS (37°C). Subsequently, three millilitres of fresh culture media containing 10 % FCS, was added to the cell culture. The cells were incubated for 23 hours and 30 minutes, at 37°C, in a humid incubator with 5 % carbon dioxide. Following this incubation period, a concentration of 10 µM BI-D1870 was inserted into the tissue culture plate (previously exposed to fresh culture media, 10 % FCS), as described by Sapkota *et al.* (2007). Simultaneously, controls for each cell line were prepared and 0.1 % DMSO added. The cell cultures were then incubated for 30 minutes at 37°C, in an incubator with 5 % carbon dioxide.

4.2.4. Whole cell protein extraction

Described previously (Chapter 2, Section 2.2.3).

4.2.5. Protein determination

Described previously (Chapter 2, Section 2.2.4).

4.2.6. Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE)

Described previously (Chapter 2, Section 2.2.5).

4.2.7. Western immunoblotting

Described previously (Chapter 2, Section 2.2.6).

4.2.8. Antibody detection

Described previously (Chapter 2, Section 2.2.7).

4.2.9. Densitometry

MATLAB® R2013a image acquisition and analysis software was used for all densitometric analyses as described previously (Chapter 2, Section 2.2.8). This provided an estimation of the relative abundance of key phospho-proteins within each of the HOSCC cell lines (described in more detail below). All densitometric data generated was compared to the optical density of A431 or WHCO6 cell lines (Appendix B, Section 2.4.). To generate accurate and comparable data, the computed optical densities (IOD) determined by MATLAB, were normalised for all western blot replicates as described in Appendix B, Section 2.6. (Degasperi *et al*., 2014)

4.2.10. Data analysis

All experiments were performed in triplicate unless otherwise stated. Results were represented as described previously (Chapter 2, Section 2.2.9). The statistical significance of the densitometric data was determined by performing a standard Student's *t*-test (Appendix B, Section 2.4.) using GraphPad Prism®, Version 7.0 (GraphPad Software, Inc., La Jolla, USA) (where $p < 0.05$ indicated statistical significance).

4.3. Results section

The levels of Phospho-GSK3β (Ser9), phospho-β-catenin (Ser33, Ser37 & Thr41) and phospho-FAK (Tyr397) were successfully detected in all of the HOSCC cell lines, post inhibition (RSK, GSK3β or RSK/GSK3β inhibition) (Figure 12). Comparative studies were then performed by relating the difference between the relative abundance of a phospho-protein within an HOSCC cell line, exposed to an inhibitor (or set of inhibitors), as opposed to its DMSO control. Overall the net changes in the phospho-protein profile (Table 3) indicated that none of the HOSCC cell lines are identical. The net change in the cellular levels of all of the key phospho-proteins, following the inhibition of GSK3β, RSK or the dual inhibition of both proteins (GSK3β/RSK) varied from cell line to cell line. Even the comparative controls had distinct responses to all three of the inhibitor combinations noted above.

4.3.1 RSK inhibition variably affected the relative abundance of a few key phospho-proteins within the HOSCC cell lines, 24 hours after the introduction of serum (control).

The exact degree to which the levels of these phospho-proteins fluctuated post RSK inhibition appeared to vary from one WHCO cell line to the next. The WHCO1, HT29 and A431 cells behaved in a similar fashion when exposed to a specific RSK inhibitor (24 hours after the introduction of FCS). The cellular levels of phospho-FAK (Tyr397) increased in these three cell lines post RSK inhibition (Figure 13 and Table 3). Interestingly, the relative abundance of phospho-GSK3β (Ser9) decreased in all of these cell lines upon the loss of RSK (BI-D1870) thereby verifying the activity of the RSK inhibitor within these cells (Figure 13). The net changes in the levels of phospho-FAK (Tyr397) and phospho-GSK3β (Ser9), within the HT29 cell line post RSK inhibition, were found to be statistically significant (Figure 13 and Table 3). Lastly the decreased levels of phospho-β-catenin (Ser33, Ser37 & Thr41) noted within the WHCO1 cell line upon the loss of RSK (BI-D1870), was also found to be statistically significant (Appendix B, Section 2.4.).

The relative abundance of all of the key phospho-proteins decreased within the WHCO3 and SNO cell lines, post RSK inhibition (24 hours after the introduction of serum, FCS). Lastly the levels of phospho-FAK (Tyr397) and phospho-GSK3β (Ser9) increased in the WHCO5 and WHCO6 cell lines (Figure 13). The increased levels of phospho-β-catenin (Ser33, Ser37

& Thr41) and phospho-FAK (Tyr397) within the WHCO6 cell line upon the loss of RSK (BI-D1870) were found to be statistically significant. The fluctuations noted in the endogenous levels of phospho-GSK3β (Ser9), within the WHCO5 and SNO cells post RSK inhibition were also found to be statistically significant.

4.3.2. The specific inhibition of GSK3β (AR-A014481) variably affected the cellular levels of a few key phospho-proteins within the HOSCC cell lines (control).

Decreased levels of phospho-β-catenin (Ser33, Ser37 & Thr41), post GSK3β inhibition, verified the activity of the GSK3β inhibitor (AR-A014481) within the WHCO5, WHCO6 and SNO cell lines (Figure 13 and Table 3). GSK3β phosphorylates β-catenin at Ser33, Ser37 and Thr41, therefore a loss of phospho-β-catenin (Ser33, Ser37 & Thr41) is indicative of a loss in GSK3β activity (Hinoi *et al*., 2000; Rask *et al*., 2003; Waaler *et al*., 2011). However, the activity of the inhibitor could not be verified within the WHCO1, WHCO3 and A431 cell line.

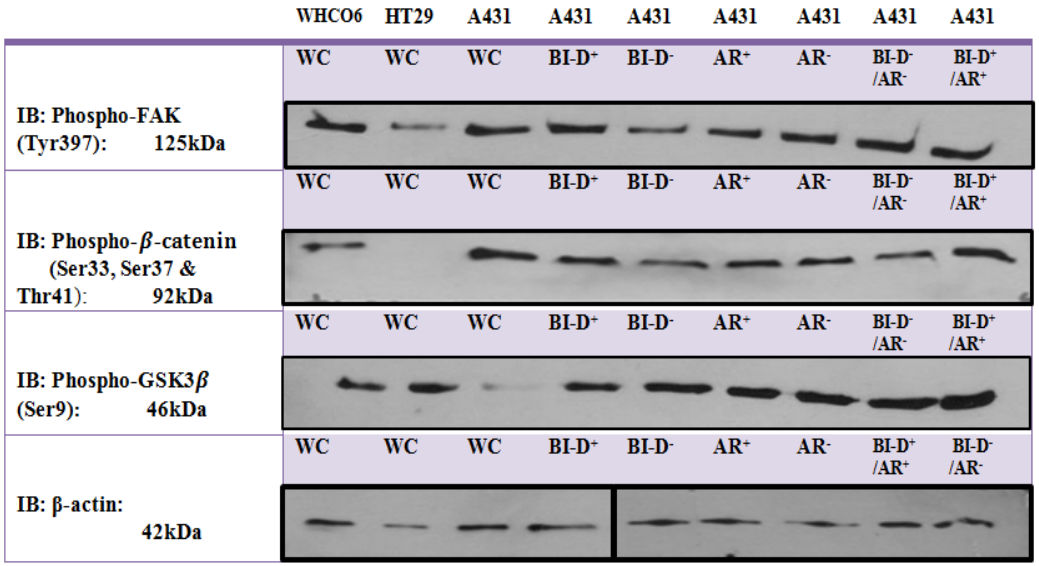
The inhibition of GSK3β (AR-A014481) decreased the cellular level of phospho-FAK (Tyr397) within A431, HT29, WHCO5 and SNO cell lines (Figure13). The cellular levels of this phospho-protein increased in the remaining three cell lines, following the inhibition of GSK3β. Interestingly, the cellular levels of phospho-GSK3β (Ser9) appeared to decrease in all of the cell lines, except the WHCO5 and WHCO1 cells, upon the loss of GSK3β (AR-A014481) (Figure 13 and Table 3).

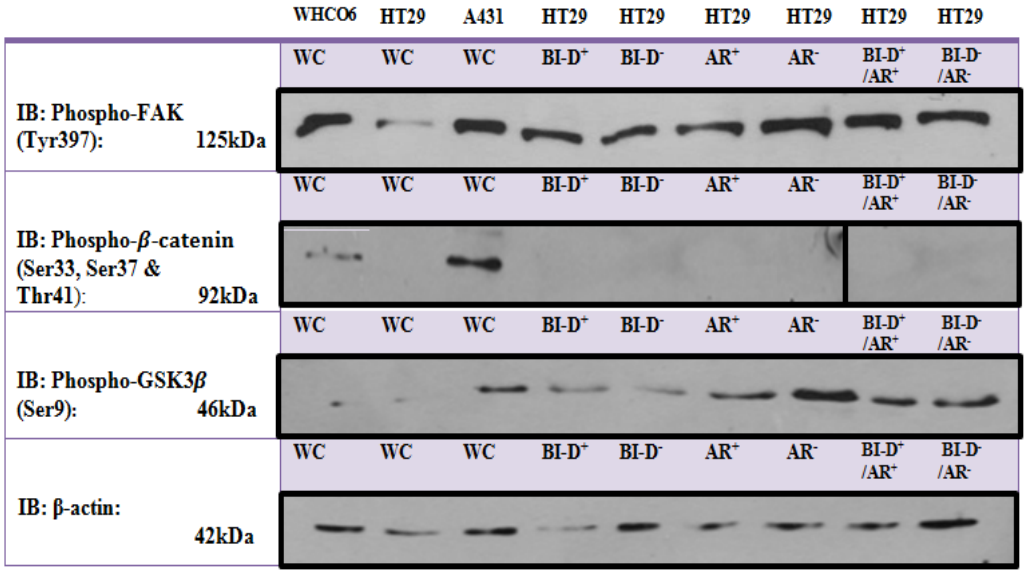
4.3.3. The dual inhibition, of GSK3β and RSK, generally resulted in decreased cellular levels of phospho-FAK (Tyr397) within the HOSCC cell lines.

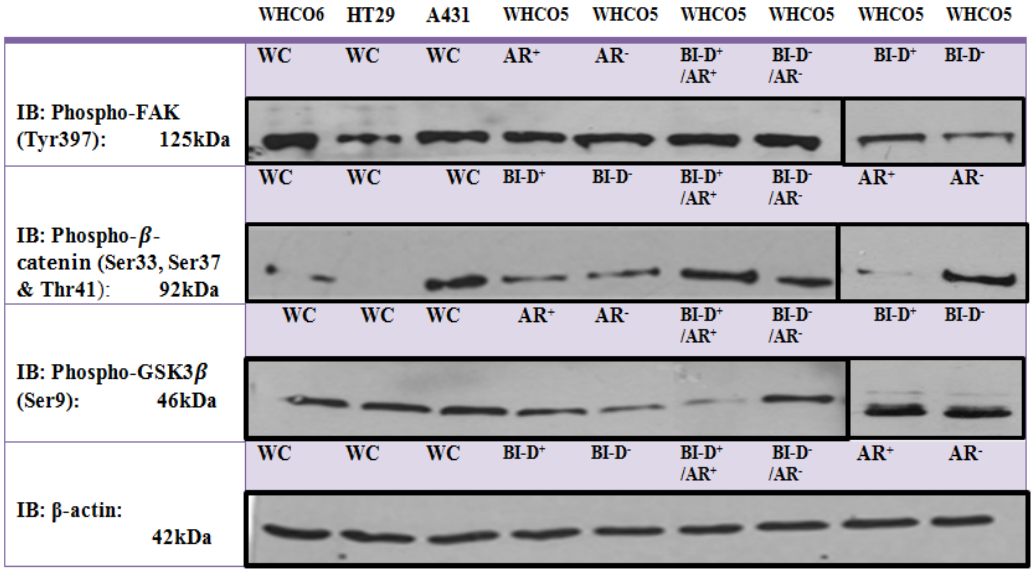
Dual inhibition, AR-A014481and BI-D1870, studies found that most of the HOSCC cell lines had diminished levels of phospho-GSK3β (Ser9) and phospho-FAK (Tyr397) when both RSK and GSK3β were inhibited simultaneously (Figure 13). Only the A431 cell line had elevated levels of GSK3β (Ser9) post RSK/GSK3β inhibition (Table 3). This suggests that the effectivity of the RSK inhibitor was verified in all of the HOSCC cell lines and one of the control cell lines. The loss of both GSK3β and RSK activity (AR-A014481and BI-D1870), within the HT29 and WHCO1 cells, appeared to have no effect on the cellular levels of phospho-FAK (Tyr397). The effectivity of the GSK3β inhibitor was verified within all of the HOSCC cell lines, except SNO and WHCO6 cells (Figure 13 and Table 3). The levels of

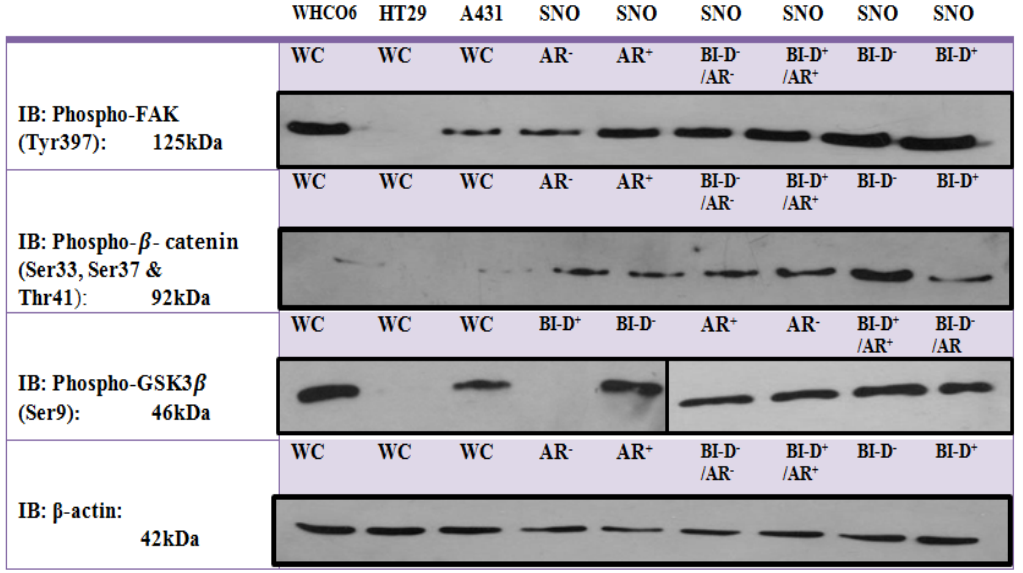
phospho-β-catenin (Ser33, Ser37 & Thr41) increased in these two cell lines, post GSK3β/RSK inhibition (Figure 13).

Fluctuations in the levels of phospho-GSK3β (Ser9), within the WHCO5 and SNO cells, post RSK/GSK3β inhibition were found to be statistically significant. The net change in the levels of phospho-FAK (Tyr397), within the WHCO3 and SNO cells, was also found to be statistically significant. The decreased cellular levels of phospho-β-catenin (Ser33, Ser37 & Thr41) within the A431 and WHCO5 cell line, upon the loss of RSK and GSK3β activity, were also found to be significantly different from their respective controls (Figure 13).









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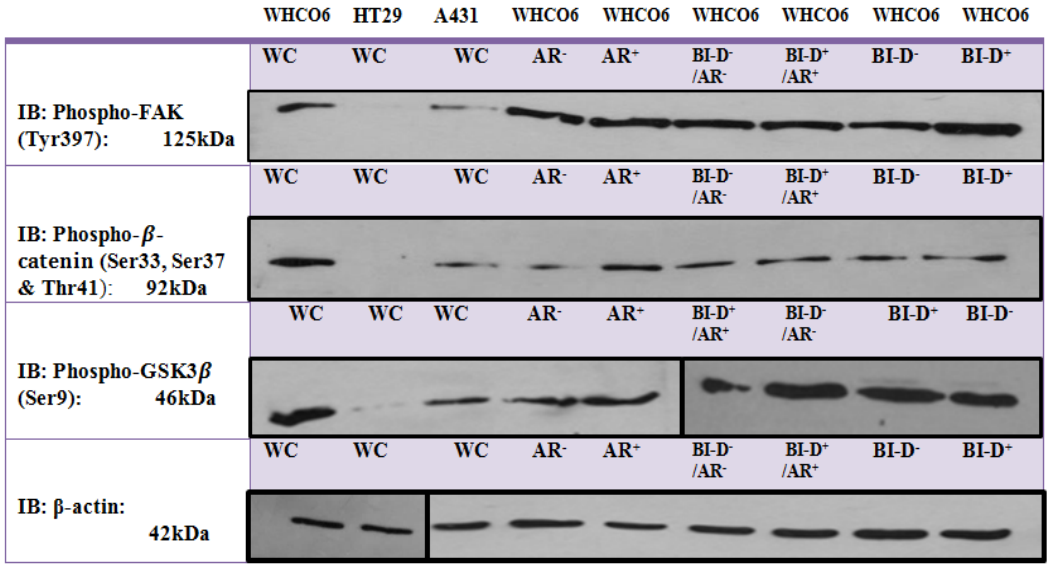
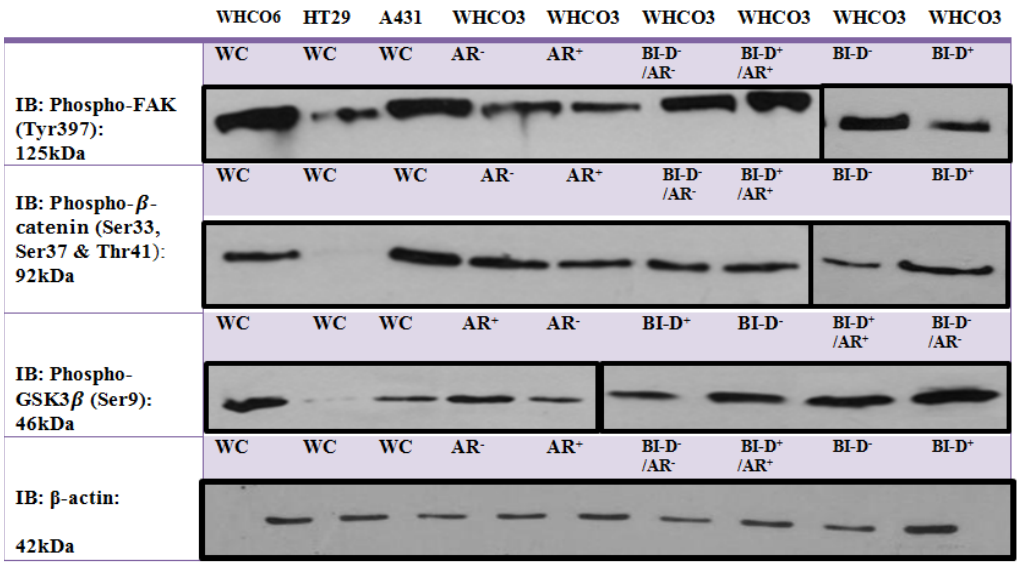




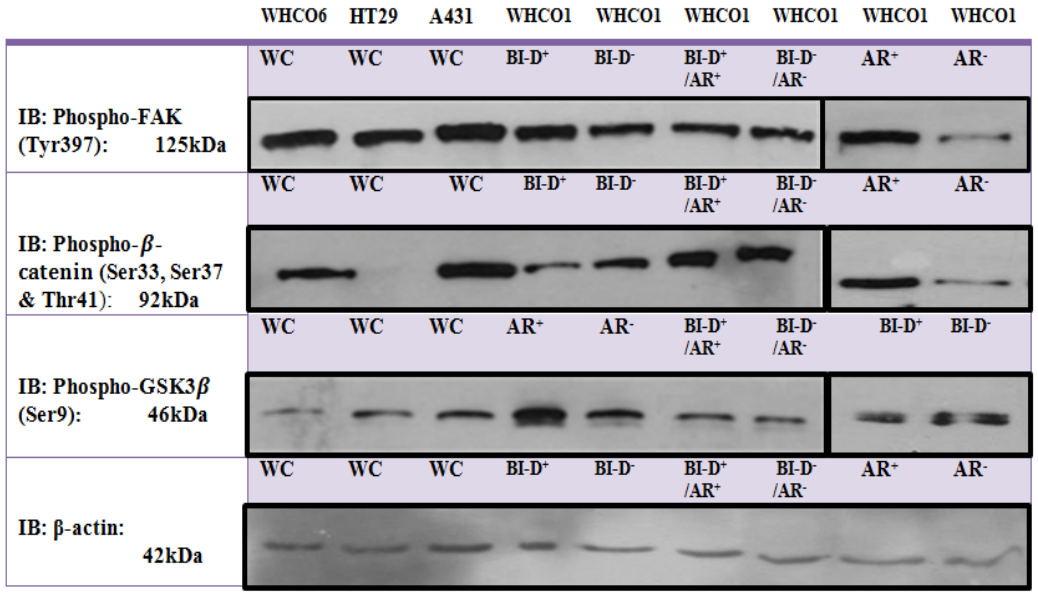
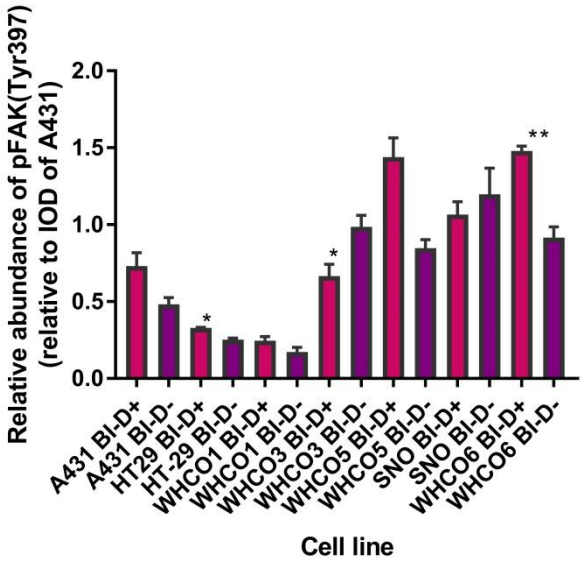
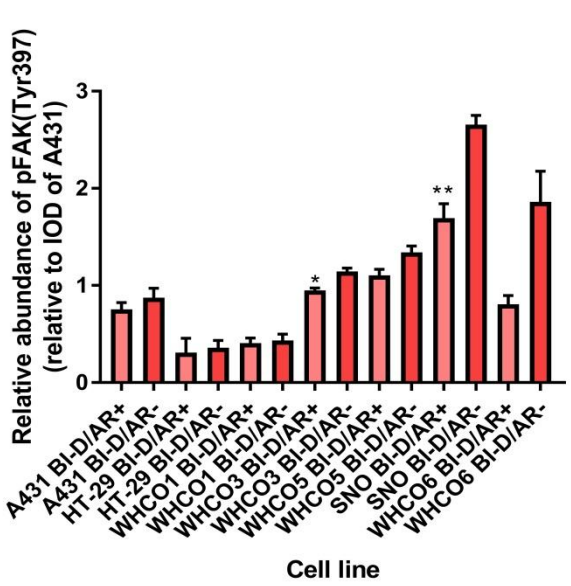
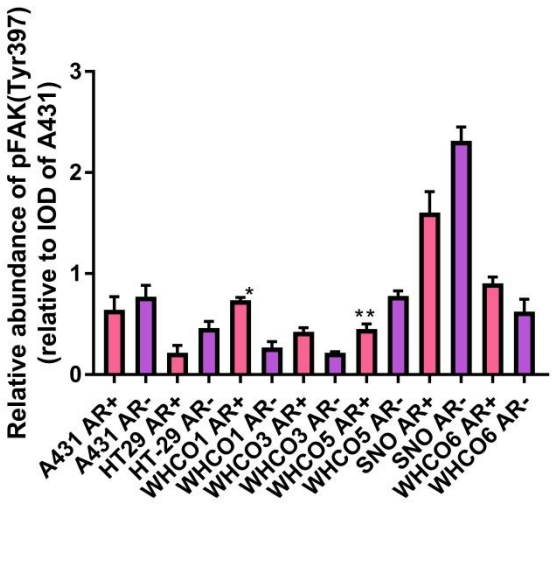


Figure 12. Representative immunoblots for the detection of a few key phospho-proteins within the HOSCC cell lines post GSK3β inhibition (10 μM AR-A014481), RSK inhibition (10 μM BI-D1870, 24 hours after serum introduction) and post RSK-GSK3β inhibition (10 μM BI-D1870 and 10 μM AR-A014418). The levels of phospho-FAK (Tyr397), phospho-GSK3*β* (Ser9) and phospho-β-catenin (Ser33, Ser37 & Thr41) were detected in the WHCO cells as well as the 2 control cell lines, HT29 and A431 cells. The relative abundance of phospho-FAK (Tyr397) within the HOSCC cell lines appeared to be variably affected by the loss of RSK and/or GSK3β. The western blot analyses, for the detection of β-actin, acted as a loading control, indicate equal protein loading, for the western blotting technique. Cells were exposed to the GSK3β inhibitor for 24 hours, the RSK inhibitor for 30 minutes and a control was added for each experiment (RSK inhibition: cells exposed to 0.1 % DMSO; GSK3β inhibition: cells exposed to 0.187 % DMSO). Three biological repeats were obtained. (BI-D$^+$: with 10 μM BI-D1870; BI-D$^-$: without 10 μM BI-D1870/containing 0.1 % DMSO; AR$^+$: with 10 μM AR-A014418; AR$^-$: without 10 μM AR-A014418/containing 0.187 % DMSO; BI-D$^+$/AR$^+$: with 10 μM BI-D1870/AR-A014418; BI-D$^-$/AR$^-$: without 10 μM BI-D1870/AR-A014418 (containing 0.287 % DMSO); WC: whole cell protein extract under standard tissue culture conditions)

A.







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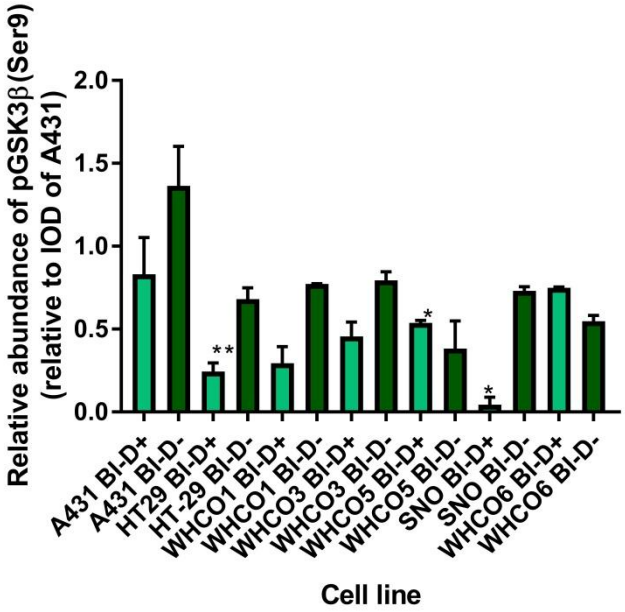

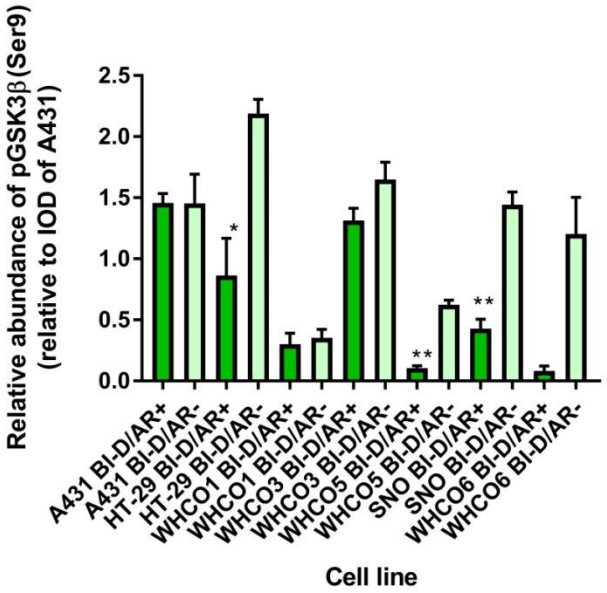

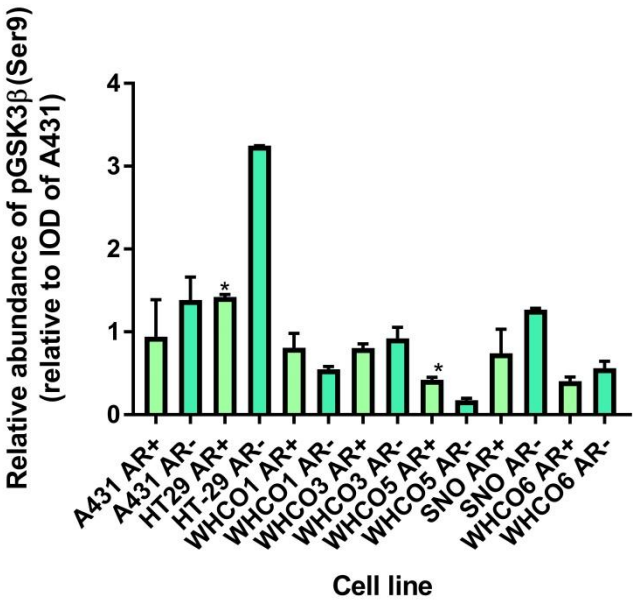

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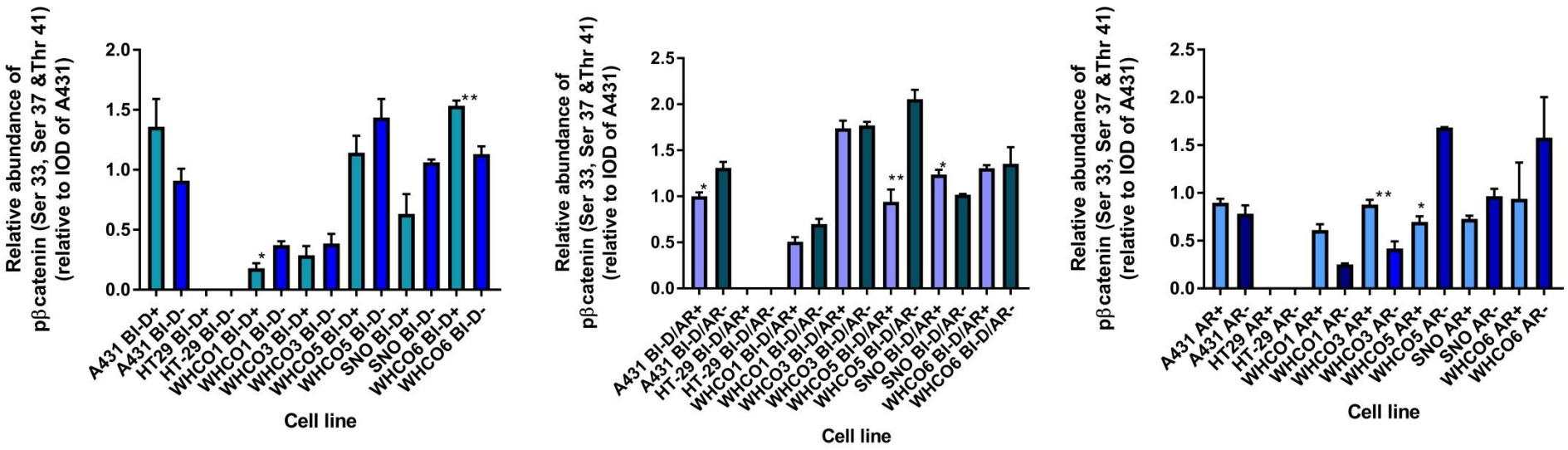

Figure 13. The relative abundance of a few key phospho-intermediates, within the HOSCC cell lines post RSK inhibition (10 µM BI-D1870, 24 hours after serum introduction), GSK3β inhibition (10 µM AR-A014481) and post dual inhibition (AR-A014481 and BI-D1870).

The relative abundance of phospho-FAK (Tyr397), phospho-GSK3*β* (Ser9) and phospho-β-catenin (Ser33, Ser37 & Thr41) were obtained by normalising semi-quantitatively determined densitometric analyses, relative to the A431 cell line (Appendix B, Section 2.6.). Net changes in the cellular levels of each phospho-protein, within the WHCO cells post RSK and/or GSK3β inhibition, were determined as outlined in Appendix B, Section 2.5. A star (*) represents any statistical difference, when compared to the control (*p <0 .05; **p < 0.01). **A.** The cellular levels of phospho-FAK (Tyr397) were variably affected by the inhibition RSK (24hours after serum introduction), the inhibition of GSK3β and the inhibition of RSK/GSK3β within the HOSCC cell lines. **B.** The relative abundance of phospho-GSK3β (Ser9) decreased within the HT29, A431, WHCO3 and SNO cells, post RSK inhibition and post GSK3β inhibition. **C.** Fluctuations in the levels of phospho-β-catenin (Ser33, Ser37 & Thr41) seem to vary from cell line to cell line, post RSK inhibition (24hours after serum introduction), GSK3β inhibition and post RSK/GSK3β inhibition. (BI-D^+: with 10 µM BI-D1870; BI-D^-: without 10 µM BI-D1870/containing 0.1 % DMSO; AR^+: with 10 µM AR-A014418; AR^-: without 10 µM AR-A014418/containing 0.187 % DMSO; BI-D^+/AR^+: with 10 µM BI-D1870/AR-A014418; BI-D^-/AR^-: without 10 µM BI-D1870/AR-A014418 (containing 0.287 % DMSO); WC: whole cell protein extract under standard tissue culture conditions)

Table 3. Net changes in the relative abundance of a few key phospho-intermediates within the HOSCC cell lines post RSK inhibition (10 μM BI-D1870, 24 hours after serum introduction), GSK3β inhibition (10 μM AR-A014481) and post dual inhibition (AR-A014481 and BI-D1870).

Net changes in the cellular levels of each phospho-protein, within the WHCO cells post RSK inhibition, were determined as outlined in Appendix B, Section 2.5. A star (*) graphically represented any statistical difference, when compared to the control (exposed to 0.1 % DMSO) for each cell line using a standard Student's *t*-test (*p <0 .05;**p < 0.01) (Appendix B, Section 2.4).(↑: Net increase in the cellular levels of a phospho-protein; ↓: Net decrease in the cellular levels of a phospho-protein; ≡: Equivalent net change in the cellular levels of a phospho-protein).

	Protein	Cell line						
		A431 ↑/↓/≡ (% change)	HT29 ↑/↓/≡ (% change)	WHCO1 ↑/↓/= (% change)	WHCO3 ↑/↓/≡ (% change)	WHCO5 ↑/↓/≡ (% change)	SNO ↑/↓/≡ (% change)	WHCO6 ↑/↓ /≡ (% change)
RSK inhibition 24 hrs after FCS introduced	Phospho-FAK (Tyr397) (Active protein)	↑ (51)	↑* (31)	↑ (47)	↓* (-33)	↑ (70)	↓ (-9)	↑** (63)
	Phospho-GSK3β (Ser9) (Inactive protein)	↓ (-41)	↓** (-63)	↓ (-62)	↓ (-42)	↑* (73)	↓* (-94)	↑ (38)
	phospho-β-catenin (Ser33, Ser37 & Thr41)	↑ (48)	0	↓* (-68)	↓ (-25)	↓ (-21)	↓ (-40)	↑** (36)
GSK3β inhibition	Phospho-FAK (Tyr397) (Active protein)	↓ (-18)	↓ (-51)	↑* (185)	↑ (99)	↓** (-13)	↓ (-31)	↑ (74)
	Phospho-GSK3β (Ser9) (Inactive protein)	↓ (-36)	↓* (-56)	↑ (45)	↓ (-10)	↑* (150)	↓ (-42)	↓ (-27)
	phospho-β-catenin (Ser33, Ser37 & Thr41)	↑ (17)	0	↑ (143)	↑** (128)	↓* (-65)	↓ (-25)	↓ (-58)
RSK/GSK 3β inhibition	Phospho-FAK (Tyr397) (Active protein)	↓ (-14)	≡ (6)	≡ (-6)	↓* (-17)	↓ (-18)	↓** (-36)	↓ (-55)
	Phospho-GSK3β (Ser9) (Inactive protein)	↑ (17)	↓* (-10)	↓ (-34)	↓ (-20)	↓** (-83)	↓** (-70)	↓ (-93)
	phospho-β-catenin (Ser33, Ser37 & Thr41)	↓* (-23)	0	↓ (-27)	↓ (-2)	↓** (-54)	↑ * (21)	≡ (4)

4.4. Discussion:

It is becoming increasingly apparent that certain signalling molecules may have a context dependent role in cancer progression and that this heterogeneity ultimately impacts the effectivity of targeted therapeutics (Languino *et al*., 2016; Kholodenko *et al*., 1997; Nakakuki *et al*., 2010; Aronchik *et al*., 2014; Rahman *et al*., 2016). The mechanisms that underlie heterogeneity within neoplastic transformations and drug resistance are still being explored today. One study found that TGF-β signalling promoted heterogeneity in squamous cell carcinoma (SCC) stem cells that were poised to metastasize, located near vascular systems (Oshimori *et al*., 2015). Oshimori and colleagues found that TGF-β signalling, alone, could encourage the activation of alternative molecular pathways, increased drug resistance and altered tumour characteristics, without ever altering the DNA profile of the SCC cells (Oshimori *et al*., 2015). This indicates the importance of studying the impact of the microenvironment on outside-in signalling and its effects of the transformed state (Oshimori *et al*., 2015). Indeed molecular heterogeneity appears to be particularly prevalent within human squamous cell carcinomas.

Oral squamous cell carcinomas (OSCC) for example often exhibit erratic behaviour and molecular heterogeneity creating fundamental challenges for targeted based chemotherapy (Severino *et al.*, 2008). Other studies conducted on HOSCC cell lines have found that there are vast differences in the pathways cancerous cells can take in order to maintain and propagate the cancerous state (Fanucchi and Veale, 2009; Kang *et al.*, 2010). Previous studies have shown that due to the nature of cancer, the step-wise accumulation of genetic defects, each cell line may be a case study in and of itself (Kang *et al.*, 2010). HOSCC cell lines are moderately differentiated carcinomas, however, the variability in the results seen in this experiment and in literature shows that there is a vast difference in the differentiation patterns of cell lines given the same grading (Fanucchi and Veale, 2009; Kang *et al.*, 2010). This diversity is further amplified by that fact that RSK activity, within cells, is context and cell lineage dependent (Aronchik *et al*., 2014). This in part explains why HOSCC cells have such high heterogeneity. In the current study not one of the HOSCC cell lines responded identically to all three inhibition studies, despite all being considered moderately differentiated carcinomas. This would suggest that an improved grading system be put in place to further subdivide moderately differentiated carcinomas. This also in part explains

the poor prognosis cancer patients have when they contract this form of cancer (Hendricks and Parker, 2002; Ohashi *et al*., 2010). The dysregulated molecular pathways that underlie/maintain the cancerous state within one patient may be vastly different to another, even though both forms of cancer appear phenotypically similar. The high level of heterogeneity from patient to patient impacts the effectivity of the selected chemotherapeutics used to combat this disease (Hendricks and Parker, 2002; Ohashi *et al*., 2010; Languino *et al*., 2016). In fact the heterogeneity, across HOSCC cancer cells, may be a marker for human oesophageal squamous cell carcinomas, not unlike the other forms of SCC. The different molecular mechanisms, that encourage molecular heterogeneity within HOSCC cells, needs to be fully defined in order to improve the grading system of moderately differentiated carcinomas and the drug targets used to treat HOSCC.

Also the combination of both RSK inhibition and serum stimulation produced a different spatiotemporal response within each of the HOSCC cells than the introduction of serum 24 hours prior to the inhibition of RSK. It is interesting the general phospho-protein trends noted in Chapter 3, were not reproduced in all of the HOSCC cell lines post RSK inhibition (24 hours after serum stimulation). Serum contains platelet derived growth factors that appear to act similarly to transforming growth factors (Childs *et al*., 1982). The presence of these growth factors, after long periods of deprivation, may trigger the MAPK pathway and the PI-3K pathway to name a few) (Clement *et al.,* 2013; Chen *et al*., 2015). This may explain some of the slight discrepancies that were noted post RSK inhibition (under 2 different environmental conditions) (Clement *et al*., 2013; Chen *et al*., 2015). This did, however, confirm that the activity of RSK, within the HOSCC cells may be variable and context specific (Aronchik *et al*., 2014). Also although not all of the observed changes, in the levels of key phospho-proteins, post RSK/GSK3β inhibition were found to be statistically significant, the alterations in the phospho-proteome did indicate the general trends HOSCC cells embody under certain cellular conditions.

4.4.1. RSK impedes FAK activation, via its inhibition of GSK3β, within a human epidermoid squamous cell carcinoma cell line.

The inhibition of RSK, when combined with serum stimulation, had a nominal/insignificant impact on the endogenous levels of FAK activation within the human epidermoid squamous

carcinoma cells. This is likely due to the activation of the PI3K/Akt salvage pathway within the A431 cells (Liu *et al*., 2015). However the loss of RSK activity, under different environmental conditions (24 hours after serum stimulation), promoted the activation of FAK (by 51 %) within the A431 cells. This suggests that RSK inhibits FAK activation, either directly or indirectly, within these cells. However the cellular levels of GSK3β activation also increased upon the loss of RSK activity (RSK-dependent GSK3β inhibition (phospho-GSK3β (Ser9) decreased 24 hours after serum/RSK inhibitor stimulation). The higher levels of GSK3β activity, post RSK inhibition, was confirmed by the increased cellular levels of β-catenin being targeted for ubiquitination (levels of phospho-β-catenin (Ser33, Ser37 & Thr41) increased by 41 %). Perhaps GSK3β may activate FAK. This was confirmed in part by the inhibition of GSK3β, within the A431 cells.

The loss of GSK3β activity decreased the cellular levels of active FAK (by 18 %). Although the levels of FAK activation were only slightly reduced, upon the loss of GSK3β, the RSK inhibition studies showed that GSK3β activation could promote increased levels of active FAK if left dysregulated. This corroborated earlier findings that suggested that the loss of GSK3β activity resulted in reduced levels of FAK activation within melanoma cells (John *et al*., 2012). However, it contradicted other studies that suggest that GSK3β directly inhibits FAK activation (Domoto *et al*., 2016; Kobayashi *et al*., 2006; Bianchi *et al*., 2005; Sutherland, 2011). This would suggest that GSK3β may indirectly promote FAK activation by modulating the activity of one of its substrates (Maccario *et al*., 2007; Sapkota *et al*., 2007). The activation of GSK3β promotes the destabilization/inhibition of PTEN, substrate of GSK3β, thereby promoting the inhibition of PTEN (Maccario *et al*., 2007; Sapkota *et al*., 2007). The loss in PTEN activity, an antagonist of FAK based signalling, may promote FAK activation within the A431 cells (Maccario *et al*., 2007; Sapkota *et al*., 2007). The inhibition of both RSK and GSK3β showed a similar decrease in the levels of FAK activation to that which was noted when GSK3β was solely inhibited, within the A431 cells.

This suggests that RSK promotes FAK inhibition by negatively regulating GSK3β activity within this cell line. It should also be noted that the role the RSK/GSK3β pathway plays in sustaining FAK activation, within this cell line, is fairly minimal, which could explain why it is not statistically significant. GSK3β-dependent FAK activation only accounted for about 14-18 % of the cellular levels of active FAK within these cells. This suggests that alternative

cellular pathways are the main driving force behind the sustained activation of FAK noted within the A431 cells. It is likely that EGF-dependent signalling may play a prominent role in FAK activation, as EGF is highly abundant within these cells and is known to promote FAK activity (Wu *et al*., 2013).

Lastly the levels of phospho-β-catenin increased in the A431 cells despite the loss of GSK3β activity (GSK3β inhibition). This could have been due to the activation of the protein kinase C (PKC) within the A431 cells (Raab *et al*., 2009). Even though GSK3β is generally known to phosphorylate β-catenin, recent studies have found that PKC also inhibits the activation of β-catenin, promoting its degradation by phosphorylating it at serine 33 and serine 37 (Raab *et al*., 2009) (Figure 14). This may explain why the levels of phospho-β-catenin increase upon the loss of GSK3β within the A431 cells.

4.4.2. RSK-dependent FAK activation: context dependent pathway that is not a commonality amongst HOSCC cells

The HOSCC cell lines can be classified into 3 different categories with regard to RSK-dependent FAK activation. RSK impeded FAK activation within the WHCO5, WHCO1 and HT29 cells, in part through its inhibition of GSK3β. This subgroup acted similarly to the A431 cells. RSK inhibition, promoted FAK activation within the WHCO5 cells, by 88 and 70 % respectively (under both environmental conditions although the extent to which this occurs varies). The effectivity of the inhibitor could not be confirmed within this cell, however, as the cellular levels of inhibited GSK3β increased post RSK inhibition. Salvage pathways, such as the Akt/PI3K pathway, may have again play a role in promoting GSK3β inhibition, in the absence of RSK, as seen in the A431 cells (BI-D1870) (Liu *et al*., 2015). Interestingly, GSK3β inhibition indicated that GSK3β promotes FAK activation (by 13 %), within these cell lines. The levels of β-catenin, targeted for ubiquitination, decreased upon the loss of GSK3β activity, confirming the effectivity of the inhibitor. Fascinatingly, the loss of both GSK3β and RSK activity within the WHCO5 cells displayed similar results to that which was obtained when GSK3β was solely inhibited (statistically significant) (Appendix B, Section 2.4.). This indicates that GSK3β plays a role in FAK activation within the HOSCC cell lines. RSK regulates FAK activation negatively by negatively regulating the activity of its activator (GSK3β) within the WHCO5 cell line (Figure 14).

The HT29 cell line and WHCO1 cell line are more complex, in that the loss of GSK3β and RSK activity simultaneously had little to no effect on FAK activation. This indicates that any role RSK or GSK3β may have on FAK activation within these cells, is likely to be the result of a substrate of either one or both of these proteins. In the HT29 cells the levels of FAK activation increased significantly, post RSK inhibition (24 hours after serum stimulation) confirming that RSK inhibits FAK activation, indirectly. As the levels of phospho-GSK3β (Ser9) decreased by 63 percent, post RSK inhibition, the active levels of this protein increased by an equivalent scale factor within the HT29 cells. This indicates that RSK may promote the inhibition of FAK indirectly by inhibiting the activity of GSK3β, within the HT29 cells. Interestingly, as with the A431 cells, GSK3β inhibition indicated that GSK3β activates FAK. This was seen by the fact that increasing levels of GSK3β inhibition resulted in decreasing levels of FAK activation, a 51 % decrease. However, the cellular levels of FAK increased only marginally within this cell line, post dual inhibition. This indicates that RSK is likely to inhibit FAK activation via the inhibition of GSK3β (an activator of FAK). RSK is also unlikely to inhibit FAK activity directly within these cell lines. GSK3β inhibition is likely to promote the activation of one of its substrates thereby encouraging FAK activation (Figure 14).

The WHCO1 cell line behaved similarly to the A431 cells, in that RSK appears to inhibit FAK activation. However in contrast to the A431 cells, the WHCO1 cell line found that GSK3β inhibited FAK activation as well. The levels of phospho-β-catenin decreased upon the loss of GSK3β activity, as seen in the A431 cells. PKC may have acted as a salvage pathway for the loss of GSK3β activity, increasing the levels of phospho-β-catenin in its absence (Raab *et al*., 2009). PKC is thought to promote FAK activation under certain circumstances (Lewis *et al*., 1996). It is possible that PKC may have been responsible; in part for the significant increase in FAK (by 185 %) activation noted upon the loss of GSK3β, within the WHCO1 cells. This activation of PKC may have masked the effects GSK3β inhibition had on FAK activation (Figure 14). It does appear as though GSK3β inhibits FAK activation within these cells. In future PKC activity should be inhibited in this cell line, in combination with GSK3β inhibition to ascertain whether this is indeed the case or not. It is possible that GSK3β may directly inhibit FAK activation, as previous studies have implied

that GSK3β may inhibit FAK activity by phosphorylation (Domoto *et al*., 2016; Kobayashi *et al*., 2006; Bianchi *et al*., 2005; Sutherland, 2011).

The loss of both RSK and GSK3β activity appeared to have no impact on FAK activation, as with the HT29 cells. This indicated that both proteins when inhibited individually caused a reduction in the levels of FAK activation but had no impact on FAK activity when inhibited simultaneously. In the same way as the HT29 cells, a substrate of either RSK or GSK3β may activate FAK within the WHCO1 cell line (Figure 14).

The loss in GSK3β activity caused by AR-A014418 variably affected phospho-GSK3β (Ser9), phosphorylation event that naturally inhibits this protein. The levels of phospho-GSK3β (Ser9) decreased in most of the HOSCC cell lines and the two comparative controls upon the loss of GSK3β activity (AR-A014418). As cells develop mechanisms to maintain homeostasis, a shift/imposed loss in GSK3β activity would naturally result in a decrease in its inhibition, phosphorylation at Ser 9, in an attempt to restore GSK3β activity within the cells (Yamada and Geiger, 1997; Fu *et al.,* 2012). It is likely that the stabilisation of PTEN, upon the loss of GSK3β activity, may suppress the activation/ activity of two of the pathways that result in its inhibition, namely the MAPK/RSK and PI3K/Akt pathways (Gu *et al*., 1999; Li *et al*., 2010; Zhang *et al*., 2014). This could explain why the levels of phospho-GSK3β (Ser9) decrease, post GSK3β inhibition. The levels of phospho-GSK3β (Ser9) increased in the WHCO1 and WHCO5 cell lines, post GSK3β inhibition. This was unexpected and the exact reasons for this occurring are not completely understood, but it may be due to the activation of other salvage pathways that inhibit GSK3β activity. Pathways that promote the activation of PKA, PKB and PKC may promote the phosphorylation of GSK3β, post GSK3β inhibition (Fang *et al*., 2000; Kim *et al*., 2007; Jope and Johnson, 2004; Stambolic and Woodgett, 1994; Pap and Cooper, 1998).

In the second subgroup, RSK activity promoted FAK activation within the SNO and WHCO3 cell lines (Figure 14). The SNO cell line was fascinating in that the levels of FAK activation decreased post RSK inhibition (under both environmental conditions) (by ±10 %), post GSK3β inhibition (by 31 %) and post RSK/GSK3β inhibition (by 36 %, statistically significant) within the SNO cell line. The cellular levels of naturally inhibited GSK3β (phospho-GSK3β (Ser9)) also decreased throughout all of the inhibition studies, indicating

that the RSK inhibitor was active within this cell line (Appendix B, Section 2.4.). The levels of β-catenin, targeted for ubiquitination decreased in SNO cells exposed to either a RSK inhibitor or a GSK3β inhibitor. When both proteins were inhibited the levels of β-catenin, targeted for ubiquitination, increased within the SNO cells. This was unexpected as the loss of GSK3β, responsible for the phosphorylation of β-catenin should result in a decrease in phospho-β-catenin (Ser33, Ser37 & Thr41). This may be due to the activation of PKC within this cell line, as discussed previously (A431 cells) (Raab *et al*., 2009).

However it appears as though GSK3β activity plays a more prominent role in FAK activation, within this cell line, than RSK. The loss of GSK3β promoted a 31 % decrease in the cellular levels of active FAK. The inhibition of both RSK and GSK3β resulted in a 36 % decrease in the levels of active FAK. It appears as though the majority of the losses in FAK activation were incurred due to the loss of GSK3β activity and not due to the loss of RSK (BI-D1870). Nevertheless as the levels of FAK activation did decrease slightly more upon the loss of both proteins, it is possible that RSK promotes FAK activation, via an alternative pathway or directly, within the SNO cells. This mechanism of FAK activation only accounted for 10 % of the active levels of FAK within this cell line. However, perhaps this pathway has a more prominent role in FAK activation when the HOSCC cells are placed under different environmental conditions. It would be interesting to see whether this RSK-dependent FAK activation pathway plays a more prominent role in maintaining FAK activation within SNO cells that are placed under conditions of anchorage independence or oxidative stress (spatiotemporal response). Therefore it appears as though RSK activates FAK within the SNO cell line, using a mechanism that is independent of its regulation of GSK3β. It is also important to note that GSK3β appears to play a prominent role in activating FAK within this cell line. This activation may in part be due to the destabilization of PTEN but as this cell line contains very low levels of this tumour suppressor, perhaps GSK3β modulates FAK activation directly (Figure 14) (John *et al*., 2012; Maccario *et al*., 2007; Sapkota *et al*., 2007; Gu *et al*., 1999; Li *et al*., 2010; Zhang *et al*., 2014).

RSK appears to activate FAK within the WHCO3 cells. The inhibition of RSK, 24 hours after serum stimulation, resulted in diminished cellular levels of active FAK and inhibited GSK3β, by 33 and 42 % respectively. The loss in FAK activation appears to be statistically significant. This suggests that RSK promoted FAK activation within the WHCO3 cells

possibly through its inhibition of GSK3β. It is possible that GSK3β inhibits FAK activation within these cells. This theory was substantiated by the increased levels of FAK activation noted when GSK3β activity was further suppressed, post RSK inhibition (serum stimulation). The activation of PKC, activated by G-protein coupled receptors, or PKA, activated via c-AMP/PKA pathways, may have been responsible for the sustained levels of GSK3β inhibition noted when the WHCO3 cells were stimulated with both serum and the RSK inhibitor (Chapter 3, Section 3.3.4.) (Fang *et al*., 2000; Kim *et al*.,2007; Jope and Johnson, 2004; Stambolic and Woodgett, 1994; Pap and Cooper, 1998). Akt is unlikely to play a role in FAK activation as the activity of this protein is minimal within the WHCO3 cells (Shaw and Veale, 2012).

Interestingly, the inhibition of GSK3β, by AR-A014481, resulted in higher levels of FAK activation being noted within the WHCO3 cells. Again the levels of phospho-β-catenin decreased upon the loss of GSK3β activity, as seen in the A431 cells. PKC may have compensated for the loss of GSK3β activity by targeting β-catenin for degradation in its absence (Raab *et al*., 2009). Should PKC be active within this cell line, as it may be within the WHCO1 cells, it could be responsible, in part for the extreme increase in FAK (by 99 %) activation, noted upon the loss of GSK3β activity (Lewis *et al*., 1996). This activation of PKC may have masked the effects GSK3β inhibition had on FAK activation. However, it does appear as though GSK3β inhibits FAK activation within these cells. It is possible that GSK3β may directly inhibit FAK activation, as previous studies have implied that GSK3β may inhibit FAK activity by phosphorylation (Domoto *et al*., 2016; Kobayashi *et al*., 2006; Bianchi *et al*., 2005; Sutherland, 2011).

The loss of both RSK and GSK3β activity, by dual inhibition, resulted in a significant decrease in the endogenous levels of active FAK, by 17 %. This suggests that the RSK/GSK3β pathway promoted FAK activation within this cell line, however the ectopic activation of FAK, via this mechanism, was only partly responsible for the activation of FAK within these cells (naturally). Here cell-ECM based signalling, EGFR based signalling and the signalling of other growth factor receptor pathways, such as the vascular endothelial growth factor, may be responsible for the remaining levels of FAK activation, within the HOSCC cell lines (Hwang *et al*., 2011; Takahashi *et al*., 1999; Yamada and Geiger, 1997; Park *et al*., 2016). Whether RSK activates FAK directly or promotes FAK activation solely

through the inhibition of GSK3β is unknown. In the future, co-immunoprecipitations should be performed to determine whether FAK directly associates with RSK or not. This will provide more insight into the mechanisms by which RSK regulates FAK activation (direct activation of FAK by RSK).

Lastly the role RSK plays in FAK activation, within the WHCO6 cell line, is not clearly defined but it is possible that RSK may act in both capacities, inhibitor/activator, within this cell line. Upon the inhibition of RSK the levels of inactive GSK3β and active FAK, significantly, increased within the WHCO6 cell line (24 hours after serum stimulation). It therefore appears as though RSK inhibits FAK activation. The loss of RSK promoted the activation of salvage pathways within the WHCO6 cells. It is likely that PI3K/Akt pathway is responsible for maintaining/upregulating the levels of GSK3β inhibition within these cells, mimicking the activity of RSK and masking the effect of the inhibitor on RSK-dependent GSK3β inhibition (Zouq *et al*., 2009). The PI3K/Akt pathway is highly active within this cell line, containing the highest levels of active Akt/PKB of all of the HOSCC cell lines (Shaw and Veale, 2012). As GSK3β inhibition was upregulated, by 38 %, within these cell lines, it is possible that the loss of GSK3β activity may have been responsible for the increase in the cellular levels of active FAK. GSK3β may promote FAK inhibition and its loss resulted in the activation of FAK. This was corroborated by the fact that increasing levels of active GSK3β resulted in decreased levels of active FAK within the WHCO6 cells, post RSK inhibition (with Serum stimulation). This was further confirmed by the inhibition of GSK3β, by AR-A014481 within these cells. The loss of GSK3β activity resulted in large increases in FAK activation within this cell line. Therefore GSK3β inhibits FAK activity within this cell line. It appears as though RSK promotes FAK activation by inhibiting GSK3β activity.

It would appear as though both proteins inhibit FAK activation, as the levels of active FAK will increase upon the loss of either protein. The loss of both RSK and GSK3β resulted in a 55 % decrease in the endogenous levels of active FAK. This implies that a combination of both proteins activity is essential for the activation of 55 % of the endogenous levels of active FAK within these cells. Had RSK promoted FAK activation solely through the inhibition of GSK3β, the loss of GSK3β/RSK (dual inhibitions studies) should have resulted in the levels of active FAK increasing in an almost identical fashion to that which was noted for the levels of FAK, post GSK3β inhibition. However, this was not the case suggesting that to a degree

both GSK3β and RSK may promote FAK activation individualy. How RSK and GSK3β regulate FAK activation within this cell line is largely unknown. It is possible that RSK may salvage FAK activation upon the loss of GSK3β and that GSK3β may salvage FAK activation upon the loss of RSK. Alternatively both of these proteins may impact the activity of other proteins that play a role in FAK activation. This would suggest that one protein may promote the activation of FAK in the absence of the other protein and vice versa, compensating for each other semi-redundantly under certain environmental conditions.

It is possible that both RSK and GSK3β activate and inhibit FAK within this cell line. RSK may promote FAK activation via its regulation of Liver kinase B1 (LKB1), which is known to inhibit FAK activity, whilst GSK3β may promote FAK activation via the inhibition/destabilisation of PTEN (Maccario *et al*., 2007; Sapkota *et al*., 2007; Martin *et al*., 2009; Sapkota *et al*., 2001; Kline *et al*., 2013; Yoneda *et al*., 2010) (Figure 14). Although this cell line has low levels of PTEN, suggesting that GSK3β activity may employ alternative pathways to activate FAK within the WHCO6 cell line (Driver and Veale, 2006). It is important to note that the role GSK3β plays in tumorigenesis appears to be two sided, as this protein appears to promote and inhibit FAK activation depending on the environmental stimuli the cells are exposed to (Grassilli *et al*., 2013; Zheng *et al*., 2007; Guturi *et al*., 2012). This duplicity may also in part explain the unusual effects the inhibition studies had on FAK activation within this cell line.

The HOSCC cell lines were therefore broadly classified into 3 different categories. The WHCO5, WHCO1 and HT29 cell lines appear to act similarly in that RSK appears to inhibit FAK activation within these cell lines, in part through its inhibition of GSK3β. RSK appeared to activate FAK within the SNO and WHCO3 cell lines. Lastly the role the WHCO6 cell line had on FAK activation is not clearly defined, but it is thought that RSK may act in both capacities, inhibitor/activator, within this cell line. However none of the above mentioned pathways resulted in vast decreases in the cellular levels of active FAK. This would suggest that these cell lines employ alternative mechanisms to promote FAK activation ectopically, over and above RSK-dependent FAK activation. Interestingly the Aurora kinase A (AURKA) is thought to promote cell migration and invasion in HNSCC by activating FAK and Akt (Wu *et al*., 2016). Perhaps the ectopic activation of FAK, within the HOSCC cells, may be caused by the activity of AURKA instead of RSK (Wu *et al*., 2016).

Lastly the role RSK plays in FAK activation appears to be context dependent. In a study conducted by Torchiaro and colleagues, RSK promoted the secretion of fibronectin, in ovarian cancer cells via the activation of the YB-1 protein and transforming growth factor-β1 (TGF-β1) (Torchiaro *et al*., 2016). The synthesis and secretion of fibronectin is known to promote cell metastasis and allow cells to survive under conditions of anchorage independence (Torchiaro *et al*., 2016). Fibronectin secretion assists in promoting cell survival in suspension by promoting the activation of α5β6 integrins (essential for cell adhesion and cell spreading) (Duperret *et al*., 2015; Torcharo *et al*., 2016). Fascinatingly, the activation of α5β6 integrins, by fibronectin, could result in the activation of FAK acting as an alternative mechanism by which RSK-dependent FAK activation may be achieved within neoplasmic cells grown in suspension.

Indeed, human oral squamous cell carcinomas cells (SCC), when placed under conditions of anchorage independence, formed multicellular aggregates that promoted Fibronectin deposition, probably via the same RSK activated pathway noted in ovarian cancer cells (Zhang *et al*., 2004; Torchiaro *et al*., 2016). These cell aggregates had high levels of FAK activation likely due to the RSK-dependent secretion of fibronectin and the subsequent activation of the α5β6 integrin. This indicated that RSK may indirectly activate FAK by promoting the transformation of the microenviroment in which the HOSCC cells are found (Zhang *et al*., 2004; Duperret *et al*., 2015). This pathway appears to play a more prominant role in sustaining the anoikis resistant phenotype of cancerous cells placed under conditions of anchorage independence (Zhang *et al*., 2004; Duperret *et al*., 2015). It is therefore possible that RSK may indirectly activate FAK within these multicellular aggregates in order to promote cell survival in suspension. This may also explain, in part, why RSK-dependent FAK activation is context dependent (a spatio-temperal response) (Zhang *et al*., 2004; Duperret *et al*., 2015).

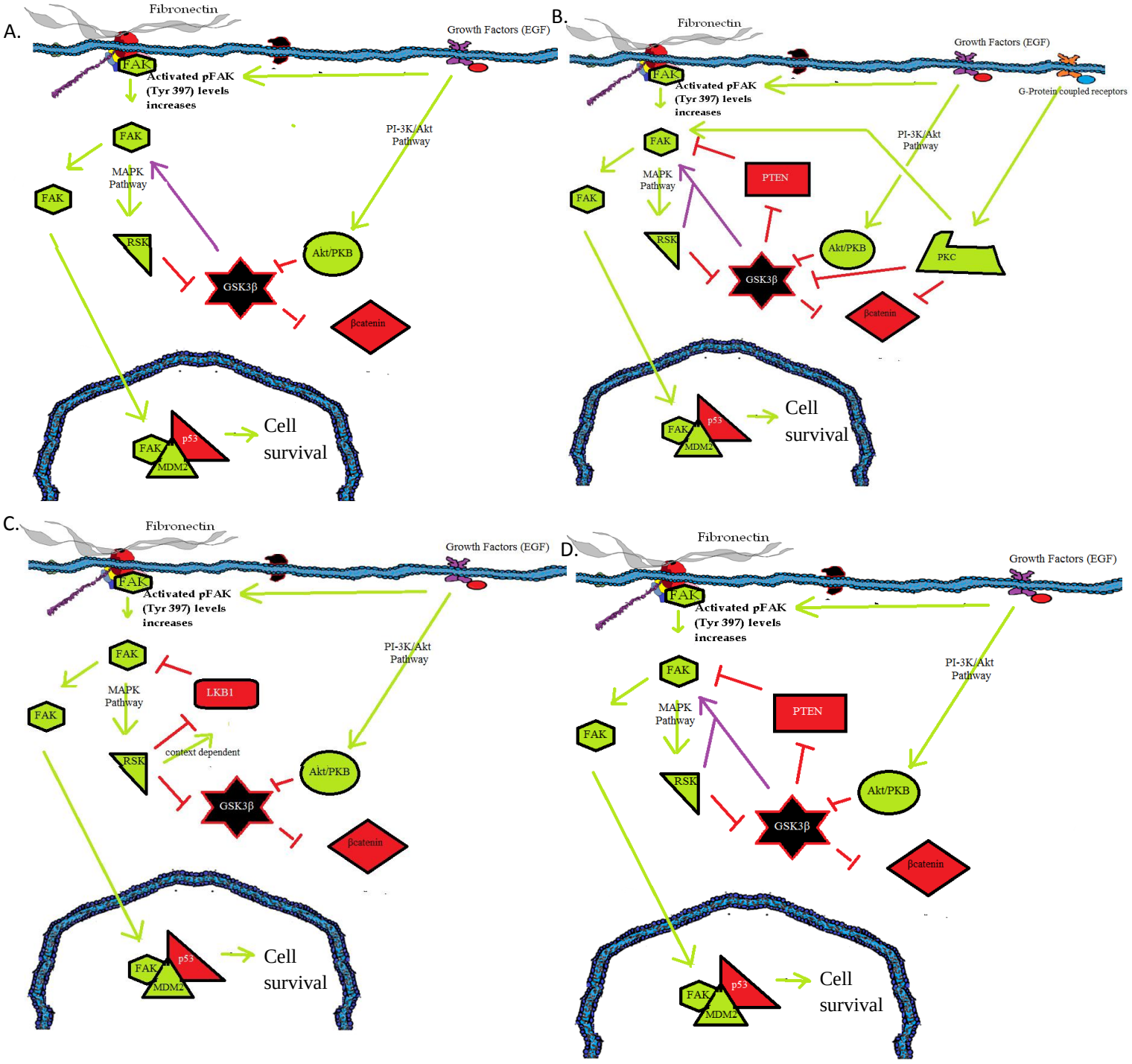

Figure 14. (A, B, C and D). A schematic representation of the possible mechanisms by which RSK regulates FAK activation within the HOSCC cell lines.

Green represents active proteins whilst red represents proteins that are inactive (images produced using Microsoft Paint ©). Red lines represent inhibition. The direct activation of FAK by RSK or GSK3β is indicated by a purple arrow. **A.** RSK impedes FAK activation, indirectly, by inhibiting GSK3β (which activates FAK). (Heading continues on the following page)

B. PKC may promote FAK activation in the absence of GSK3β activity. RSK still hinders FAK activation by inhibiting GSK3β, thus allowing PTEN to remain active (a known antagonist of FAK). **C.** GSK3β activity promotes FAK activation by destabilising PTEN, a known inhibitor of FAK. **D.** RSK regulates LKB1 activity and may therefore either hinder or promote FAK activation via this protein. The image was produced by making use of the information provided from papers published from Maccario *et al*., 2007; Sapkota *et al*., 2007; Martin *et al*., 2009; Sapkota *et al*., 2001; Kline *et al*., 2013; Yoneda *et al*.,2010; Raab *et al*., 2009 Lewis *et al*., 1996. (LKB1: Liver kinase B1; PKC: Protein kinase C; PTEN: Phosphatase and tensin homolog deleted in chromosome 10 FAK: Focal adhesion kinase; RSK: p90 Ribosomal S6 kinase; PI-3K: Phosphatidylinositol-3 kinase)

4.4.3. Link to therapeutics and future prospects:

FAK activity is associated with EMT, cancer stem cells, metastasis, invasion, cell motility, anoikis resistance and cell survival based signalling (Infusino and Jacobson, 2012; Golubovskaya, 2014; Ho *et al*., 2009) . In HOSCC cells the reduction of FAK activation and Rac1, by introducing microRNA-92b-3p, decreased lymph node metastases by supressing cell motility (Ma *et* al., 2016). Cancer cells that have high levels of EGFR, like the HOSCC cell lines and the non-small cell lung cancers, are known to become resistant to chemotherapeutics that target this receptor by undergoing an EMT transition (Wilson *et al*., 2014; Fanucchi and Veale, 2009; Veale and Thornley, 1989). The FAK/Src pathway is thought to promote this EMT-associated resistance to chemotherapeutics (Wilson *et al.*, 2014). Therefore the inhibition of FAK may also render cells susceptible to anti-cancer agents (Wilson *et al*., 2014). In fact the inhibition of FAK in 3D human and neck squamous cell carcinoma cells rendered them susceptible to radiotherapy indicating that FAK activation also promotes chemo-resistance (Hehlgans *et al*., 2009). There is a large body of evidence to support the notion that FAK is a promising drug target for anti-cancer therapeutics (Kurio *et a*l., 2012). The loss of this protein, by use of inhibitors such as TAE226, inhibited cell proliferation, invasion and anchorage independent growth in oral squamous cell carcinomas (Kurio *et al*., 2012). However FAK is a challenging protein to target. Predominantly as FAK acts as both a scaffolding protein and non-receptor tyrosine kinase. In its scaffolding capacity it is known to mediate the degradation of p53 and is even known to mediate endophilin A2 phosphorylation, which promotes invasion, in mammary tumours (Fan *et al*., 2013; Lim *et al*., 2008). FAK inhibitors have already began clinical trials however the efficacy of these

inhibitors are underwhelming, with acquired resistance to the inhibitor being responsible for the poor inhibitor responses (Marlowe *et al*., 2016). It is interesting that some have found that receptor tyrosine kinases can by-pass the inhibition by promoting the direct phosphorylation of Tyr397. This would suggest that receptor tyrosine kinases may impact patient responses to FAK inhibitors (Marlowe *et al*., 2016). As FAK inhibition is challenging, and yields undesirable side effects, scientists have looked at the inhibition of its activator, β1 integrin, as a possible alternative to blocking the protein directly (Li *et al*., 2015).

For this reason the role RSK plays in the FAK activation pathway was explored. RSK promotes metastasis, EMT and invasion making it a promising target for anti-cancer therapeutics (Sulzmaier and Ramos, 2013). In breast cancer cells the inhibition of RSK, and hence the inhibition of YB-1, sensitised breast cancer cells to chemotherapy (Davies *et al*., 2015). This suggests that RSK activity promotes cancer relapses and ultimately links to poor prognoses (Davis *et al*., 2015). p90RSK is a more attractive target for anticancer therapeutic, than FAK, as it is thought that the inhibition of this protein may have less harmful side effects (Neise *et al.*, 2013). RSK is a promising target for anti-cancer therapeutics, despite only promoting FAK activation within 2 of the 5 HOSCC cell lines. However, as its role in the HOSCC cells also appears to be context specific, it may be more appropriate for RSK inhibitors to be used in combination with other targeted chemotherapeutics in an attempt to ward of cancer resurgence. Also since RSK inhibits FAK activation in most of the HOSCC cell lines, it would appear that the inhibition of GSK3β may be a more promising therapeutic target for the inhibition of FAK activity within the HOSCC cell lines.

GSK3β is a mysterious protein in that it appears to be both anti-tumorigenic and pro-tumorigenic. Cancers with inhibited GSK3β and active EGFR are thought to correlate with higher mortality rates as the suppression of GSK3β promotes the Wnt/β-catenin pathway (Zheng *et al*., 2007). Interestingly a further link between GSK3β inhibition, EGFR overexpression and β-catenin was made. In a study conducted by Guturi and colleagues, cross talk between β-catenin and EGFR was found in GSK3β inactivated prostate cancer (Guturi *et al.*, 2012). It is suggested that the loss of GSK3β promotes β-catenin activity. β-catenin was found to transcribe EGFR leading to increased cell survival and proliferation (Guturi *et al*., 2012). In contrast the inhibition of GSK3B in pancreatic cancer cells promoted apoptosis and

forced the cells into senescence (Zhou *et al*., 2012). Globally the inhibition of GSK3B also reduced tumour growth size and angiogenesis (Zhou *et al*., 2012)

In the current study FAK activation appears to be promoted in part, by the activation of GSK3β in some of the HOSCC cells. Fascinatingly, GSK3β promotes that activation of MDM2, by phosphorylation in the central kinase domain of MDM2 (Kulikov *et al*., 2005). The phosphorylation, of MDM2 by GSK3β, promoted its activity resulting in MDM2-mediated p53 degradation (Kulikove *et al*., 2005). It is interesting that the same protein that activates MDM2 also promotes the activation of FAK, a scaffolding protein that assist in MDM2-mediated p53 degradation (Kulikove *et al*., 2005; Lim *et al*., 2008). Therefore GSK3β promoted cell survival based signalling by targeting p53 for degradation (Kulikove *et al*., 2005; Lim *et al*., 2008). This was corroborated by a study that found that GK3β sustained chemo-resistance by promoting the loss of p53 (Grassilli *et al*., 2013). The inhibition of this protein sensitized cancer to chemotherapeutics such as 5-fluorouracil. Fascinatingly the FAK/MEK/ERK pathway has also been linked 5-fluorouracil resistance (Yang *et al*., 2016). This would suggest that GSK3β may play a prominent role in chemo resistance through the activation of FAK.

As GSK3β activity acts more as a master switch that either promotes or inhibits neoplasmic transformations depending on the environment in which it is found, the inhibition of this protein as a target for anti-cancer therapeutic may be challenging. There is no doubt that the inhibition of this protein may impede cancer growth and improve overall patient prognoses by inhibiting FAK activation and p53-mediated degradation (Grassilli *et al*., 2013). However this may only occur in cancers with a specific proteome profile. Cancer cells that are more reliant on the Wnt/β-catenin pathway are unlikely to benefit from the loss of GSK3β, within these cells, as that would further promote Wnt pathway based signalling (Zheng *et al*., 2007). Therefore although GSK3β activates FAK, within some of the HOSCC cell lines, the inhibition of this protein may be too hazardous to capitalize on. Perhaps GSK3β may act in combination with other chemotherapeutics such as 5-flourouracil to improve its efficacy under specific circumstances.

The combination of GSK3β and RSK inhibition resulted in reduced levels of FAK inactivation in most of the HOSCC cell lines. This suggests that the combination of these

inhibitors could be used to inhibit FAK activation within most of the HOSCC cells. However, as predicted above, the loss of GSK3β did promote β-catenin stabilization in some of the HOSCC cells. This may counteract anti-proliferative effects the dual inhibition is likely to have on the HOSCC cells. Experiments should be performed to determine whether the dual inhibition of RSK/GSK3β results in reduced cell viability, reduced invasion or promotes anoikis susceptibility within the HOSCC cell lines. If this were indeed the case then the dual inhibition of both of these proteins may be a promising anti-cancer therapeutic for the treatment of most HOSCC cell lines.

Future work may also involve looking at the role RSK and GSK3β play in anchorage independent growth. This is particularly interesting as RSK is thought to play a more prominent role in pro-survival based signalling under conditions of anchorage independence (Aronchik *et al*., 2014). Therefore the loss of cell-ECM based attachment could result in RSK- dependent FAK activation. In an attempt to ascertain the exact effects serum plays in promoting spatiotemporal responses, further research will have to be performed to determine how/if growth factors rescue the effects of the GSK3β and RSK inhibitors within HOSCC cell lines. Lastly as RSK-mediated fibronectin secretion is thought to promote sustained FAK activation in cells placed in suspension; it would be interesting to see if supressing RSK/fibronectin secretion would diminish the HOSCC cells ability to survive in suspension (Duperret *et al*., 2015; Torchiaro *et al*., 2016; Meng *et al*., 2009). Lastly the role PKC pathway plays in the HOSCC cells should be elucidated.

5. Conclusion

p90RSK does not appear to activate or inhibit FAK directly, in most of the HOSCC cells, but rather modulates FAK activation through the inhibition of GSK3β. The role RSK, or GSK3β, plays in FAK activation, within HOSCC cells, cannot be generalised as it appears to be context dependent. The dysregulation of certain molecular pathways which underlie the diseased state within HOSCC cell lines appears to be variable. Here the effects the RSK/GSK3β pathway had on FAK activation was partially dependent on the HOSCC cells containing active levels of PTEN. It appears as though the inhibition of both GSK3β and RSK reduced the levels of active FAK in 3 of the 5 HOSCC cell lines, suggesting that this might be a good anti-cancer therapeutic. The general trends in the current paper suggest that the grading system for moderately differentiated carcinomas needs to be improved. RSK appears to play a context specific role in FAK activation and this adds an additional layer of complexity that will challenge scientists that are searching for targeted anti-cancer therapeutics. This paper also highlights the importance of studying the effects the microenvironment has on neoplasmic transformations.

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1. Appendix A

1.1. Protein extraction:

1.1.1. Phosphate buffer saline (1X), pH 7.3:

136.9 mM	Sodium chloride
2.68 mM	Potassium chloride
10.1 mM	Disodium hydrogen phosphate decahydrate
1.76 mM	Potassium dihydrogen phosphate

Make up final volume with distilled water

Autoclave to sterilize (store at 4°C)

1.1.2. Laemmli double lysis buffer:

125 mM	Tris-HCl, pH 6.8
4.0 %	Sodium Dodecyl Sulphate
20.0 %	Glycerol
10.0 %	β-mercaptoethanol

Make up to final volume with distilled water

Autoclave to sterilize (store at 4°C)

1.1.3. Sodium Orthovanadate:

100 mM	Sodium Orthovanadate

Make up to final volume with distilled water, pH 10

1.1.4. Sodium Fluoride:

1 M	Sodium Fluoride

Make up to final volume with distilled water

1.1.5. Phenylmethylsulfonyl Fluoride (PMSF):

100 mM	PMSF

Make up to final volume with Methanol

1.2. Protein determination:

1.2.1. 95 % Ethanol:

95.0 % Ethanol

Make up to final volume with distilled water

1.2.2. 7.5 % Trichloroacetic Acid (TCA):

7.5 % TCA

Make up to final volume with distilled water

1.2.3. Coomassie Blue solution (0.25 %):

0.25 % Coomassie brilliant blue powder

50.0 % Methanol

10.0 % Glacial acetic acid

Make up to final volume with distilled water

1.2.4. Destain solution:

12.0 % Glacial acetic acid

10.0 % Methanol

Make up to final volume with distilled water

1.2.5. Elution solution:

66.0 % Methanol

33.0 % Distilled water

1.0 % Ammonia

1.3. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

1.3.1. Buffers:

1.3.1.1. Electrophoresis tank buffer, pH 8.3:

3.74 mM SDS

25.0 mM Tris-HCl, pH 8.3

192.5 mM Glycine

The pH of the solution was adjusted to 8.3 by using 5 N HCl

Make up to final volume with distilled water

1.3.1.2. Separating Buffer:

18.12 g Tris

The pH of the solution was adjusted to 8.8 by using 5N HCl

Make up to final volume with distilled water

1.3.1.3. Stacking Buffer:

6.04 g Tris

The pH1 of the solution was adjusted to 6.8 by using 5 N HCl

Make up to final volume with distilled water

1.3.2. 50 mg/ml Sodium Dodecyl Sulphate Solution:

50 mg/ml SDS

Make up to final volume with distilled water

1.3.3. Gel solutions:

1.3.3.1. Separating Gel:

10.0 % Acrylamide

0.1 % N,N'-methylenebisacrylamide

375.0 mM Tris-HCl, pH 6.8

0.2 % SDS

Make up to final volume with distilled water

Add 1 mM Ammonium Persulphate (APS) and 0.25 % N',N',N',N'-Tetramethylethylene-diamine (TEMED) just before use

1.3.3.2. Stacking Gel:

5.0 % Acrylamide

0.1 % N,N'-methylenebisacrylamide

125 mM Tris-HCl, pH 6.8

0.2 % SDS

Make up to final volume with distilled water

Add 1 mM Ammonium Persulphate (APS) and 0.25 % N',N',N',N'-Tetramethylethylene-diamine (TEMED) just before use

1.3.4. SDS Overlay:

400 µl 50 mg/ml SDS

Make up to 10ml with distilled water

1.3.5. 0.25 % Coomassie Brilliant Blue Stain:

As previously described (Appendix A, Section 2.3.)

1.3.6. Destain solution:

10 % Acetic Acid
10 % Methanol

Make up to final volume with distilled water

1.4. Immunoblotting (Western Blot Analyses):

1.4.1. Buffers:

1.4.1.1. Transfer Buffer:

25.0 mM Tris-HCl, pH 8.3

1.41 % Glycine

20.0 % Methanol

Make up to final volume with distilled water

1.4.1.2. Tris-Buffer Saline (TBS) (1X):

50.0 mM Tris-HCl, pH 7.8

150.0 mM Sodium Chloride

2.0 mM Anhydrous Calcium Chloride

Make up to final volume with distilled water

Autoclave to sterilize (store 4°C)

1.4.1.3. Tris-Buffer Saline-Tween (TBS-T):

50.0 mM Tris-HCl, pH 7.8

150.0 mM Sodium chloride

2.0 mM Anhydrous calcium chloride

0.1 % Tween

Make up to final volume with distilled water

Store at 4°C

1.4.1.4. TBS-for-Blotto Blocking buffer:

50 mM Tris-HCl, pH 7.8

2.0 mM Anhydrous Calcium Chloride

5.0 % Non-fat Milk Powder

0.05 % TritonX-100

Make up to final volume with distilled water

1.4.1.5. Blocking Solution:

5.0 % Non-fat milk powder

Make up to final volume with appropriate buffer

1.4.1.6. Phosphate buffer saline (1X), pH 7.3

As previously described (Appendix A, Section 1.1.)

1.4.2. Developer:

6.40 M Metol

0.60 M Sodium sulphite (anhydrous)

80.0 mM Hydroquinone (Quinol)

0.45 mM Sodium Carbonate (anhydrous)

34.0 mM Potassium Bromide

Make up to final volume with distilled water

Store at room temperature in the dark

1.4.3. Fixer:

0.8 M Sodium Trisulphate

0.2 M Sodium Metasulphite

Make up to final volume with distilled water

Store at room temperature in the dark

1.4.4. SuperSignal ® West Pico Chemiluminescent Substrate Kit:

50 %	Luminol Enhancer Solution
50 %	Stable Peroxide Buffer

Mix in a 1:1 ratio

Store in the dark

1.5. Specific Inhibition of p90RSK with BI-D1870:

1.5.1. BI-D1870 working dilution

10 µM BI-D1870 in 100 % Dimethyl Sulfoxide (DMSO)

1.5.2. BI-D1870 Stock solution

1 mg/ml	BI-D1870

Make up to final volume with 100 % DMSO

1.6. Specific Inhibition of GSK3β with AR-A014418

1.6.1. AR-A014418 working dilution

10 µM in 100 % Dimethyl Sulfoxide (DMSO)

1.6.2. AR-A014418 Stock solution

5 mg/ml	AR-A014418

Make up to final volume with 100 % DMSO

1.7. Tissue culture:

1.7.1. Dulbecco`s Modified Eagles Medium (DMEM):

1.370 %	DMEM
0.370 %	Sodium Bicarbonate
2.0 %	Penicillin (500 U/ml)/Streptomycin (0.5 %) solution

1.7.2. Hams F12 Medium Solution:

1.070 %	Hams F12 Medium
0.118 %	Sodium Bicarbonate
2.0 %	Penicillin (500 U/ml)/Streptomycin (0.5 %) solution

1.7.3. DMEM/Hams F12 Medium Solution:

DMEM/Hams F12 Medium solutions mixed in a 3:1 ratio

Filter sterilize (Store at 4°C)

1.7.4. Trypsin Solution:

0.010 % Trypsin

Make up to final volume with PBS

1.7.5. Ethylenediaminetetra-acetic acid (EDTA):

0.004 % EDTA

Make up to final volume with PBS

1.7.6. Trypsin/Ethylenediaminetetra-actetic acid (EDTA):

Trypsin Solution/EDTA mixed in a 1:1 ratio

Store at 4°C

2. Appendix B

2.1. Representation of a standard curve:

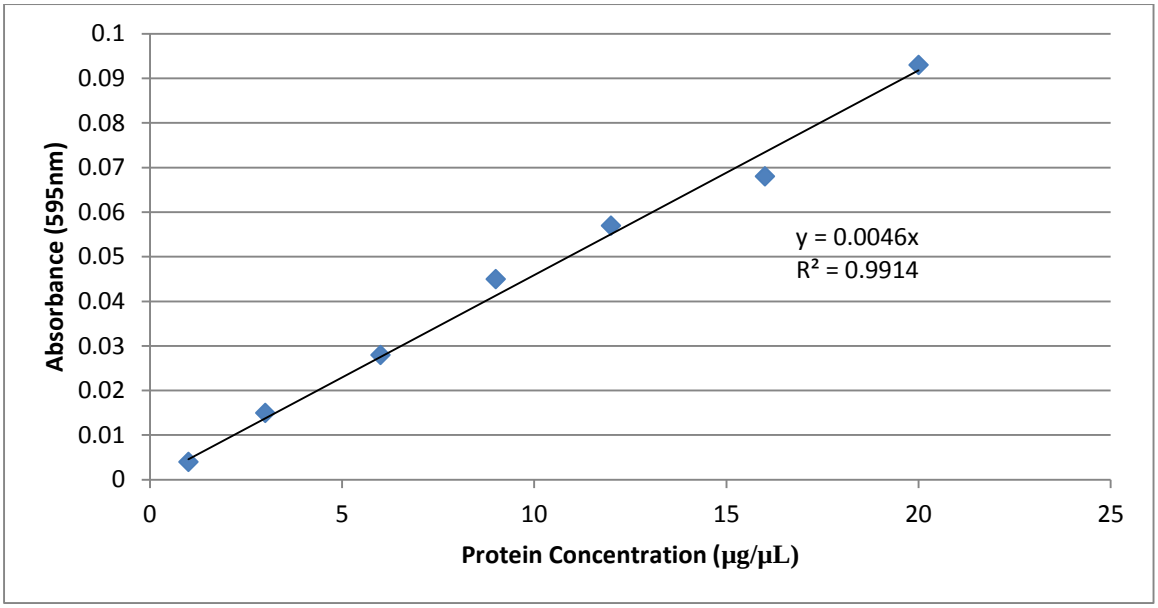


Figure B1. Representation of a standard curve used to estimate the concentration of protein within HOSCC whole cell extracts. Seven BSA standards of known concentration (1, 3, 6, 9, 12, 16 and 20 µg/µl) were plotted in conjunction with their respective absorbance readings (at 595 nM). This was done to obtain a standard curve with the equation y = 0.0046x. The equation of the standard curve was then used to calculate the protein concentrations of the unknown HOSCC whole cell protein lysates. The R^2 value indicates how well the data is represented by the graph (linear regression).

2.2. The relative abundance of key phospho-proteins under standard tissue culture conditions.

Table 4. Standard error in the mean (SEM) relative abundance of a few key phospho-proteins (expressed as % IOD) within the HOSCC cells under standard tissue culture conditions.

Phospho-protein	Cell line- SEM						
	A431	HT29	WHCO1	WHCO3	WHCO5	SNO	WHCO6
Phospho-FAK (Tyr397)	0	13	7	3	11	13	0
Phospho-GSK3β (Ser9)	0	4	6	4	6	9	0
Phospho-β-catenin (Ser33, Ser37 & Thr41)	0	N/A	0	7	4	8	8

Table 5. Standard Student's *t*-test on the relative abundance of a few key phospho-proteins within the HOSCC cell lines when compared to the A431 cell line under standard tissue culture conditions.

A star (*) represents statistical significance when compared to the A431 cell line (SS: Statistically significant; NS: not statistically significant; *: p <0 .05; **: p < 0.01; ***: p < 0.001)

Phospho-protein	Cell line																	
	HT29			WHCO1			WHCO3			WHCO5			SNO			WHCO6		
	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value
Phospho-FAK (Tyr397)	NS	4.54	0.1380	**	11.02	0.0081	**	14	0.005	NS	2.82	0.0668	NS	4.193	0.0525	***	1493762	□0.0001
Phospho-GSK3β (Ser9)	**	12.48	0.0064	**	10.46	0.009	**	17.69	0.0032	*	5.579	0.0307	NS	2.499	0.0878	***	5881	□0.0001
Phospho-β-catenin (Ser33, Ser37 and Thr41)	N/A	N/A	N/A	*	5	0.0154	NS	4.124	0.0541	*	5.227	0.0347	NS	1.833	0.2082	*	9.62	0.0106

2.3. The relative abundance of key phospho-proteins within the HOSCC cells post RSK inhibition (10 μM BI-D1870).

Table 6. Standard Student's *t*-test on the relative abundance of a few key phospho-proteins within each HOSCC cell line post RSK inhibition (10 μM BI-D1870) when compared to its equivalent comparative control (0.1 % DMSO).

A star (*) represents statistical significance when compared to the A431 cell line (SS: Statistically significant; NS: not statistically significant; *: p <0 .05; **: p < 0.01; ***: p < 0.001)

Phospho-protein	Cell line																				
	A431			HT29			WHCO1			WHCO3			WHCO5			SNO			WHCO6		
	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value
Phospho-FAK (Tyr397)	NS	0.1571	0.8833	*	3.682	0.0254	NS	0.09051	0.9323	NS	1.88	0.1420	NS	2.025	0.1344	NS	0.5663	0.5920	NS	2.484	0.0726
Phospho-GSK3β (Ser9)	NS	0.4106	0.6956	NS	0.8233	0.4888	NS	1.758	0.1574	NS	1.758	0.1574	NS	2.052	0.1546	**	5.124	0.0022	NS	0.9103	0.4568

Table 7. The mean and standard error in the mean (SEM) of the cellular levels of a few key phospho-proteins (expressed as % IOD) within the HOSCC cells post RSK inhibition (BI-D1870).
(BI-D^+: with 10 µM BI-D1870; BI-D^-: without 10 µM BI-D1870/containing 0.1 % DMSO).

Phospho-protein		Cell line													
		A431		HT29		WHCO1		WHCO3		WHCO5		SNO		WHCO6	
		mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	mean	SEM	mean	SEM	mean	SEM
Phospho-FAK (Tyr397)	BI-D^+	35	9	28	4	50	11	51	4	53	5	137	19	126	20
	BI-D^-	37	12	53	6	48	10	41	3	33	9	153	21	210	27

2.4. The relative abundance of key phospho-proteins within the HOSCC cells post RSK inhibition (10 µM BI-D1870, 24 hours after serum stimulation), GSK3β inhibition (10 µM AR-A014418) and RSK/GSK3β inhibition (10 µM BI-D1870/ 10 µM AR-A014418).

Table 8. The mean and standard error in the mean (SEM) of the cellular levels of a few key phospho-proteins (expressed as % IOD) within the HOSCC cells post RSK inhibition (10 μM BI-D1870, 24 hours after serum stimulation), GSK3β inhibition (10 μM AR-A014418) and RSK/GSK3β inhibition (10 μM BI-D1870/ 10 μM AR-A014418).

(BI-D^+: with 10 μM BI-D1870; BI-D^-: without 10 μM BI-D1870/containing 0.1 % DMSO; AR^+: with 10 μM AR-A014418; AR^-: without 10 μM AR-A014418/containing 0.187 % DMSO; BI-D^+/AR^+: with 10 μM BI-D1870/AR-A014418; BI-D^-/AR^-: without 10 μM BI-D1870/AR-A014418 (containing 0.287 % DMSO))

Phospho-protein	Inhibitor	Cell line													
		A431		HT29		WHCO1		WHCO3		WHCO5		SNO		WHCO6	
		Mean	SEM	mean	SEM	mean	SEM	mean	SEM	Mean	SEM	mean	SEM	mean	SEM
Phospho-FAK (Tyr397)	BI-D^+	73	9	33	1	25	3	67	8	144	12	107	8	148	3
	BI-D^-	48	4	25	1	17	3	99	7	85	6	120	17	92	7
	AR^+	64	13	22	7	74	3	42	4	45	5	160	21	90	6
	AR^-	77	11	46	6	27	6	22	1	78	5	231	14	62	12
	BI-D^+/ AR^+	75	7	31	15	41	5	95	3	110	6	169	15	81	8
	BI-D^- /AR^-	87	10	36	7	43	6	114	4	134	7	266	9	186	31
Phospho-GSK3β (Ser9)	BI-D^+	83	22	24	5	29	10	46	8	54	1	4	4	75	1
	BI-D^-	137	23	68	7	77	0,1	79	5	38	16	73	3	55	4
	AR^+	94	44	142	3	81	17	80	5	42	3	74	29	41	5
	AR^-	139	28	325	1	55	4	92	13	17	2	127	2	56	8
	BI-D^+/ AR^+	146	8	86	30	30	8	131	10	10	2	43	7	8	4
	BI-D^- /AR^-	145	23	219	11	35	6	165	14	62	4	144	10	120	30
Phospho-β-catenin (Ser33, Ser37 & Thr41)	BI-D^+	136	23	N/A	N/A	18	4	29	7	114	14	63	16	154	4
	BI-D^-	91	9	N/A	N/A	37	3	39	8	144	15	106	2	113	7
	AR^+	90	4	N/A	N/A	61	6	88	5	70	6	73	3	94	37
	AR^-	78	9	N/A	N/A	25	1	42	7	168	4	97	8	158	40
	BI-D^+/ AR^+	100	4	N/A	N/A	51	5	174	8	94	13	124	5	131	3
	BI-D^- /AR^-	130	7	N/A	N/A	70	6	177	4	206	10	102	1	135	18

Table 9. Standard Student's *t*-test on the relative abundance of a few key phospho-proteins within each HOSCC cell line post RSK inhibition (10 μM BI-D1870, 24 hours after serum stimulation) when compared to its equivalent comparative control (0.1 % DMSO).

A star (*) represents statistical significance when compared to the A431 cell line (SS: Statistically significant; NS: not statistically significant; *: p <0 .05; **: p < 0.01; ***: p < 0.001)

Phospho-protein	Cell line																				
	A431			HT29			WHCO1			WHCO3			WHCO5			SNO			WHCO6		
	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value
Phospho-FAK (Tyr397)	NS	2.534	0.0875	*	7.463	0.0105	NS	1.887	0.1332	*	2.988	0.0405	NS	4.77	0.0896	NS	0.6927	0.5399	**	7.361	0.0064
Phospho-GSK3β (Ser9)	NS	1.639	0.1770	**	5.165	0.0083	NS	4.753	0.1319	NS	0.8174	0.4826	*	6.802	0.0226	*	13.42	0.0121	NS	5.52	0.1092
Phospho-β-catenin (Ser33, Ser37 and Thr41)	NS	1.785	0.1818	-	N/A	N/A	*	3.681	0.0242	NS	0.8875	0.4687	NS	1.404	0.2964	NS	2.587	0.1179	**	5.203	0.0099

Table 10. Standard Student's *t*-test on the relative abundance of a few key phospho-proteins within each HOSCC cell line post GSK3β inhibition (10 μM AR-A014418) when compared to its equivalent comparative control (0.1 % DMSO).

A star (*) represents statistical significance when compared to the A431 cell line (SS: Statistically significant; NS: not statistically significant; *: p <0 .05; **: p < 0.01; ***: p < 0.001)

Phospho-protein	Cell line																			
	A431			HT29			WHCO1			WHCO3			WHCO5			SNO			WHCO6		
	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value
Phospho-FAK (Tyr397)	NS	0.756	0.5300	NS	2.526	0.0657	*	7.374	0.0397	NS	4.978	0.0978	**	4.978	0.0089	NS	2.849	0.0543	NS	2.044	0.1358
Phospho-GSK3β (Ser9)	NS	0.8438	0.5019	*	56.46	0.0108	NS	1.492	0.2648	NS	0.8174	0.4826	*	6.802	0.0226	NS	1.814	0.3194	NS	1.612	0.1964
Phospho-β-catenin (Ser33, Ser37 and Thr41)	NS	1.169	0.3294	-	N/A	N/A	NS	5.905	0.0965	**	5.182	0.0098	*	17.01	0.0365	NS	2.861	0.1589	NS	1.123	0.3249

Table 11. Standard Student's *t*-test on the relative abundance of a few key phospho-proteins within each HOSCC cell line post RSK/GSK3β inhibition (10 μM BI-D1870/ 10 μM AR-A014418) when compared to its equivalent comparative control (0.1 % DMSO).

A star (*) represents statistical significance when compared to the A431 cell line (SS: Statistically significant; NS: not statistically significant; *: p <0 .05; **: p < 0.01; ***: p < 0.001)

Phospho-protein	Cell line																				
	A431			HT29			WHCO1			WHCO3			WHCO5			SNO			WHCO6		
	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value	SS	t-value	P-value
Phospho-FAK (Tyr397)	NS	1.009	0.756	NS	0.3078	0.7788	NS	0.3354	0.7548	*	4.483	0.0136	NS	2.654	0.0569	**	5.518	0.0083	NS	3.221	0.1638
Phospho-GSK3β (Ser9)	NS	0.01016	0.9933	*	4.084	0.0352	NS	0.4605	0.6705	NS	1.934	0.1325	**	12.45	0.0010	**	7.922	0.0020	NS	3.703	0.0623
Phospho-β-catenin (Ser33, Ser37 and Thr41)	*	3.933	0.0239	-	N/A	N/A	NS	2.527	0.0666	NS	0.3449	0.7546	**	6.636	0.0034	*	4.23	0.0479	NS	0.2675	0.8127

Table 12. The relative abundance of key proteins within the HOSCC cells as determined by Shaw (2012) and Driver (2006).

Protein	Cell line					
	WHCO1 (%)	WHCO3 (%)	WHCO5 (%)	SNO (%)	WHCO6 (%)	HT29 (%)
Phospho-PKB (Ser473)	10	0	50	22	100	30
PTEN	100	80	50	30	20	N/A

2.5. Net change in the cellular levels of key phospho-proteins.

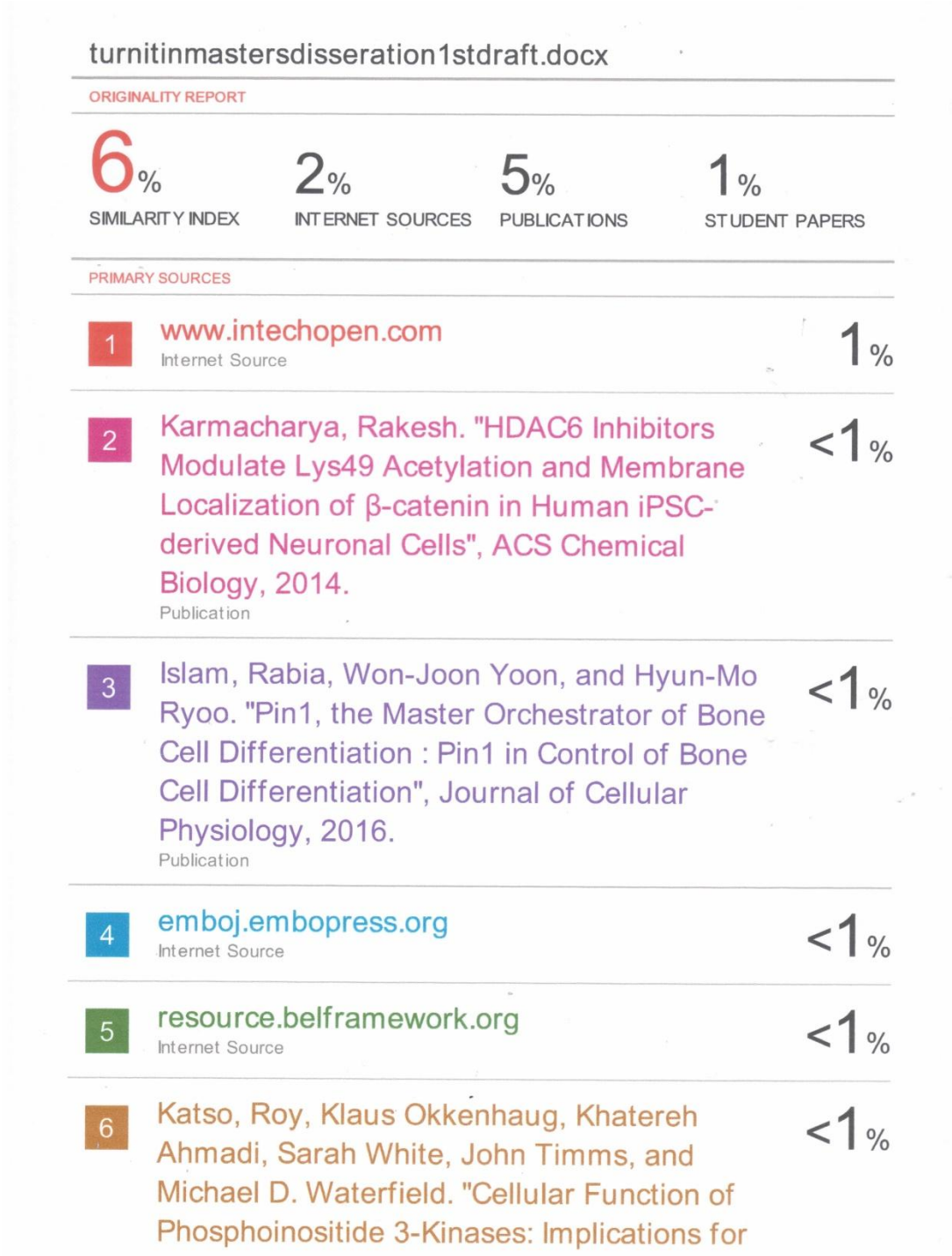
Comparative studies were performed obtaining the percentage difference between the relative abundance of a phospho-protein when an HOSCC cell line is exposed to an inhibitor (or set of inhibitors) (IOD^T) as opposed to DMSO (control) (IOD^C) (exposed to 0.1 % DMSO). The net change in the cellular levels of a specific phospho-protein, post inhibition, was determined as outlined below. Normalized densitometric data, optical density, obtained by western blot analyses for each experimental condition, was utilized to determine the net change in the cellular levels of a specific phospho-protein as outlined below:

$$Net\ change\ in\ the\ cellular\ levels\ of each\ phospho-protein\ (\%) = \frac{(IOD^T - IOD^C)}{IOD^C} \times 100$$

2.6. Normalisation of the densitometric data by fixed point.

The data was normalised as outlined by Degasperi and colleagues (2014). The Normalisation by fixed point was employed in the current study. However, instead of dividing all the replicated data by a measurement of a single condition, all of the data was normalised using a ratio two measurements, the whole cell A431 cells/Whole cell WHCO6 cells. This made use of the densitometric data of both the loading control and the comparative control to normalize the data. Normalising the data based on 2 measurements reduced some of the biological variability of using the reference point mechanism to normalise data.

2.6. Plagiarism Report



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Note: The methods and materials, appendices and reference list were omitted to reduce the risk of false positives.