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The integrative role of Glycogen Synthase Kinase 3β (GSK3β) in adhesion-originating signals human oesophageal squamous cell carcinoma cells

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"Too many stars, too many dreams. The reality is that in front of these things, I'm just a speck of dust."

-Kim Nam Joon

ii. ABSTRACT

The serine-threonine kinase glycogen synthase kinase 3β (GSK3β), has, in recent years, become established as hub for a myriad of intracellular signalling pathways. Many of these pathways have been implicated in cell cycle progression and proliferation in a multitude of carcinomas. GSK3β is ubiquitously expressed and considered to be constitutively active, and phosphorylation at the N-terminus serine 9 residue results in the inhibition. Interestingly, another prominent phosphorylation at tyrosine 216 in the activation loop has been reported to enhance GSK3β activity 200-fold. Its' role in human oesophageal squamous cell carcinoma (HOSCC) migration, however is not well characterised. This study established that both active and inactive GSK3β are present in high abundance in HOSCC cells at rest. In order to characterise the influence of GSK3β on the migratory phenotype of HOSCC, focal adhesion kinase (FAK), a focal adhesion-associated protein known to be increasingly activated during cell migration (FAK(Tyr397)) was used as a marker for cell migration. The abundance of active GSK3β (pGSK3β(Tyr216)) was found to fluctuate during cell migration into a wound over 24 hours. Further investigation via the abrogation of GSK3β revealed that the observed variation was not a result of migration. Instead active GSK3β was found to differentially influence the migratory response observed in HOSCC cells by either 1.) promoting laemellipod extension and cell migration or 2.) partially-abrogate these processes. These findings however, did not produce the expected biochemical alterations with respect to the abundance of pFAK(Tyr397). Moreover, the effect of GSK3β-inhibition on HOSCC was shown to be dependent on the order in which wound initiation and GSK3β occurred, as abrogation of GSK3β prior to wound initiation was seen to either 1.) simply sustain the changes in the migratory response or 2.) produce little variation in the migratory response, suggesting the existence of "rescue-signalling". These influences thus present GSK3β as a key regulator in HOSCC migration. Additionally culturing HOSCC cells on either collagen I of fibronectin, presented general decreases in the abundance of activated FAK, suggesting that varying suggesting that no one ECM-component, but rather the cell surface receptor distribution has become more diverse. This diversity may be a contributing factor to the varied influence of active GSKβ on the migratory response observed in HOSCC.

iii. Conference Output -Poster presentations:

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Mahomed, S., and Veale. R.B. (2016) Inhibition of GSK3β influences pFAK levels during HOSCC cell migration. . University of the Witwatersrand. 7th Annual Cross-Faculty Graduate Symposium. Johannesburg. 1-2 March 2016

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v. LIST OF ABBREVIATIONS AND SYMBOLS

ABD:	Adaptor-binding Domain
AJs:	Anchoring Junctions
AP1:	Activator Protein 1
Apaf-1:	Apoptotic Protease activating factor-1
APC:	Adenomatous Polyoptis Coli
BCL:	B-cell Lymphoma protein
BH:	Bcl2 Homology domain
BSA:	Bovine Serum Albumin
Ca2+:	Calcium ions
cAMP:	cyclic Adenosine Monophosphate
CK:	Casein Kinase
CRC:	Colorectal Carcinoma
DISC:	Death-inducing Signalling Complex
DMEM:	Dulbecco's Modified Eagle medium
DNA:	Deoxyribonucleic Acid
Dvl:	Dishevelled
ECM:	Extracellular Matrix
EDTA:	Ethylenediaminetetraacetic acid
EGF:	Epidermal Growth Factor
EGFR:	Epidermal Growth Factor Receptor
eIF2B:	Eukaryotic initiation factor 2B
EMT:	Epithelial-Mesenchymal Transition

ER:	Endoplasmic Reticulum
ERK:	Extracellular signal-related kinase
FAK:	Focal Adhesion Kinase
FCS:	Fetal calf serum
Fz:	Frizzled
GC:	Gastric Carcinoma
GIT:	Gastrointestinal Tract
GSK3β:	Glycogen Synthase Kinase 3 Beta
GTPase:	Guanosine Triphosphatase
HOSCC:	Human Oesophageal Squamous Cell Carcinoma
ILK:	Integrin Linked Kinase
LRP5/6:	Low Density Lipoprotein-receptor-related protein 5/6
MAPK:	Mitogen activated protein kinase
MAPKAP:	Mitogen activated protein kinase-activated protein
MDCK:	Madin Darby canine kidney
MEK:	MAPK/ERK Kinase
Mg2+:	Magnesium ions
mTOR:	Mammalian Target of Rapamycin
NEDD4-1:	Neural precursor cell expressed, developmentally downregulated 4, E3 ubiquitin ligase-1
NFATc:	Nuclear factor of activated T-cells c
NFκB:	Nuclear Factor kappa light chain enhancer of activated B-cells
p70S6K:	70 kDa Ribosomal protein S6 Kinase

p90S6K:	90 kDa Ribosomal protein S6 Kinase
PAGE:	Poly-acrylamide gel electrophoresis
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PDK1:	Phophoinositide-dependant Kinase 1
PI-3,4-P2:	Phosphatidylinositol-3,4-biphosphate
PI3K:	Phosphatidylinositol-3-Kinase
PIP:	Phosphatidylinosotol-4-Phosphate
PIP2:	Phosphatidylinositol-4,5-biphosphate
PIP3/PI-3,4,5-P3:	Phosphatidylinositol-3,4,5-triphosphate
PKA:	Protein Kinase A
PKB:	Protein Kinase B
PP1:	Protein Phosphatase 1
PP2A:	Protein Phosphatase 2A
PPAR:	Peroxisome Proliferator activated receptor
PTEN:	Phosphatase and tensin homologue deleted on chromosome ten
Raf-1:	Rapidly Accelerated Fibrosarcoma-1
RNA:	Ribonucleic acid
RSP6:	Ribosomal Protein S6
SDS:	Sodium dodecyl sulphate
Ser:	Serine
SH2:	Src Homology 2
SNAIL:	Zinc Finger protein SNAI1

STAT:	Signal transducer and activator of transcription
TBS:	Tris-buffered saline
TCA:	Trichloro-acetic acid
TCF/LEF:	T-cell like Factor/Lymphoid Enhancing-binding Factor
Thr:	Threonine
TNF:	Tumour Necrosis Factor
TNFR:	Transforming Nuclear factor receptor
TSC:	Tuberous Sclerosis Complex
Tyr:	Tyrosine
UV:	Ultraviolet
WHCO:	Wits Human Carcinoma of the Oesophagus
α:	Alpha
β:	Beta
γ:	Gamma

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The integrative role of Glycogen Synthase Kinase-3β (GSK3β) in adhesion-originating signals in Human Oesophageal Squamous Cell Carcinoma cells

Chapter 1: Introduction and Literature Review

1.1. Cellular Signalling integrates a myriad of different pathways

Extensive study into the nature of multicellular organisms has revealed that within such organisms no single cell is entirely self-sufficient. These cells are both dependent on, and have the ability to influence, the environment in which they are found (Singer et al., 1988). This dependence and influence extends to neighbouring cells as well as the extracellular matrix (ECM) and is facilitated by the formation of adhesions between adjacent cells as well as between the cells and the ECM (Ruoslahti and Pierschbacher, 1987). There are four functional classes of junctions which are known to mediate cellular adhesions. These are (1) anchoring junctions, which mediate both cell-cell and cell-ECM adhesions (Takeichi, 1991); (2) occluding junctions, which contribute to making the cell sheet into a selectively permeable or impermeable barrier by sealing gaps between cells in epithelia (Madara and Dharmsathaphorn, 1985), (3) channel-forming junctions, that link the cytoplasm of adjacent cells through the formation of passageways (Kumar and Gilula, 1996) and (4) signal-relaying junctions, which allow for the relay of signals across the plasma membrane at sites of cell-cell contact (Haydon, 1988) e.g. at neuronal chemical synapses. The integrin superfamily of anchoring junctions is central to the transduction of signals across the cell-ECM interface (refer to Fig. 1.1.). Signals originating at these junctions ultimately impact the migration and metastasis of cancer cells. However, before the implications of aberrant signal transduction can be investigated, it is first important to provide perspective on normal integrin-signalling. Particular emphasis will be placed on the integrin-associated protein, focal adhesion kinase (FAK) (Fig. 1.1), and the serine/threonine kinase, glycogen synthase kinase 3β (GSK3β) (Fig. 1.1.) which is proposed to contribute to the migratory capacity of oesophageal squamous cells carcinoma (OSCC).

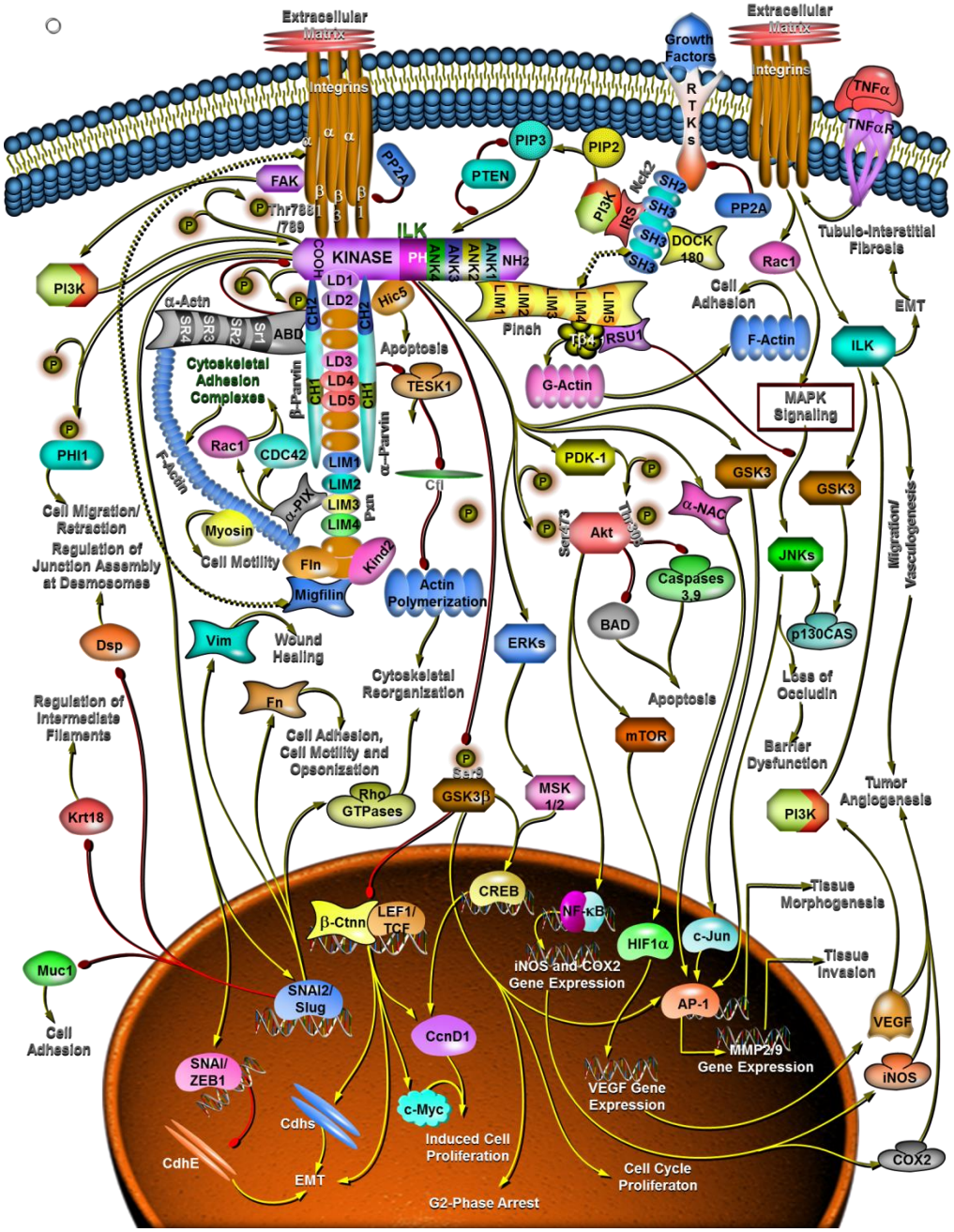


Fig. 1.1. Integrated integrin-related signalling pathway. Signals are relayed from the ECM to the cell via transmembrane integrin receptors. These conformational changes in these integrins are then facilitate the activation of plasma membrane-localised protein tyrosine kinases (PTK) which influence multiple cellular processes. *Adapted from SABioSciences ILK signalling pathway array*.

1.1.1. Signalling at the cell-ECM interface

The survival of epithelial cells is heavily reliant on contact with the extracellular matrix (ECM). These cells form contacts to the extracellular matrix for the dual purpose of communication and adhesion (Harburger and Calderwood, 2009). It is these connections that allow the cell to undergo changes as a result of variation in the ECM-environment which it inhabits (Takada et al, 1997). Thus the ECM conditions and the signals which are relayed to the cell may influence cell growth, proliferation and migration (Takada et al., 2007), this type of signalling is termed "outside-in" signalling as signals generated outside of the cells result in alterations in the intracellular environment (Law et al. 1996). Similarly, in what is termed "inside-out" signalling, the cell, in turn, exhibits bipartite control of ECM structure by the production and secretion of the molecules which are found in the ECM and the export of cellular signals that induce changes in the ECM (Chen et al., 1994 and O'toole et al., 1994).

The basal-surface of epithelial cells rests on an intricate matrix of interwoven laminin, perlecan, entactin and type IV collagen fibres (Timpl, 1989; Timpl and Aumailley, 1989). This layer is known as the basal lamina which serves to anchor epithelial cells and serves as a selective barrier which filters signals to the epithelium above (De Luca et al. 1990). Thus the basal lamina serves as the ECM for epithelial cells. Cell-ECM contacts occur at anchoring junctions called integrins. Integrins are known to cluster at sites of cell-ECM contact called focal adhesions. At focal adhesions the ECM is linked to actin filaments, and hemidesmosomes (Sonnenburg et al.,1991; Van der Nuet et al., 1996), which tie the intermediate filaments of the cell's basal surface to the underlying basal lamina. Clustered integrins in turn interact with a variety of non-receptor protein tyrosine kinases (PTK) localised at the interior plasma membrane resulting in the activation of multiple signal transduction pathways which influence cellular processes such as proliferation and survival.

1.1.2. Integrin-mediated cell adhesion and migration via focal adhesion kinase (FAK).

The integrin family of cell surface receptors are heterodimers made up of α- and β-subunits. Therefore integrin heterodimer formation requires a combination of one of 24 α-subunits with one of 9 β-subunits (Alonso, 2002). The different α-β integrin combinations are able to bind to different cytoskeletal elements as seen with $\alpha_5\beta_1$ and $\alpha_6\beta_1$ intergrins which are ubiquitiously expressed but bind only fibronectin (Akiyama *et al*., 1990) and laminin respectively (Gehlben *et al*., 1988). Integrins also exhibit tissue specificity such that different integrins can bind the same ECM protein but in different cell types as seen with $\alpha_7\beta_1$ and $\alpha_6\beta_4$ integrins which, like the $\alpha_6\beta_1$ integrins, bind laminin but are only expressed in muscle cells and at epithelial hemidesmosomes respectively (as reviewed by Heynes, 2002).

Migrating cells need to be able to rapidly form and disassemble interactions with the ECM to facilitate movement (Palecek *et al*., 1999). This is achieved through the integrin ability to switch between active and inactive states which result in changes on both sides of the plasma membrane such that, detachment from ECM molecules result in a loss of association with intracellular anchor proteins resulting in a "folded" inactive structure whereby the intracellular regions of the integrin sub-units lie close to and interact with one another (Takada *et al*., 2007). Upon ligand binding, the heterodimer assumes an extended conformation which results in the binding domain increasing its affinity for the ligand, while the transmembrane domains separate allowing for the intracellular interaction with talin. Talin binding then initiates the intracellular assembly of anchoring actin filaments (Takada *et al*., 2007). This serves as an example of "outside-in" signalling. Conversely "inside-out" signalling entails talin binding to the β-chain which then initiates α-β dissociation resulting in the extracellular regions of the heterodimer adopting the active formation and binding to ECM ligands. It is also imperative to understand that attachment to the ECM, i.e. the basal lamina with respect to epithelial cells, is required for the normal growth and proliferation of many cell types, including cells of endothelial, muscle and epithelial origin (Frisch and Francis, 1994). These cells exhibit integrin-mediated anchorage dependence whereby loss of substrate attachment results in apoptosis and thus can only grow and proliferate under the appropriate

conditions. Invasive cancer cells often exhibit mutations which cause a loss of anchorage dependence (Marshall *et al*. 1977), which combined with other mutations, allows for the uncontrolled, growth and proliferation characteristic of cancer cells.

Upon ECM-ligand binding to a typical integrin, the cytoplasmic tail of the β-subunit interacts directly with intracellular anchor proteins namely, talin (Horwitz *et al*., 1986), filamin (Pavalko *et al*., 1989) and α-actinin (Otey *et al*., 1990) which in turn either bind directly to the actin cytoskeleton or bind to other proteins such as vinculin (Ezzell *et al*. 1997). Under the appropriate conditions, this cytoplasmic linkage, results in integrin clustering at the membrane which leads to the formation of multi-integrin complexes known as focal adhesions (Jockush *et al*., 1995). Focal adhesion formation, however is not essential to the proper functioning of integrins as this type of clustering is absent in migrating cells despite the adhesion of the ECM to the cytoplasm via integrins at sites referred to as focal complexes. Activation of the small GTPase Rho results in an increase in integrin and actin filament recruitment which stimulates the maturation of focal complexes to focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996).

Focal adhesions present prominent sites of tyrosine phosphorylation and are thus known to attract a number of tyrosine-phosphorylated proteins (Burridge et al., 1988). One such protein is focal adhesion kinase (FAK) which binds the talin molecule attached to the tail of most β-integrins. Thus integrin clustering at focal adhesions causes the clustering of FAK molecules which cross-phosphorylate each other creating a phospho-tyrosine docking-site for the Src-family of cytoplasmic tyrosine kinases (Schlaepfer et al., 1999). In addition to phosphorylating other substrates, these tyrosine kinases then phosphorylate additional tyrosine residues on FAK creating docking sites for additional signalling proteins via "outside-in" signalling. It is well established that FAK plays a vital role in cell motility and migration by diminishing the number of focal adhesions formed with the ECM (Hauck, 2002) and that FAK inhibition results in decreased rates of cell migration (Bianchi et al., 2005). This is in consensus with the elevated levels of FAK observed in cancer cells, particular those that are metastatic in nature.

1.2. Changes in cell adhesion influence a multitude of pathways in the cell

Cadherins and integrins, the functional proteins of anchoring junctions are much more than simple adhesion molecules. In addition to mediating the formation of cell-cell and cell-ECM junctions, they also act as conduits for signal transduction. Thus, changes in the adhesive status of the cell, such as loss of ECM-contact may direct cells either down a route to survival whereby it attempts to postpone the initiation of apoptosis in favour of autophagy (Mizushima et al., 2004) or simply allow for the initiation of anoikis (a pathway to apoptosis which is induced upon loss of cell-ECM contact) (Frisch and Ruoslahti, 1997). This illustrates that changes at the cell-cell and cell-ECM interface have an influence on the intracellular environment (Galbraith and Sheetz, 1998). It therefore follows, that signals produced within the cell should then also have an effect on the activity and structure of adhesive sites. While there exist a multitude of signalling events which can be explained in a cause and effect manner and further cross-talk between the pathways which they initiate, of particular interest to this study are pathways affecting proliferation and differentiation, as a result of changes in cell adhesion e.g. MAPK, PI3K/PKB, mTOR. It is important to note that the serine threonine kinase glycogen synthase kinase-3β (GSK3β) is an element common to all of these pathways.

1.3. Glycogen Synthase Kinase 3β

1.3.1. Structure Mechanism and Isoforms

The protein Glycogen Synthase Kinase-3 (GSK3) is a multi-function protein kinase named for its ability to phosphorylate ser/thr residues on and thus inactivating the enzyme Glycogen Synthase (Woodgett and Cohen, 1984). GSK3 has two known isoforms, these are the 51 kD GSK3α and the 47 kD GSK3β, however a further variant to the *beta*-isoform has been identified (Woodgett, 1990; and Woodgett 1991). This variant, termed GSK3β2 contains a 13 amino acid insertion in the kinase domain of GSK3β (Mukai *et al*., 2002), however, unlike GSK3β, it is poorly characterised in terms of functional kinase activity. There exist two post-translational modifications which are believed to regulate GSK3β activity, these are the "activating" phosphorylation at Tyrosine 216 (Tyr216) (Hughes *et al*., 1993) and the "inactivating" phosphorylation at Serine 9 (Ser9)

(Sutherland *et al*., 1993) (fig 1.3.1.1.). While the presence of a phosphate group at Tyr216 is a well-established hallmark of active GSK3β the mechanism by which this phosphorylation occurs though, remains unclear. Despite this, studies have shown Tyr216 phosphorylation appears to be essential for GSK3β activity by inducing three different mutations at different points within the kinase domain of the protein (Bhat *et al*., 2000). None of the GSK3β-mutants where able to be phosphorylated at Tyr216, thus none were able to catalyse substrate phosphorylation.

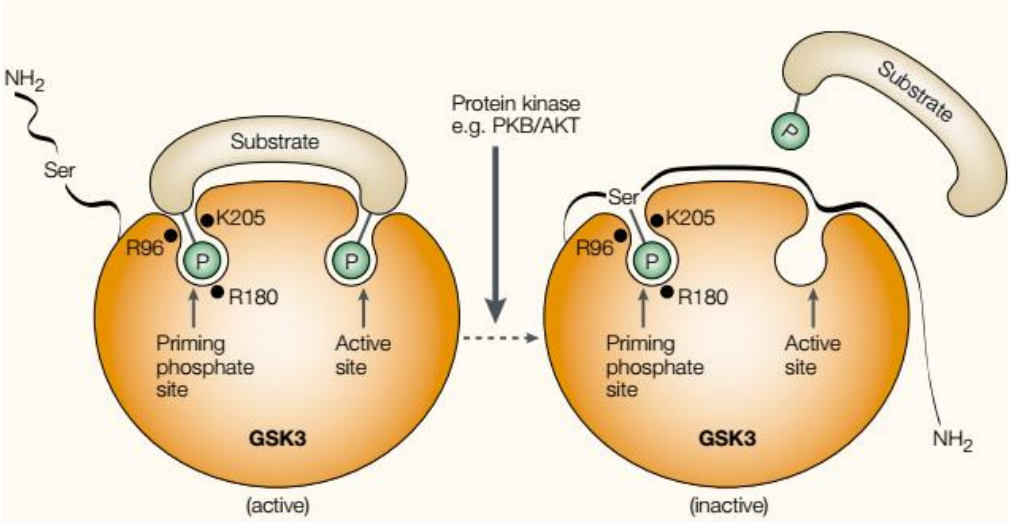


Fig. 1.2. The inactivation of GSK3β by phosphorylation at Ser9. A requirement for phosphorylation by GSK3βis the presence of either a phosphorylated seine or threonine residue 4 residues N-terminal of target residue termed the priming phosphorylation. Docking of this priming phosphate aligns the substrate with the active site of GSK3β. Phosphorylation at the N-terminus Serine 9 residue of GSK3β , results in the phospho-Ser9 acting as a priming phosphate while then-terminal tail wraps around and blocks the active site of GSK3β Adapted from Cohen and Frame (2001).

Mouse studies have revealed that although the α- and β- isoforms exhibit over 80% homology within the respective kinase domains, the isoforms are by no means redundant (Hoeflich *et al*. 2000) as mouse embryos deficient in GSK3β underwent premature death in the early embryo stage while those deficient in the α-isoform did not. However, both isoforms require the presence of a priming phosphate in the form of a phosphorylated Ser/Thr residue. This should located four amino acids amino-terminal of the phosphorylation site of the substrate (Fiol *et al*., 1987). Binding of the substrate's

priming phosphate to the priming phosphate binding site of the GSK3 enzymes allow for the Ser/Thr residue to align with the active site of the enzyme allowing phosphorylation to occur (ter Haar *et al*., 2001). There are instances, however, in which a substrate does not have to be primed for phosphorylation. One such instance occurs when GSK3β phosphorylates the first PPPSP region on LRP6 during Wnt signalling (Section 1.2.2 above) as studies by Zeng *et al*. (2005) showed that mutant GSK3β with an alanine in place of an arginine GSK3β(R96A) at the priming phosphate binding site is still capable of phosphorylating LRP 6 at the first PPPSP repeat in the presence of a Wnt signal but incapable of phosphorylating CK1α-primed β-catenin as part of the destruction complex in a Wnt "off" situation (Zeng *et al*., 2005). This study will thus focus on GSK3β, the more investigated of the two isoforms which has been shown to play a role in the regulation of multiple pathways and is believed to be ubiquitously expressed with differential regulation depending on cell type.

The multitude of substrates available for GSK3β phosphorylation ranges to include a large number of transcription factors; such as c-Jun (Nikolakaki *et al*., 1993) and CREB, both of which exhibit decreased DNA binding ability upon phosphorylation, c-Myc and β-catenin which are marked for ubiquitination, while NFATc and SNAIL (SNA1) (Cano *et al*., 2000), like cyclin D are marked for nuclear export by GSK3β phosphorylation (Ryves and Harwood, 2003). Active GSK3β has also been shown to inhibit the function of adhesion-linked molecules such as FAK (as mentioned previously,) as well as play a role in the initiation of apoptosis by phosphorylating and inhibiting the translation initiation factor eIF2B (Welsh and Proud 1993) while nuclear GSK3β promotes the expression of the STAT, AP1 and NFκB transcription factors (Grimes and Jope, 2001).

1.3.2. The upstream effectors and downstream targets of GSK3β inactivation

GSK3β inactivation occurs as a result of the phosphorylation of serine 9, a residue located on the N-terminal tail of GSK3β. The addition of a phosphate group coupled with the flexibility of the N-terminal tail region allows for the phospho-Serine residue to act as the priming phosphate and thus bind to the priming phosphate binding site of GSK3β while the rest of the tail region is believed to wrap around GSK3β and block the kinase domain

thereby completely inhibiting kinase activity (Frame *et al*., 2001) as GSK3β can no longer bind and phosphorylate primed or unprimed substrates.

There exist a number of ser/thr kinases which are capable of initiating Ser9 phosphorylation of GSK3β under normal physiological conditions. These include Akt/PKB in response to insulin signalling (Cross *et al*., 1995), PKA as a result of increased cAMP levels as well as to mitogen activated protein kinase-activated protein kinase 1 (MAPKAP protein kinase 1 also known as ribosomal protein S6 related kinase-2 or Rsk2 or p90Rsk but will henceforth be referred to as p90Rsk) (Sutherland *et al*., 1993) and p70S6K in response to epidermal growth factor (EGF) and other growth factor signalling (Saito *et al*. 1994). ILK has also been identified as an inhibitor of GSK3β, both directly and indirectly through the activation of Akt/PKB (Armelle, 2003).
Inhibition of such a multifunctional kinase could produce a vast number of cellular responses under normal physiological conditions thus the implications of its aberrant activity and regulation in cancer cells present a large number of possible responses. Specific signalling events considered to be of relevance to this study, based upon their common interaction with GSK3β will be further expanded upon. These include β-catenin dependent Wnt signalling and the well-established 'hub' of cellular signalling the PI3K/PKB pathway.

1.3.2.1. The Phosphatidylinositol-3-Kinase/Protein kinase B (Akt) pathway

Phosphatidylinositol-3-Kinase is a multimeric protein complex responsible for the phosphorylation of a number of substrates including the phosphatidylinositols (PtdIns/PIPs); phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-phosphate (PIP_2) (Tolias and Cantley, 1999). There are three known subclasses of PI3K, however, Class I_A PI3K is the most well studied of the three, being known to have an extensive role in the regulation of cell signalling within both normal and cancerous cells (Cantley, 2002). Membrane-bound PI3K is capable of catalysing the transfer of the γ-phosphate of ATP to PIP2 producing the second messenger PIP3 which in turn phosphorylates a number of substrates including integrin-linked kinase (ILK) (discussed below) and phosphoinositide-dependant protein kinase 1 (PDK1) (Leevers *et al*., 1999) both which lead to the activating phosphorylation of protein kinase B (PKB/Akt) (Coffer and Woodgett, 1991; Jones *et al.*, 1991a and Brazil and Hemmings, 2001) by inducing

conformational changes after recruiting it to the plasma membrane (Song *et al*., 2005). However, the major activating phosphorylation of PKB, at Ser473, has been shown to be catalysed by the mammalian target of rapamycin complex 2 (mTORC2) (Chen and MacKintosh, 2009).

The serine/threonine kinase protein kinase B/Akt is a member of the cAMP-dependant protein kinase A/protein kinase G/protein kinase C (AGC) super family of protein kinases. Initially identified based on its similarity to protein kinase A and protein kinase C (Jones *et al*., 1991b). PKB consists of multiple domains namely, an N-terminal Pleckstrin Homology (PH) domain characteristic of most phosphinositide binding proteins (Ferguson *et al*., 2000), a central kinase domain and a C-terminal regulatory domain that is largely conserved through the AGC superfamily (Nicholson and Anderson, 2002). Two major sites of PKB activation have been identified, these are at serine 473 and threonine 308 (Alessi *et al*., 1996) The aforementioned Ser/Thr kinases; ILK and PDK1 are known to phosphorylate PKB at serine 473 (Delcommenne *et al*., 1998) and threonine 308 respectively (Williams *et al*., 2000).

Active Akt/PKB is capable of phosphorylating a vast number of substrates resulting in a range of cellular responses (Song *et al*., 2005). These responses range from the inhibition of apoptosis via the inhibitory phosphorylation of the pro-apoptotic proteins BAD, Caspase-9, to the promotion of cell cycle progression by the inhibitory phosphorylation the cell cycle; inhibitors p21, p27 and GSK-3β as well the activating phosphorylation of various inhibitors of tumour suppressor genes (Cantley, 2002). Thus constitutive activation of PKB and its ability to phosphorylate GSK3β at Ser9 in cancerous cells has been identified as an integral player in tumorigenesis (as reviewed by Carnero, 2010).

1.3.2.2.The effect of GSK3β on β-catenin

β-catenin, which plays a major role in the stability and function of the cadherin-linked junctional complex (as mentioned in 1.1.1 previously) has also been identified as an integral part of Wnt-mediated gene transcription. Initially, it was believed that β-catenin localised constitutively to adherens junctions, while any remaining cytoplasmic β-catenin was destroyed by the destruction complex in the absence of Wnt signalling (as reviewed by Harris and Piefer, 2005). However Gottardi and Gumbiner (2004) showed that Wnt

signalling causes β-catenin to adopt a closed conformation which possibly blocks a number of the 12 Armadillo repeats required for Cadherin binding, thus increasing its affinity for the transcription factor T-cell like factor (TCF), which only requires 8 repeats. Furthermore it was found that α-catenin-bound β-catenin was more likely to localise to adherens junctions than monomeric β-catenin which showed a greater affinity for TCF (Gottardi and Gumbiner, 2004). Decreased β-catenin-Cadherin associations may result in the weakening of cell-cell adhesions increasing cell motility and possibly triggering anoikis under normal cellular conditions. Thus follows an overview of β-catenin dependant-Wnt signalling.

The wingless integrated signalling site or Wnt signalling pathway is activated by the binding of a lipid-modified glycoprotein of the Wnt family (termed a Wnt signal) to the seven-transmembrane cell surface Frizzled (Fz) receptor. In the absence of a Wnt-signal, cytoplasmic β-catenin is recruited to a destruction complex (also termed the axin complex) consisting of the enzymes glycogen synthase kinase-3β (GSK3β), casein kinase 1α (CKIα), the scaffolding protein axin, and the tumour suppressor protein adenomatous polyoptis coli (APC) (Kimelman and Xu, 2006). Axin binds to each member of the destruction complex via a different binding domain in a conformation which allows for both CKIα and GSK3β to come into contact with and sequentially phosphorylate β-catenin (Kimelman and Xu, 2006). CKIα mediated phosphorylation occurs at serine 45 (Ser45) while GSK3β mediates phosphorylation at Thr41, Ser37 and Ser33. Phosphorylation of the latter two residues prime β-catenin for ubiquitination and subsequent proteosomal degradation (Kimelman and Wu, 2006). GSK3β further contributes to the functionality of the destruction complex by phosphorylating both APC and Axin. This phosphorylation in turn increases the binding affinity of these tumour suppressors for β-catenin which results in increased rates of β-catenin turnover (Huang and He, 2008). β-catenin degradation can however, be abrogated in the absence of a Wnt-signal by the relatively abundant serine/threonine (Ser/Thr) phosphatases protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), which serve to dephosphorylate axin and β-catenin respectively (Su *et al*., 2008 and Luo *et al*., 2007). The dephosphorylation of axin promotes destruction complex disassembly thus dephosphorylated β-catenin cannot be marked for degradation (Su *et al*., 2008). Furthermore, mutations at the phosphorylation sites of β-catenin and APC, as commonly

seen in colorectal cancer, result in β-catenin escaping degradation (Kimelman and Xu, 2006).

The binding of a Wnt signal to a Frizzled (Fz) receptor results in its association with a co-receptor, membrane-bound Low Density Lipoprotein-receptor-related protein 5/6 (LRP5/6) (Zeng *et al*., 2005). This induces the dual phosphorylation and activation of LRP5/6 by either the α- or the β- isoform of glycogen synthase kinase-3 (GSK3) and Casein Kinase 1 (CKI) at the conserved PPPSPxS motifs (P-proline, S-serine/threonine, x-any residue) where GSK3 mediated phosphorylation of PPPSP primes LRP6 for phosphorylation at the xS by CKI (MacDonald *et al*., 2009), where GSK3 phosphorylation occurs in a priming-phosphorylation independent manner as shown by Zeng *et al*. (2008) (discussed in further detail below). Wnt signal-binding results in the binding of the protein Dishevelled (Dvl) to the Fz receptor which in turn recruits the Axin-GSK3β complex to the membrane (Schwarz-Romond *et al*., 2007). Furthermore, following Dvl mediated Axin-GSK3 recruitment to the inner plasma membrane, the protein kinase CK1γ, phosphorylates, a threonine residue (Thr1479) located upstream of the first PPPSP site on LRP6 which appears to prime LRP6 for further phosphorylation by GSK3β (Davidson *et al*., 2005). The membrane recruitment of Axin-GSK3 prevents the formation of the destruction complex that leads to β-catenin ubiquitination and degradation. This allows for the cytoplasmic accumulation of β-catenin, its association with the TCF transcription factor and subsequent nuclear translocation where it induces the transcription of pro-cell cycle regulators such as c-Myc, as well as other Wnt target genes (Gottardi and Gumbiner, 2004). Thus the Axin-GSK3 complex appears to play a dual role within in the cell being capable of both positively or negatively regulating β-catenin levels, governed by the presence or absence of a Wnt signal (Zeng *et al*., 2008).

1.3.3. Behaviour of GSK3β in tumour cells

The effect of GSK3β within the progression of various disease signalling pathways has gained increasing attention (Luo, 2008), while only within the last 20 years has it come to the forefront as a major point of study with regard to cancer pathogenesis. As such a multifunctional kinase, which has been implicated in both cell proliferation and adhesion pathways, it stands to reason that the dysregulation of its activity, either by hyper-

activation or inhibition, may be responsible for some of the characteristics associated with the different cancer phenotypes.

Being a known down-regulator of various cell proliferation pathways it is expected that most cancer types would exhibit a marked inhibition of GSK3β. While it was shown that GSK3β inactivation increased significantly upon the initiation and progression of mouse skin tumorigenesis (Leis *et al*., 2002) and a study conducted on human skin cancer tissue revealed similar increase in pGSK3β(Ser9) in squamous cell carcinoma (Ding *et al*., 2007), a study conducted by Ma *et al*. (2007) showed a significant decrease in levels of inactivated GSK3β when compared to non-transformed keratinocytes. This illustrates that the role of GSK3β in tumorigenesis remains to be fully understood. A similar contrast in GSK3β regulation exists in cancers of the gastro-intestinal (GI)-tract where studies of the same colorectal cancer (CRC) and gastric cancer (GC) cell lines produced conflicting results (Shakoori *et al*., 2005; Mai *et al*., 2009; Zheng *et al*., 2013).

Darrington *et al*. (2012) reported an overexpression of active GSK3β in prostate cancer cells observing that silencing of GSK3β resulted in a decrease in levels of Akt/PKB phosphorylation. This divergent behaviour, coupled with findings that GSK3β over-expression in ovarian cancer stimulates cell cycle progression via increased expression of cyclin D (Cao *et al*., 2006) suggest at the existence of possible gain of function or loss of function GSK3β mutants, though no such mutation has yet been documented.

There have been various studies into the role of GSK3β in oesophageal squamous cell carcinoma via its ability to activate cytokine transcription (as reviewed by Gao *et al*., 2013). Conversely, there is very little known with regard to the effect of changes in GSK3β regulation on the adhesion status of human oesophageal squamous cell carcinoma despite having known influences on the regulation of adhesion-related molecules such as FAK (Bianchi *et al*., 2005), E-cadherin (Bachelder *et al*., 2005) and on the process of epithelial-mesenchymal transition (EMT) (Doble and Woodgett, 2007).

GSK3β has long been used as a marker for the cellular presence and functional activity of Akt/PKB and only in recent years has it begun to emerge as a kinase which in itself has the ability to influence changes in the cellular environment (Wakefield *et al*., 2003). As a result of the emphasis placed on the role of GSK3β in the survival and continued proliferation of cancer cells, its signalling in response to changes in adhesion and migration are often overlooked. However, it is well established that many of the

hallmarks of epithelial cancers, in particular, involve aberrations in the mechanisms of cell adhesion, such as loss of anchorage dependence, anoikis resistance and EMT (Polyak and Weinberg, 2009).

A study by Zheng *et al*. (2013) has shown that aberrations in death receptor signalling in colorectal cancer (CRC) and gastric cancer (GC) cells, both in cultured cell lines and biopsies, due to oncogenic K-ras and raf-1 have resulted in decreased transcription of E-cadherin which in turn contributes to EMT in these cells. The decrease in E-cadherin expression has been attributed to the ERK/MAPK mediated inhibition of GSK3β which abrogates its nuclear translocation preventing its phosphorylation of the transcriptional repressor SNAIL. SNAIL binds to the promoter region of the gene responsible for E-cadherin transcription and prevents its transcription (Cano *et al*., 2000; Zheng *et al*., 2013). Additionally GSK3β phosphorylation allowed for the nuclear shuttling of β-catenin which in complex with SNAIL, promoted the transcription of Vimentin, an intermediate filament characteristic of mesenchymal cells and the matrix metalloproteinase (MMP) Type IV collagenase, which contribute to EMT and metastasis respectively. Similar to Zheng's group, Mai *et al*. (2009) (as reported by Zheng *et al*., 2013) and Wang *et al.* (2009) found increased levels of pGSK3β(Ser9), indicating increased inhibition of GSK3β in gastro-intestinal (GI) cancers. This contrasts with earlier findings of Shakoori *et al*. (2005) who reported increased levels of the active pGSK3β(Tyr216) and no detectable pGSK3β(Ser9) in all the CRC cell lines used in the study. The findings of Shakoori *et al*. (2005) are also in disagreement with those of Leis *et al*. (2002) who found that upon induction of skin tumorigenesis in mice, there was an increase in levels of the inactive pGSK3β(Ser9) and a decrease in pGSK3β(Tyr216) levels.

The differences in the findings of the three groups (Shakoori *et al*., 2005; Wang *et al*., 2009; Zheng *et al*., 2013), all of whom assessed levels of GSK3β in the lower GI-tract cancers (GC and CRC), poses an interesting question about the presence and phosphorylation status of GSK3β in oesophageal squamous cell carcinoma. While it is already known that GSK3β inhibition plays a role in cell cycle progression of these cancers (as reviewed by Gao *et al*., 2013), its effect on the migratory response stimulated by changes in cell-ECM adhesive in African cancer cell lines presents an interesting conundrum.

1.4. Human Oesophageal Squamous Cell Carcinoma

Oesophageal cancer is the eighth highest cause of cancer related deaths in the world (GLOBOCAN 2012; Ferlay *et al., 2015*) with the highest incidence rates occurring in Southern and Eastern Africa (Jemal et al., 2011) as well as in East Asian countries such as China and Japan (Ferlay *et al*., 2010). The causative agents of this cancer vary from vitamin E deficiency and poor oral hygiene in developing and underdeveloped countries to increased rates of tobacco and alcohol intake more frequently noted in developed nations (Kamanagar *et al*., 2006). This cancer type can further be divided into two subtypes, namely, oesophageal adenocarcinoma, the better characterised of the two sub-types, which occurs in the lower third of the oesophagus or at the oesophageal-stomach junction and oesophageal squamous cell carcinoma (OSCC) which occurs in the middle or upper third of the oesophagus (Jemal *et al*., 2010).

In the South African context OSCC is the third highest cause of cancer related deaths and is the leading cancer affecting males and the second highest in females in the Eastern Cape (Somdyala *et al*., 2010). In 1976 Bey *et al*. isolated the first South African immortal oesophageal squamous cell carcinoma cell line from a tumour ressected from an African male after it was noted that the disease was already in an advanced stage by the time symptoms were presented (Bey *et al*., 1976). Thus the need for further studies into the nature of this disease was identified. After the isolation of the Wits Human Cancer of the Oesophagus (WHCO) cell lines by Veale and Thornley in 1989, it was found that the expression of a single class of low affinity epidermal growth factor receptors (EGFR) are conserved across the isolated cell lines.

All five HOSCC cell lines (SNO, WHCO1, WHCO3, WHCO5, WHCO6) have been histologically defined as moderately differentiated tumours. However, each cell line presents a different degree of loss of differential markers and thus the different cell lines may react differently to the same stimuli. This proves to be an invaluable resource as it provides a type of spectrum of expected responses from cell lines with are more differentiated than others within the moderately differentiated class of oesophageal squamous cell carcinoma.

1.5. Existing perspectives on GSK3β signalling in HOSCC

Preliminary laboratory studies have shown that inactive GSK3β (pGSK3β(Ser9)) is present in all 5 HOSCC cell lines (Shaw, PhD Thesis, 2011, unpublished data). Initially GSK3β inactivation in HOSCC cells was attributed almost exclusively to Akt/PKB mediated phosphorylation, however, abrogation of the PI3K/PKB pathway (often referred to as a signalling axis) resulted in decreased Ser9 phosphorylation in only the teo of the cell lines, suggesting that GSK3β inhibition may occur via an Akt/PKB-independent mechanism (Shaw, PhD Thesis, 2011, unpublished data).

Additionally, further study revealed that inhibition of p90Rsk resulted in a dramatic decrease in the levels of phospho-GSK3β(Ser9) detected in HOSCC cells (Buthelezi, PhD in preparation). These findings, coupled with the differences in morphology and growth patterns observed between the WHCO and SNO cell lines, provide a strong foundation for further study into the regulation and involvement of GSK3β signalling events in response to changes in the adhesive interactions of oesophageal squamous cell carcinoma cells. While the inactive form of GSK3β has been examined as a downstream control for various proteins, active GSKβ itself and its role with respect to the invasive nature of HOSCC is yet to be characterised.

1.6. Aims and Objectives

Main Aim: The main aim of this study is to investigate the influence of glycogen synthase kinase 3β in signals leading to cancer cell migration and to assess whether the extracellular source of these signals affects this pathway in human oesophageal squamous cell carcinoma (HOSCC) cells.

Objectives:

1. Establish the abundance of p GSK3β (Ser9) and p GSK3β (Tyr216) as a measure of active versus inactive GSK3β in HOSCC cells.
2. Manipulate GSK3β in order to identify its role in the migratory response exhibited by HOSCC.

3. To understand the impact of GSK3β -mediated signalling in response to ECM-originating signals with special reference to collagen and fibronectin.

Chapter 2: Materials and Methodology

2.1.Cell lines and Tissue Culture

The five human oesophageal squamous cell carcinoma (HOSCC) cell lines, WHCO1, WHCO3, WHCO5, WHCO6 (Veale and Thornley., 1989) and SNO (Bey *et al*., 1976), all exhibiting varying degrees of moderate neoplastic differentiation, were obtained from the Cell Biology Research Laboratory, School of Molecular and Cell Biology, University of Witwatersrand, Johannesburg. Additionally, the HT29 colorectal adenocarcinoma, BxPC-3, pancreatic adenocarcinoma and A431, epidermoid carcinoma cell lines were introduced as comparative controls representing a range of epithelial cancers. All cells were cultured in 10cm tissue culture dishes and maintained in a 3:1 mixture (Appendix A; section 1.2) of Dulbecco's modified eagle medium (DMEM) and Hams F12 (Sigma-Aldrich, USA), with a 10% supplement of fetal calf serum (FCS)(Biowest, South America), at 37 ^{o}C in a 5% CO_2-containing atmosphere. The BxPC-3 pancreatic cancer cell line (a kind gift from Prof S.F.T. Weiss of the Laminin Receptor Research Group at The University of the Witwatersrand, Johannesburg) was used as a control for elevated levels of tyrosine-phosphorylated GSK3β (Kitano *et al*., 2013) and the HT29 colorectal carcinoma cell line was used as a two part control, firstly as a control for the elevated abundance of serine-phosphorylated GSK3β (Wang *et al*., 2009) and secondly as a negative control for β-catenin degradation due to its truncated APC (Rowan *et al*., 2000).

When cells reached 80-85 % confluency, the medium was aspirated and the cells washed twice in phosphate buffered saline (PBS) pH 7.2 (Appendix A; section 1.1.1), and incubated in 2 ml of Trypsin/EDTA (Sigma-Aldrich, USA) at 37 ^{o}C to facilitate cell detachment from the surface. Thereafter medium containing 10 % FCS were cultured as specified above. Unless otherwise stated, all experiments were conducted on cells at 80 % confluency.

2.2. Immunodetection of signalling intermediates

Different primary antibodies were required for the immunodetection of the two GSK3β phosphorylated forms, pβ-catenin and actin (β-isoform), which served as a control for equal loading. Inhibited GSK3β was detected with a rabbit polyclonal antibody designed to target phosphorylated Ser9 residues in the C-terminal tails of GSK3β molecules (Cell Signalling Technology,USA). Activated GSK3β was identified with a rabbit polyclonal antibody, produced by Abcam, UK, which targeted the phosphorylated Tyr216 residue near the active site of the protein. Phospho-β-catenin, was detected using a rabbit polyclonal antibody from Cell Signalling Technology specific for sequence of three phosphorylated residues, serine 33, serine 37 and threonine 41 (Ser33/37/Thr41). Additionally, a rabbit monoclonal anti-phospho-FAK antibody designed to specifically target the Tyrosine 397 residue, produced by Cell Signalling Technology,USA, was introduced in order to detect pFAK(Tyr397). The cytoskeletal protein, Actin, was detected using a polyclonal rabbit anti-actin antibody from Sigma-Aldrich. As all three primary antibodies were of rabbit origin, a goat anti-rabbit horseradish peroxidase-conjugated (HRP) secondary antibody from Sigma-Aldrich (USA) was used to catalyse the chemiluminescent reaction which would allow for the visualisation for the detected proteins.

2.3. Wound-healing assay (24 hour time-course)

To assess the migratory response of HOSCC cells at 75% confluency, in 60 mm dishes, were wounded 30 times using a sterile 10 µl micropipette tip. This created a clear zone on the tissue culture dish. Cell extension into this clear region was visualised using light microscopy following incubation for 0, 6, 12 and 24 hours under normal tissue culture conditions (see section 2.1.) which provided a meand to track cell migration. While the actual width of a wound was arbitrary, the same pipette-tip was sterilised and reused to produce all the wounds, thereby controlling for variation in wound-width. Whole cell lysates (see 2.6 below) were also extracted at each time point.

2.4. The specific abrogation of GSK3β

Cells at 75 % confluency were treated with 10 µM of GSK3β specific inhibitor (AR-A014418, BioVision, USA) in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA), alongside an inhibitor-free and DMSO-only control for 24 hours and incubated for 24 hours at 37^{o}C in a humidified incubator at 5% CO_2. Additionally the monolayer was wounded as described above (2.3.) to allow for the observation of cell migration for 24 hours in addition to migration in the presence of GSK3β inhibition under 2 conditions; (1) the concurrent induction of cell migration and GSK3β inhibition and (2) the induction of cell migration under conditions of sustained GSK3β inhibition, achieved by treating the cells with GSK3β inhibitor 1 hour prior to wound initiation and then incubating for 24 hours in medium containing 10µM AR-A014418.

2.5. The effect of ECM composition in HOSCC

2.5.1. Collagen as an ECM substrate

For growth of cells on a Collagen I-coated surface, between 600000-680000 cells (depending on cell line) were dispensed into sterile, 6 cm tissue culture dishes which had been pre-coated with 600 µl of collagen I by swirling until the surface of the dish was fully covered. The collagen which had previously been extracted from rat-tail tendon as described by Teng *et al* (2006) was diluted to a final concentration of 100 µl/ml in 0.2 N acetic acid. The dishes were then left to dry completely under the ultraviolet (UV) light for 2.5 hours. The surface of each dish was then washed 3 times with sterile (autoclaved) dH_2O in order to dissolve and remove any residual acetic acid so that only the collagen, which is insoluble in water remained on the surface of the dish. The dishes were then washed 3-times with PBS in order to remove any residual H_2O from the previous dissolution step. Following the last PBS wash cells were dispensed into the approximately 650 000 cells were dispensed into the coated dishes and supplemented with 6 ml of 3:1 DMEM:F12 (10% FCS). Cells were allowed to grow for 24 hours before extraction.

2.5.2. Fibronectin as an ECM substrate

In preparation for fibronectin-supplemented cell culture, 10 µl of a 1 mg/ml stock solution of fibronectin (Roche, Switzerland) was diluted in sterile PBS to make a 10 µg/ml working solution of fibronectin. Each 6 cm, sterile tissue culture dish was diluted by gently swirling 1 ml of media in it until the surface was fully covered. The dish was left in the laminar flow, under the UV light with the lid on for 45 minutes. After 45 minutes, the remaining fibronectin solution was aspirated from the dish without, making contact with the bottom of the dish or allowing the dish to dry out.

Immediately after removing the excess fibronectin, between 600000 and 680000 cells were seeded into the dish and supplemented with 6 ml 3:1 DMEM:Ham F12 (10% FCS). Cells were allowed to grow for 24 hours before extraction.

2.6.Whole cell Protein Extraction

Cells at 80% confluency, were washed twice with phosphate buffered saline (PBS, pH 7.2). This was followed by a single wash in PBS containing 10 µl/ml of the protease-inhibitor, phenylmethanesulphonylflouride (PMSF; Sigma-Aldrich, USA), as well as 5 µl/ml and 100 µl/ml of the phosphatase inhibitors sodium flouride (Sigma-Aldrich, USA) and sodium orthovanadate (Sigma-Aldrich, USA) respectively. A thin layer of PBS-inhibitor mixture was left on the cells to facilitate cell extraction by scraping. Cells were then pelleted in an HF-120 TOMY 1200 X g and resuspended in Laemmli double lysis buffer in a 2:1 ratio based on pellet volume. Once fully-resuspended, the whole-cell lysates were boiled at 100 ^{0}C for 5 minutes and centrifuged at 12 000 X g for 15 minutes at 4 ^{0}C in a PRISM™ Refrigerated Microcentrifuge in preparation for gel electrophoresis (SDS-PAGE). The samples were then aliquoted in order to preserve protein integrity for longer and stored at -20 ^{0}C.

2.7. Determination of Protein Concentration

To ensure equivalent loading, the protein content of each sample was determined using a method established by Bramhall et al. (1969). Whatmann filter paper was

hydrated in dH_2O for 20 minutes with gentle agitation, followed by dehydration by gentle washing in 95 % ethanol, 100 % ethanol, and 100 % acetone for 5 minutes each. The filter paper was then air-dried in an extraction hood for 10-15 minutes after which a standard curve was set up by dotting varying amounts (1 µl, 3 µl, 6 µl, 12 µl, 16 µl and 20 µl) of 1 mg/ml BSA in Laemmli double lysis buffer (See appendix A; section 1.3.1) onto the filter paper. Additionally, 2 µl of each whole cell lysate was also dotted onto the filter paper and air-dried. The filter paper was then washed gently in 7.5 % Trichloroacetic acid (TCA, See appendix 1.4.2), for 45 minutes in order to precipitate the protein onto the paper. A five minute wash in Coomassie Brilliant Blue G250 stain allowed for the removal of any remaining TCA and sodium dodecyl sulphate (SDS) from the filter paper. Subsequently, the filter paper was stained in Coomassie Brilliant Blue (G250) for 1 hour, before being destained for one hour. After air-drying the protein spots were cut out and the bound stain was eluted from each spot was eluted into 5 ml of elution solution overnight. The absorbance of each solution was read on an Abbota SV1100 Spectrophotometer at 595 nm. A standard curve of absorbance vs amount of protein (µg) was constructed using the absorbance values from the solutions of known protein concentration on Microsoft Excel. The constructed standard curve was then used to determine the protein content of the extracted whole-cell lysates.

2.8. SDS-PAGE (Sodium-dodecyl sulphate polyacrylamide gel electrophoresis)

The proteins in the extracted whole cell lysates were separated by size using discontinuous Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). The gel of 0.5 mm thickness was set using the Mighty Small SE 245 Dual Gel Caster (Hoefer Scientific, USA). First the 10 % (w/v) polyacrylamide separating gel (pH 6.8, Appendix A, section 1.5.2.2.) was poured between a set of glass and silica plates, overlaid with 200 µl of 0.2 % SDS, to prevent oxidation of the top layer of the gel which inhibits polymerisation, and allowed to polymerise for 30 minutes. Once polymerised, the SDS overlay was removed and the 5 % (w/v) polyacrylamide stacking gel (pH 6.8, Appendix A; 1.5.2.2) was poured on top of the separating gel. A comb was inserted into the liquid

stacking gel to form uniform wells, each with a maximum capacity of 20 µl, for sample loading. The stacking gel was left to polymerise for 25 minutes.

Following polymerisation, the plates containing the polyacrylamide gel were transferred to the Mighty Small II SE 250 Mini Vertical Electrophoresis Unit (Hoefer Scientific, USA), which was filled with Electrophoresis Buffer (pH 8.3, Appendix A; 1.5.1.5). The comb was then removed allowing buffer to flood the wells and the appropriate volume for each of the samples was loaded into the wells, alongside 1 µl of PageRuler Plus Prestained Protein Ladder (ThermoScientific, USA) and an equal amount of whole cell lysate which served as a loading control. The samples were then resolved at a constant current of 21 mA per gel until the lowest band on the molecular weight marker was approximately 1 cm from the end of the gel.

The gel was then stained in 0.25 % Coomassie Brilliant Blue G250 solution (Appendix A; 1.4.3.) for an hour with gentle agitation and then destained for 1 hour in destain solution (Appendix A; 1.4.4).

2.9.Western Blot Analysis

Using the bands of the molecular weight marker as a guide the 35-55 kD and the 55-100 kD portions of the gel were excised and transferred onto a nitrocellulose membrane (PALL, BioTrace…, PALL Corporation, USA) at 400 mA at 4 ^{0}C in a Criterion Blotter transfer tank (BioRad, USA) in western blot transfer buffer (pH 8.3, Appendix A; 1.6.1.1) for either 2 hours (actin, pGSK3β(Y216), and pGSK3β(Ser9)) or 3 hours (pβ-catenin). After transfer, the membranes were rinsed once in ice cold Tris Buffered Saline (TBS, pH 7.8) (Appendix A; 1.1.2.) for 5 minutes (pβ-Catenin and both pGSK3β forms) or subjected to 6 quick washes in ice-cold PBS (Actin) before being blocked in the appropriate blocking buffers (Appendix A, 1.6.1) for 1 hour. After blocking, the pGSK3β and β-catenin membranes were washed two times for 5 minutes in TBS containing 0.1% Tween-20 (TBS/T, Appendix A; 1.1.3), while the membrane used for actin detection underwent 6 quick washes before being transferred to a 1 in 1000 solution of anti-actin antibody in PBS. pGSK3β(Ser9) was detected using a primary antibody dilution of 1 in 1000; pGSK3β(Tyr216), by 1 in 2000, and pβ-catenin(Ser33/37/Thr41) by 1 in 1000 antibody dilution. All antibodies,

with the exception of actin which was diluted in PBS, were diluted in 2.5% BSA in TBS/T solution and incubated at 4 ^{0}C, overnight on a rotary shaker.

The following day, the antibody solutions were removed and the nitrocellulose (PALL Corporation, USA) membranes were washed to remove excess primary antibody. Actin was washed 6 times for 5 minutes each in PBS while pGSK3β(Ser9), pGSK3β(Tyr216) and pβ-catenin(Ser33/37/Thr41) were washed 3 times in TBS/T for 5 minutes each before a 1 hour incubation in HRP-linked secondary antibody diluted in PBS for actin and 2.5% Fat-free milk (Clover, South Africa) – BLOTTO (Appendix A; 1.6.1.) solution. Secondary antibody dilutions were as follows; Actin, 1 in 40000 at 30 ^{0}C; pGSK3β(Ser9), 1 in 3000 at room temperature; pGSK3β(Y216), 1 in 10000 at room temperature and pβ-catenin(Ser33/37/Thr41) 1 in 2500 at room temperature. The membranes were then washed to eliminate any unbound secondary antibody, 6 x 5 minutes in PBS for actin and 3 x 5 minutes in TBS/T followed by one 5 minute wash in TBS for pGSK3β(Ser9), pGSK3β(Tyr216) and pβ-catenin(Ser33/37/Thr41). All membranes were incubated for 5 minutes in WestPico chemiluminescent substrate (ThermoScientific, USA) before being individually sealed in a piece of polyethylene and exposed to CL exposure film (Pierce Biotechnology, USA) for 10 minutes. Each piece of film was then developed (using Developer, see Appendix A, 1.6.1.4) and fixed (in Fixer, see Appendix A, 1.6.1.5) to allow for the visualisation of the chemiluminescent bands formed by the reaction between the HRP and the chemiluminescent substrate in areas where the desired protein was detected.

2.10. Imaging

Cells were viewed using a Leitz Waltzlar Inverted light microscope and imaged using a DCE-2 digital camera eyepiece (Gentuar). Images were captured using ScopePhoto 3.0 at 135X magnification. All western blot results were scanned using ScanJet G3110(Hewlett-Packard, USA) to produce a soft copy for densitometric analysis.

2.11. Quantitation

Western Blots were analysed in order to obtain a semi-quantitative measure of the abundance of the target proteins. RK Densitometry Analysis Version 1.1. © (School

of Computer Science and Applied Mathematics, University of the Witwatersrand, Johannesburg, South Africa) was utilised for densitometric analysis. The software is designed to automatically identify the bands on the images of x-ray film by differentiating between pixel margins which allows for the selection of individual bands regardless of length or width. The pixel densities of each band are then presented as a percentage in relation to a "standard" selected by the user, with the standard being assigned the value of 100 %. For this study the a whole cell lysate of WHCO6 cells was used as a standard for all experiments with the exception of for those depicting the baseline abundance of the target proteins where BxPC-3 cells were used instead.

Chapter 3: Results

3.1. HOSCC cells possess a high abundance of both pGSK3B(Ser9) and pGSK3B(Tyr216)

GSK3β, in both its activated (pTyr216) and inactive (pSer9) forms, was revealed to be present in varying amounts throughout the WHCO and SNO oesophageal cancer cell lines (Fig. 3.1.). The BxPC-3 pancreatic, and HT29 colorectal, adenocarcinoma cell lines are respectively known to possess elevated levels of the active, and inactive, phosphorylated forms of GSK3β. Interestingly, all the WHCO cell lines exhibit an almost 2-fold or greater abundance of pGSK3β(Ser9) than HT29 colorectal adenocarcinoma cells (fig 3.2.c.). While A431 epidermoid carcinoma cells also showed a greater abundance of pGSK3β(Ser9) than HT29s, SNO cells were shown to possess slightly lower amounts. Predictably, BxPC-3 pancreatic adenocarcinoma cells, which are known to have a high abundance of activated GSK3β showed the lowest abundance of inhibited GSK3β.

Interestingly, the WHCO1, WHCO3 and the WHCO6 cell lines, all of which possess large amounts of inactive GSK3β were found to have a high abundance of the activated form, pGSK3β(Tyr216), only slightly lower than BxPC-3s. WHCO5, SNO and A431 cells showed approximately 30 % less pGSK3β(Tyr216) than the BxPC-3 cells, while HT29 cells were found to possess 80 % less of the active form of GSK3β when compared to BxPC-3 cells.

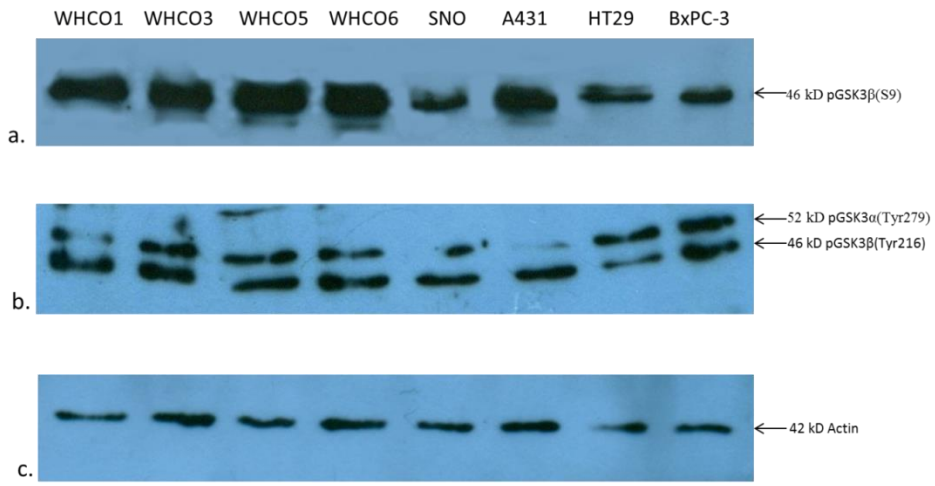


Fig. 3. 2. Immunodetection of the two phosphor-forms of GSK3β. a.) pGSK3β(Tyr216), b.) pGSK3β(Ser9). Whole cell protein extracts were quantified and resolved on a 10% SDS-PAGE gel prior to Western Blot analysis. The A431, HT29 and BxPC-3 cell lines are included as comparative control cell lines for increased EGFR expression, increased levels of pGSK3β(Ser9) and increased levels of pGSK3β(Tyr216) respectively. All cell lines within the HOSCC cell series appear to possess, elevated of both the inactive (pSer9) and the active (pTyr216) forms of GSK3β. The SNO cell line however, appears to produce lower levels of pGSK3β(Ser9), than the HT29 cell line which is known to contain high levels of inhibited GSK3β. c.) β-actin was used as a control to ensure equal loading.

Active GSK3β is known to phosphorylate β-catenin at Serine 33, Serine 37 and Serine 41, thereby marking it for ubiquitination and subsequent degradation. Thus the presence of β-catenin phosphorylated at these residues can be used as a measure for GSK3β activity.

Following protein separation using SDS-PAGE and western blot for the detection of pβ-catenin(Ser33/37/Thr41) it was found that not all the cell lines in which high levels of active GSK3β were reported showed high levels of GSK3β activity. WHCO1 cells, which possess the highest levels of pGSK3β (Tyr216) (Fig. 3.2.a.) exhibit the lowest level of pβ-catenin (Fig.3.2.b.) amongst the experimental and control cell lines. The highest levels of β-catenin phosphorylation, were reported in the WHCO3 cell line which also contains a relatively high, but not the highest abundance of active GSK3β (Fig. 3.2.a). .

It is important to note that the levels of pβ-catenin reported for the HT29 cell line, cannot be used as a measure of GSK3β activity, as HT29 cells contain a mutation which results in the

formation of a truncated APC (Morin *et al*., 1996). This truncation prevents the formation of the β-catenin destruction complex into which GSK3β is recruited under WNT-off conditions.

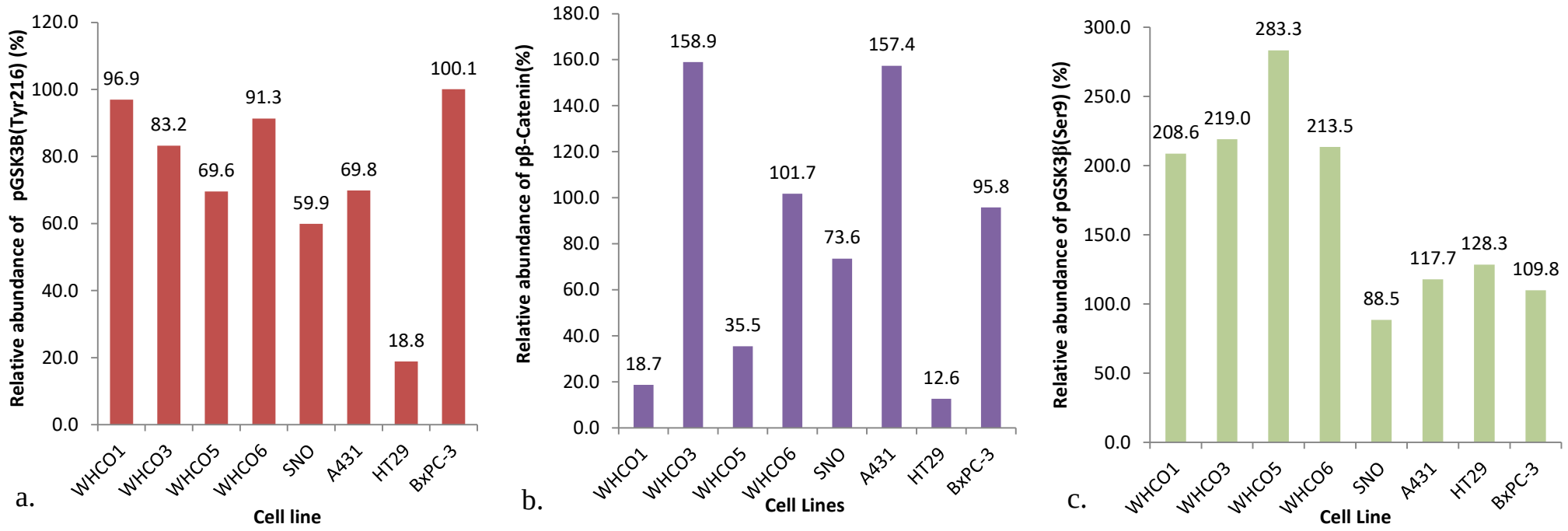


Fig. 3. 2. Comparison of GSK3β and pβ-catenin abundance in HOSCC.. a.)pGSK3β(Tyr216), b.) pβ-Catenin(Ser33/37/Thr41). and c.) pGSK3β(Ser9) HOSCC cells show variation in the abundance of the phosphorylated forms of GSK3β. All five HOSCC cell lines have a higher abundance of each pGSK3β(Ser9) than the designated high abundance control while the abundance of pGSK3β(Tyr216) in HOSCC cell lines appears relatively similar to the abundance in BxPC-3 cells. (pGSK3β(Ser9) control = HT29; pGSK3β(Tyr216) control = BxPC-3) as such it can be said that both active and inactive forms are present in high abundance in all HOSCC cell lines. The abundance of pβ-catenin does not show the expected relationship with the levels of active GSK3β in the WHCO1and WHCO5 cell lines.

3.2. Levels of active FAK and GSK3β are altered during cell migration

3.2.1. 24-hour migration assay

During cell migration the non-receptor tyrosine kinase FAK is known to be activated by phosphorylation at the tyrosine 397 residue (Zhao and Guan, 2011). Understanding the abundance of total FAK and activated FAK (pFAK(Tyr397)) in each of the cell lines provides a foundation for the observation of the alterations that take place when these cells undergo migration. Before this could be expanded upon, however, it was necessary to first obtain an understanding of the relative abundance of FAK and its active form, pFAK(Tyr397) across the experimental cell lines.

Western blot analysis revealed some variation in abundance of the FAK protein within the WHCO and SNO cell series in relation to the abundance observed in the BxPC-3 control cell line (Fig.3.3.a). The HT29 colorectal cancer and WHCO1 and SNO cell lines exhibited the lowest abundance, while the WHCO6 cell line was shown to have the most FAK. It is interesting to note that, the WHCO5 and not the WHCO6 cells (Fig.3.3.b) were shown to possess the greatest abundance of pFAK(Tyr397) amongst the oesophageal cancer cell lines. Generally, the HOSC cells, with the exception of the WHCO1 and SNO cell lines, display greater levels of FAK activation by phosphorylation at the tyrosine 397 than BxPC-3 pancreatic cancer cells. It is important to note that the anti-FAK polyclonal antibody detects both phosphorylated and non-phosphorylated forms of FAK. This suggests that FAK activation occurs minimally in all cell lines, as the HT29 cells, which exhibit the lowest abundance (less than 0.1 times that of BxPC-3) of total FAK are shown to have levels of pFAK(Tyr397) which do not differ as greatly from levels of pFAK(Tyr397) detected in the HOSC cell series or the BxPC-3 cells.

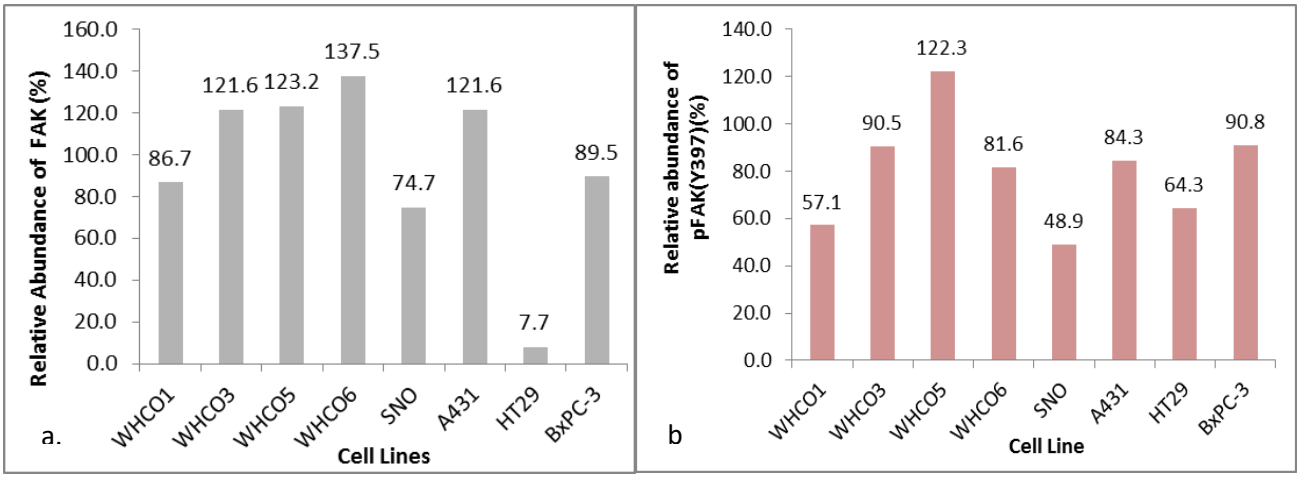


Fig.3.3. Abundance of the non-receptor tyrosine-kinase focal adhesion kinase (FAK) in a range of oesophageal and other epithelial (epidermoid, colorectal and pancreatic) carcinomas. a.) The abundance of total FAK shows a large amount variation in HOSCC. The SNO, WHCO5 and WHCO6 contain elevated levels of total FAK protein. While levels are extremely low in the HT29 cell line. b.) The abundance of tyrosine-397 phosphorylated FAK in HOSCC varies, but the variation is mostly dissimilar to the pattern observed in FAK abundance as SNO and WHCO1 cells exhibit very low levels of pFAK(Tyr397), while HT29 cells exhibit higher abundance of pFAK(Tyr397) despite having the lowest levels of FAK.

To track the changes in cell morphology as well as determine the amount of time required for adjustments in levels of FAK activation to be sufficiently observed, artificial wounds were created on the surface of 60mm culture dishes when the cells had reached approximately 80% confluency. This would cause the formation and clustering of focal contacts resulting in focal adhesion formation due to cell extension onto an uninhabited region of the same environment. The WHCO5, WHCO6, SNO and BxPC-3 cells were specifically selected for this purpose. The WHCO6 and WHCO5 cell lines were selected as they displayed the greatest abundance of total FAK and phosphoFAK(Tyr397) respectively (Fig. 3.3.) . The SNO cell line, because both FAK and pFAK are present in neither high nor low abundance (fig 3.3.) and BxPC-3 pancreatic cancer cell line was selected as a standard of comparison for the HOSCC lines.

HOSCC cells grow in the paving stone-like manner (see Appendix B, Fig. B4) characteristic of squamous cell epithelium (Tanaka *et al*., 1997), however immediately following wound initiation, the ordered nature of the entire monolayer becomes disordered. This disorder is generally maintained until laemellipod formation or the time during which cells begin to migrate into the open space

created by wounding the monolayer becomes evident. The time at which this occurs was shown to vary differentially in the three HOSCC cell lines; after 6 hours in SNO cells, 12 hours in WHCO6 cells but only after 24 hours in WHCO5 cells (Fig 3.4.). The BxPC-3 cells show laemellipod formation by 6 hours, extension and migration by 12 hours and by 24 hours clear migration across the wound (Fig 3.4. d). Interestingly, while all 3 of the HOSCC cell lines showed cell migration into the interrupted region of the monolayer after 24 hours, the degree to which migration occurred varied in each of the cell lines. The WHCO5 cells showed laemellipod extension into the wound, however, after 24 hours, the cells were unable to extend across the entire wound (Fig. 3.4.a.). The WHCO6 (Fig. 3.4.b.) and SNO (Fig. 3.4.c.) cells instead covered large regions of the wound after 24 hours, adopting a fibroblast-like morphology before returning to the canonical morphology for each cell line as the wounded region grew more confluent.

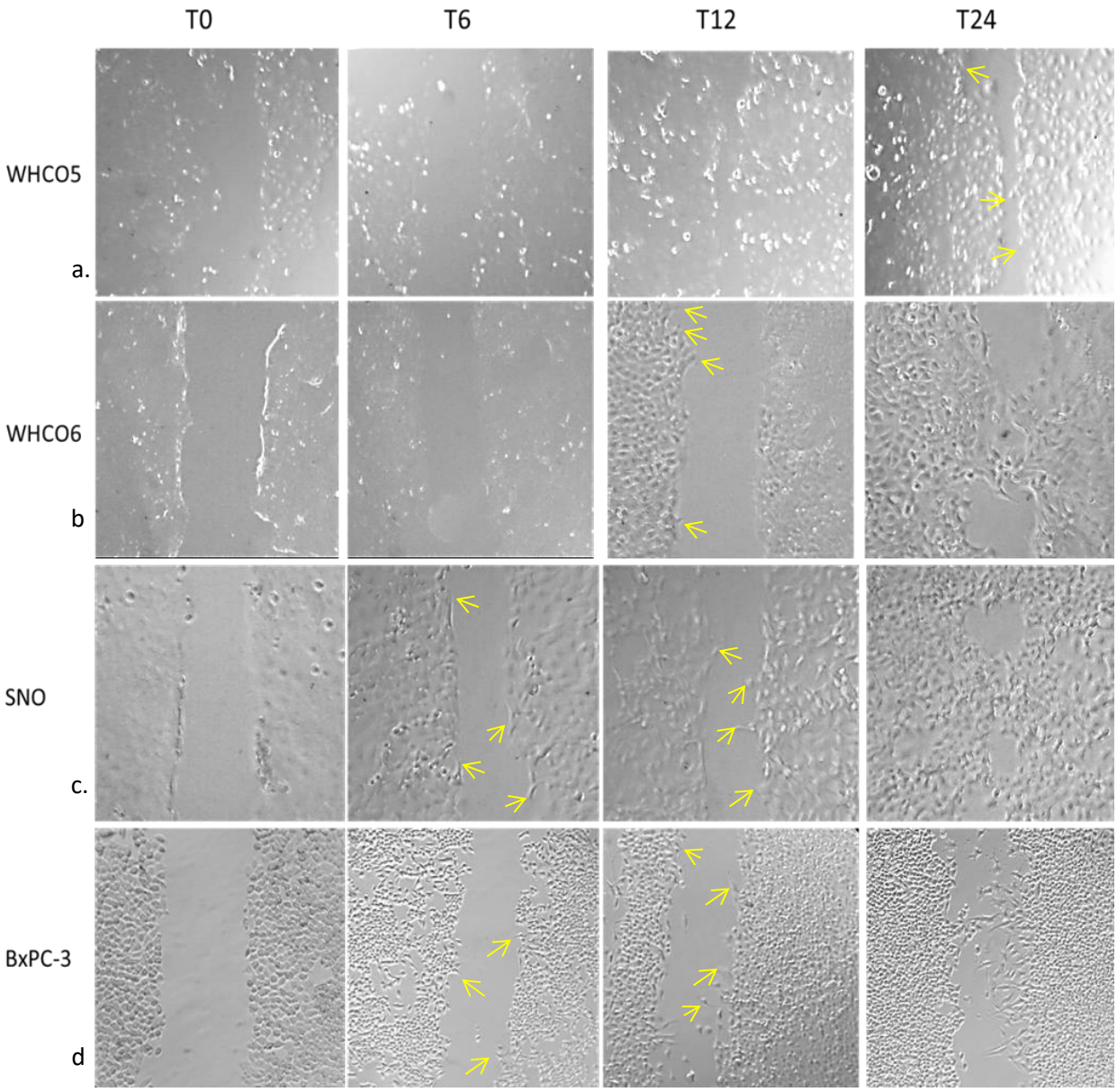


Fig. 3.4. 24-hour Time-course assaying cell migration into a wound in the monolayer. Moderately differentiated HOSCC cells show laemellipod extension differentially through the time-course. All three HOSCCcell lines appear to enter a state of disorder (defined by indistinct cell boundaries) immediately following wound initiation. (all images shown at 135X magnification, yellow arrows indicate points of laemellipod extrusion) a.) WHCO5 HOSCC cells show very little migration into the wound, over the course of 24 hours, however, the presence of laemellipodi does become evident. b.) WHCO6 cells show a return to order and clear laemellipod extrusion by 12 hours. c.) SNO cells show signs of a return to order and laemellipod formation by approximately 6 hours d.) BxPC-3, retains "paving stone-like" appearance following wounding; show laemellipod extension by 6 hours although migration into the wound appears slow over time. Images were reduced due to allow everything to fit on one page for comparative purposes.

The migration of adherent cells would result in the formation of focal contacts and the subsequent recruitment of FAK. Thus pFAK(Tyr397) (activated FAK) was detected for at each time-point in the 24 hour time-course. The WHCO5 and WHCO6 oesophageal carcinoma (Fig.3.5.a.) cell lines show little change in the levels of total FAK detected while the SNO and BxPC-3 cell lines show only a slight variation (visible to the naked eye) in the abundance of FAK over the time course. Interestingly, The WHCO6 and SNO cells show a decrease in the abundance of activated FAK between 6 and 12 hours (Fig.3.5.b.), these levels however, appear to have increased over the course of the final twelve hours such that they appear either partially (WHCO6) or entirely recovered at 24 hours (SNO). The BxPC-3 cells, display an increase in levels of pFAK(Tyr397), while conversely, the abundance of active FAK in WHCO5 cells appears to decrease as time progresses. While the importance of FAK during cell migration is well established (Cary *et al*., 1996, and Sieg *et al*., 1999), this study is aimed at identifying the possible role of GSK3β in oesophageal cancer cell migration, as such the effect of migration on levels of both tyrosine-phosphorylated and serine-phosphorylated GSK3β need to be assessed.

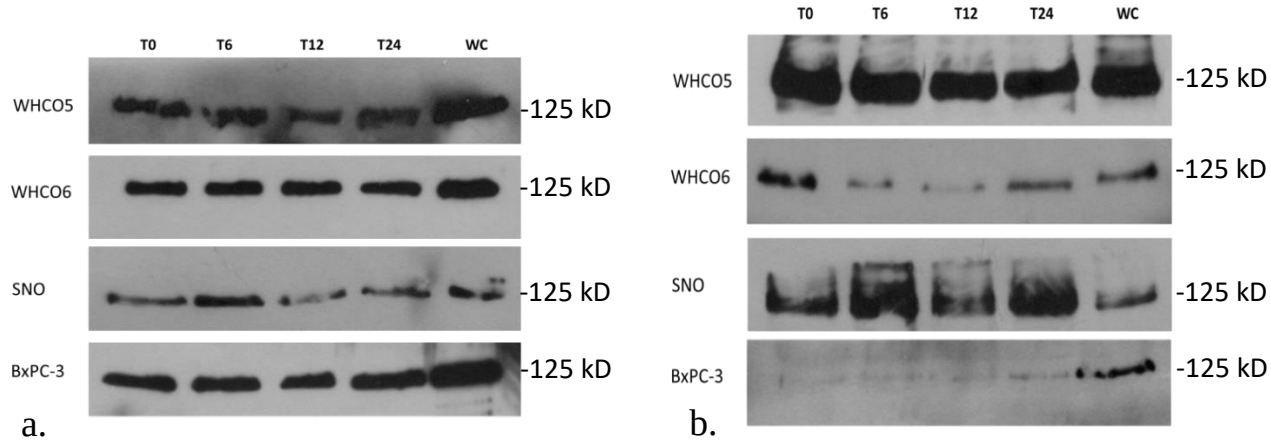


Fig. 3.5. Immunodetection of total and active focal adhesion kinase through a 24 hr time-course migration assay. Protein was extracted at time intervals of 0,6,12 and 24 hours (T0-T24) and resolved against a whole cell samole which served as a loading control (WC) a.) Total FAK, in all cell lines except for SNO, the abundance remains relatively constant. b.) Active FAK (pFAK(Tyr397) abundance varies most prominently in the WHCO6 and SNO cell lines, while steadily increasing in BxPC-3 cells.

To determine whether or not pGSK3β abundance is affected during cell migration, levels of pGSK3β(Ser9) and pGSK3β(Tyr216) were assessed by immunodetection. Alterations in levels of p GSK3β (Tyr216) are evident in all three oesophageal cancer cell lines (Fig. 3.6.). While only the WHCO6 cells show a clear decrease in the abundance of pGSK3β (Ser9), the levels in both WHCO5

and SNO cells appear relatively unchanged over 24 hours. Interestingly, both WHCO6 (Fig.3.6.a) and SNO (Fig.3.6.b) show a marked decrease in pGSK3β (Tyr216) abundance at 24 hours, which contrasts with the increase in levels of pFAK(Tyr397) observed in Fig. 3.5.b above. This, in conjunction with the slight recovery of pGSK3β (Tyr216) levels and the slight decrease in the abundance of pFAK(Tyr397) detected in WHCO5 cells, imply that an association between GSK3β and the process of cell migration may exist in HOSCC.

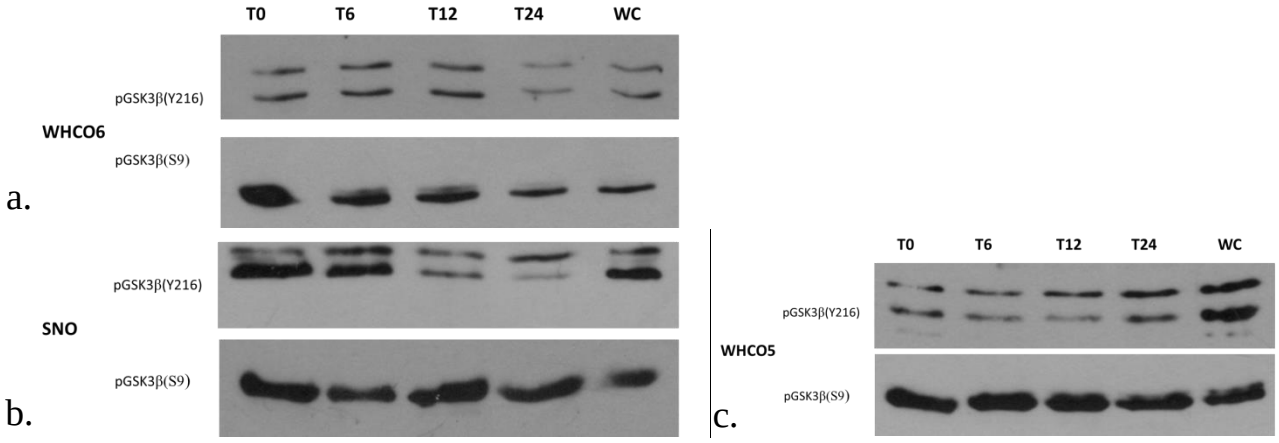


Fig. 3.6. The effect of cell migration on the abundance of pGSK3β(Tyr216) and pGSK3β(Ser9) in HOSCC. a.) WHCO6 – both active(Tyr216) and inactive(Ser9) GSK3β decrease over 24 hours, however pGSK3β(Tyr216) abundance remains constant until 12 hours before declining between 12 and 24 hours whil pGSK3β(Ser9) appears to decrease steadily b.)SNO cells exhibit a decrease in active GSK3β abundance after 6 hours which continues until pGSK3β(Tyr216) is almost entirely diminished at 24 hours. The inactive form appears relatively unchanged. c.) WHCO5 cells interestingly show decreases in the abundance of pGSK3β up to 12 hours before increasing again between 12 and 24 hours. Inactive GSK3β abundance seems unchanged.

3.3. Abrogation of GSK3β function in HOSCC

3.3.1. Impeded GSK3β function alters resting levels of pFAK(Tyr397) and pβ-catenin(Ser33/Ser37/Thr41)

GSK3β is a serine-threonine kinase; as such its inhibition may result in various changes in cell signalling. Before assaying the effect of this inhibition during cell migration it is first important to assess the effect of the GSK3β specific inhibitor (AR-A014418) on non-migrating cells in culture.

The AR-A014418 inhibitor has been shown to reduce levels of pGSK3β (Tyr216) in various pancreatic cancer cell lines (including BxPC-3; Kitano *et al*., 2013). As the analysis to follow relies heavily on the ability to visualise cells clearly in order to obtain an idea of the physical migratory response, the decision was taken to exclude the WHCO5 and A431 cells from this step in the study as the borders of individual cells are known to be indistinct which made it a challenge to identify laemellipod formation and observe morphological changes via light microscopy, without the aid of a dye.

Densitometric analysis of the immunoblots obtained from cells in which GSK3β was abrogated indicated that after the cells were challenged with AR-A014418 for 24 hours, the abundance of tyrosine phosphorylated GSK3β decreased in all four HOSCC cell lines. The most pronounced change was observed in the WHCO1 cell line, where pGSK3β (Tyr216) is almost entirely diminished (Fig.3.7. a.) and the WHCO3 cells where a decrease of approximately 80% was seen. Additionally, the WHCO6 and HT29 cells also showed a marked reduction in levels of active GSK3β (see Appendix B, Section 6, Table B2)

GSK3β-inhibition has a minimal (< 20 % decrease) influence on total FAK abundance in the WHCO1 and WHCO3 cell lines (Fig. 3.7. b.). SNO and HT29 both exhibit an approximately 30% change in active FAK levels in response to GSK3β-inhibition, although the former increased while the latter decreased by that amount (Fig. 3.7. c.). Similarly, levels of tyrosine 397-phosphorylated FAK (Fig.3.7. c.) in WHCO6 cells also decreased greatly due to GSK3β inhibition. The SNO cell line, however, exhibited an increase (Fig. 3.7. c.) in the abundance of pFAK(Tyr397) despite the relatively unchanged (Fig. 3.6. b.) abundance of total FAK. Furthermore, SNO cells exhibit the highest levels of FAK phosphorylation following GSK3β inhibition (see Appendix B, Section 6, Table B3)

As mentioned previously the Wnt associated protein β-catenin, is marked for ubiquitination by the β-catenin destruction complex, GSK3β forms a key part of that complex by phosphorylating β-catenin at the Ser33, Ser37 and Thr41 residues. Thus pβ-catenin can therefore be used as a measure of the presence of active GSK3β, and its abundance is expected to decrease in the presence of a GSK3β -inhibitor. Curiously, only the WHCO1, WHCO6 and HT29 cell lines show decreases (Fig.3.7.d.) in the abundance of pβ-catenin following GSK3β inhibition by the selective inhibitor AR-A014418. It must also be noted that the abundance of pβ-catenin was not expected to decrease in the HT29 cell line as a result of GSK3β inhibition, due to the presence of truncated APC present in this cell line which disrupts the formation of the β-catenin destruction complex. The WHCO3, SNO and BxPC-3

cell lines all show increases in the abundance of pβ-catenin (Fig. 3.7. c). Interestingly, these three cell lines also show increases in the abundance of pFAK(Tyr397) in response to GSK3β inhibition, suggesting an association may exist between FAK activation and GSK3β activity within the cell. Understanding the effect of cell migration on these three proteins both in the presence and absence of GSK3β inhibition may help in part to either validate or disprove the existence of an association between them.

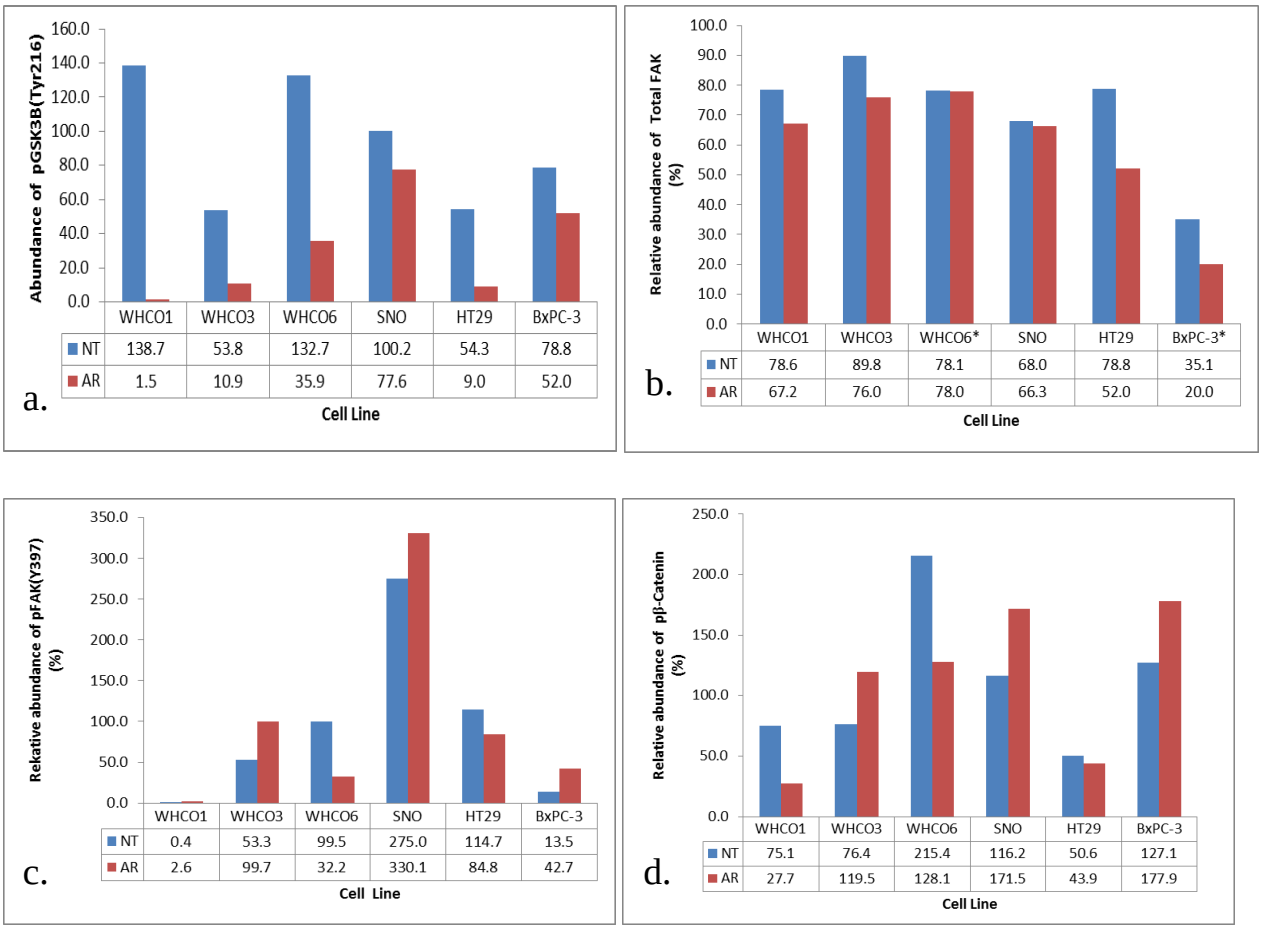
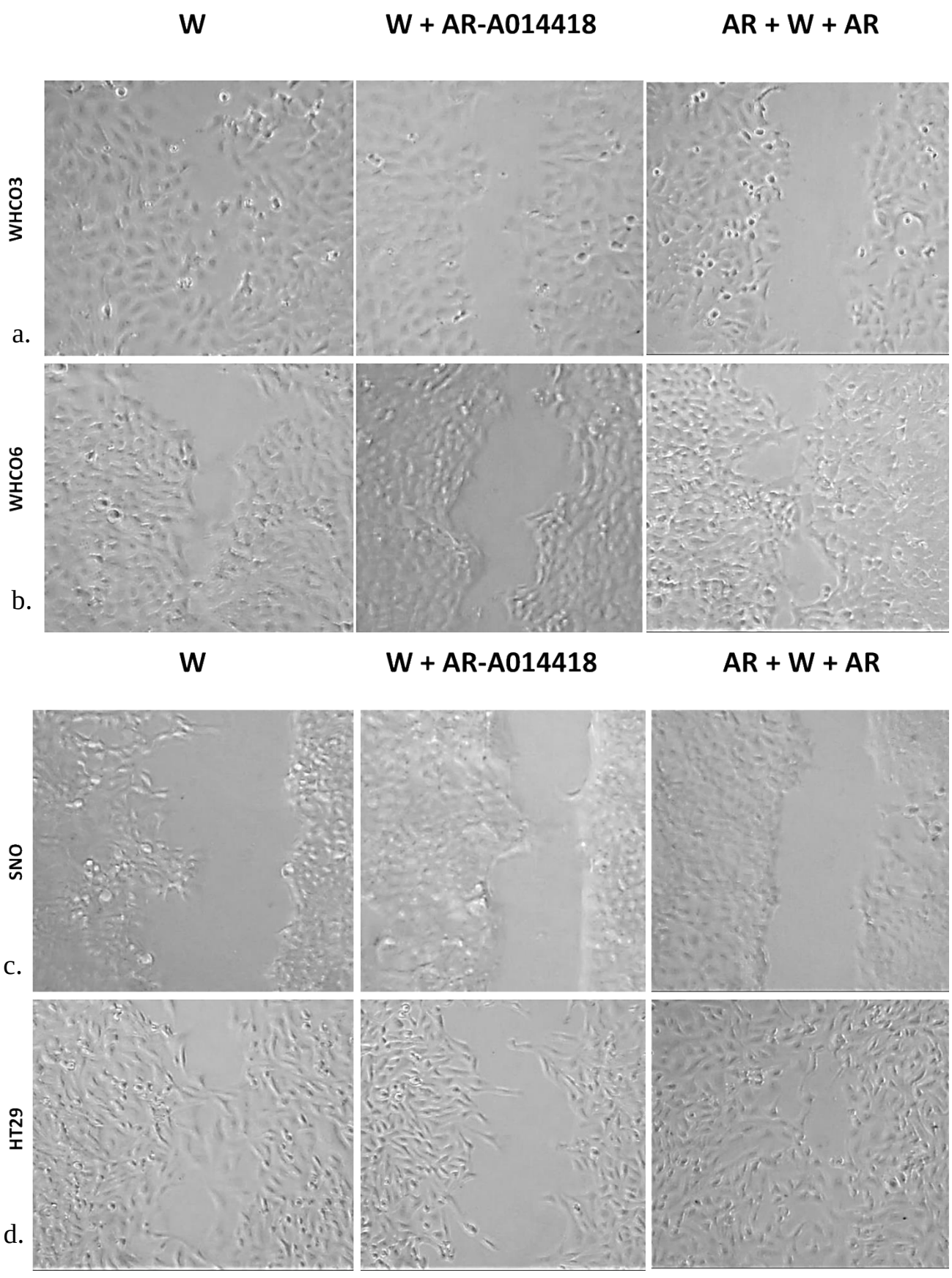


Fig. 3.7. The Influence of GSK3β inhibition on the abundance of total and activated FAK, as well as on the phosphorylation of the GSK3β-substrate, β-catenin. 40 µg of whole cell protein lysate was resolved on 10% gel a.) pGSK3β(Tyr216) decreased as expected in all cell lines in response to the AR-A014418 (AR) GSK3β inhibitor when compared to untreated (NT) cells b.) total FAK exhibits varying changes in abundance in response to GSK3β abrogation . c.) phospho-FAK(Tyr397) levels in HOSCC vary with WHCO3, SNO and BxPC-3 cells showing increases in levels of active FAK, while WHCO6 and HT29 cells show decreases d.) levels of phopho –β-catenin were expected to decrease in HOSCC in response to GSK3β abrogation. This is only observed in the WHCO1 and WHCO6 cell lines.

3.3.2. Inhibition of GSK3β alters the migratory response in HOSCC

There are multiple pathways which link both upstream and downstream effectors of GSK3β to cell migration, however, it is imperative to determine whether the changes in the levels of the phosphorylated forms of GSK3β observed during cell migration over 24 hours (Fig. 3.5.) occurred as a result of cells migrating, or rather occurred in order to either impede or facilitate migration into the wound. To do this end, cells were wounded as described previously (2.3.1.) and subsequently treated with 10μM AR-A014418 (AR) specific GSK3β inhibitor and incubated for 24 hours to allow for sufficient cell migration into the wound.

The inhibition of GSK3β has varying effects on epithelial cancer cell migration, with distinct differences between the different HOSC cell lines. In the WHCO3 (Fig. 3.8. a.), WHCO6 (Fig. 3.8. b.), and SNO (Fig. 3.8. c.) cell lines, active GSK3β appears to favour the promotion of cell migration into the wound (Fig. 3.8.). This was inferred from the prominent decrease in the number of cells which have breached the wound "boundary" to enter the uninhabited region (as seen with the HT29 cells Fig. 3.8. d.) when GSK3β is inhibited. Additionally, these cells, when treated with the inhibitor (W + AR-A014418) have fewer laemellipodia (SNO, Fig. 3.8.c.) and also display slight changes in morphology, moving away from the "flattened" configuration associated with cell migration onto a surface (WHCO3, Fig. 3.8.a.). The WHCO1 (Fig.3.8.e.) and BxPC-3 cells (Fig.3.8.f.) on the other hand, behave contrarily whereby the inhibition of GSK3β results in increases in laemellipod formation and migration into the wound when compared to the migration in the absence of GSK3β inhibition. This suggests that GSK3β antagonises cell migration in these cell lines. Active GSK3β appears to promote cell migration in the WHCO6, WHCO3 and SNO cell lines; a characteristic shared with the HT29 colorectal carcinoma cells, while the WHCO1 and BxPC-3 cell lines show an increase in cell migration following GSK3β inhibition suggesting that active GSK3β counteracts cell migration. It can therefore be inferred that different GSK3β signalling pathways may be active in the different HOSCC cell lines.



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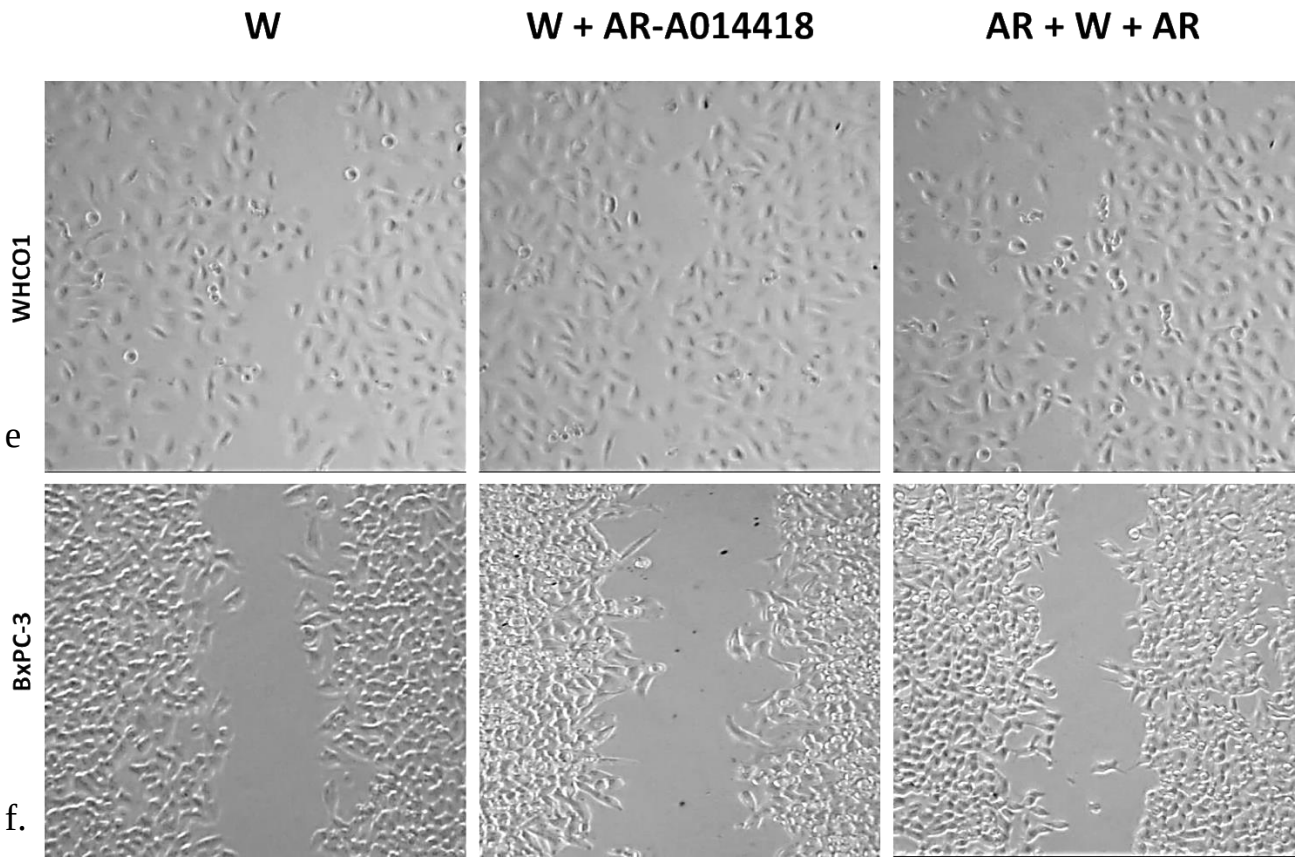
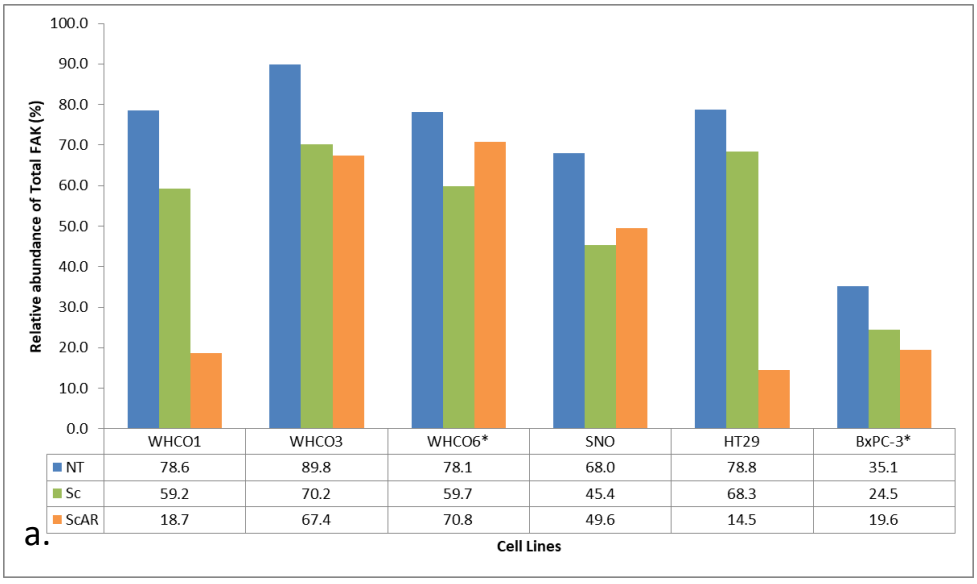


Fig. 3.8. The influence of GSK3β-inhibition on cancer cell migration in a range of epithelial cancers. A wound was created on the surface of a 75% confluent 60 mm dish of cells to initiate cell migration following which the cells were supplemented with 3 ml of DMEM:F12(3:1) and incubated immediately for 24 hours at 37^{O}C and 5% CO_2 (W), or wounded and treated with 10μM AR-A014418 inhibitor in DMEM before incubation (W+AR-A014118) or treated with AR-A014418 1 hour prior to wound initiation and then being treated again with AR-A014418 and incubated for 24 hours (AR + W +AR) a.) WHCO3 b.)WHCO6 c.) SNO d.) HT29 e.) WHCO1 f.) BxPC-3. GSK3β inhibition enhanced the migratory response observed in WHCO1, SNO and BxPC-3 cells, but abrogated it in WHCO3, WHCO6 and HT29 cells. In the WHCO6, HT29 and WHCO1 cells inhibition prior to wounding appeared to reverse the effects activate alternate pathways which resulted in a migratory response similar to cells with uninhibited GSK3β.

Immunoblot analysis revealed that the observed morphological changes were partially accompanied by alterations at a biochemical level. In the absence of GSK3β inhibition all the cell lines showed a decrease in levels of total FAK when compared to the unwounded, untreated cells (NT; Fig.3.9.a). GSK3β inhibition coupled with cell migration (ScAR) resulted in negligible differences in the

abundance of FAK in WHCO3, BxPC-3 and WHCO6 cells when compared to the abundance of FAK in uninhibited cells (Sc). The WHCO1 and HT29 cell lines produce notable differences of 68 % and 78 % respectively, while FAK abundance increased by approximately 44 % in SNO cells. Interestingly, abundance of active FAK in the WHCO1 cells is barely detectable (Fig. 3.9.b), becomes apparent during migration for 24 hours, but is diminished again upon AR-A014418 treatment of migrating cells. It should be noted that only the WHCO3 cells exhibit minor elevation in the abundance of pFAK(Tyr397) (Fig. 3.9.b) while in all the other HOSCC cell lines (Fig. 3.9.b), the abundance appears to decrease consistently (see Appendix B, Section 6, Table B3).

A further point of interest is that, similar to the initial inhibition of HOSCC cells with a GSK3β inhibitor only the WHCO1, HT29 and WHCO6 cells show decreases in the abundance of pβ-catenin(Ser33/Ser37/Thr41) (Fig.3.9.c) following the canonical phosphorylation pathway. However, the absence of the expected decrease in the other cell lines further supports the existence of an alternate "rescue" pathway within the HOSCC cell series. Moreover, this presents the question as to whether these cells would be able to activate a rescue pathway in order to counteract the effects of GSK3β inhibition on cell migration.



	WHCO1	WHCO3	WHCO6*	SNO	HT29	BxPC-3*
NT	78.6	89.8	78.1	68.0	78.8	35.1
Sc	59.2	70.2	59.7	45.4	68.3	24.5
ScAR	18.7	67.4	70.8	49.6	14.5	19.6

a.

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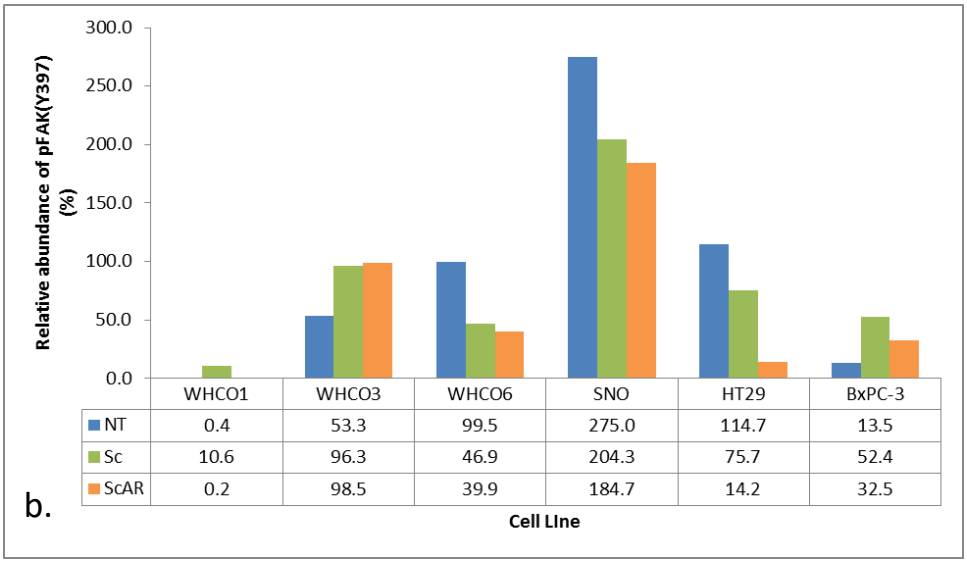


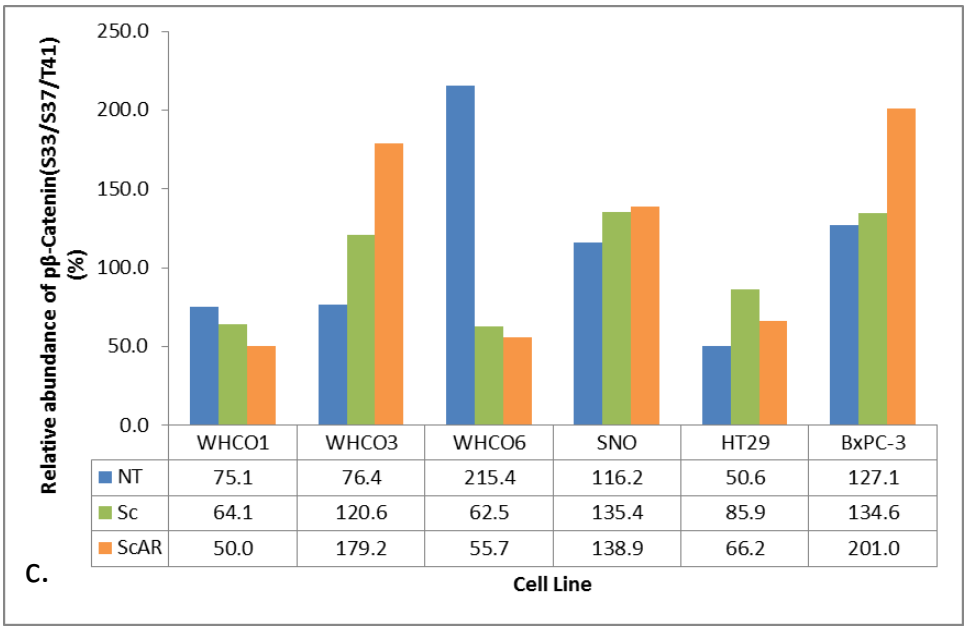
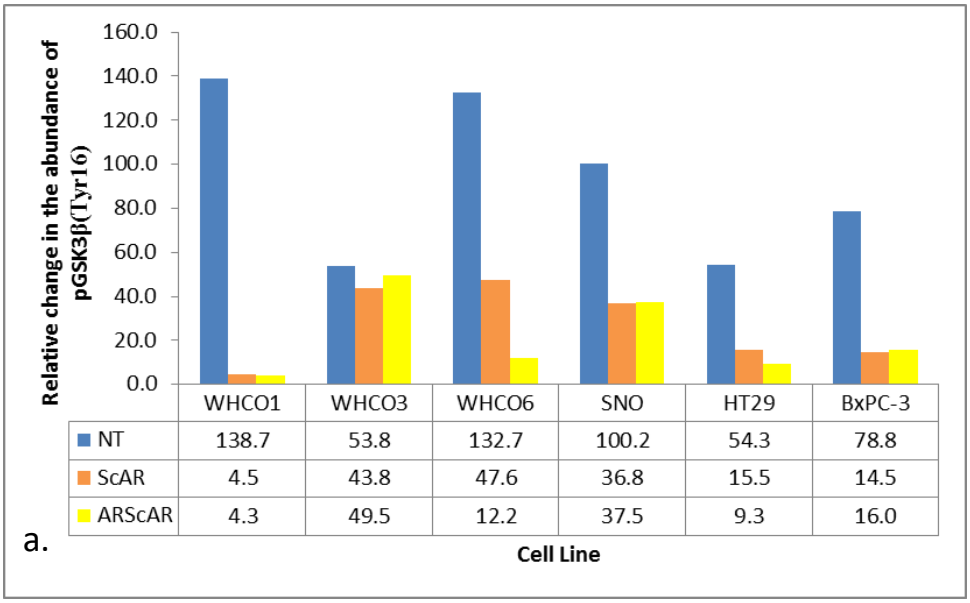


Fig. 3.9. The effect of GSK3β-inhibition on the protein abundance during the migratory response. "Sc" represents cells where migration was stimulated by wounding and "ScAR" represents cells treated with 10 μM AR-A014418 GSK3β inhibitor post wounding. a.) Total FAK abundance decreases in all cell lines in as a result of migration, most noticeably in WHCO6 and BxPC-3 cells. GSK3β inhibition enhances this decrease in WHCO1 and HT29 cells in particular. b.) pFAK(Tyr397) decreases in abundance in the WHCO6, SNO and HT29 cells but increases in the WHCO1,WHCO3 and BxPC-3 cells following the stimulation of migration. Coupled with GSK3β inhibition, migrating HOSCC cells show negligible changes in active FAK abundance. c.) Changes in pβ-catenin(Ser33/37/Thr41) abundance is observed in all cell lines in response to cell migration. Only the WHCO3 and BxPC-3 cell lines exhibit changes in the abundance of pβ-catenin in response to GSK3β inhibition, both cell lines exhibit increases in abundance.

Inhibiting GSK3β prior to the stimulation of cell migration and subsequently maintaining that inhibition for 24 hours resulted in prominent changes in the migratory response observed in HOSCC cells (Fig.3.8.). This sustained inhibition caused an increase in laemellipod extrusion and overall migration into the uninhabited region of the dish in the WHCO6 (Fig. 3.8.b.) and HT29 (Fig.3.8.d.) cells, thereby mimicking the response seen when GSK3β is uninhibited (refer to the images marked "W" in Fig. 3.8.) Conversely, the WHCO1 (Fig.3.8.e) cells show a sustained degree of migration (compared to "W", Fig. 3.8.e) into the wound site but the cell population away from the wound-edge appears less dense than when GSK3β was inhibited following the stimulation of migration. The migratory response in WHCO3 (Fig. 3.8.a), SNO (Fig. 3.8.c) and BxPC-3 (Fig. 3.8.f.) cell lines however, appear unchanged by the timing of GSK3β abrogation as neither laemellipod formation nor migration into the wound appears enhanced or diminished.

These trends are possibly reflected by differences in the abundance of both pGSK3β(Tyr216) and pβ-catenin(Ser33/37/Thr41) observed in cells wounded before GSK3β inhibition versus those wounded one hour after GSK3β was inhibited. The epithelial carcinoma cell lines that did not show sequence-dependent changes (dependent on the order in which the stimulation of migration and GSK3β inhibition occurred) in the migratory response, i.e.WHCO3, SNO and BxPC-3 cell lines, showed negligible changes in the abundance of tyrosine-phosphorylated GSK3β (Fig. 3.10. a.) and decreases in the abundance of p pβ-catenin(Ser33/37/Thr41) that were less than 25 % (Fig. 3.10. b.; see Appendix B, section 6, Table B2). The cell lines which exhibited changes in migration, however, showed changes in pβ-catenin(Ser33/37/Thr41) levels of approximately 50% in the WHCO1 cells and a major 270% increase in WHCO6 cells (Fig.3.10. b.). Interestingly, the increase observed in WHCO6 cells is accompanied by a dramatic 74% decrease in the abundance of pGSK3β(Tyr216) while the abundance of active GSK3β in WHCO1 cells shows no observable change (Fig.3.10.a). Furthermore, levels of pFAK(Tyr397) (see Appendix B; section 6) appear relatively unchanged in the WHCO1 and WHCO6 cells but levels of total FAK fluctuate in these cell lines(Appendix B; section 6). WHCO1 cells which show a decrease in the density of cell population show and almost 100% increase in the abundance of total FAK, while WHCO6 cells which show increased migration into the wound, display a decrease in the total FAK abundance (approximately 30%) (see Appendix B, section 6, Table B3). As FAK is known to localise to sites of cell-ECM contact (Takada *et al*., 2007) it is possible that the composition of the ECM may affect cell-ECM attachment and subsequently affect the migratory response observed in HOSCC.



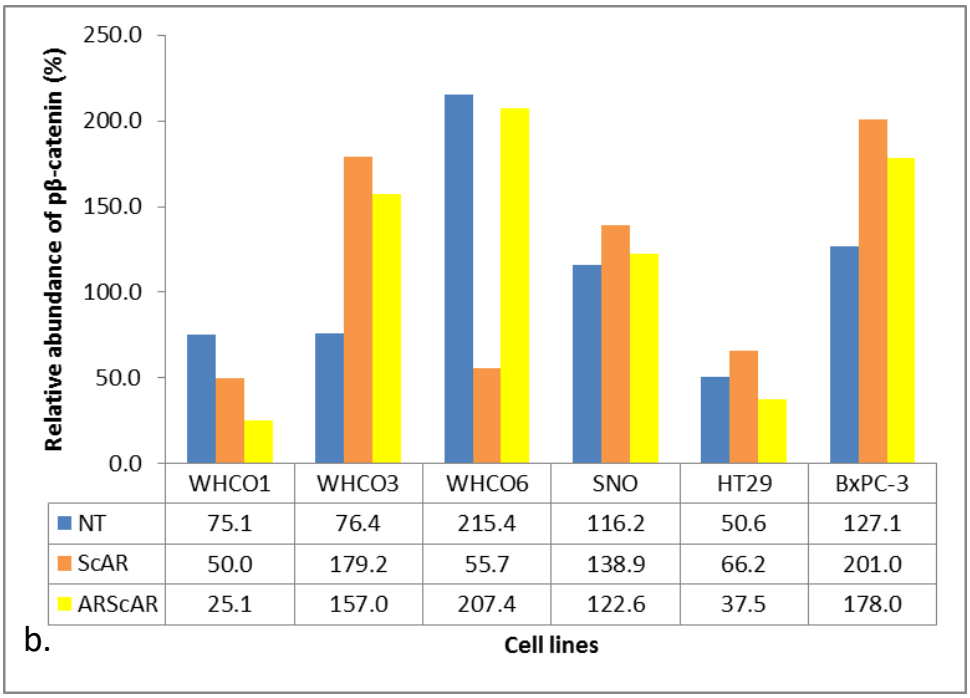


Fig. 3.10. Abrogation of GSK3β, prior to the stimulation of cell migration, alters the abundance of pGSK3β(Tyr216) and pβ-catenin(Ser33/37/Thr41). "NT" represents, untreated cells, "ScAR" denotes cells which were wounded and then immediately treated with 10 μM AR-A014418, GSK3β inhibitor and "ARScAR" denotes cells exposed to 10 μM AR-A014418 for 1 hour prior to, and then immediately after wounding. a.) The abundance of pGSK3β(Tyr216) in WHCO6 and HT29 cells is diminished to a greater extent as a result of result of prior-inhibition of GSK3β. b.) Prior inhibition of GSK3β results in a major increase In the abundance of pβ-catenin(Ser33/37/Thr41) in WHCO6 cells, decrease was observed in HT29 cells, while WHCO1 cells exhibited a more pronounced decrease in the abundance of pβ-catenin(Ser33/37/Thr41).

3.4. HOSCC cells display ECM-substrate specific activation of pFAK and GSK3β

3.4.1. Collagen and Fibronectin differentially affect FAK activation in Human Oesophageal squamous cell carcinoma

Grown on the ECM substrate, collagen I for 24 hours, the HOSCC cell lines showed variation in the levels of pFAK(Tyr397). These results are presented as a percentage change in the relative abundance of the protein, extracted from cells cultured on substrate-coated dishes, to the abundance of the same protein extracted from cells cultured on uncoated dishes.

With the exception of the WHCO5 and WHCO6 cell lines, a collagen-rich substrate was observed to cause the level of pFAK(Tyr397) to decrease in all the oesophageal cancer cell lines in addition to the A431, HT29 and BxPC-3 cell lines. A decrease of 96 % can be seen in the WHCO1cells (Fig.3.11.a.). This drastic decrease makes the presence of pFAK(Tyr397) almost undetectable in these cells. Furthermore, collagen-supplemented growth resulted in a 40 % decline in levels of pFAK(Tyr397) observed in WHCO3 cells, while pFAK(Tyr397) in SNO cells appears to be the least diminished when cultured on collagen decreasing in abundance by approximately 26 %. The HT29, A431 and BxPC-3 cell lines exhibit pFAK(Tyr397) depletion in the range of 60 % to 40 %. Although levels of active FAK were noted to increase in the WHCO5 and WHCO6 cell lines, these increases were only of 13.9 % and 15.8 % (Fig.3.11.a.) respectively.

Cell growth on a fibronectin-supplemented surface (Fn) produced the same general profile as cells cultured on the Cn-coated dishes. It should be to noted that, with the exception of the WHCO5 and A431cell lines (3.11.b.) the variation in the abundance of pFAK(Tyr397) as a result of growth on a fibronectin surface is slightly less pronounced for some of the cell lines than that of those observed after Cn-treatment (Fig.3.11.a.). This is illustrated by the 88 % reduction in the abundance of active FAK in the WHCO1 cell line and the 23 % reduction observed in WHCO3 cells. The WHCO5 and A431 cell lines which show an 82 % and 122 % increase in active pFAK. The WHCO6 cell line exhibited a negligible increase of 1.3%.

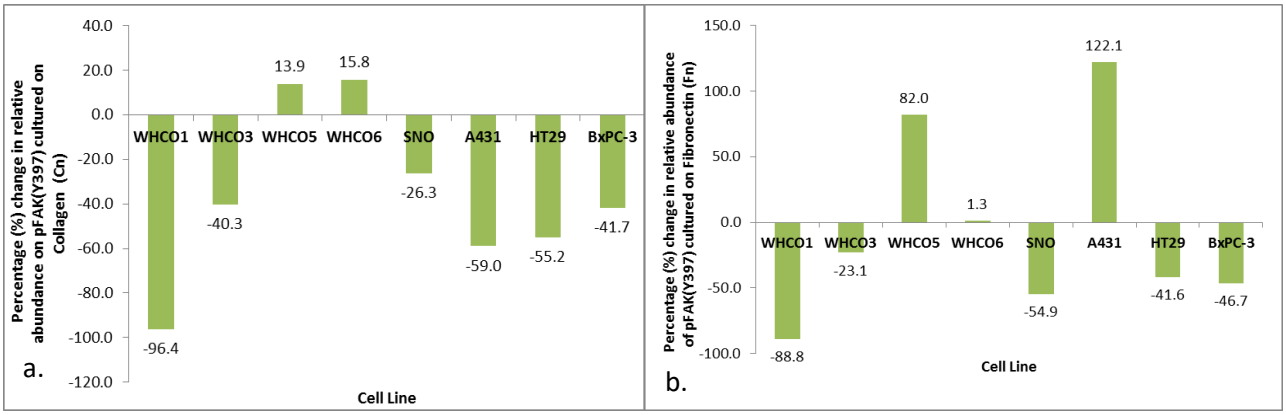


Fig.3.11.The effect of cell growth on an ECM-substrate supplemented surface on the abundance pFAK(Tyr397). Values are presented as a percentage change in protein abundance compared to cells cultured on normal TC dishes a.) Cultured on 100 µg/ml rat-tail tendon Collagen I (Cn), all tumorigenic cell lines, excluding WHCO5 and WHCO6 cells, showed greater than 25 % decreases in pFAK(Tyr397) abundance. b.) Cultured on 10 µg/ml Fibronectin (Fn, Roche) reduced levels of WHCO1 almost entirely while decreases in abundance also occurred in WHCO3, SNO, HT29 and BxPC-3 cells. Negligible change was observed in the WHCO6 cells while the abundance of activated FAK was increased by 80 % and 120 % in WHCO5 and A431 cells respectively.

3.4.2. ECM-substrate supplemented growth differentially affects the abundance of active and inactive GSK3β

To determine the influence of ECM composition on the activation and inactivation of GSK3β, HOSCC cells were cultured on the ECM-substrates collagen I and fibronectin. Levels of pGSK3β(Ser9) and pGSK3β(Tyr216) were assessed by western blot analysis.

Thus, in response to collagen-supplemented growth the WHCO5, WHCO6 and A431 cells all showed decreases ranging less than 20 % while a 67 % decline in the abundance of inactive GSK3β was observed the HT29 cell line (Fig 3.12.a.). Interestingly, the abundance of inactive GSK3β in BxPC-3 and SNO cells was relatively unchanged, while the WHCO3 cell line underwent a 40 % increase in pGSK3β(Ser9) abundance (Fig 3.12.a.). Using fibronectin as an ECM substrate resulted in increases of 5.3 % and 13.1 % in the abundance of Ser9 phosphorylated GSK3β in WHCO3 and

WHCO5 cells respectively (Fig 3.12.b). The WHCO6, HT29 and BxPC-3 cells all exhibit decreases in the abundance of inactive pGSK3β (Fig 3.12.b). The most pronounced decreases were observed in the HT29 and WHCO6 cells. To fully understand the influence of ECM composition on GSK3β phosphorylation in HOSCC, it is necessary to also assess changes in the abundance of the active GSK3β phosphor-species.

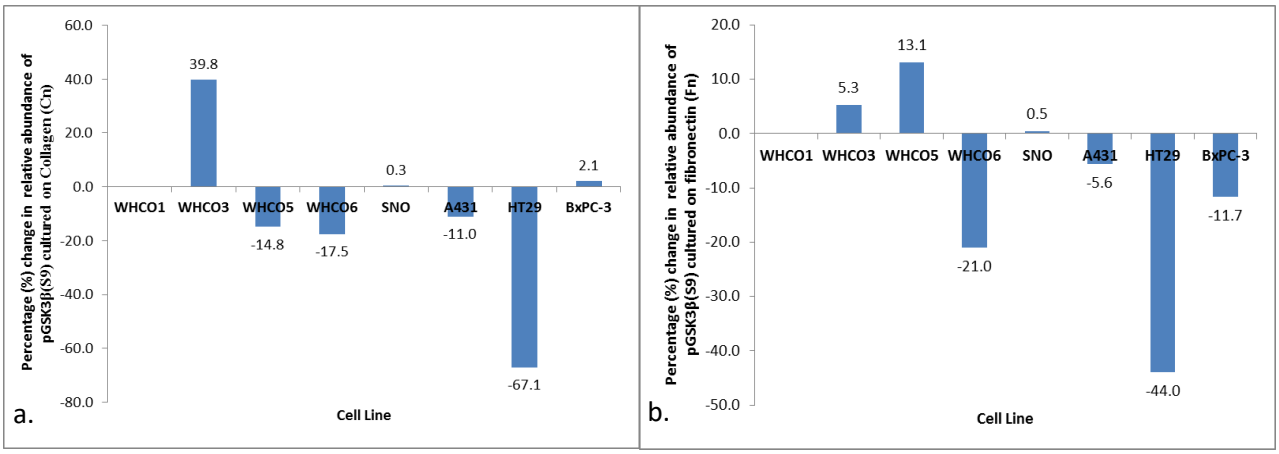


Fig. 3.12. The effect of cell growth on an ECM-substrate supplemented surface on the abundance of pGSK3β(Ser9). Values are presented as a percentage change in protein abundance compared to cells cultured on normal TC dishes a.) Cultured 100 µg/ml rat-tail tendon collagen I (Cn), decreases in the abundance of pGSK3β(Ser9) were observed in WHCO5, WHCO6 and A431 cells (<20 %), the HT29 cells showed a decrease of 67 %. WHCO3 cells exhibited an increase of approximately 40% b.) Culture 10 µg/ml fibronectin (Fn, Roche) for 24 hours resulted less pronounced changes in the abundance of pGSK3β(Ser9) observed in all cell lines compared to collagen supplemented growth, except for BxPC-3 cells which appeared further decreased and WHCO5 cells where the abundance of inactive GSK3β increased. Both of these changes were less than 20 %.

Similar to serine phosphorylated GSK3β, marked changes in the abundance of pGSK3β(Tyr216) were not observed when the WHCO, SNO and control cell lines were cultured on ECM-substrate coated tissue culture plates.

However, a decrease of 53.3 % in the abundance of pGSK3β(Tyr216) was observed in WHCO3 cells cultured on collagen (Fig. 3.13.a.), while SNO cells exhibit a major increase (248 %) in the abundance of pGSK3β(Tyr216) (Fig. 3.13.a.). This massive increase is conserved in cells cultured on fibronectin (Fig.3.13.b.) where the abundance of pGSK3β increases 2.5 times (by 250%) in SNO

cells but only 35% in BxPC-3 cells. HT29 cells, exhibit a 67% increase in tyrosine-activated GSK3β when cultured on fibronectin compared to "untreated" cells but decreased by 39.7 % on collagen.

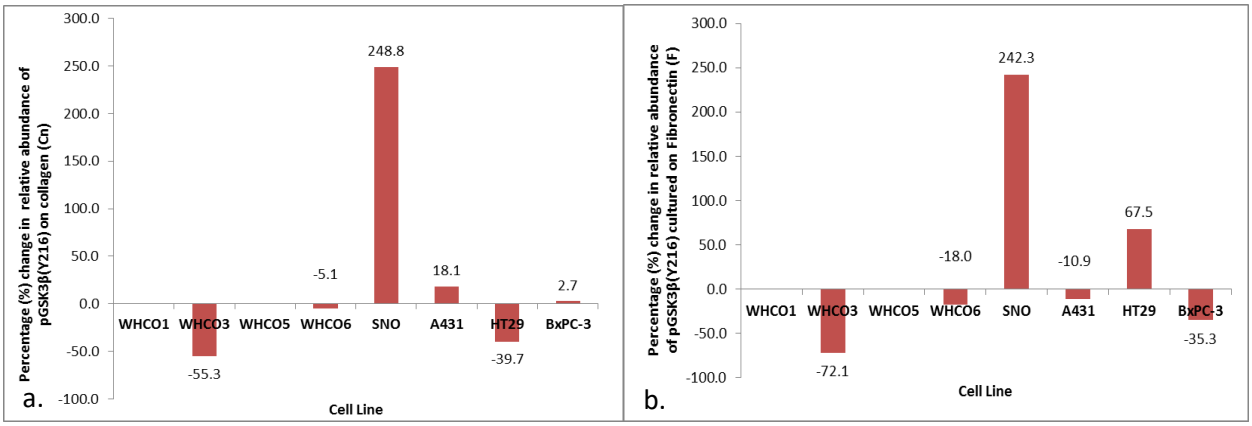


Fig. 3.13. Levels of pGSK3β(Tyr216) as a result of ECM-substrate supplemented growth. Values are presented as a percentage change in protein abundance compared to cells cultured on normal TC dishes. a.) Culture on 100 µg/ml rat-tail tendon Collagen I (Cn) resulted in decreases in the abundance of pGSK3β(Tyr216) in WHCO3 and HT29 cells and a massive increase was seen in the SNO cells (approximately 250%) and b.) Culture on 10 µg/ml Fibronectin (Fn, Roche) resulted in a more pronounced decrease in the abundance in pGSK3β(Tyr216) than was observed on collagen, but the major increase observed in SNO cells remains similar to collagen (250%) while the HT29 cells now exhibit a 67 % increase in active GSK3β abundance and the BxPC-3s exhibit a 35 % decrease.

Chapter 4: Discussion

The serine-threonine kinase Glycogen Synthase Kinase 3β (GSK3β), has, in recent years, become established as hub for a myriad of intracellular signalling pathways (Plyte *et al*., 1992; Frame and Cohen, 2001; Ferkey and Kimelman, 2007; Wu and Pan, 2009). Many of these pathways have been implicated in cell cycle progression, proliferation and migration in highly tumorigenic cancers (Mishra, 2010; Wang *et al*., 2011; Zheng *et al*., 2013). GSK3β is ubiquitously expressed and considered to be constitutively active, and phosphorylation at the N-terminus Serine 9 residue results in its inhibition (ter Haar *et al*., 2001). Interestingly, another prominent phosphorylation at tyrosine 216 in the activation loop has been reported to increase GSK3β activity 200-fold (Hughes *et al*., 1993), as such pGSK3β(Tyr216) is often referred to as active GSK3β. The understanding of its role in human oesophageal squamous cell carcinoma however, has been limited to its incorporation as part of the β-catenin destruction complex in the Wnt pathway. Studies in other epithelial carcinomas have revealed that GSK3β is a prominent regulator of the actin cytoskeleton during cell migration (Koivisto *et al*., 2009). Additionally focal adhesion kinase (FAK), a non-receptor tyrosine kinase, which mediates signalling at focal adhesions (Sieg *et al*., 1999), has the ability to phosphorylate GSK3β at tyrosine 216, thereby favouring the Wnt-on pathway (Gao *et al*., 2015). This allows for the cytoplasmic accumulation and subsequent nuclear translocation of β-catenin, where it behaves as a transcription factor resulting in cell cycle progression and the production of matrix metalloproteases. As such it is pertinent to understand the influence of GSK3β on cell migration in human oesophageal squamous carcinomas.

4.1. The abundance of active and inactive GSK3β appears tightly controlled in HOSCC

Analysis of phospho-GSK3β levels in the WHCO and SNO cell series revealed that the serine-phosphorylated form (pGSK3β(Ser9)) is present in high abundance in all the WHCO cell lines, as the levels of pGSK3β detected in these cell lines was more than two times greater than the level detected in the HT29 colorectal cancer cell line, known for elevated levels of pGSK3β(Ser9) (Wang *et al*., 2009). Similarly, the BxPC-3 pancreatic cancer cell line displays elevated levels of tyrosine-phosphorylated GSK3β (pGSK3β(Tyr216); Kitano *et al*., 2013) and diminished levels of the inactive pGSK3β(Ser9). Interestingly, all the WHCO cell lines, despite the presence of increased quantities of pGSK3βSer9), also possess levels of pGSK3β(Tyr216), almost equivalent to that of the BxPC-3

cells. This suggests that the moderately differentiated HOSCC favours neither phosphorylated form and instead GSK3β is phosphorylated as required by the cell for survival.

While characterising the occurrence of GSK3β in oesophageal cancer cells, it is also pertinent to monitor its effects on its direct downstream targets. The most well-established of which is β-catenin. Formerly established as only a cadherin-associated scaffolding protein (see review by Kemler, 1993), β-catenin is now also known to translocate to the nucleus and initiate the transcription of a number of genes responsible for cell cycle progression (Nusslein-Volhard and Wieschaus, 1980). This is however, precluded by its incorporation and subsequent phosphorylation by what is known as the B-catenin destruction complex. GSK3β plays an integral role in this complex by directly phosphorylating β-catenin at the Thr41, Ser37 and Ser33 residues, thereby marking it for ubiquitination by the beta-transducin repeat containing protein (B-TrCP) (Yost *et al*, 1996 and Lui *et al*, 2002). Thus it stands to reason that measuring the abundance of pβ-catenin(Ser33/37/Thr41) would serve as a measure of uninhibited GSK3β signalling within the cell. As the occurrence of β-catenin destruction complex formation and the subsequent ubiquitination and degradation of β-catenin have been confirmed throughout the HOSCC cell series (Bezuidenhout, 2008), this signal transduction pathway (signalling axis) appears uncompromised by carcinogenesis.

Varying levels of pβ-catenin(Ser33/37/Thr41) was detected at in all the HOSCC cell lines. Interestingly, the WHCO3 and WHCO6 cell lines showed the largest amount of β-catenin phosphorylation. While the WHCO1 cells have the lowest level of pβ-catenin(Ser33/37/Thr41) of all the HOSCC cell lines, only slightly higher than the HT29 cells which serve as a negative control for destruction complex formation. It cannot be said, however, that WHCO1 cells behave in a similar manner to HT29 colorectal cancer cells as the HT29 cells possess a truncated isoform of APC, which impedes the formation of the β-catenin destruction complex, thereby preventing β-catenin phosphorylation at the Ser33/37 and Thr41 residues. As destruction complex formation and the subsequent degradation of β-catenin have been shown to occur in WHCO1 cells (Bezuidenhout, 2008), the reduced phosphorylation of β-catenin in WHCO1 cells may be an artefact of the fact that a largest portion of cellular B-catenin is plasma membrane associated in WHCO1 cells despite the highest abundance of total β-catenin being detected WHCO3 and WHCO6 cell lines (Bezuidenhout, 2008). It is well established that, within the cell, different "pools" of β-catenin are dedicated to different cellular functions (Harris and Peifer, 2005). Thus it stands to reason that in general, cytoplasmic, not plasma-membrane (or cadherin)-associated β-catenin would be marked for ubiquitination and degradation. The variation in the abundance of pβ-catenin(Ser33/37/Thr41) within

the HOSCC cell series, may be attributed to the fact that GSK3β is known to localise to the cytoplasm (Leis *et al*., 2002 and Diehl *et al*., 1998).

4.2.Active GSK3β negatively regulates FAK activation during cell migration

4.2.1. An inverse association exists between active FAK and tyrosine-phosphorylated GSK3β in HOSCC

The focal adhesion associated, non-receptor tyrosine kinase (RTK), focal adhesion kinase (FAK) is a key component in the formation of focal contacts facilitated by integrin-mediated cell-ECM adhesion. In adherent cells, FAK co-localises with integrins to points of focal adhesions (Schlaepfer *et al*., 1999) where conformational changes in integrin-structure result in the activation of FAK by phosphorylation at the tyrosine 397 residue (Giancotti and Ruoslahti, 1999). As such changes in FAK and pFAK(Tyr397) were used to monitor the influence of GSK3β on cell-ECM interactions.

Baseline abundance reveals that FAK and activated FAK (pFAK(Tyr397)) are present in all the HOSCC cell lines. The variation in levels serves an early indicator for the presence of differences in signal transduction within members of the cell series. Nonetheless, FAK was found to be most abundant in the WHCO6 cell line and the least abundant in the HT29 cell line. Curiously, activated FAK (pFAK(Tyr397)) was found to be the most abundant in the WHCO5 cells. It is important to note that the anti-FAK polyclonal antibody detects both phosphorylated and non-phosphorylated forms of FAK, this suggests that FAK activation, by phosphorylation at tyrosine 397 (Giancotti and Ruoslahti, 1999), occurs minimally in all cell lines (see Results, section 3.2.1) This raises the question of, to what extent are both FAK and pFAK(Tyr397) altered during cell migration.

One of the most prominent alterations observed when monitoring cell migration over the course of 24 hours was the disorder introduced to the entire cell population as a result of wounding, which caused the monolayer to lose its typical paving stone-like (Tanaka *et al*., 1997) appearance as even cells distant from the wound-boundary become disordered. This suggests that the morphological effects of mechanical stress-induced changes in the adhesive-phenotype of HOSCC are not only localised to the individual cells in the immediate vicinity of the mechanical-stressor, in this respect; cells on the wound-edge, but are rather translated throughout the entire cell population.

Laemellipod extension into the wound was observed to occur at 12 hours in the three HOSCC cell lines, while extension was observed in the BxPC-3 control cells within the first 6 hours. This suggests that existing cellular FAK and pFAK may not influence the rate at which cellular migration

occurs in HOSCC as both WHCO5 and WHCO6 cells have higher levels of total and activated FAK than BxPC-3 cells yet initial laemellipod formation occurs much later than in BxPC-3 cells. Interestingly, the SNO and WHCO6 cells showed the highest degrees of wound-coverage which may be consistent with the findings of Freidl *et al.* (1995) in that epithelial carcinoma cells more commonly migrated as a sheet in during wound-healing. The prominently visible "cell-free zone" present in WHCO5 and BxPC-3 cells further implies that the process of cell migration is influenced by the entire cell environment and that cell migration may proceed in a haptotactic manner (Kobayashi *et al,* 2006). Moreover, it highlights the possibility that a positive feedback mechanism may control cell migration via the inactivation of GSK3β; similar to the function of the mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinase (ERK1/2) in MDCK normal canine keratinocytes (Matsubayashi *et al*., 2004). This mechanism would proceed until contact-inhibition of growth is initiated. Densitometric analysis revealed little change in the abundance of total FAK across the BxPC-3 and all three HOSCC lines. While the cell lines which showed maximum wound-coverage (SNO and WHCO6), exhibited elevated levels of activated FAK at 24 hours consistent with enhanced ERK-mediated positive feedback (Matsubayashi *et al*., 2004), the abundance of pFAK(Tyr397) increases slightly in pancreatic cancer and decreases in the WHCO5 cell line. This, however, may be consistent with the slower process of cell migration into an uninhabited region of the dish, observed in WHCO5 cells, whereby the cells have not extended onto the empty surface despite laemellipodia formation, thus new focal adhesions, to which FAK could be recruited and activated, are unable to form. Additionally, changes were observed in the abundance of the one of the two GSK3β phosphorylated species. The almost complete depletion of pGSK3β(Tyr216) after 24 hours of cell migration, contrasts strongly with the elevation in levels of activated FAK also observed in these cells. Similarly, the WHCO5 cells showed levels of pGSK3β(Tyr216) increased over the course of 24 hours while pFAK(Tyr397) decreased, suggesting an inverse association between pGSK3β(Tyr216) and active FAK similar to the mechanism proposed by Bianchi *et al*. (2005), whereby tyrosine-phosphorylated GSK3β triggers a series of phosphorylation steps which eventually leads to the dephosphorylation and resultant deactivation of FAK at tyrosine 397. This model, however suggested that this occurs as the result of a collagen-rich ECM (see below).

4.2.2. Inhibition of GSK3β alters the migratory response observed in HOSCC cells.

The highly metastatic quality of HOSCC (Tew *et al*., 2005) is thought to be a result of dysregulation in a multitude of cell signalling pathways (Kato *et al*., 2002; Miyazaki *et al*., 2003; further reviewed by Enzinger and Mayer, 2003) arising from a host of factors (Holmes and Vaughn; Umar and Fleischer, 2008). As such elucidating the driving forces behind the invasive nature of these cells becomes of key importance to further understand and possibly abrogate the overall signalling mechanisms of this metastatic potential. Using the moderately differentiated WHCO and SNO cell series as a model, it was established that that the abundance of tyrosine-phosphorylated, "active" GSK3β (pGSK3β(Tyr216)) is altered during the cell migration and that changes observed appeared to correlate negatively with changes in the levels of activated FAK(see section 4.2.1. above). Abrogation of GSK3β suggests that these variations do not occur as a result of cell migration but rather that GSK3β may be an essential signalling intermediate facilitating the migratory response in HOSCC.

Variation in the abundance of activated FAK and pβ-catenin(Ser3/37/Thr41) as a result of the inhibition of GSK3β in HOSCC cells at rest serves as an indicator of the dynamic influence of GSK3β in oesophageal squamous carcinoma. The abundance of pGSK3β(Tyr216) decreased across the range of cancer-types examined, however only two (WHCO1 and WHCO6) of the four HOSCC cell lines showed the expected decrease in the phosphorylation of β-catenin following GSK3β-abrogation. While it may be argued that GSK3β is said to be constitutively active (Papkoff and Aikawa, 1998), and that phosphorylation at Tyr216 serves only to enhance the activity of GSK3β (Hughes *et al*., 1993), it must be stressed that the GSK3β-specific inhibitor (AR-A014418, see section 2.4. above) is a non-competitive inhibitor as it binds to the ATP pocket of the enzyme (Bhat *et al*., 2003). This means that the inhibitor is able to bind to both the GSK3β and the GSK3β-substrate complex, regardless of the phosphorylation status of GSK3β itself, ultimately preventing substrate phosphorylation (Bhat *et al*., 2003). Interestingly, the alterations in the migratory responses observed in HOSCC cells in the absence and presence of GSK3β inhibition suggest that active GSK3β plays a prominent role in OSCC cell migration.

The findings suggest that active GSK3β may function to either 1.) promote cell migration and facilitate the migratory response (as seen in Fig 3.8.a-d.) whereby abrogation of GSK3β appears to stunt laemellipod extrusion and migration or 2.) repress cell migration (3.8.e-f), thus inhibition of GSK3β caused more aggressive cell migration into the wound. While the first model (1, above) appears consistent with the findings of Kobayashi *et al* (2005), which showed that GSK3β inhibition caused a reduction in cell migration in human cervical cancer and mouse mammary epithelium. The

observed decrease in migration though, was found to be coupled with decreases in FAK activation (pFAK(Tyr397); Kobayashi *et al*., 2005). Immunodetection however, revealed that marked changes in the abundance of pFAK(Tyr397) occur only in the WHCO1, HT29 and BxPC-3 cell lines. Interestingly, WHCO1 and BxPC-3 cells show increased cell migration while pGSK3β(Tyr216) is almost entirely diminished following GSK3β-inhibition. Contrarily, migration is largely diminished in the HT29 cell line, which displays the same decreases in the abundance of both pFAK(Tyr397) and activated GSK3β. Thus it can be said that while GSK3β appear to effect the migratory response of select HOSCC cells in a manner morphologically consistent with the findings of Kobayashi *et al*., (2005), the biochemical mechanism appears wholly different. This highlights that not only the multifaceted nature of GSK3β as a regulator of cellular processes, but also presents the possibility of cross-talk between these pathways and other migration and proliferation-related cellular processes (Wu and Pan, 2010).

Further insight into the nature of the effect of active GSK3β on oesophageal cancer cell migration was obtained by inhibiting GSK3β prior to the stimulation and sustaining the inhibition for 24 hours (for ease of understanding and explanation, this process will subsequently be referred to as "prior-inhibition").

The influence of active GSK3β in the migratory response was emphasised, as all the GI-tract cancer types, regardless of origin and initial response to GSK3β abrogation were found to present with one of two responses. Prior-inhibition caused cells to either revert to the migratory response observed in cells not exposed to the GSK3β inhibitor, suggesting that prior-inhibition allows cells time to adapt to an environment without GSK3β such that the subsequent stimulation of cell migration will activate alternate or "rescue" pathways in order to achieve the desired effect. Alternatively, cells show little to no difference when compared to the migratory response observed from cells in which GSK3β was inhibited post-wounding. This may be as a result of the cells being unable to initiate a rescue pathway, or alternatively, the pathways initiated in order to maintain cell survival do not translate to changes in the migratory response as survival has been prioritised over migration.

Immunoblot analysis appears to reinforce the aforementioned mechanisms, as the cell lines which displayed changes in the migratory response also displayed marked increases in the abundance of pβ-catenin(Ser33/37/Thr41). Furthermore, these increases were accompanied by a large decrease in active GSK3β as well as fluctuations in the abundance of total FAK. In a similar manner, cells which did not exhibit a change in the migratory response following the early-abrogation of GSK3β, also support the idea that alternate pathways prioritising survival over migration were activated. As such,

alterations in the abundance of signalling intermediates associated with the migratory response would not be observed. Consequently, it can be said that the HOSCC cell lines are able to bypass GSK3β inhibition if exposed the GSK3β inhibitor prior to wound initiation. Furthermore, the aforementioned increase in pβ-catenin abundance notwithstanding the decrease in pGSK3β(Tyr216) seen in WHCO6 cells, serves as evidence for supposition that a non-destruction complex–related mechanism of β-catenin phosphorylation exists in HOSCC and WHCO3 in particular. Moreover, it is proposed that the WHCO6 cell line may adopt a similar signalling pattern as the WHCO3 cells as a result of prior-inhibition of GSK3β, given the similarities in the abundance of pGSK3β(Tyr216) and pβ-catenin(Ser33/37/Thr41).

4.3.HOSCC cells do not display ECM-substrate specific activation of pFAK and GSK3β

Human oesophageal squamous cell carcinomas (HOSCC) are established as being both highly tumorigenic and metastatic (Tew *et al*., 2005). While these properties occur as the result of a multitude of aberrations in various signal transduction pathways governing cell cycle regulation and cell survival (Kato *et al*., 2002; Miyazaki *et al*., 2003; further reviewed by Enzinger and Mayer, 2003), it must be emphasised that HOSCC retain their cell polarity with respect to orientation in the ECM (Christiansen and Rajasekaran, 2006). As such, specialised ECM receptors (integrins) still localise to the basal surface in order to associate with specific components of the basal lamina. The WHCO cell series has been shown to alter the integrin sub-unit combinations expressed on the basal surface in order to broaden the range of tissues to which individual cells can bind thereby facilitating metastasis (Miller and Veale, 2001). Miller and Veale (2001) found that the non-canonical α_v integrin subunit is increasingly expressed on the surface of the WHCO1, WHCO3, WHCO5 and WHCO6 cells. This subunit is known to pair with a multitude of β integrin subunits thereby expanding the range of ECM substrate specificity exhibited by HOSCC (Miller and Veale, 2001). As such the overall reduction in the activation of FAK at Tyr397, may be an effect of the reduced expression of the established collagen-specific $\alpha_1\beta1$ and $\alpha_2\beta2$ receptors on the HOSCC cell surface.

Similarly, fibronectin-stimulated cell growth also resulted in a general reduction in levels of active FAK detected. Notable exceptions to this general decrease in FAK activation are the WHCO5 cell line which, previously been shown to possess exceptionally high levels of activated FAK in cells at rest (see section 3.2.1), and the A431, epidermoid cancer cell line which both exhibit major increases in the abundance of active FAK. The general decrease observed in all the other cell lines including the BxPC-3, well-differentiated pancreatic carcinoma, and HT29 colorectal adenocarcinoma cell

lines, may be attributed tissue culture plates being cultivated specifically to mimic the ideal ECM-composition for a variety of adhesion dependant cell types (converse to the findings of Vlodavsky *et al*., 1980). This, combined with the broadened ECM-substrate specificity exhibited by HOSCCs makes it extremely unlikely that one or two specific substrates would be identified as being solely responsible for the activation of FAK, and its downstream signalling pathways (Dotomo *et al*., 2016).

Along this vein, the same may be true for the changes observed in the abundance of both GSK3β phosphor-species. The phosphorylation of which is triggered by a multitude of upstream kinases (refer to figure 1.1.).

4.4. Summary and Conclusion

The functionality of the serine-threonine kinase GSK3β is known to be governed by phosphorylation at two residues, serine 9 and tyrosine 216 (ter Haar *et al*, 2001). Both these phosphor-forms have been shown to be highly abundant in HOSCC cells.

It has been revealed that the abundance of GSK3β fluctuates during HOSCC cell migration and that this fluctuation occurred in tandem with alterations in the abundance of activated FAK, the overexpression of which is associated with increased HOSCC migration (Miyazaki *et al*., 2003). Specific abrogation of GSK3β, using the small molecule inhibitor AR-A014418 (Bhat *et al*., 2003), has been shown to variably affect the migratory response observed across the HOSCC cell series with respect to laemellipod extrusion and over-all cell extension into the wound. Thus active GSK3β has been implicated as a differential regulator of migration and laemillipod formation in these cells. This varied influence was observed in that active GSK3β favoured migration in the WHCO3, WHCO6, SNO and HT29 cell lines in a manner that was morphologically similar to the observations of Kobayashi *et al*. (2005), but proved to be mechanistically dissimilar by way of protein abundance. Conversely WHCO1 cells exhibited alterations in protein abundance consistent with the Kobayashi *et al*. (2005) model but GSK3β was found to impede the progression of cell migration.

Interestingly, the effects of GSK3β inhibition appears to be subverted in the WHCO6 and WHCO1 cell lines when migration is initiated after GSK3β inhibition, suggesting that while GSK3β is a regulator of cell migration, cells still are able to function in a GSK3β-null environment. Conversely, SNO and WHCO3 cells appeared unable to overcome the loss of GSK3β in order to advance cell migration, which may be due to the non-existence of alternate pathways. Alternatively, GSK3β signalling may be more prominent in cell survival within these cell lines, such that "rescue" would entail favouring survival over maintaining cell migration.

Additionally it was shown that while cell polarity is largely maintained in HOSCC cells, the ECM-substrate specificity of these cells may be altered. This would create an affinity for a wider range of ECM substrates and thus facilitating invasion and metastasis.

In Conclusion: The data presented in this study emphasises the multifunctional influence of GSK3β signalling within human oesophageal carcinoma. This influence, with respect to cell migration and the biochemistry of the migratory response is both complex and multi-layered owing to the nature of GSK3β as a kinase. Despite the mechanisms responsible for the alterations observed in the migratory

response being unknown, GSK3β has presented itself as a key-component in HOSCC migration. Identifying the pathways responsible for such changes in cell behaviour during migration presents an interesting conundrum, due the myriad of upstream regulators and downstream targets of GSK3β. Further elucidation and characterisation of GSK3β function in HOSCC, may provide valuable insight into mechanistic interactions responsible for HOSCC progression and invasion.

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6. APPENDICES

APPENDIX A

1.1. Commonly Used Solutions

1.1.1. Phosphate Buffered Saline (PBS) (1X)

136.9 mM Sodium Chloride
2.68 mM Potassium Chloride
10.1 mM Disodium Hydrogen Phosphate Dodecahydrate
1.769 mM Potassium Dihydrogen Phosphate
Adjust to a pH between 7.2 – 7.3
Make up to final volume with dH2O
Autoclave to sterilize
Store at 4 °C

1.1.2. Tris Buffered Saline (TBS) (1X)

50 mM Tris-HCl (pH 7.8)
147 mM Sodium Chloride
2 mM Anhydrous Calcium Chloride
Make up to final volume with dH2O
Autoclave to sterilize.
Store at 4 °C

1.1.3. Tris Buffered Saline with Tween (TBS-T)

50 mM Tris-HCl (pH 7.8)
147 mM Sodium Chloride
2 mM Anhydrous Calcium Chloride
0.1 % Tween
Make up to final volume with dH2O
Store at 4 °C

1.1.4. 10 % Sodium dodecyl Sulphate (SDS)

10 % SDS
Make up to final volume with dH2O
Heat to assist dissolution
Adjust to pH 7.2
Store at room temperature

1.2. Tissue Culture

1.2.1. DMEM/Hams F12 Medium Solution

Mix DMEM/Hams F12 Medium Solutions in a 3:1 ratio
Filter sterilize
Store at 4 °C

1.2.1.1. Dulbecco's Modified Eagles Medium (DMEM)

1.37 % DMEM
0.37 % Sodium Bicarbonate
2 % Penicillin (500 U/ml)/Streptomycin (0.5 %) Solution

1.2.1.2. Hams F12 Medium Solution

1.07 % Hams F12 Medium
0.118 % Sodium Bicarbonate
2 % Penicillin/Streptomycin (0.5 %) Solution

1.2.2. Trypsin/Ethylenediaminetetra-acetic Acid (EDTA)

Mix Trypsin Solution/EDTA in a 1:1 ratio
Store at 4 °C

1.2.2.1. Trypsin Solution

0.01 % Trypsin in PBS

1.2.2.2. Ethylenediaminetetra-acetic Acid (EDTA)

0.004 % EDTA in PBS

1.3. Protein Extraction

1.3.1. Laemmli Double Lysis Buffer (2X)

123.8 mM Tris-HCl (pH 6.8)
4.000 % SDS
20.00 % Glycerol
10.00 % β-mercaptoethanol
Make up to final volume with dH2O
Store at 4 °C

1.4. Protein Determination

1.4.1. 95 % Ethanol

95 % Ethanol
Make up to final volume with dH2O

1.4.2. 7.5 % Trichloroacetic Acid (TCA)

7.5 % TCA
Make up to final volume with dH2O

1.4.3. 0.25 % Coomassie Brilliant Blue Stain

0.25 % Coomassie Brilliant Blue Powder
50.0 % Methanol
Dissolve, and then add:
10.0 % Glacial Acetic Acid
Make up to final volume with dH2O

1.4.4. Destain Solution

12 % Glacial Acetic Acid
10 % Methanol
Make up to final volume with dH2O

1.4.5. Elution Solution

66 % Methanol
33 % dH2O
1.0 % Concentrated Ammonia

1.5. Sodium dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1.5.1. Buffers

1.5.1.1. Running Buffer

25 mM Tris-HCl (pH 8.3)
192.5 mM Glycine
3.74 mM SDS
Adjust solution to pH 8.3 using 5 N HCl
Make up to final volume with dH2O
Store at 4 °C

1.5.1.2. Separating Buffer

18.12 g Tris

Adjust solution to pH 8.8 using 5 N HCl
Make up to final volume with dH2O

1.5.1.3. Stacking Buffer

6.04 g Tris
Adjust solution to pH 6.8 using 5 N HCl
Make up to final volume with dH2O

1.5.2. Working Solutions

1.5.2.1. Separating Gel

375 mM Tris-HCl (pH 6.8)
10 Acrylamide
0.100 % N,N'–methylenebisacrylamide
0.200 % SDS
Make up to final volume with dH2O
Just prior to use add:
1 % Ammonium Persulphate Solution (APS)
0.25 N',N',N',N'–tetramethylethylene-diamene (TEMED)

1.5.2.2. Stacking Gel

125 mM Tris-HCl (pH 6.8)
5 – 8 % Acrylamide
0.1 % N,N'–methylenebisacrylamide
0.2 % SDS
Make up to final volume with dH2O
Just prior to use add:
1 % Ammonium Persulphate Solution (APS)
0.25 % N',N',N',N'–tetramethylethylene-diamene (TEMED)

1.5.2.3. SDS Overlay

400 µl of 50mg/ml SDS
Make up to 10 ml with dH2O

1.5.3.4. 0.25 % Coomassie Brilliant Blue Stain

As previously described (see Appendix A, Section 1.4.3)

1.5.3.5. Destain Solution

10 % Acetic Acid
10 % Methanol

Make up to final volume with dH2O

1.6. Western Immunoblot Analysis

1.6.1. Buffers

1.6.1.1. Transfer Buffer

25 mM Tris-HCl (pH 8.3)
20.0 % Methanol
1.41 % Glycine
Make up to final volume with dH2O

1.6.1.2. TBS–for–Blotto Blocking Buffer

50 mM Tris-HCl (pH 7.8)
2.0 mM Anhydrous Calcium Chloride
0.05 % TritonX-100
Make up to final volume with dH2O

1.6.1.3. 5 % Non-fat Milk Powder Blocking Solution

5.0 % Non-fat Milk Powder
Make up to final volume with appropriate buffer

1.6.1.4. Developer

6.4 M Metol
0.6 M Sodium Sulphite (Anhydrous)
80 mM Hydroquinine
0.45 mM Sodium Carbonate (Anhydrous)
34 mM Potassium Bromide
Made up to final volume with dH2O. Stored in the dark

1.6.1.5. Fixer

0.8 M Sodium Thiosulphate
0.2 M Sodium Metasulphite
Made up to final volume with dH2O. Stored in the dark

APPENDIX B

1. **Representative Standard curve used for the determination of protein content (µg).**

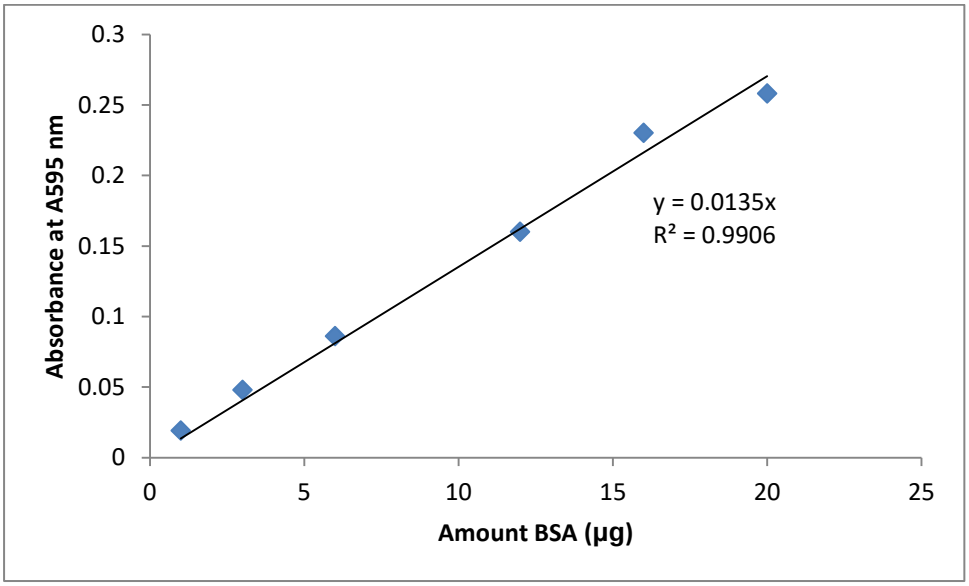


Fig. B1. Standard curve for determination of protein abundance. Absorbance readings were taken at 595 nm for coomassie brilliant blue stain eluted from 1, 3, 6, 12, 16 and 20 µg of BSA protein immobilised on cellulose filter paper. The readings where then used to construct a standard curve, the equation of the trend line was used to determine the protein abundance of the cell extracts. Readings were obtained in triplicate for each amount of protein and the average of the three readings was subsequently plotted on the standard curve. R^2 = Linear regression.

2. **Representative 10 % SDS-PAGE gel used to resolve proteins**

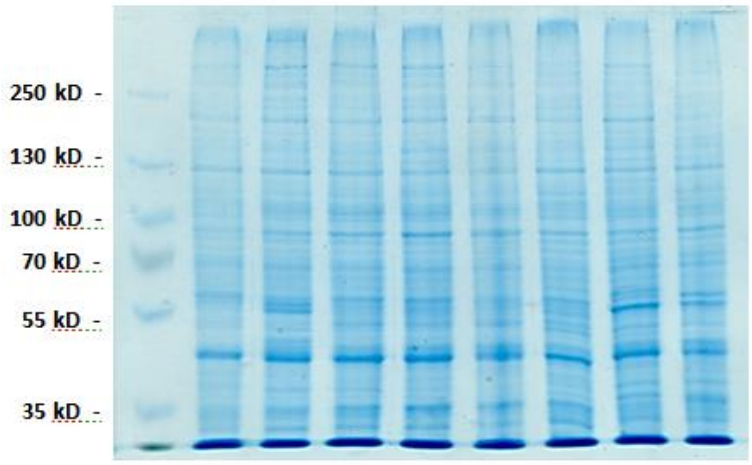


Fig. B2. Resolution of whole cell extracts on a 10 % SDS-PAGE gel. Resolution of 40 µg of protein at 21 mA per gel, produced clear sharp bands. The gel was prepared as specified by Laemmli (1970). Samples were resolved along side 1 µl of PageRuler Prestained Plus molecular weight marker

(ThermoScientific, USA) to allow for the identification and excision of the correct gel regions for western blot analysis.

3. **Base line figures were produced by averaging results from 3 repeats.**

	pGSK3β (Y216)	pGSK3β (S9)	pβ-catenin (S33/37/Thr41)	total FAK	pFAK (Y397)
WHCO1	114.1	179.8	15.7	140.5	69.3
	98.1	221.5	21.8	90.0	6.9
	78.6	224.5	Bands not visible	29.7	95.1
WHCO3	108.1	231.8	179.9	189.5	86.5
	92.9	213.6	138.0	121.3	66.4
	48.7	211.4	Bands not visible	53.8	118.6
WHCO5	87.7	275.3	32.4	151.5	71.9
	75.3	257.2	38.5	107.3	133.3
	45.8	317.4	Bands not visible	110.7	161.8
WHCO6	107.8	191.0	103.4	156.2	72.9
	92.6	248.7	100.0	100.0	72.9
	73.5	200.7	Bands not visible	156.2	99.0
SNO	70.4	99.4	79.9	77.3	83.8
	60.5	94.2	67.2	49.5	26.5
	48.9	71.8	Bands not visible	97.2	36.3
A431	83.7	55.1	160.0	148.2	100.0
	71.9	183.4	154.7	105.5	64.9
	53.8	114.6	Bands not visible	111.2	88.0
HT29	30.4	100.0	10.3	10.6	62.4
	26.1	100.0	15.0	6.8	83.0
	0.0	185.0	Bands not visible	5.9	47.4
BxPC-3	100.0	92.2	100.0	100.0	100.0
	100.0	73.8	91.6	64.0	35.3
	100.0	163.4	Bands not visible	104.4	137.1

Table B1. Values used to produce the figures seen for the abundance of each protein in HOSCC cells at rest. While repeats of three are preferred, only 2 are observed for the pβ-catenin for statistical purposes.

4. **HOSCC cells are ordered in a paving stone-like manner.**

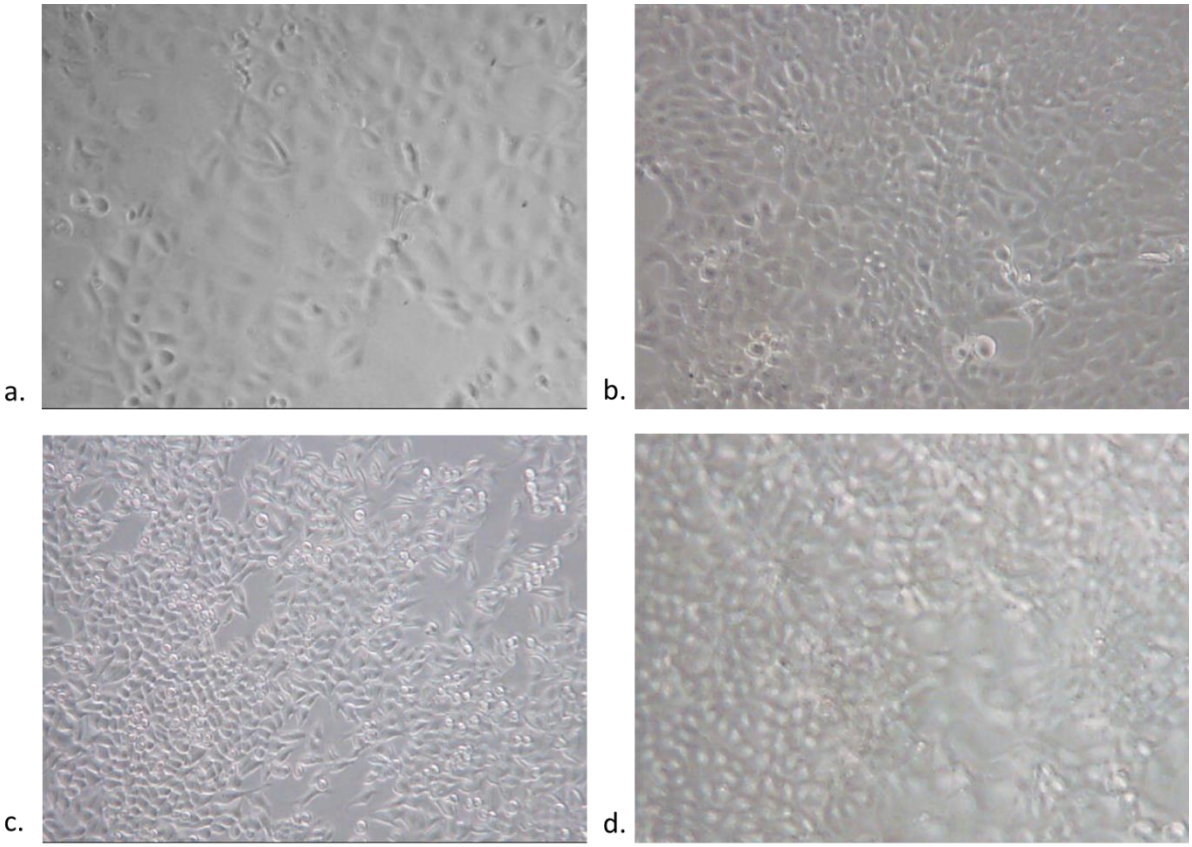


Fig. B4. Paving stone-like order displayed by HOSCC cells at rest. HOSCC cells retain the ability form an ordered monolayer in the transformed state. Cells are observed to fit together with minimal overlap. a.) despite being faint, the cell boundaries can still be partially identified in WHCO5 cells. b.) the WHCO6 cells grow in an ordered manner, with clear distinctions between individual cells. c) BxPC-3 pancreatic cells do not appear to show similar levels of order cell growth. Cells appear elongated, and regions of overlap can be seen even though the surface of the dish is not entirely covered. d.) SNO cells are seen retain the ordered, flattened epithelial cell morphology.

5. Western blots for the detection of actin during 24 hour wound-healing and GSK3β-inhibition migration assays.

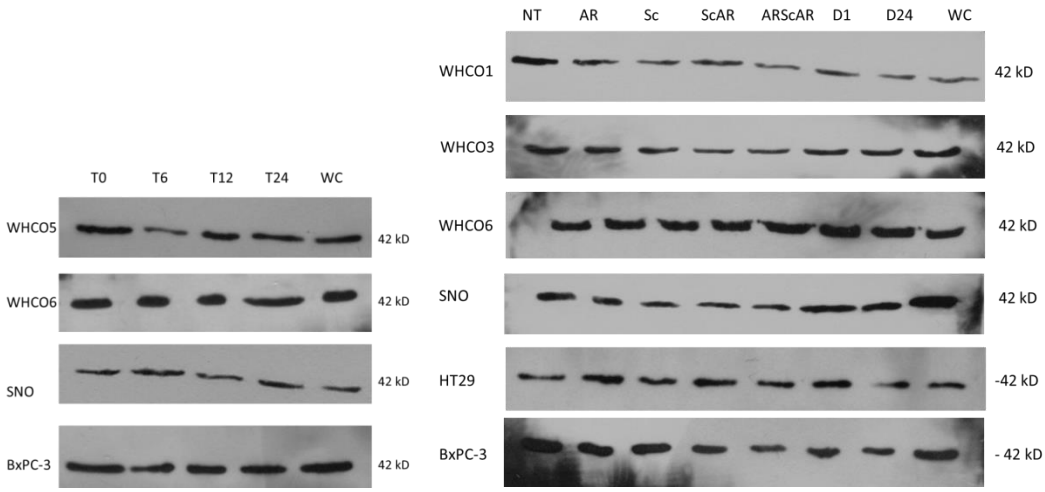
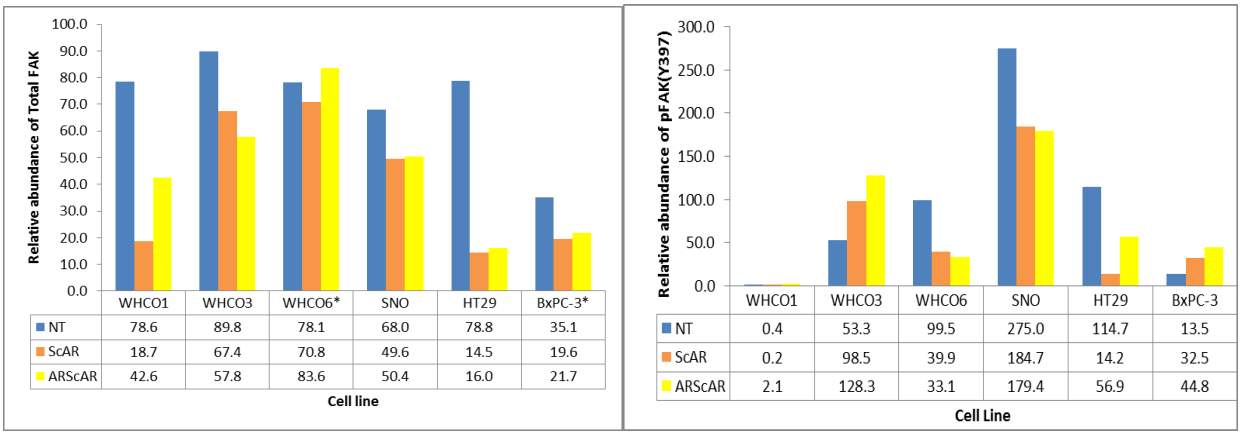


Fig B5. Representative actin blots. Analysis was conducted on total protein from cells extracted during the 1.) 24hour wound healing and 2.) GSK3β-inhibition –wound healing assay. Detection of actin serves to confirm the equal loading of 40 µg of total protein

6. Sustained GSK3β-inhibition alters the abundance total FAK



	WHCO1	WHCO3	WHCO6*	SNO	HT29	BxPC-3*
NT	78.6	89.8	78.1	68.0	78.8	35.1
ScAR	18.7	67.4	70.8	49.6	14.5	19.6
ARScAR	42.6	57.8	83.6	50.4	16.0	21.7

	WHCO1	WHCO3	WHCO6	SNO	HT29	BxPC-3
NT	0.4	53.3	99.5	275.0	114.7	13.5
ScAR	0.2	98.5	39.9	184.7	14.2	32.5
ARScAR	2.1	128.3	33.1	179.4	56.9	44.8

Fig. B6. Alterations in the abundance of total and activated FAK as a result of prior-inhibition of GSK3β. While abrogation of GSK3β, was observed to produce changes in the migratory response observed in HOSCC cells. The introduction of GSK3β inhibition prior to wound initiation was observed to allow the WHCO6, HT29 and WHCO1 cells to somewhat return to a "normal" migratory response. This response was only observable, on a chemical level however in the HT29 cancer cells by changes in the abundance of 1.) total but not 2.) active FAK.

*Regions marked with an asterisks, represents instances whereby the figure was constructed using the mean abundance of that protein (obtained from 3 repeats) in that particular cell line. Furthermore, the sheer scale of

the preparation for each experiment, coupled with the number of variables associated with the process of western blotting and subsequent immunodetection made obtaining usable replicates a challenge. This is because often, to random, uncontrollable variation in our immediate environment, such that changing weather patterns or excessive heat, shaking that is too vigorous or not vigorous enough, a band that is well established, may appear very faint or indistinct may even disappear entirely.

The changes in the abundance of pGSK3β(Tyr216), pβ-catenin, total FAK and pFAK have been summarised into Tables B2 and B3, with arrows representing the percentage change to simplify and amalgamate the effects of the different experiments performed. The percentage change was calculated by obtaining the percentage difference between the densitometry values, thus allowing for the determination of either an increase or a decrease, and furthermore, providing a measure of the "scale" of the increase/decrease in protein abundance.

	Active GSK3β (pGSK3β(Tyr216))						pβ-catenin					
	WHCO1	WHCO3	WHCO6	SNO	HT29	BxPC-3	WHCO1	WHCO3	WHCO6	SNO	HT29	BxPC-3
NT vs AR	↓	↓	↓	↓	↓	↓	↓	↑	↓	↑	↓	↑
NT vs Sc	↓	↓	↓	↓	↓	↓	↓	↑	↓	↑	↑	–
Sc vs ScAR	↓	↑	↓	↑	↓	↓	↓	↑	↓	–	↓	↑
ScAR vs ARscAR	–	↓	↑	–	↓	↓	↓	↓	↑	↓	↓	↓

Key
- ↑ - increase>65%
- ↑ - increase 30-65%
- ↑ - increase 10-30%
- ↓ - decrease >65%
- ↓ - decrease 30-65%
- ↓ - decrease 10-30%
- – - Change< 10%

Table B2: The changes in abundance of pGSK3β(Tyr216) and pβ-catenin in HOSCC cells in response to different stimuli. The change in abundance of the target proteins are shown by arrows which represent a % change the abundance of the protein between two experiments i.e. Untreated (NT) Vs GSK3β inhibition (AR); NT vs Scratch only (Sc); Sc vs Scratch and subsequent inhibition of GSK3β (ScAR); and ScAR vs cells treated with inhibitor before and after wounding (ARScAR).

	Total FAK						pFAK(Tyr397)					
	WHCO1	WHCO3	WHCO6	SNO	HT29	BxPC-3	WHCO1	WHCO3	WHCO6	SNO	HT29	BxPC-3
NT vs AR	↓	↓	–	–	↓	↓	↑	↑	↓	↑	↓	↑
NT vs Sc	↓	↓	↓	↓	↓	↓	↑	↑	↓	↓	↓	↑
Sc vs ScAR	↓	–	↑	↑	↓	↓	↓	–	↓	–	↓	↓
ScAR vs ARscAR	↑	↓	–	–	↑	↑	↑	↑	↓	–	↑	↑

Table B3: The changes in abundance Total FAK and pFAK(Tyr397) in HOSCC cells in response to different stimuli. The change in abundance of the target proteins are shown by arrows which represent a % change the abundance of the protein between two experiments i.e. Untreated (NT) Vs GSK3β inhibition (AR); NT vs Scratch only (Sc); Sc vs Scratch and subsequent inhibition of GSK3β (ScAR); and ScAR vs cells treated with inhibitor before and after wounding (ARScAR).

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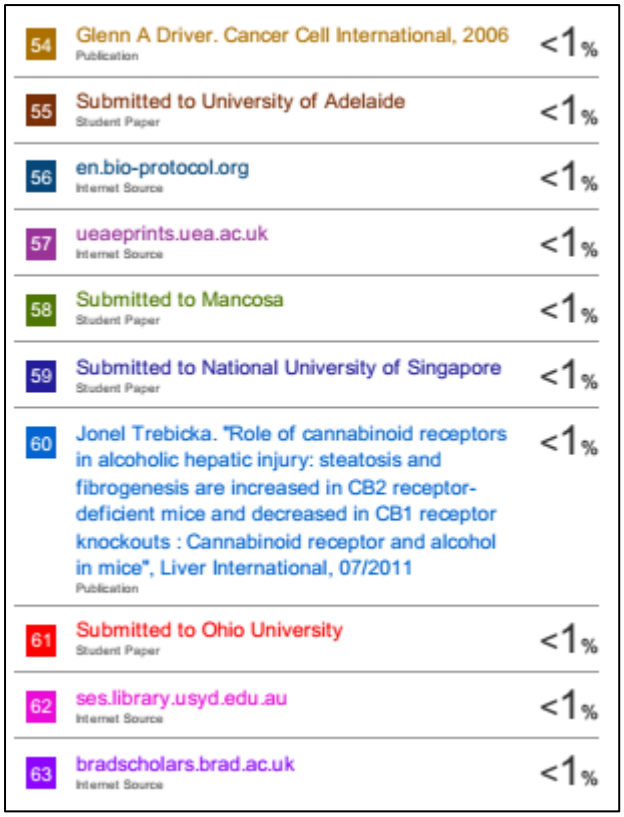
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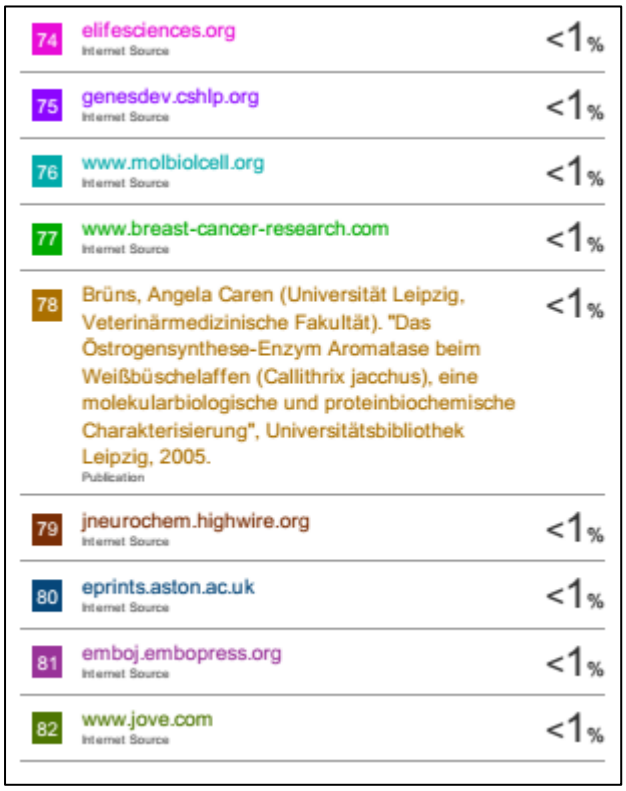
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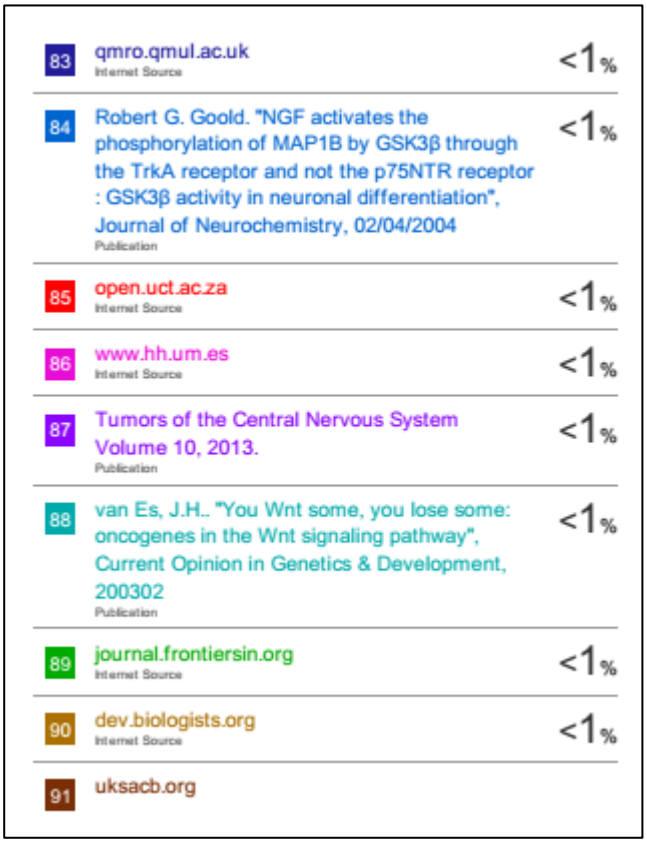
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