

https://dspace.org/

Modulation of adhesion: dependent signalling by CK2 and its associated regulators in hoscccells

Buthelezi, Sandile

2022
https://hdl.handle.net/10539/34481

Downloaded from a DSpace repository. https://dspace.org/

UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

MODULATION OF ADHESION–DEPENDENT SIGNALLING BY CK2 AND ITS ASSOCIATED REGULATORS IN HOSCC CELLS

by

Sandile Buthelezi

(0717048R)

(ORCiD: orcid.org/0000-0002-4004-3991)

Thesis

Submitted in fulfilment of the requirements for the degree

Philosophiae Doctor

in

Molecular and Cell Biology

in the Faculty of Science, University of the Witwatersrand, Johannesburg, South Africa

Supervisor: Prof. R. B. Veale

January, 2022

Declaration

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



(Signature of candidate)

21st of January 2022; at Johannesburg

“Bekezela,

Uphumelele”

Mevane!

Abstract

CK2 introduces a supplementary regulatory influence on proliferative pathways, aberrant in oncogenic signalling. This, with the nodal positioning of PKB in proliferative signalling, necessitated the elucidation of signalling routes employed by the EGFR overexpressing oesophageal squamous carcinoma cells. Furthermore, differential regulation of the PI3K/PKB proliferative signalling axis, within the series of oesophageal carcinoma cells, potentiates significant implications for the resistance to therapeutic interventions. Western blotting and densitometry were used to measure changes in pathway-specific protein concentrations in response to stimulation by EGF, or specific blockade of signalling intermediates. Whilst proliferation assays were conducted using the MTT method. The activation of EGFR significantly increased the concentration of CK2α in a time-dependent manner in the SNO oesophageal carcinoma cells ($p < 0,05$) and A431 epidermoid carcinoma cells (control) ($p < 0,05$) only, while phospho-GSK3β levels were only significantly increased SNO cells ($p < 0,05$). Concurrent blockade of CK2 function (TBB) with the specific inhibition of PI3K (LY294002) led to decreased abundance of active PKB and phospho-GSK3β concentrations in the WHCO1, SNO and A431 cell lines. However, this dual inhibition in the WHCO3 cells produced an increase in phospho-GSK3β in the absence of phospho-PKB. Strikingly, in the WHCO3 cells, there was a depression of phospho-GSK3β levels when CK2 was abrogated concurrently with specific RSK inhibition (BI-D1870) – this is further supported by a 46% loss of proliferative activity ($p < 0,05$) when WHCO3 cells were challenged with the RSK inhibitor, concurrent with EGF stimulation. Such changes were less noticeable in the WHCO1 and SNO cell lines, indicative of complementary routes to effect GSK3β inhibition in the WHCO1 and SNO cells. These data demonstrate that the WHCO1 and SNO cells (like the A431 cells) primarily employ the PI3K-PKB signalling axis to inhibit GSK3β. In contrast, the WHCO3 use a RSK-associated pathway which is chaperoned by CK2. Together, these differentially regulated EGFR-derived cues depend on the influence of CK2 to effect inhibition of GSK3β, for proliferative signalling in the oesophageal cancer cells. While the salvage pathway involving the ERK/RSK axis being used by cells

that maintain low levels of active PKB. Finally, for these and other reasons CK2 is an important regulator of both the PI3K/PKB and ERK/RSK proliferative pathways in adherent HOSCC cells and it may enhance the resistance to therapeutic interventions that are dependent on PKB.

Research Outputs

Manuscripts:

Buthelezi, S. and Veale, R. B. (2022). The dependable shepherd: CK2 shepherds an alternative to the PI3K-PKB route in proliferating oesophageal cancer cells (*submitted to International Journal of Biochemistry and Cell Biology: BC-S-21-01066*)

Conferences:

Buthelezi, S. and Veale, R. B. (2014). HOSCC identifies the influence of CK2 in Akt-integrated EGFR-derived proliferative signals. University of the Witwatersrand 6th Cross Faculty Symposium. Johannesburg. 28-29 October 2014. p87 [TALK]

Buthelezi, S. and Veale, R. B. (2014). A regulatory role for CK2 in EGFR-derived proliferative signalling alternatives in oesophageal squamous carcinoma cells. The South African Society of Biochemistry and Molecular Biology Conference 2014. Goudini Spa, Rawsonville. 6-9 July 2014. p68 [POSTER]

Buthelezi, S. and Veale, R. B. (2013). Akt-ing up: CK2 influences the node of proliferative signal integration in WHCO3 Oesophageal cancer cells. Molecular Biosciences Research Thrust Annual Research day. University of the Witwatersrand, Johannesburg. 04 Dec 2013. p13 [POSTER]

Buthelezi, S. and Veale, R. B. (2012). Defining a role for CK2 in PKB-independent survival signalling in oesophageal cancer. Molecular Biosciences Research Thrust Research day. University of the Witwatersrand, Johannesburg. 05 Dec 2012. p14 [TALK]

Buthelezi, S. and Veale, R. B. (2012). The influence of CK2 on the diminished PKB signalling in Human oesophageal cancer cells. 4th Cross-Faculty postgraduate symposium. University of the Witwatersrand, Johannesburg, 19-22 October 2012. p25 [POSTER]

Acknowledgements

First, and most important, I would like to thank my supervisor, Prof. Rob B. Veale for all his academic guidance, support – You have shown me the greatest level of kindness and understanding, even 'doing things in the background' when I had gone AWOL.. For all the army stories, and even for calling from the ground floor whenever I would get carried away with the radio in the lab… and obviously, the zamaleks as well ☺

I would also like to thank all the people I have met during my postgraduate studies under Prof Veale's supervision. From Mrs Elsabe Scott (it took be a while to be comfortable to call her by her first name), for all her training and always being happy to assist when I get stuck with a technique. Many thanks to all the older members of the Cell Biology Research Lab (Yael, Steph, Nicolene, Ari) from whom I learnt a great deal. Bottomless gratitude to all those who found me in the lab as well, specifically – Keren, Sabeeha, Embeth + Candice, Claire , Lauren and Kampi; all those coffee walks and breaks are cherished.

A special thanks to the Alex to my Meredith; when I started slipping away from reality, and battling darkness, listening to you fangirl over K-pop bands would always remind me of a much lighter side of everything.

Not forgetting Ekene: THANK YOU! You have been a great friend.

To my Family and all my other friends (Cristina, B, Mzala, The-Nomonde One, Otuz, Hloni, Moose-man), thank you for being able to pull me in and for the love.

To all those that funded my studies over the years: Wits PMA, NRF, GDARD, TATA, Wits Staff Bursary; I am eternally grateful .

Finally, thank you to Dr. D. W. Litchfield (University of Western Ontario) for the very kind gift of an aliquot of their anti-CK2α antibody which I used in this research.

Table of Contents

Declaration ii
Abstract iv
Research Outputs vi
Acknowledgements viii
Table of Contents x
List of Figures xv
List of Tables xvii
List of Abbreviations and Symbols xviii
Chapter 1: General Introduction (Literature Review) 1
1.1 Signal integration: the adhesion of cells to substrates and each other, together with soluble factor inputs are vital in the regulation of normal epithelial cells and their carcinomas 1
1.1.1 Cell-extracellular matrix (ECM) interactions 2
1.1.2 Cell-Cell interactions 4
1.1.3 The role played by soluble factors in cell behaviour 5
1.1.4. PKB: The hub of signal integration 7
1.2 The ubiquitous CK2: A prospective coordinator of signals which converge at PKB in oesophageal carcinoma 11
1.3 Signal integration and elucidating a role for the lateral kinase (CK2), in HOSCC 12
1.3.1 Human Oesophageal Squamous Cell Carcinoma 12
1.4 Aims and Objectives of the study 13
Chapter 2: Oesophageal carcinoma cells contain elevated levels of endogenous CK2 and variable abundance of proliferative signalling intermediates 14
2.1 Introduction 14
2.2 Materials and Methods 15
2.2.1 Cell Lines and Standard Tissue Culture Conditions 15
2.2.2 Whole Cell Extraction of Protein from Adherent Cells 15
2.2.3 Determination of Protein Content in Cell Lysates 16
2.2.4 Polypeptide Resolution using SDS-PAGE and Western Blotting 17
2.2.5 Image Capture and Semi-quantitative Densitometric Analyses 20
2.2.6 Statistical Analyses of Data 21
2.3 Results 22
2.3.1 Oesophageal carcinoma cells contain great amounts of endogenous CK2, but with varying concentrations of phospho-PKB (Ser473) and phospho-GSK3β (Ser09) 22

2.4 Discussion 26

Chapter 3: Influence of Epidermal Growth Factor on CK2 in proliferating oesophageal carcinoma cells 28
3.1 Introduction 28

3.2 Materials and Methods 29

3.2.1 Cell Lines and Standardised Tissue Culture Conditions 29
3.2.2 Stimulation of Cells Using Exogenous EGF 29
3.2.3 Extraction of protein from adherent cells 30
3.2.4 Determination of protein content in cell lysates 30
3.2.5 Polypeptide Resolution using SDS-PAGE and Western Blotting 30
3.2.6 Image Capture and Semi-quantitative Densitometric Analyses 30
3.2.7 Statistical Analyses of Data 31
3.3 Results 31

3.3.1 Only the SNO cells in the HOSCC series increase their concentration of CK2 in cells treated with EGF 31
3.3.2 There are varying responses to EGF treatment in HOSCC cells 34
3.4 Discussion 37

Chapter 4: EGF is able to sustain the proliferation of oesophageal carcinoma cells 38
4.1 Introduction 38

4.2 Materials and Methods 39

4.2.1 Cell Lines and Standardised Tissue Culture Conditions 39
4.2.2 Stimulation of Cells Using Exogenous EGF 39
4.2.3 Proliferation Assays: The stimulation on HOSCC cells using EGF 39
4.2.4 Statistical Analyses of Data 40
4.3 Results 40

4.3.1 Exposure to EGF alone did not significantly affect proliferative activity in the 6 hour period 40
4.4 Discussion 43
Chapter 5: The dependable shepherd: CK2 supports an alternative to the PI3K-PKB route in proliferating monolayer adherent HOSCC cells 44
5.1 Introduction 44

5.2 Materials and Methods 46

5.2.1 Cell Lines and their Maintenance 46
5.2.2 Stimulation of HOSCC Cells Using Exogenous EGF 46
5.2.3 Blockade of the Activity of Signalling Intermediates Using Specific Molecular Inhibitors 46

5.2.4 Whole Cell Extraction of Protein from Adherent Cells 47
5.2.5 Determination of Protein Content 47
5.2.6 Polypeptide Resolution using SDS-PAGE and Western Blotting 47
5.2.7 Image Capture 48
5.2.8 Proliferation Assays: The effects of EGF stimulation and/or specific pathway inhibition 48
5.2.9 Statistical Analyses of Data 48
5.3 Results 49
5.3.1 Differing proliferative responses to specific pathway inhibitors suggests varied routes to effect proliferation in oesophageal cancer 49
5.3.2 Oesophageal Carcinoma Cells are Differentially Dependent on CK2 for their Maintenance of Proliferative Signals 53
5.4 Discussion 59
Chapter 6: General Discussion - Defining a role for CK2 in HOSCC 61
6.1 Synthesis 61
6.2 Conclusions 65
6.3 Prospects 65
References 66
Appendix A: Laboratory and Tissue Culture Solutions 81
1.1. Standard Solutions 81
1.1.1 Phosphate Buffered Saline (PBS) (1X) 81
1.1.2 Tris Buffered Saline (TBS) (1X) 81
1.1.3 Tris Buffered Saline with Tween (TBS-T) 81
1.1.4 10% Sodium dodecyl Sulphate (SDS) 82
1.2. Tissue Culture Solutions 82
1.2.1. DMEM/Hams F12 Medium Solution 82
1.2.2. Trypsin/Ethylenediaminetetra-acetic Acid (EDTA) 82
1.3. Protein Extraction Solutions 83
1.3.1. Laemmli Double Lysis Buffer (2X) 83
1.4. Protein Determination Solutions 83
1.4.1. 95 % Ethanol 83
1.4.2. 7.5 % Trichloroacetic Acid (TCA) 83
1.4.3. 0.25 % Coomassie Brilliant Blue Stain 83
1.4.4. Destain Solution 84
1.4.5. Elution Solution 84

1.4.6. Example of Standard Curve for Protein Determination 84
1.5. Sodium dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) 85
1.5.1. Buffer Solutions 85
1.5.2. SDS-PAGE Working Solutions 86
1.5.3. SDS Overlay Solution 86
1.5.4. Tracking Dye 87
1.5.5. 0.25 % Coomassie Brilliant Blue Stain 87
1.5.6. Destain Solution 87
1.6. Western Blotting Solutions 87
1.6.1. Buffers 87
1.6.2. SuperSignal® West Pico Chemiluminescent Substrate Kit 88
1.6.3. Developer 88
1.6.4. Fixer 88
1.7. Co-Immunoprecipitation Analysis 88
1.7.1. Radio-immunoprecipitation Assay (RIPA) Buffer 88
1.7.2. Immunoprecipitation (IP) Buffer 89
Appendix B: Proliferative Pathway Manipulation in Tissue Culture 90
2.1. Stimulation of cells using EGF (Sigma) 90
2.1.1. EGF Stock 90
2.2. MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) Proliferation Assays 90
50 mg MTT (Sigma) 90
Make up to final concentration of 5 mg/ml with 10 ml of PBS. 90
Store at -20 °C. 90
2.3. Specific Blockade of CK2 (CK2α) using 4,5,6,7-tetrabromo-1H-benzatriazole (TBB) (Sigma – Cat. No. T0826) 90
2.4. Specific Blockade of PI3K using LY294002 (Sigma – Cat No. 440204) 90
2.3.1. LY294002 Stock 90
2.5. Specific Blockade of p90RSK using BI-D1870 (BioVision- Cat. No. 1924-1) 91
2.5.1. BI-D1870 Stock 91
Appendix C: Sample Data (or Analyses) and Sample Statistics 92
3.1 Protein Sample Blot Data 92
3.2 Protein Sample Densitometry Analyses Sample Data 95

3.3 Proliferation Assays Data.....103

List of Figures

Figure 1.1: Signals that are associated with the IPP complex and its downstream physiological influences……...**3**

Figure 1.2: The E-cadherin-catenin complex involved in the facilitation of cell-cell adhesions……..**5**

Figure 1.3: Cellular physiological processes, as mediated by active PKB/Akt……**8**

Figure 1.4: Multiple players involved in the regulation of PKB activity and stability to effect processes that are involved in cell survival……**10**

Figure 2.1: Oesophageal cancer cells contain high endogenous levels of CK2, while they have varying amounts of active PKB and inhibited GSK3β. ……**23**

Figure 3.1: The effect of EGF on CK2α concentration over a 24-hour period…..**33**

Figure 3.2: EGF stimulated HOSCC cells contain different amounts of active PKB and inhibited GSK3β……..**35**

Figure 4.1: Serum starved oesophageal carcinoma cells are able to use EGF only to stay viable and proliferate……..**41**

Figure 5.1: EGF stimulated oesophageal cancer cells differentially respond to proliferative pathway inhibitors……**51**

Figure 5.2: Differential responses of the PI3K-PKB signalling axis' activity in oesophageal cancer cells in response to concurrent blockade of CK2 and PI3K……..**53**

Figure 5.3: RSK's provide an alternative route to effect GSK3β inhibition which is facilitated by CK2 activity in oesophageal cancer cells……**56**

Figure 6.1: A summary of EGFR-derived proliferative signals in oesophageal cancer cells, with the proposed influence of CK2 and the integration of these proliferative signals……**62**

Figure A1: An Example of a Standard Curve used for to Determine the concentration of protein in cell lysates……**78**

Figure C1: Sample blots of CK2α showing the effect of EGF on protein abundance over a 24-hour period…….**88**
Figure C2: Sample Blot of EGF stimulated WHCO1 HOSCC cells showing the effect of protein abundance over a 24-hour period…….**89**
Figure C3: Sample Blot of EGF stimulated WHCO3 HOSCC cells showing the effect of protein abundance over a 24-hour period……**89**
Figure C4: Sample Blot of EGF stimulated SNO HOSCC cells showing the effect of protein abundance over a 24-hour period…….…..**90**
Figure C5: Sample Blot of EGF stimulated A431 cells showing the effect of protein abundance over a 24-hour period……**90**

List of Tables

Table 2.1: Summarised Western Blotting Protocols as Optimised for Each Protein Target…….**20**
Table 2.2: Oesophageal cancer cells contain high endogenous levels of CK2, while they have varying amounts of phospho-PKB (Ser473) and phospho-GSK3β (Ser09)……**24**

List of Abbreviations and Symbols

APS	Ammonium persulphate
ATP	Adenosine triphosphate
Cdc37	Cell division cycle 37
CK2	Protein kinase CK2 (Casein kinase 2; previously known as Casein Kinase II)
CKIP-1	Casein kinase-interacting protein-1
Co-IP	Co-Immunoprecipitation
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagles Medium
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular-signal-regulated kinase
FAK	Focal adhesion kinase
FCS	Foetal calf serum
GSK3β	Glycogen synthase kinase-3β
HEK293	Human embryonic kidney 293
HCl	Hydrochloric acid
HOSCC	Human oesophageal squamous cell carcinoma
HRP	Horseradish peroxidase
ILK	Integrin-linked kinase
kDa	kilodalton
MAPK	Mitogen activated protein kinase
mTOR	Mammalian target of rapamycin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
pCDC37	Ser13 phosphorylated cdc37

PDK1	Phosphoinositide-dependent protein kinase 1
pGSK3β	Ser9 phosphorylated GSK3β
PKB	Protein kinase B (also known as Akt)
PH	Pleckstrin homology
PI3K	Phosphoinositide-3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PMSF	Phenylmethanesulphonylflouride
pPKB	Ser473 phosphorylated PKB
PTEN	Phosphatase and tensin homologue deleted on chromosome ten
RTK	Receptor tyrosine kinase
RT	Room temperature
SDS	Sodium dodecyl sulphate
SEM	Standard Error of Mean
Std Dev	Standard deviation
TBB	4,5,6,7-Tetrabromobenzatriazole
TBS	Tris buffered saline
TEMED	N,N,N‘,N‘-tetramethylethylene-diamine
TCA	Trichloro-acetic acid
TGF-β	Transforming growth factor β
Tris	Tris(hydroxymethyl)aminomethane
WHCO-	Wits Human Carcinoma of the Oesophagus-

Chapter 1: General Introduction (Literature Review)

1.1 Signal integration: the adhesion of cells to substrates and each other, together with soluble factor inputs are vital in the regulation of normal epithelial cells and their carcinomas

Functional epithelia are formed and maintained by the interactions cells make with both the extracellular matrix (ECM) (cell-ECM contacts) and their adjoining cells (cell-cell contacts) - these interactions also influence cellular behaviour (Fristrom, 1988; Gumbiner, 1992). These interactions are primarily facilitated by transmembrane proteins that further direct the specificity of the cell-cell and/or cell-ECM interactions. A number of these proteins have been described, but it is the integrins, cadherins and receptor tyrosine kinases (RTKs) which have the most significant influences on the development and maintenance of epithelial tissue and as such, the cancers arising from this particular tissue (Albelda & Buck, 1990; Braga, 2000; Gumbiner, 1996; Malik & Parsons, 1996; Hynes, 1999; Stewart *et al*., 2004; Nair *et al*., 2005; Perez-Moreno et al., 2003). The understanding of these interactions is very important in the elucidation of mechanisms that underlie the pathology of carcinomas. These events together with the influence of soluble factors provide great insights into the integration of signalling pathways, and altogether, they can also contribute to maintaining the cancerous state. Therefore, in the next sections canonical and well-studied cell-ECM and cell-cell interactions, together with the role of soluble factors will be briefly discussed, especially in the context of oesophageal carcinoma.

1.1.1 Cell-extracellular matrix (ECM) interactions

The extracellular matrix (ECM) is comprised of a network of proteins and polysaccharides that underline tissues (most importantly epithelial and endothelial), they provide mechanical support to these tissues and further serve as a repository to allow for the presentation of signals (such as ECM molecules and soluble factors) that stimulate the cells (Fristrom, 1988; Gumbiner, 1992; Katz & Streuli, 2007). The ECM interacts with the cell via specific cell surface receptors, influencing various downstream cellular signalling pathways that are important for the regulation of events such as proliferation, survival, cell spreading and differentiation (Fristrom, 1988; Gumbiner, 1992; Katz & Streuli, 2007). Integrins are probably the widely studied of these adhesion molecules as they appear to have the greatest influence on cell behaviour.

Integrins are a class of heterodimeric adhesion receptors comprised of α and β subunits, and they have characteristic large extracellular domains and relatively small cytoplasmic domains (Barkan *et al.*, 2010; Brunton *et al.*, 2004; Gilcrease, 2007; Katz & Streuli, 2007). Integrin β1 subunits are said to be the most common in heterodimer formation and along with the β3 subunit they have provided much insight into the role played by integrins in adhesion-based signalling (Brunton *et al.*, 2004; Geiger *et al.*, 2001; Giepmans & van Ijzendoorn, 2009; Katz & Streuli, 2007). Furthermore, there are reports of integrins mediating both cell-cell and cell-ECM adhesions (Gilcrease, 2007), but their activity has been greatly characterised in cell-ECM interactions. The latter influence has been the major focus in the study of their role in the pathogenesis of human oesophageal carcinoma, since integrin subunits were found to have deregulated expression patterns in oesophageal carcinoma and other cancers and thus making the understanding of signalling important (Cooper *et al.*, 2002; Gilcrease, 2007; Lössner *et al.*, 2008; Miller & Veale, 2001).

The ILK-PINCH-Parvin (IPP) complex is one of the most important complexes recruited to the site of activated integrins. This complex, IPP, is comprised of

integrin-linked kinase (ILK), particularly interesting Cys-His-rich protein (PINCH) and parvin, and it is named after its components in the order of their discovery, and it is an important component of focal adhesions (**Figure 1.1**) (Legate *et al.*, 2006; Wozniak *et al.*, 2004). ILK is the central component of this IPP complex and it interacts with PINCH via its N-terminal ankyrin-repeat domain and with parvin through the kinase domain (Legate *et al*., 2006). In oesophageal carcinoma cells it has been shown to play very central roles in the activation of protein kinase B (PKB) by phosphorylation, and this is consistent with findings in other studies (Driver & Veale, 2006).

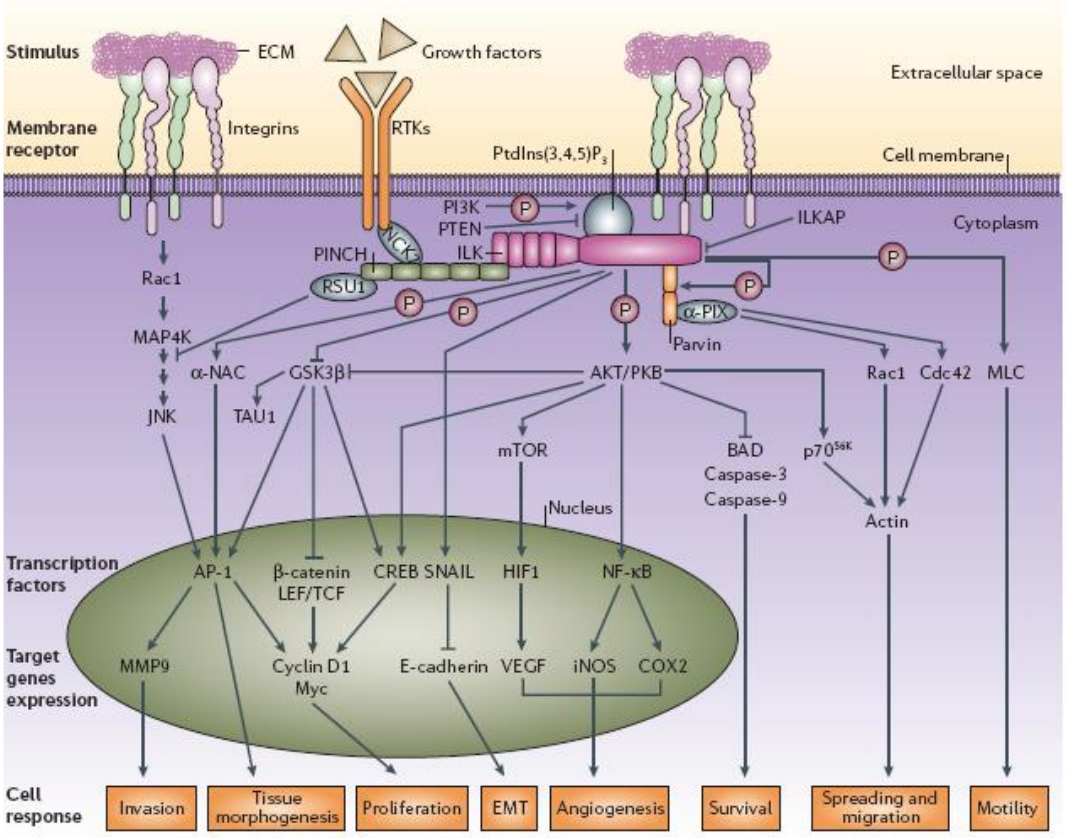


Figure 1.1: Signals that are associated with the IPP complex and its downstream physiological influences. Depicted here are the various signalling pathways that are known to be regulated by integrin-linked kinase (ILK) activation. The relevant signals are those that converge on glycogen synthase kinase 3-β (GSK3-β) (inhibitory phosphorylation at Ser09) and PKB (activating phosphorylation at Ser473). (adapted from Legate *et al.*, 2006)

In the same study it was found that exogenous soluble factors significantly enhanced the abundance and activity of ILK (Driver & Veale, 2006). Further to this, these cancer cells also have elevated levels of integrins, including the *de novo* expression of the αv integrin subunit (Miller & Veale, 2001). This increased abundance of integrin subunits has been reported in a variety of cancers and this associate with aggressive metastasis and tumour progression (Cooper e*t al.*, 2002; Lössner *et al*., 2008; Miller & Veale, 2001; Varadarajulu *et al*., 2005).

1.1.2 Cell-Cell interactions

In epithelial cells, the cadherin (e.g. E-cadherin) family of proteins are responsible for the formation and maintenance a cell-cell adhesion sites, using α- and/or β-catenin (**Figure 1.2**) (van Aken *et al.*, 2001). This promotes cell-cell communication as required for development and it leads to the establishment of tight associations as facilitated by these cadherins (Jeanes *et al.*, 2008; van Aken *et al*., 2001). These are vital the differentiation and polarisation of epithelial cell (Jeanes *et al*., 2008). Owing to this importance, a lot of aberrations in these associations have been linked to a number of developmental disorders (Jeanes *et al*., 2008; van Aken *et al.*, 2001). Most relevant, the dysregulation of these adhesions is exploited by carcinomas, via epithelial-mesenchymal transitions (EMT) (Jeanes *et al.*, 2008; van Aken *et al.*, 2001).

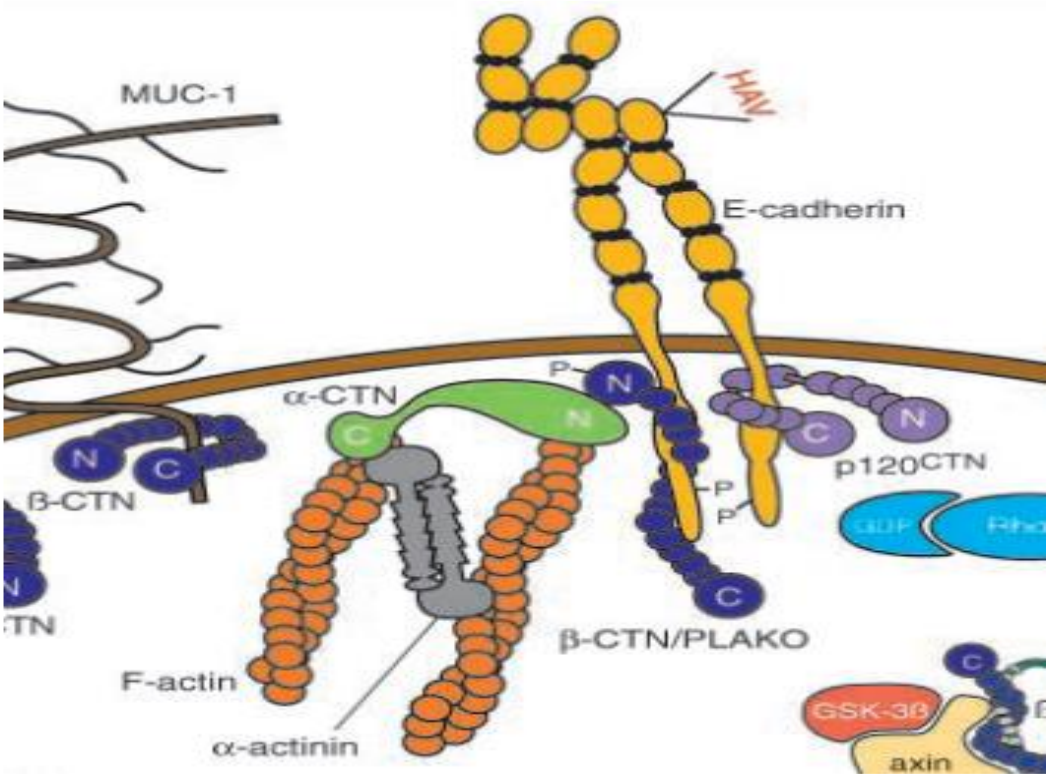


Figure 1.2: The E-cadherin-catenin complex involved in the facilitation of cell-cell adhesions. At these adhesion sites α and β catenin (α-CTN and β-CTN respectively) can be seen associating with the E-Cadherin. Also depicted, is a complex that includes GSK3-β, a well-known downstream target of active PKB. (adapted from van Aken *et al.*, 2001)

1.1.3 The role played by soluble factors in cell behaviour

Soluble factors (viz, growth factors), are key players in cell survival and the promotion of cell-cycling (in proliferating cells) (Yun *et al*., 2009). These signals activate an assortment of pathways, including the canonical mitogen-activated protein kinase (MAPK), and the PI3K/PKB pathways (Brunton *et al*., 2004; De Luca *et al*., 2008; Delcommenne *et al.*,1998; Dong & Liu 2005; Kandel & Hay, 1999; Marte & Downward, 1997; Stiles, 2009). In oesophageal carcinoma, the epidermal growth factor (EGF) are known to contribute greatly in the neoplastic transformation of epithelial tissues where the EGF-receptor (EFGR) is available in great abundance (Arteaga, 2003; Mendelson & Baselga, 2000). However, the

transforming growth factor-β (TGF-β) pathway has also been linked to the tumour development and progression, but the mechanisms cited differ considerably from those used by EGF (Rahimi & Leof, 2007).

1.1.3.1 The influences of EGF in oesophageal cancer cells

EGF is a ligand for the EGF receptor (EGFR) and EGF-dependent activation of the PI3K/PKB pathway is an important regulator of neoplastic growth and drug resistance (Thakkar *et al.*, 2001). Moreover, it has been demonstrated that EGFR is elevated in the oesophageal carcinoma cells that are used in this research (Veale & Thornley, 1989). It has also been shown that the integration and convergence of signals from ILK-mediated signals on the activity of PI3K in this carcinoma influence PKB activity and is also differentially stimulating a number of pathways that are vital for maintaining the cancerous state (Driver & Veale, 2006; Shaw, 2013).

1.1.3.2 An integrated influence of soluble factors: defining roles in PI3K-PKB proliferative signalling in adhesion-dependent oesophageal cancer cells

Insulin-like growth factor (IGF) and TGF-β pathway canonically stimulate different pathways, but these have been shown to converge to effect similar stimulatory influences on the PI3K/PKB proliferative pathway (Danielpour & Song, 2006). A similar response was observed in oesophageal carcinoma cells, where EGF and TGF-β were used (Driver &Veale, 2006). However, one of the oesophageal cancer cell lines had a contrasting response to the growth factors – the WHCO3 cell line. This meant that a different influence could provide an explanation or that a salvage pathway may be used to effect proliferative signalling in these carcinoma cells.

1.1.4. PKB: The hub of signal integration

Protein kinase B (PKB), also known as Akt, is constitutively activated or aberrantly regulated in many cancers and for this reason, a lot of its regulators and effectors have been targeted for therapeutic interventions (**Figure 1.3**) (Hemmings & Restuccia, 2012). Additionally, the three known isoforms (Akt1, Akt2, Akt3) provide an extra hurdle to therapeutic interventions, since these are also reported to confer resistance to radio- and chemotherapeutic interventions in varying degrees (Hwang *et al.*, 2020; Kishor *et al.*, 2017). Altogether, these have driven a concerted global focus on strategies that target this signaling integrator and recent advances are promising in this regard (Hwang *et al.*, 2020; Lapierre *et al.*, 2016; Shariati & Meric-Bernstam, 2019). In this study, this kinase has been previously demonstrated as an important intermediate in the survival and proliferation of adhesion-dependent oesophageal cancer cells (Driver & Veale, 2006).

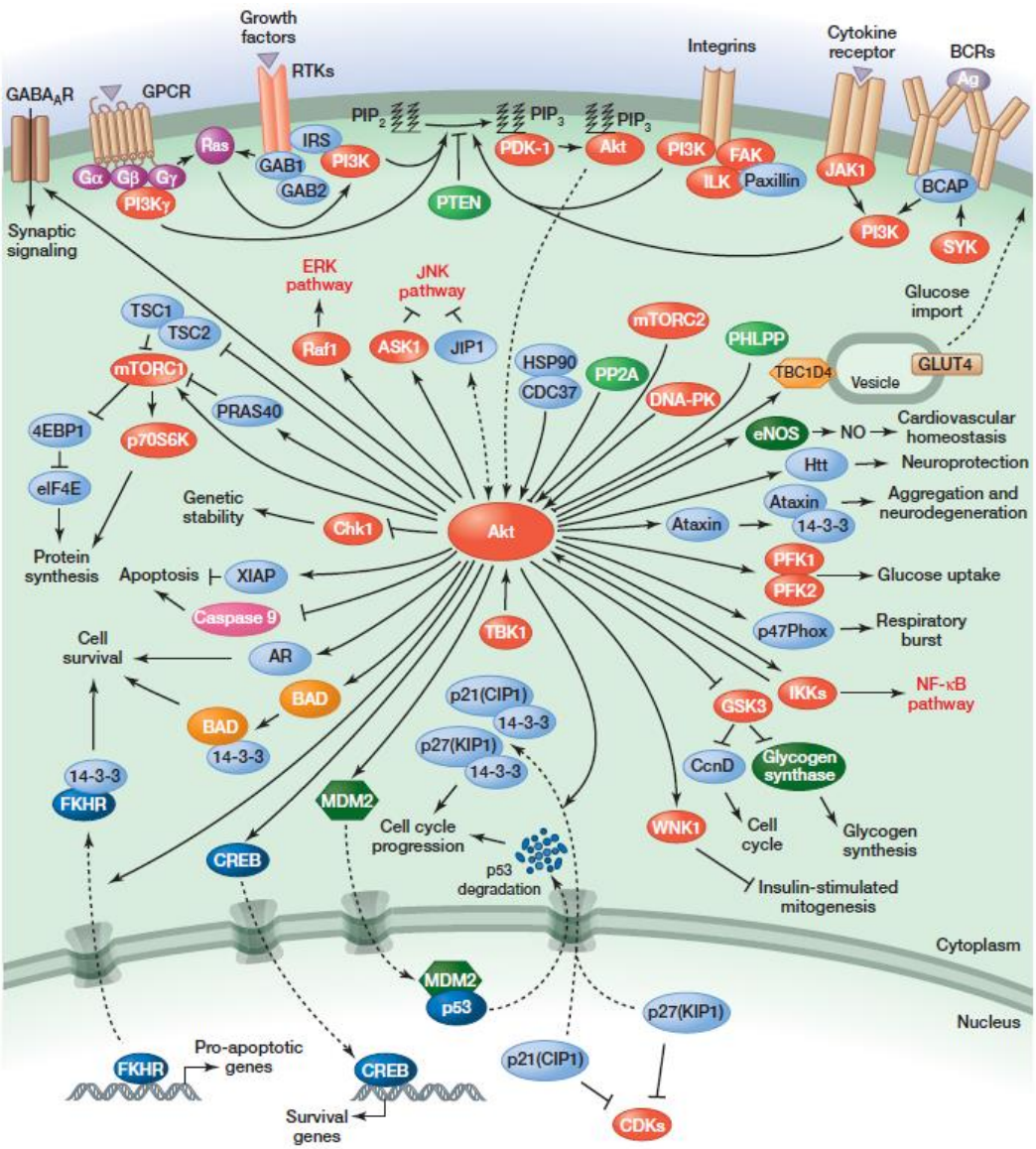


Figure 1.3: Cellular physiological processes, as mediated by active PKB/Akt. Once activated, as depicted in the figure, Akt is suitably positioned at the hub for the integration of signals which can influence a range of cellular behaviours such as cell cycle progression, cell proliferation, cell survival, insulin-mediated mitogenesis, etc**.** (Adapted from Hemmings & Restuccia, 2012)

1.1.4.1. Signals as integrated by PKB

The convergence of these EGFR originating signals at Protein kinase B (PKB, also known as Akt), canonically directs the maintenance of proliferation and other related physiological processes (Brazil & Hemmings, 2001). The sequential phosphorylations of PKB at Thr-308 and then at Ser-473 are required for its full-activation (Dong & Liu, 2005; Kandel & Hay, 1999; Marte & Downward, 1997). These phosphorylation events are canonically regulated by phosphatidylinositol-3 kinase (PI3K) activity. It's important to note there are other proteins and intermediates that support the full activation of PKB (**Figure 1.4** below), but most of these are not directly influenced by soluble factors (Brazil & Hemmings, 2001; Hemmings & Restuccia, 2012). One such protein is the peptidyl-prolyl cis/trans isomerise, Pin1, and it has been shown to facilitate further phosphorylation of PKB, leading to its stabilisation (Messenger *et al*., 2002).

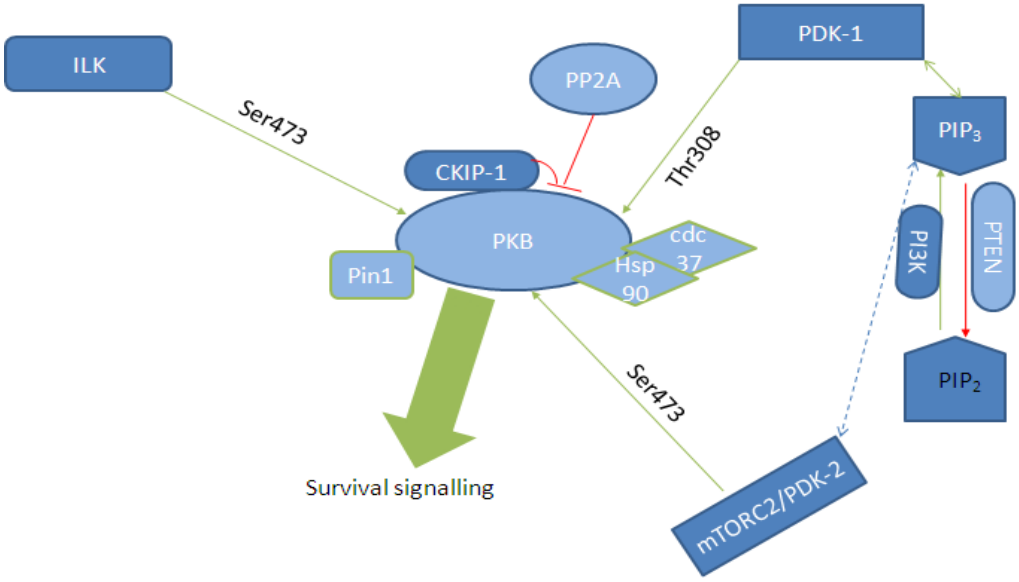


Figure 1.4: Multiple players involved in the regulation of PKB activity and stability to effect processes that are involved in cell survival. Primary phosphorylation of protein kinase B (PKB) is at Thr308 and it is facilitated by 3'-dependent phosphoinositide kinase-1 (PDK-1) in response to membrane accumulation of 3'-OH phosphorylated phosphoinositides (such as PIP_3). PDK-2 and/ or the mammalian target of rapamycin complex 2 (mTORC2) are also recruited to the membrane but phosphorylate PKB at Ser473, resulting in its full activation, this site is also phosphorylated by active integrin linked kinase (ILK). Its activity can be inhibited by protein phosphatase 2A (PP2A) as well as its association with CK2 interacting protein-1 (CKIP-1). The peptidyl-prolyl cis/trans isomerase, Pin1, and the heat shock protein 90 (Hsp90)/ cell cycle division protein 37 (cdc37) co-chaperone complex are important in maintaining the stability of PKB. The proteins represented by shapes shaded in light blue are CK2 targets/ or known CK2 interacting proteins (Di Maira *et al.*, 2005; Gao & Wang, 2006; Klumpp *et al.*, 2004; Meggio & Pinna, 2003; Torres & Pulido, 2001; Wang & Jones, 2006).

As noted above, PKB is tightly regulated by several interacting proteins, a few of these have been previously studied in the oesophageal cancer model that are used in this research. It has also been previously established that tracking the

phosphorylation of GSK3-β, a downstream effector of active PKB was a good marker of the activation of proliferative signalling in response to environmental stimuli (ECM or growth factors). Despite there being several investigations on the control and regulation of PKB activity, the isolated data does not fully explain some of the varying influences observed to date in many cancers and even within oesophageal cancer. This allows for the postulation of a larger, coordinating influence by CK2, on the signalling events that converge at PKB, and by extension, those events that would be targeted by active PKB.

1.2 The ubiquitous CK2: A prospective coordinator of signals which converge at PKB in oesophageal carcinoma

Originally identified as Casein kinase II (protein kinase CK2), this serine/threonine kinase is now simply known by the acronym CK2 (Ahmed *et al.*, 2002; Duncan & Litchfield, 2008; Hanif *et al.*, 2010; Olsten & Litchfield, 2004). It is a ubiquitously expressed, highly conserved tetramer made up of two catalytic subunits, the α and/or and a shorter α′ (42kDa and 38kDa) (Grasselli *et* al., 2004; Olsten & Litchfield, 2004; Duncan & Litchfield, 2008; Hanif *et al.*, 2010). The full tetramer also has two regulatory β subunits (28kDa) (Ahmed *et al.*, 2002; Olsten & Litchfield, 2004; Duncan & Litchfield, 2008; Hanif *et al.*, 2010). The elevated abundance of CK2 (in subunit and tetrameric form) is well documented in solid tumours such as kidney, lung, breast and colon cancers (Lamprecht *et al.*, 1990; Stalter *et al.*, 1994; O-charoenrat *et al.*, 2004; Zhu *et al.*, 2010). This great abundance is thought to have important physiological benefits to cancers.

Expanding from the current knowledge base, defining a role for this kinase will contribute to a greater in understanding of the molecular underpinnings of cancers with a poor prognosis, such as human OSCC (HOSCC), it will be interesting to study the role played by this kinase in proliferative signalling in this cancer.

1.3 Signal integration and elucidating a role for the lateral kinase (CK2), in HOSCC

Integration or crosstalk in signalling pathways has provided a new body of knowledge, even for signalling molecules whose roles had been clearly defined. The integration of signals is particularly important in cancer. PI3K and ILK activity, which are important mediators of adhesion-based signalling, have both been shown to inhibit GSK3-β activity, resulting in Wnt-independent accumulation of cytoplasmic β-catenin (Clevers, 2006; Legate *et al.*, 2006; Voskas *et al.*, 2010). Moreover, in oesophageal cancer it has been shown that EGF and lithium signalling result in the redistribution of β-catenin from the membrane where it is involved in cell-cell adhesions, to the cytoplasm (Jones & Veale, 2003).

1.3.1 Human Oesophageal Squamous Cell Carcinoma

HOSCC is a highly metastatic tumour with very poor prognosis, it is reported that over 80% of oesophageal neoplasms are strongly malignant and it is the second most prevalent cancer in the South African black male population (Enzinger & Mayer, 2003; McCabe & Dlamini, 2005; Pickens & Orringer, 2003; Zhang, 2013). Current therapeutic strategies are ineffective and thus new avenues need to be explored (Ekman *et al.*, 2007; Enzinger & Mayer, 2003). The main challenges are brought about by the disease's puzzling aetiology; genetic, geographic and racial disparities; as well as the lack of information regarding the survival and transmission of this cancer (Bye *et al*., 2011; Enzinger & Mayer, 2003; McCabe & Dlamini, 2005). Oesophageal cancers are known for defying most known solid tumour norms, but the elevated EGFR can be associated with the cancer's phenotype and high proliferative (Veale & Thornley, 1989).

Having briefly noted the links CK2 has with cancers, it is expected that this kinase could have an important role in maintaining the cancerous state. Thus, together with

the support provided by the PI3K-PKB axis to this cancer, the elucidation of the influence of CK2 is important in the efforts to understand the molecular underpinnings of oesophageal cancer.

1.4 Aims and Objectives of the study

There is a vast body of knowledge that details the regulation of from the extracellular matrix (adhesion) and soluble factors in human cancers and this picture is a continuous "work in progress" in oesophageal carcinomas. These signals influence multiple physiological processes and proliferation is one such process. Further to this, the convergence of these signals at the PI3K-PKB signalling axis provides an important focus point in human cancers and as already discussed above, this is also the case in oesophageal cancer. An aspect driven by protein kinase CK2 and associated regulators is obviously absent in oesophageal cancer. This project aimed to elucidate the contribution by this signalling orchestrator in oesophageal carcinoma, especially in the context of proliferative signalling. To do this, the study undertaken here had to:

- Confirm the presence and quantify the abundance of CK2 in oesophageal carcinoma cells.
- Measure and track the influence of CK2 in proliferating oesophageal carcinoma cells.
- Identify and describe the mechanism/s used by CK2 to influence the proliferation of HOSCC cells by using specific molecular inhibitors.
- Measure the effect of inhibitors on the proliferation of oesophageal carcinoma cells that have been treated with pathway inhibitors, concomitantly with stimulation by EGF.

Chapter 2: Oesophageal carcinoma cells contain elevated levels of endogenous CK2 and variable abundance of proliferative signalling intermediates

2.1 Introduction

There is a growing urgency to investigate the importance of CK2's influence on the PI3K-to-PKB signalling axis, as well as on the maintenance of aberrant oncogenic signalling. This kinase is said to offer augmenting regulatory influences of the PI3K-PKB signalling axis in order to maintain proliferative signalling (Di Maira *et al.*, 2005; Torres & Pulido, 2001). However, it remains unknown whether this influence is in any way affected by either the elevated growth factor levels present in a number of carcinomas, or the hyperactivity of canonical oncogenic pathways such as the PI3K-PKB proliferative signalling axis. Moreover, the PI3K/PKB pathway has long been demonstrated to be the preferred route for EGFR derived proliferative survival signals in a number of epithelial tumours. Skin epidermoid carcinomas are very well studied in this regard and the A431 cells, which were derived from this cancer have been used as a reliable model cell line when assaying EGF and EGFR influences on cellular physiology.

In order to better understand the measured responses of signalling intermediates in oesophageal cancer cells, the A431 epidermoid carcinoma cells were used as a comparative control. These cells also overexpress EGFR (Kawamoto, 1984); with most of the EGFR-responsive proliferative pathways having been well characterised in them. Importantly, the non-existence of an established normal cell line of epithelial cells from the oesophagus motivates for the use of such a comparative solid tumour cell line so that a reliable relative measured cellular response can be attained. Additionally, whole cell lysates from H1299 non-small cell lung carcinoma cells, over-expressing CK2α (O-charoenrat *et al.*, 2004) were

used as positive control to help us establish a baseline of CK2 abundance in these oesophageal carcinoma cells.

2.2 Materials and Methods

2.2.1 Cell Lines and Standard Tissue Culture Conditions

This study made use of three HOSCC cells (these will also be referred to interchangeably as 'oesophageal carcinoma cells' in this research): the WHCO1 and WHCO3 (Veale &Thornley, 1989) cell lines, and the SNO cell line (Bey *et al.*, 1976). All three cell lines were derived from moderately differentiated tumours and they were maintained in Dulbecco's Modified Eagles Medium (DMEM)/Hams F12 (3:1) supplemented with 10% Foetal Calf Serum (FCS) in a humidified incubator at 37ºC with 95% air and 5% CO_2. The A431 cells were used as a comparative control cell line and they are derived from human epidermoid carcinoma; a lot has been studied on these cells and because they are also from a solid tumour, it was speculated that the oesophageal carcinoma cells will exhibit comparable behaviour. The H1299 cell line, derived from human non-small cell lung cancer, was used as the positive control in the experiments carried out in this study, because they have been demonstrated to have a hyperactive PI3K-PKB signalling axis (Soria, *et al.*, 2002; Torres & Pulido, 2001). Both control cell lines were cultured and maintained in the same way as the HOSCC cells. All cells were passaged once they had reached about 80% confluency using Trypsin/EDTA, and plated in fresh tissue culture plates as needed. Seeding for experiments is detailed in the relevant methodology sections of this study.

2.2.2 Whole Cell Extraction of Protein from Adherent Cells

Standardly cultured cells that have reached about 70 to 80% confluency were rinsed twice with 1X PBS (pH 7.2), and then once in a final PBS rinse containing a Protease inhibitor cocktail (Sigma-Aldrich – Cat. No. P8340). The monolayer of adherent cells was scraped into a thin layer of the last PBS rinse and then transferred

into an Eppendorf tube using a pipette. This was followed by brief centrifugation on a desktop centrifuge (TOMY HF-120) at 1145 *x* g, so that whole cells and other cell debris can pellet. The supernatant was decanted, and the pellets were reconstituted in double Laemmli Lysis buffer (Laemmli, 1970), this was followed by boiling for 5min and then centrifugation for 5 min at 4 ºC (12000 *x* g). Samples were then stored at -20 ºC until they could be used for western blotting.

2.2.3 Determination of Protein Content in Cell Lysates

In order to make sure that equivalent amounts of protein were loaded onto polyacrylamide gels for all HOSCC cell lysate, the concentration of protein within each sample had to be determined. This was achieved by making use of a modified Bramhall *et al.* (1969) method, which incorporates the principle of protein-dye binding, as in the Bradford (1976) method; whilst allowing for more precise determination of the amount of protein in samples which contain a lot of SDS. To start this, the filter paper (Whatman®) was hydrated by rinsing in distilled water (dH_2O) for 20 min and then subjected to a series of dehydration steps, in the following order, for 5 min each: 95 % ethanol rinse, 100 % ethanol and finally a rinse in 100 % acetone (Appendix A, 1.4). Following this, the filter paper was air-dried by leaving it on a clean surface in a sterile fume hood. Whilst the filter paper was drying, a solution for a protein standard curve was set up by solubilising Bovine Serum Albumin (BSA) (BDH Laboratory reagents) in Laemmli double lysis buffer (1µg/µl). This BSA stock was then spotted onto the dry filter paper as a series of dots using a micropipette, such that the standard curve would have the following amounts: 1 µg, 3 µg, 6 µg, 9 µg, 12 µg, 16 µg and 20 µg. Additionally, cellular samples prepared as above (section **2.2.3**) were spotted onto the filter paper as 2 µl duplicates. The filter paper was air-dried in a sterile fume hood again. Once the spots had dried up, the filter paper was placed in a solution of 7.5 % trichloroacetic acid (TCA) for 40 min; this allows for the precipitation of the protein onto the filter paper. The TCA was then decanted; and the excess TCA was rinsed off by the addition of 0.25 % Coomassie Blue stain for 5min (Appendix A, 1.4.3), and this

was also decanted. Following this, the filter paper was placed in fresh 0.25 % Coomassie Blue stain for a period of 45 min. This was followed by placing the filter paper in a destain solution (Appendix A, 1.4.4) for about 1 hour or until the background stain was sufficiently washed off. The circles containing the protein retained the blue; and after air drying the filter paper for about 15 minutes, they were cut out and placed, individually, into 5 ml elution solution (Appendix A, 1.4.5) in the dark overnight. Subsequently, the absorbance (Abs) of each solution was determined at 595 nm (Abbota SV1100 Spectrophotometer), with the elution solution used as the "blank" (so that Abs values are due to the protein-dye complexes). A standard curve of Abs versus the amount of protein (μg) was then constructed using the BSA protein standards (see Appendix A, 1.4.6 Figure A1). From this, the amount of protein in the whole cell lysates was determined.

2.2.4 Polypeptide Resolution using SDS-PAGE and Western Blotting

Equivalent amounts of protein, as quantified in section 2.2.3 above were then resolved on a discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel, following the protocol proposed by Laemmli (1970). These were then subjected to wet transfer onto nitrocellulose membranes (Hybond -C; Sartorius). A β-actin loading control (on blots) was used for each lysate, as confirmation that equivalent amounts of protein were loaded.

2.2.4.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis of Protein Lysates

Equivalent amounts of the protein lysates were resolved on a polymerised discontinuous SDS-PAGE, incorporating a 5 % stacking gel (Appendix A, 1.5.2.2) as well a 10 or 12 % resolving gel (Appendix A, 1.5.2.1). A molecular weight marker (MWM, PageRuler^{TM} Prestained Protein Ladder (Fermentas Life Science),) as well as tracking dye (Appendix A, 1.5.4) for the protein samples were used to track the resolution of the protein, as well as to allow for the precise sectioning of the gels using the range within which specific target proteins are found. The gels

were cast using Mighty Small™ SE245 Dual Gel Caster, with the corresponding well combs. Once polymerised, transferred into an electrophoresis tank Mighty Small™ SE250 electrophoresis tank filled with electrophoresis buffer (Appendix A, 1.5.1.1). Combs could then be removed, and the buffer overflow filled up the wells created. Once samples and molecular weight marker were loaded into different wells, the electrophoresis tank was connected to a power pack. Gels were resolved at a constant current of 20 mA per gel, with the voltage allowed to vary (limited to 400 volts for the electrophoresis tank). The separation was allowed until the dye front was about 5mm from the bottom edge of the plate. Gels were then carefully sectioned into regions where different target proteins were expected, using the known molecular weights of the MWM.

2.2.4.2 Western Blotting of SDS-PAGE Gels

Sectioned gels were then sandwiched, together with a Hybond™-C nitrocellulose membrane (Sartorius), in a cassette of filter paper, and then inserted into the Bio-Rad 19 Criterion™ Blotter. The blotter was filled with transfer buffer (Appendix A, 1.6.1.1), and then connected to a power pack, to facilitate the transfer of protein from the gels onto the membranes at 400 mA (Table 2.1).

Once the membranes were ready, they were removed from the cassette and then quickly rinsed in wash buffer (either TBS or PBS, based on Table 2.1 below), then non-specific binding sites on the membrane were blocked by pre-incubation in a casein-based blocking buffer (Appendix A; 1.6.1.2 - *for β-Actin*, 1.6.1.3 - *for the other protein targets*) for 1 hour; followed by three 5 min washes.

Then, each blot was incubated in target specific polyclonal primary antibody, specific for their targets: anti-CK2α (a gift from Dr. D.W. Litchfield – University of Western Ontario) – it is widely accepted to use the presence of only the α subunit as a measure of the presence of the enzyme , anti-phospho-CDC37 (Ser13) (Cell Signalling Technology – Cat. No. 8733), anti-phospho-PKB (Ser473) (Cell Signalling Technology – Cat No. 9271), phospho-GSK3β (Ser09) (Cell Signalling

Technology– Cat. No. 9336) and anti-β-actin (Sigma-Aldrich – Cat. No. A2066) (Table 2.1). Following which, after three 5 min washes, blots were incubated in horseradish peroxide (HRP)-conjugated secondary antibodies (Table 2.1), respective to the source of the primary antibodies, and then detected using the Supersignal West Pico Chemiluminescent substrate kit (Thermo Scientific) (Appendix A, 1.6.3).

After the final washes, following incubations in secondary antibodies as set out below (Table 2.1), the membranes were incubated in SuperSignal™ West Pico Chemiluminescent Substrate (Sigma) (see Appendix A, 1.6.2) for 5 minutes after which it was removed. These were then sealed using clear cling-wrap (Versafilm®) and exposed to clear-blue CL-XPosure™ X-ray film (Thermo Fischer) for 10 min in the dark. The X-ray film was developed by initially gently swirling in developer solution (Appendix A, 1.6.3), followed by a water rinse/stop and then a gentle swirl in fixer solution (Appendix A, 1.6.4) and then finally a water rinse. These X-ray films (the blots) were then allowed to air dry, before the capturing of images (section **2.2.6** below).

Table 2.1: Summarised Western Blotting Protocols as Optimised for Each Protein Target.

Target Protein	Wet Transfer Time	Primary Antibody Conditions	Secondary Antibody Conditions*
CK2α	1 hour	1: 10 000 in 1% BSA/TBS-T; 2 hours at room temperature	1: 5 000 in 5% Milk Powder /TBS-T
phospho-CDC37 (Ser13)	2 hours	1:2000 in 2.5% BSA/TBS-T; overnight at 4 ºC	1: 5 000 in 5% Milk Powder /TBS-T
phospho-PKB (Ser473)	2 hours	1:1000 in 5% BSA/TBS-T	1:2000 in 5% Milk Powder/TBS-T at 30 ºC
phospho-GSK3β (Ser09)	2 hours	1: 1000 in 2.5% BSA/TBS-T; overnight at 4 ºC	1: 3000 in 2.5% Milk Powder/TBS-T
β-Actin	2 hours	1: 3 000 in PBS-T; overnight at 4 ºC	1: 20 000 in PBS at 30 ºC

*All secondary antibody incubations (for 1 hour) were in the dark (dark room) at room temperature, unless otherwise stated, so was the processing of the resulting X-ray films

Important Note: Western Blot data in chapters that follow on from this one (*from Chapter 3 onwards*) will only be represented by the corresponding graphical figures/summary tables and sample images or sample statistics of the blots will be in corresponding appendices (Appendix C) – where blot data, as physical evidence is important , it will be included in the main body of the Thesis.

2.2.5 Image Capture and Semi-quantitative Densitometric Analyses

Images of the X-ray films were captured using the Hewlett Packard ScanJet G3110 desktop scanner. The Labworks™ Image Acquisition and Analysis software (Version 4.5) and RK Densitometry Analysis Version 1.1. © (School of Computer

Science and Applied Mathematics, University of the Witwatersrand, Johannesburg, South Africa) were used for densitometric analysis. Both software applications allow for a semi-quantitative comparison of relative protein expression levels as determined by western blotting, by measuring pixel intensity of the bands on scanned x-ray films. The intensities of bands from H1299 samples were used as the maximum (set at 100 % or 1).

2.2.6 Statistical Analyses of Data

The graphical and tabulated results presented here are expressed as the mean ± S.E.M from three independent biological replicates (n =3, unless otherwise stated). Student's t-tests were performed on the GraphPad Prism version 4.00 statistical program as well as Microsoft Excel (Microsoft Office 2013), to determine the statistical significance; as represented by p-value of differences at different levels; $p < 0.05$ (*), $p < 0.001$ (**), $p < 0.0001$ (***). This data is presented in tables or as part of figures; with the supplementary data in Appendix C for this chapter, and all the chapters that follow.

2.3 Results

2.3.1 Oesophageal carcinoma cells contain great amounts of endogenous CK2, but with varying concentrations of phospho-PKB (Ser473) and phospho-GSK3β (Ser09)

Although the natural variance of protein abundance from different tissues is widely established, the H1299 cells are a reliable comparative positive control because they have already been reported to contain significantly higher levels of CK2α when compared to normal lung cells (O-charoenrat *et al.*, 2004).

Relatively, the HOSCC series of cells have at least 1.4 times more CK2α than the H1299 cells (Figure 2.1 and Table 2.2) and it is hypothesized that these high levels of CK2α potentiate an important role for active CK2 in oesophageal cancer. The concentrations of phosphorylated cdc37 (on serine 13: pCDC37-S13) are comparable to those of the H1299 cells (no significant differences, Figure 2.1 and Table 2.2). As an endogenous substrate of CK2, it serves as confirmation of CK2 activity within the HOSCC cells. At this stage of the study it is speculated that CK2 influences the PI3K-PKB proliferative signalling axis in a manner that is comparable to studies that have been briefly reviewed in Chapter 1, especially those that used H1299 cells (NSCLC cells). These NSCLC cells also have a hyperactive PI3K-PKB signalling axis and this is due to the inactive tumour suppressor and antagonist of this signalling axis, phosphatase and tensin homolog (PTEN) (Soria, *et al.*, 2002; Torres and Pulido, 2001).

The distinctly great abundance of active PKB (phosphorylated at Serine 473, pPKB-Ser473) in these lung cancer cells when compared to the oesophageal cancer cells is evidence of a possibly very active PI3K to PKB signalling (Figure 2.1). Furthermore, the great abundance of the inhibited (by phosphorylation at serine 9) form of glycogen synthase kinase 3- beta (pGSK3β-Ser09), an important downstream substrate of active PKB are good support for a hyper-active PI3K-PKB signalling axis in this comparative control cell line. However, the oesophageal carcinoma cells have variably lower amounts of the inactive GSK3β (Figure 2.1),

with the WHCO1 and WHCO3 containing the lowest levels (Table 2.2, statistically significant low levels). These data, in pattern are not contradictory to what is already known in these oesophageal carcinoma. Only the endogenous levels of CK2 had never been studied in these cells. Furthermore, all three oesophageal cancer cell lines have comparable levels of all intermediates, except for the phospho-GSK3β (Ser09) as described above. This is an important difference and it will be discussed below.

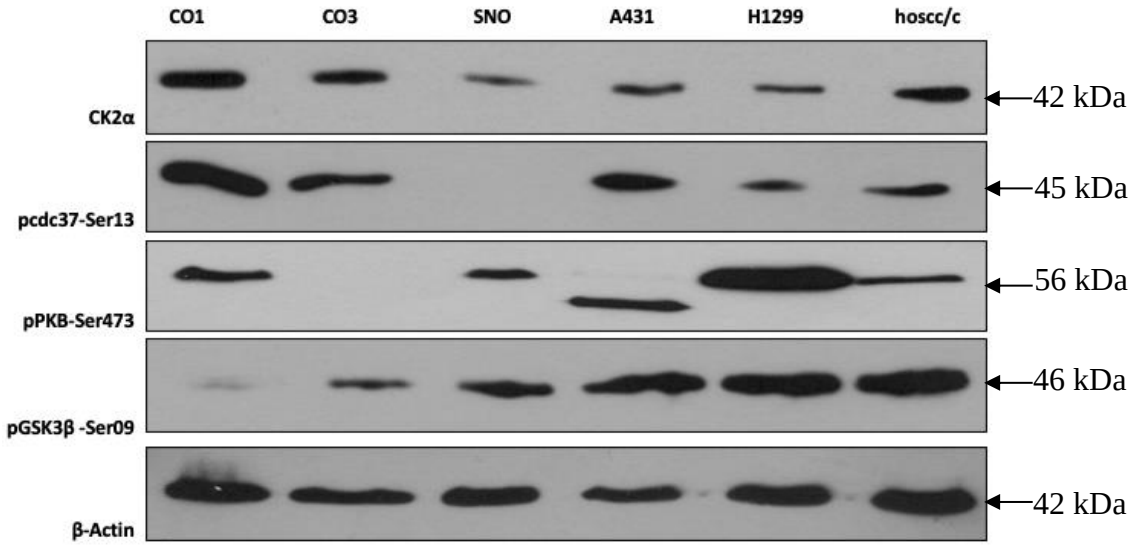


Figure 2.1: Oesophageal cancer cells contain high endogenous levels of CK2, while they have varying amounts of active PKB and inhibited GSK3β. 20µg of protein lysates were resolved on a 5-12% SDS-PAGE gel. These endogenous proteins were detected by western blotting; CK2α (42 kDa); phospho-CDC37 -Ser13 (45 kDa); active PKB (phospho-PKB, Ser473, 56 kDa); inhibited GSK3β (phospho-GSK3β, Ser13, 46 kDa); in oesophageal cancer cells. A standardized amount of protein was loaded from lysates prepared from cells grown under standard tissue culture conditions as described in the materials and methods section; and a β-Actin (42 kDa) loading control was used. The hoscc/c lane contains a sample that is similar to the oesophageal cancer cells used here, and it is known to contain copious amounts of protein and reacts very well with most known antibodies, and it is used as an internal control (additional to the H1299 positive control) when comparing or pooling data from biological replicates. Band intensities and size are an indicator of the amount of protein present; and these were subjected to semi-quantitative analyses, with the data (taken from biological replicates) presented in Table 2.2.

Table 2.2: Oesophageal cancer cells contain high endogenous levels of CK2, while they have varying amounts of phospho-PKB (Ser473) and phospho-GSK3β (Ser09)

	Relative Protein Abundance (± S.E.M) [1]			
	CK2α	**pCDC37-S13**	**pPKB-S473**	**pGSK3β-S09**
WHCO1	1,9 (± 0,07) **	1,7 (± 0,37)	0,6 (± 0,15)	0,1 (± 0,02) **
WHCO3	1,6 (± 0,25)	1,1 (± 0,38)	n. d	0,3 (± 0,15) **
SNO	1,4 (± 0,24)	n. d	0,2 (± 0,02) **	0,6 (± 0,1)
A431	1,2 (± 0,15)	1,2 (± 0,14)	0,9 (± 0,09)	0,9 (± 0,05)

[1]These values are averages (means) relative to the H1299 abundance (intensity in Figure 1 above); this sample was set as the max value (1.00). The standard error of mean (S.E.M) for each value is enclosed in parentheses.

**Denotes values which were significantly statistically different from the H1299 control as determined using a student's t-test ($p < 0,05$)

n.d – protein was not detectable (no bands on the corresponding blot in Figure 1 above)

2.4 Discussion

The diverse roles of CK2 in tumours are fast growing; they range from anti-apoptotic to pro-survival roles, depending on the signalling intermediate it associates with. These roles are also very specific with each cancer as it has already been observed in different studies (Ahmed *et al.,* 2002; Di Maira *et al.*, 2005; Klumpp *et al.*, 2004; Ponce *et al.*, 2010). What initially directed the focus of this research was the mounting evidence that introduces CK2's role in augmenting the activity of PKB to promote and maintain aberrant signalling in cancer (Di Maira *et al.,* 2005). In figure 1.4 above (Chapter 1), a number of regulatory proteins are mentioned, such as the Pin1 and the Hsp90/cdc37 complex. Both of these stabilise natively folded client proteins (PKB in this context); and CK2-dependent phosphorylations of the isomerase (Pin1) and cdc37 of the chaperone complex are reported to enhance their regulation of client proteins (Messenger *et al.*, 2002; Miyata & Nishida; 2005). Additional to how CK2 may contribute to maintaining the cancerous state, PKB is frequently implicated in the resistance to therapeutic interventions (Winograd-Katz & Levitzki, 2006). Taken together, it is not surprising that CK2 has attracted significant and promising attention as a therapeutic target (D'Amore *et al.,* 2017; Martins *et al.*, 2014; Pierre *et al.*, 2011; Siddiqui-Jain *et al.*, 2010) and that targeting the regulation of PKB upstream, or its effectors downstream still forms part of treatment regimens.

In oesophageal carcinoma, the elevated amount of EGFR is an important prognostic indicator, and high levels are usually linked to a poor prognosis. However, what is recently emerging is that where there is reduced activation of PKB, this may contribute to development of more effective treatment regimens (Wang *et al.*, 2015). This does not state how, with elevated levels of EGFR, these cancers are able to have reduced activation of PKB. In the oesophageal carcinoma cells used here, phospho-PKB (Ser473) levels are low when compared to the comparative A431 cells (Table 2.2). In the WHCO3 cells, which normally have undetectable PKB under standard conditions; there was a dose-dependent accumulation of active PKB during conditions that mimic oxidative stress, because this deactivates PTEN

(Shaw, 2013). This deactivation is known to be reversible and in cellular environments where there isn't uncontrolled accumulation of reactive oxygen species (ROS') this effect was reported to be insignificant (Shaw, 2013). When navigating downstream of active PKB, only the WHCO1 and the WHCO3 cells have low levels of inhibited GSK3β; and this could be seen to due to the low active PKB. These then establish the basal levels of activity of the PI3K-PKB proliferative signalling axis, in response to soluble factors that are available in standard tissue culture conditions.

The CK2 levels detected for WHCO1 are an early indicator that even within this series of oesophageal carcinoma cells, there is differential regulation or influence from this pleiotropic kinase. Taken together with previous investigations involving EGF, ILK, PI3K and PTEN (Driver & Veale, 2006; Shaw, 2013), it is becoming clear that oesophageal carcinoma cells do not propagate proliferative signals in the same way in response to soluble factors, even under standard tissue culture conditions. This has serious implications in developing therapeutic strategies, as it is becoming clear that no two carcinomas are the same. Lastly, there is still a clear gap in the relationship between soluble factor inputs and CK2 in oesophageal carcinoma; and an even bigger question on whether, if at all, CK2 plays an important role in this cancer.

Chapter 3: Influence of Epidermal Growth Factor on CK2 in proliferating oesophageal carcinoma cells

3.1 Introduction

Mitogenic stimulation is an important input to a number of physiological processes, especially proliferation. In epithelial cancers, like in HOSCC, there are elevated levels of the receptor and this is unfortunately linked with the aggressiveness of the disease.

Since oesophageal cancers are known over expressers of EGFR and with the stimulation of this receptor known to augment (ILK) activation and β-Catenin nuclear translocation in the HOSCC cells (Driver & Veale, 2006; Jones & Veale, 2003). There is an unquestionable also influence from PI3K – it is well documented in HOSCC cells that PI3K is vital for the maintenance of integrin (Cell-ECM) and growth factor signalling (EGF and TGFβ) (Driver & Veale, 2006; Jones & Veale, 2003; Shaw, 2013; Veale &Thornley, 1989) . Interestingly, however, the activated form of PKB (Ser473 phosphorylation; phospho-PKB) is absent in WHCO3 cells when they are compared to the other oesophageal carcinoma cells (Figure 2.1; Shaw, 2013). This is already evidence of the differential reliance on active PKB within the HOSCC cell series that were used in this study.

As mentioned in the introductory chapter of this thesis (Chapter 1) CK2 may pose new, unique regulatory influences on the canonical PI3K/PKB proliferative signalling axis. To set the scene, it is important to establish how CK2 abundance responds to mitogenic stimulation (EGF stimulation in this study). Signalling intermediates that fluctuate in response to mitogenic stimulation are usually very important in cycling cells. This was predicted to be the case for CK2 in studies where CK2 catalytic subunits where knocked out lead to cell death made knockout of this kinase an unreliable strategy (Sedlin *et al*., 2008). Emerging research findings have proposed a coordinating role for the regulatory β subunits in directing CK2 activity to its targets (Franchin *et al.*, 2018). This is consistent with earlier

proposals of the spatial or local regulation of CK2 activity (Guerra *et al.*, 1999; Ahmed *et al.*, 2002). Since the regulation CK2 activity is still being debated widely, one way of studying its influence is by impeding its catalytic activity by using a specific molecular inhibitor that targets this kinase. However, before assaying that component specifically, it was important to establish how CK2 concentrations are affected in cells that are stimulated using exogenous growth factors. Additional to this, measuring the effect of EGF on PKB and GSK3β in the same conditions as the assay for CK2 concentrations, will add much needed context on the speculated influence CK2 may have on proliferative signalling.

3.2 Materials and Methods

3.2.1 Cell Lines and Standardised Tissue Culture Conditions

The oesophageal cancer cells (WHCO1, WHCO3 and SNO), as well as the control cells (A431 and H1299) were cultured as in section **2.2.1** above.

3.2.2 Stimulation of Cells Using Exogenous EGF

Cells were cultured, as in section **2.2.1** above, to about 70% confluency. Following this, the culture media were carefully decanted and the adherent monolayer of cells were subjected to the following rinses, by gentle swirling: two 1X PBS rinses, then two rinses with serum-free DMEM/F-12 culture medium and finally one rinse with DMEM/F-12 with 10% FCS. After these rinses, fresh DMEM/F-12 (with 10% FCS) that was supplemented with 10 ng/ml EGF (Appendix B, 2.1) as previously optimised for these cancer cells was added to the cultures (Jones & Veale, 2003). It must be noted that aliquots from the same stock of DMEM/F-12 media were used so that any possible stock-stock variations did not affect cell behaviour, and thus the behaviour of cellular cultures could be attributable to the supplementary EGF. These cells were then incubated as above, for periods of, 1, 3, 6, 12 and 24 hours.

Thereafter, cells were harvested for protein lysates that will be applied in analyses as set out below.

3.2.3 Extraction of protein from adherent cells

The extractions of protein from adherent cells that were stimulated with EGF or standardly cultured were carried out as in section **2.2.2** above.

3.2.4 Determination of protein content in cell lysates

The determination of protein content from lysates of cells that were stimulated with EGF or standardly cultured cells was carried out as in section **2.2.3** above.

3.2.5 Polypeptide Resolution using SDS-PAGE and Western Blotting

Lysates from experimental and control samples were then analyses by Western Blots, for CK2α, phospho-PKB (Ser473), phospho-GSK3β and β-Actin as set out in section **2.2.4** above.

3.2.6 Image Capture and Semi-quantitative Densitometric Analyses

Images of X-ray films and the subsequent densitometric analyses were performed as per section **2.2.5** above.

3.2.7 Statistical Analyses of Data

The data in this chapter was also analysed as in section **2.2.7** above. Sample/Representative statistical data is available at the end of this thesis in Appendix C. **Important Note**: *The data representing the changes in the abundance of pCDC37-Ser13 in response to the treatment of cells with EGF will not be shown (for ease of reading of results within the body of this thesis) – the changes were not significant (See Appendix C, 3.2) and this could be attributed to the importance of the co-chaperone in the stabilisation of Hsp90 client proteins as briefly introduced in Chapter 1 and 2 above.*

3.3 Results

3.3.1 Only the SNO cells in the HOSCC series increase their concentration of CK2 in cells treated with EGF

There were marked increases (statistically significant changes) in the levels of CK2α in the A431 comparative control cell line after the first hour and third hours of the cells being treated with 10 ng/ml EGF (Figure 3.1 and Appendix C, Figure C1). This is followed by a return to basal levels of CK2α concentrations until the end of the 24 hour period. These observations are supported by the cell cycle-dependent regulation of the CK2's subunits reported by Litchfield and colleagues (1991 and 1992). The physiological relevance of this, however, is still unclear.

The HOSCC cells had varied responses to EGF stimulation where the abundance of CK2α is concerned. In two of the cell lines, the WHCO1 and the WHCO3 cells, there were no statistically significant changes in the concentration of CK2α over the 24 hour period (Figure 3.1 and Appendix C, Figure C1). Interestingly, all cell lines that were used here presented strikingly similar CK2α curves. That said, the

SNO cells were the only HOSCC cells that had increased concentrations CK2α, at hours 1, 3 and 6, following their treatment with exogenous EGF (Figure 3.1 and Appendix C, Figure C1). As mentioned in Chapter 1 and 2 above, the concentration of proteins found in this section and elsewhere in this study are compared to the concentrations of the same protein in H1299 cell lysates (set at 1 – the maximum). In this context all HOSCC cells are able to maintain elevated levels of CK2α and some may also increase the abundance of the protein in response to EGS stimulation.

The observed elevations in concentration are a unique response in cultured mammalian cells, with the exceptions of studies that modify protein expression levels by transfecting inducible vectors. Drawing from such studies, where is has been demonstrated that overexpression of CK2 promote cancer cell growth, proliferation and evasion of apoptosis (Di Maira *et al.*, 2005; Torres and Pulido, 2001; Wang and Jones, 2006), a similar role for CK2 is hypothesised for oesophageal cancer. At this point, a brief look at PKB and GSKβ concentration is necessary.

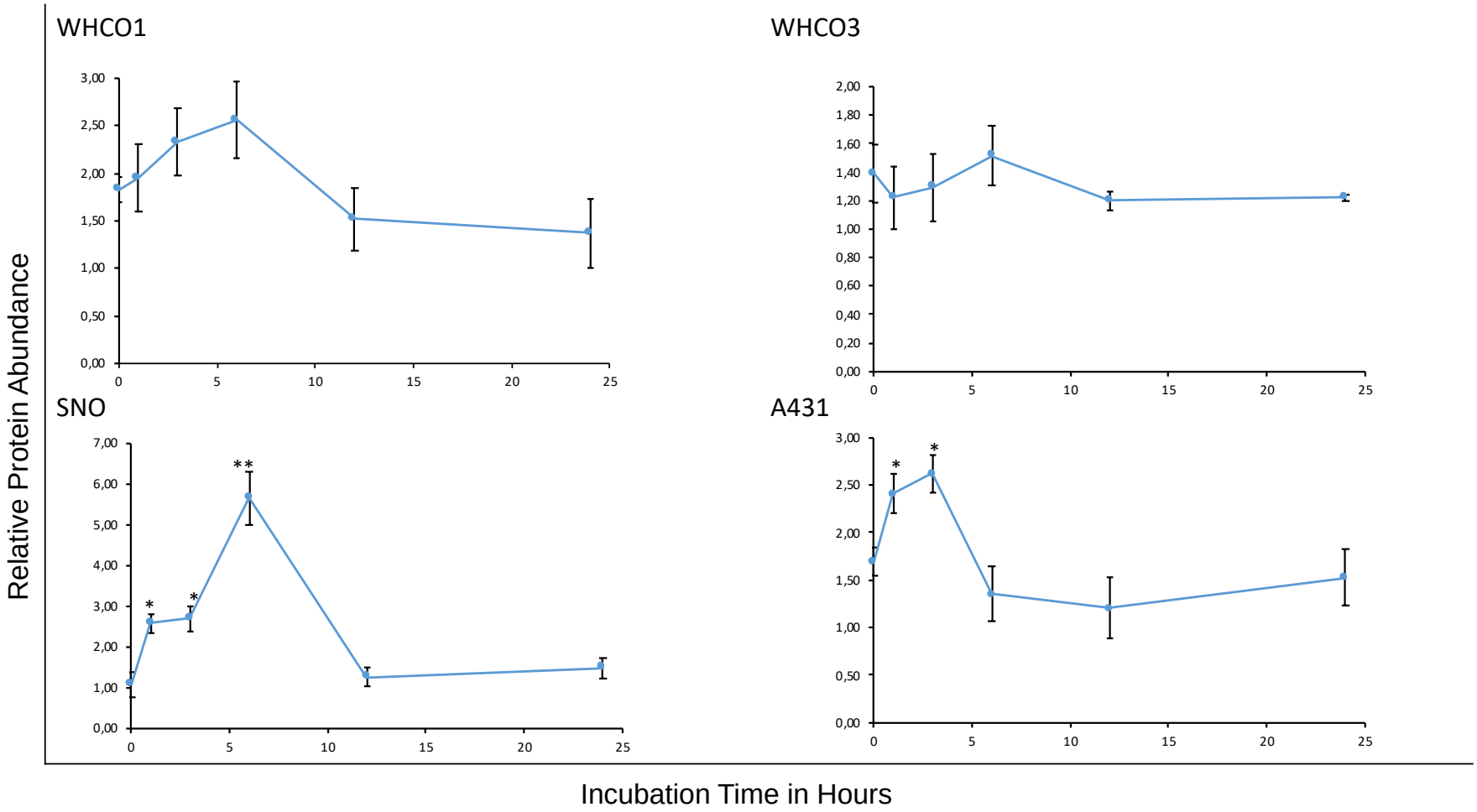

WHCO1
WHCO3
SNO
A431
Relative Protein Abundance
Incubation Time in Hours

Figure 3.1: The effect of EGF on CK2α concentration over a 24-hour period. Oesophageal cancer cells (WHCO1, WHCO3 and SNO) and epidermoid carcinoma cells (A431) cultured under standard conditions were supplemented with 10 ng/ml EGF and incubated for 1, 3, 6, 12 and 24 hours. (The presence of bands in the 42 kDa region as demonstrated by Western Blotting confirmed the presence of CK2α in all cell lines under the incubation times noted above. P/C denotes the H1299 cell lysate that was used as a positive control for the detection and subsequent quantification of CK2α. Densitometric analysis revealed significant increases in CK2α concentration at 1, 3 and 6 hours of exposure to exogenous EGF for the SNO cells, comparable to the significant increases at 1 and 3 hours in the A431 cells. The other HOSCC did not show significant changes in CK2α concentrations. The blots (appearing in Appendix C, Figure C1) are representative of three independent biological replicates. The bars on the trendline represent the S.E.M and treatments were compared to their respective time zero to determine statistical significance (* symbolising $p < 0.05$; ** symbolising $p < 0.01$)

3.3.2 There are varying responses to EGF treatment in HOSCC cells

It was widely anticipated early in this study that EGF stimulation of HOSCC cells would readily increase the concentrations of both active PKB and inhibited GSK3β. Even with all the background preceding this study, a favourable positive response to the stimulation of cultured cells with exogenous was most welcome, as this was observed in only the SNO cells. These cells have significantly elevated levels of inhibited GSK3β (phospho-GSK3β-Ser09) as seen in figure 3.2 below. This is unlike the negligible fluctuations in the other cell lines, including the comparative control cells (the A431 cells), as these point to point differences in abundance are not statistically significant. Densitometric analyses (Figure 3.2 and Appendix C Figure C3) couldn't detect any active PKB in the WHCO3 cells, and this is not unexpected, as they have been shown to be without phospho-PKB(Ser473) under these conditions.

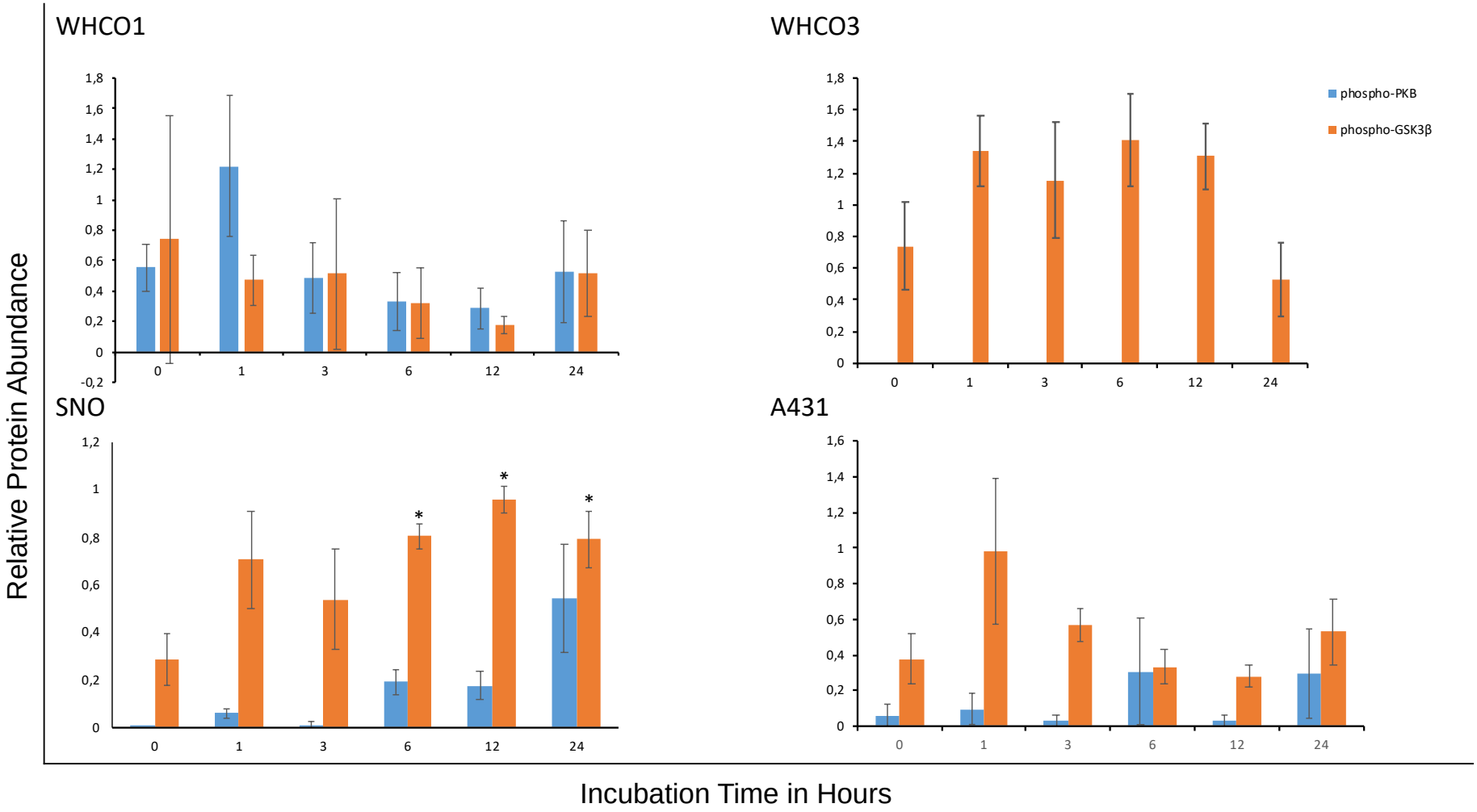

WHCO1
WHCO3
SNO
A431
phospho-PKB
phospho-GSK3β
Relative Protein Abundance
Incubation Time in Hours
0
1
3
6
12
24
*

Figure 3.2: EGF stimulated HOSCC cells contain different amounts of active PKB and inhibited GSK3β. Oesophageal cancer cells (WHCO1, WHCO3 and SNO) and epidermoid carcinoma cells (A431) cultured under standard conditions were supplemented with 10 ng/ml EGF and incubated for 1, 3, 6, 12 and 24 hours. Densitometric analysis revealed significant increases in CK2α concentration at 1, 3 and 6 hours of exposure to exogenous EGF for the SNO cells, comparable to the significant increases at 1 and 3 hours in the A431 cells. The other HOSCC did not show significant changes in CK2α concentrations. The blots (appearing in Appendix C, Figure C2-5) are representative of three independent biological replicates. The bars on the trendline represent the S.E.M and treatments were compared to their respective time zero to determine statistical significance (* symbolising $p < 0.05$; ** symbolising $p < 0.01$). On the Western Blots that correspond to these figures here (See Appendix C, Figures C2, C3, C4 and C5): The presence of bands in the 46 kDa region as demonstrated by Western Blotting confirmed the presence of phopho-GSK3β (Ser09) in all cell lines under the incubation times noted above. The presence of bands in the 56 kDa region as demonstrated by Western Blotting confirmed the presence of phospho-PKB (Ser473). P/C denotes the H1299 cell lysate that was used as a positive control for the detection and subsequent quantification of CK2α.

3.4 Discussion

The time-dependent accumulation of CK2, at least in SNO cells, in response to EGF stimulation will now become the focal point. It appears that in all cells, there is a general trend that revolves around the 6-hour time point. There is either a sudden drop from a 3-hour peak, to return to more basal levels; or this timepoint is the starting point for an upward trend that seemingly lasts until the end of the 24-hour period. This is obviously not simple to confirm as the data were statistically inconclusive. However, the consistency in the SNO cells, of following this idea around the 6-hour time point is the only one that provides an immediate step to follow. Probing proliferative signalling events at 6 hours, in what can only be thought to be cells that are cycling could help in understanding why there are such stark observations in the SNO cells, and perhaps also expose why the other cells behave the way they did.

Chapter 4: EGF is able to sustain the proliferation of oesophageal carcinoma cells

4.1 Introduction

Proliferation is a an essential hallmark of cancers that usually present with an aggressive phenotype (Hanahan & Weinberg, 2011). They tend to evade cell death pathways and rely on aberrant proliferative signalling, among other processes. It has also been previously demonstrated that the oesophageal cancer cells in this study have increased proliferative activity in response to a number of extracellular matrices and soluble factor inputs (Driver & Veale, 2006; Jones & Veale, 2003; Shaw, 2013). The proposed route was thought to involve the PI3K-PKB axis, with some of the downstream targets being GSK3β, β-catenin and related transcription factors (Driver & Veale, 2006; Jones & Veale, 2003). The PI3K-PKB axis was proving to be of great significance and it led to the interest around the regulation of these signals at the membrane becoming a core focus. With studies that followed, it also became important to understand how fast some of these proliferative pathways could be activated; and the regulation thereof (Shaw, 2013). Along with other data leading up to this study (Dahan, 2013; Driver & Veale 2006; Shaw, 2013; *Chapter 2 to 3 of this research*), it became important to understand what happens to these cells when their exposure to an exogenous stimulus is limited (6 hours), instead of allowing the cells to grow for a duration that is the equivalent of their doubling time. Additionally, if indeed some of these cells are cycling and require mitogenic stimulation to stay on course, then the signaling intermediates that will be assayed in succeeding chapters should reveal that. For the moment, perhaps just checking if EGF is adequate as a viable stimulant that can be used to exploit the known great abundance of EGFR on the surfaces of both the HOSCC cells as well the A431 comparative control.

4.2 Materials and Methods

4.2.1 Cell Lines and Standardised Tissue Culture Conditions

This was carried out like in section 2.2.1 above.

4.2.2 Stimulation of Cells Using Exogenous EGF

This was carried out like in 3.2.2 above, but only incubated for 6 hours.

4.2.3 Proliferation Assays: The stimulation on HOSCC cells using EGF

A cell proliferation assay was performed on all HOSCC cell lines as well as the A431 comparative control cells using the MTT (Appendix B, 2.2) based method. This gives a measure to the number of viable cells which is related to the number of active (proliferating) cells.

Day 1: The cells were passaged at about 70 % confluency with trypsin/EDTA solution. The cell suspension was mixed in tissue culture medium supplemented with either 10% serum or 1% BSA. The cells were counted using a haemocytometer. The cell suspension was centrifuged briefly and the supernatant containing trypsin solution was removed. 8000 cells/well were resuspended in 200 µl of standard tissue culture medium supplemented with 10% FCS and were seeded on non-coated wells. The cells were then incubated in the same standard tissue culture conditions as above.

Day 2: The cells were rinsed with 100 µl of 1X PBS and Serum-Free or Standard (with 10 % FCS) was added to different well in triplicate for each biological replicate. These were then left to incubate for 6 hours under standard tissue culture

conditions. The number of viable cells were counted by MTT method. 100 µl of 0.5 mg/ml MTT in filter sterilized PBS was added to the adherent cells and the cells were incubated for 3 hours at 37ºC. The MTT solution was removed and 100 µl of DMSO was added to dissolve the formazan crystals. After an hour the absorbance reading was determined using a microtiter plate reader at 570 nm. The proliferation assays were performed in triplicates, under the same tissue culture conditions and in the same time frame. The number of proliferating cells was measured at the end point of an experiment. Clearly detachment may affect the proliferation responses, however in this instance data were generated for comparative purposes rather than an absolute measure.

4.2.4 Statistical Analyses of Data

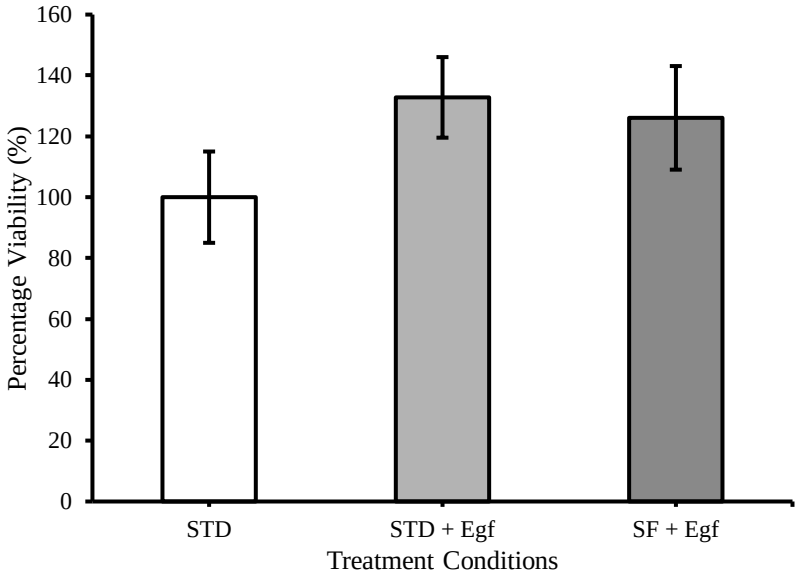
See section 2.2.7 above.

4.3 Results

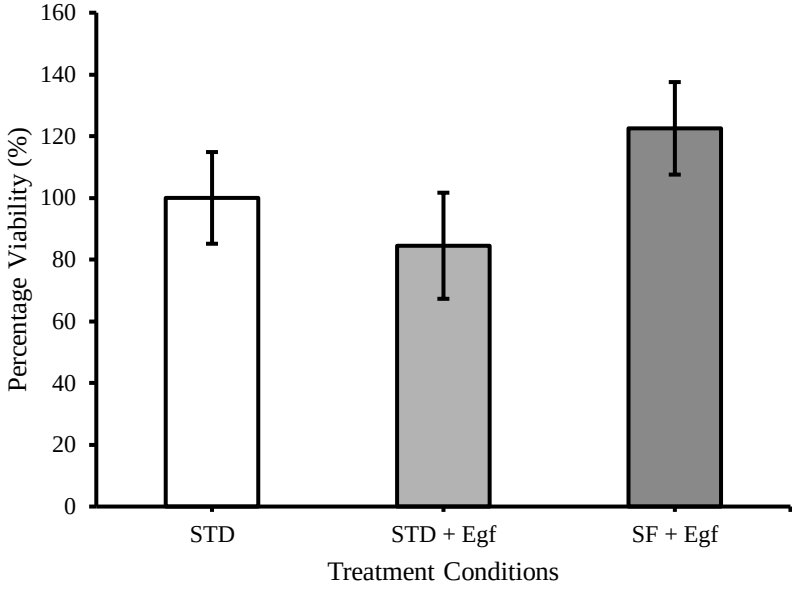
4.3.1 Exposure to EGF alone did not significantly affect proliferative activity in the 6 hour period

It was not surprising that none of the cells used here, including the A431's had significant changes in proliferative activity when cells that were treated with additional 10 ng/ml EGF in the standard tissue culture medium (Figure 4.1). However, it is also worth noting that all oesophageal cancer cells, as well as the A431 cells were able to maintain their basal proliferative activity even when cultured in serum-free media that was supplemented only with 10 ng/ml EGF (Figure 4.1). For this reason, it is possible that EGF alone, for 6 hours, is enough to maintain HOSCC cells in their viable state. thus further supporting earlier studies in this laboratory where ILK concentration and the cytoplasmic localisation of β-catenin could be stimulated by EGF (Driver & Veale, 2006; Jones & Veale, 2003).

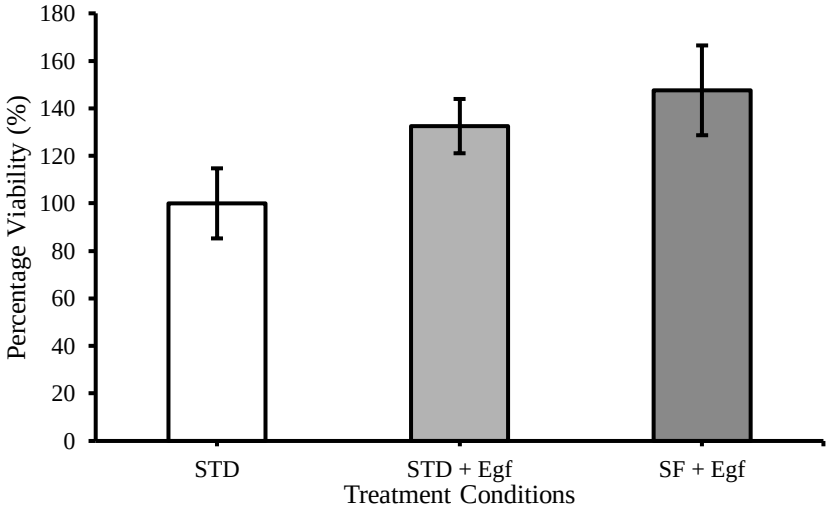
A – WHCO1



B – WHCO3



C – SNO



D – A431

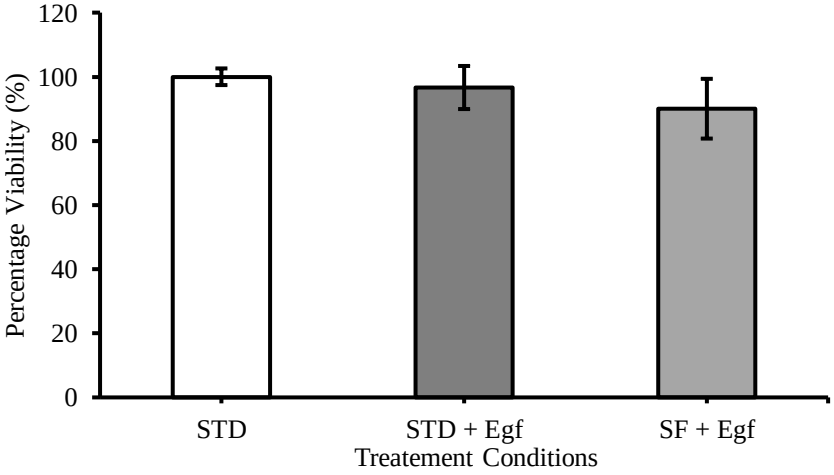


Figure 4.1: Serum starved oesophageal carcinoma cells are able to use EGF only to stay viable and proliferate. Proliferating oesophageal carcinoma cells were exposed to exogenous EGF either in the presence (STD – standard) or the absence (SF – serum free) of 10 % Foetal Calf Serum. Independent biological replicates ($n \geq 3$) of each cell line were incubated with their respective treatments in a sterile humidified CO_2 incubator at 37ºC. STD – Standard, for cells exposed to standard tissue culture medium (DMEM/F-12 with 10% FCS); STD+Egf – Standard medium supplemented with Egf; SF+Egf – Cells cultured in serum free medium (no FCS) supplemented with 10 ng/ml Egf. There were no significant differences in the proliferative activity of all the oesophageal cancer cells (WHCO1, WHCO3 and SNO cells, **A, B, C**, respectively), under all three culture conditions after 6 hours of incubation. This was also the case in the comparative cancer cells, the A431 cells, **D**. ***Note***: *A student's t-test was used to compare the proliferation of cells exposed to EGF (with or without FCS) to cells that were cultured standardly. S.E.M. was used for the error bars and statistical significance is denoted using an asterisk* (* = $p < 0.05$) *versus the respective controls.*

4.4 Discussion

The assay used here assay does not accurately measure cells that are cycling, nor does the time-point used in this study offer conclusive insights on proliferation of the cells under the different experimental conditions. The investigation was carried out to assess whether the changes in CK2 concentration in response to EGF stimulation also influenced the concentration of the P13K-PKB signalling pathway intermediates. The abnormal abundance of the epidermal growth factor receptor (EGFR) is a very important prognostic marker for a number of epithelial cancers, including oesophageal carcinoma. This is also particularly linked to aberrant and uncontrolled proliferation, growth and survival of these cancers. Specifically, for oesophageal carcinoma, with its molecular underpinnings still poorly defined, the amount of EGFR plays an important role. There are also no reports on mutations for the major oncogenes involved in proliferative signalling, including the EGFR gene (Shaw, 2013). Thus, the hopes of understanding this cancer are in the understanding of molecular signalling pathways which are activated by EGFR. Pertinently then, is the understanding of the contribution of proliferative signalling pathways; to the maintenance of this cancer. Specifically, in the context of the EGFR-PI3K-PKB proliferative signalling pathway, as it has been demonstrated to be the preferred route for the transduction of signals from EGFR in epithelial cancers.

Chapter 5: The dependable shepherd: CK2 supports an alternative to the PI3K-PKB route in proliferating monolayer adherent HOSCC cells

5.1 Introduction

Preceding chapters (Chapter 2 to 4) set up context for this part of the investigation, because it was noted that WHCO3 cells require a different route, that may not directly involve active PKB, for proliferative signalling. This is further supported by evidence elsewhere (Shaw, 2013); however here, only the reversible oxidation of PTEN was considered as a contributing factor. In Chapter 2 and 3, this study reports the relatively great abundance of CK2 in oesophageal carcinoma cells; additionally, reporting the increased accumulation of CK2 in response to the stimulation of these cells by EGF. This kinase has also been demonstrated to contribute to the regulation of this PI3K-PKB axis by either facilitating the recruitment of Hsp90 client proteins (PKB in this case) via cdc37, a co-chaperone that is phosphorylated by CK2 on Ser-13, forming a complex (Miyata & Nishida, 2005). This complex stabilises and protects active client kinases from degradation (Miyata & Nishida, 2005). Alternatively, CK2 has been shown to phosphorylate PKB at Ser-129, hyper activating it to induce increased cellular proliferation (Di Maira *et al.*, 2005).

As already introduced in the introductory chapter 1 (section 1.2) and Chapter 3, the regulation of CK2 activity is not well defined, owing to its pleiotropic and constitutively active nature, as well as its presence in discreet populations at subcellular level (Niefind & Issinger, 2010; Olsten & Litchfield, 2004; Tokuda *et al.*, 2007). It is also widely reported that the regulatory β subunits may be expressed more than the α and α' catalytic subunits, but this has limited functional significance since the catalytic subunits can also function outside the tetrameric form of the

enzyme (Olsten & Litchfield, 2004). This means that the regulation of this kinase is still not well understood, even more than 60 years after its discovery (Burnett & Kennedy, 1954). Altogether, these contribute to the ambiguous (or 'puzzling') understanding about its contribution to neoplastic transformation and/or the maintenance of the cancerous state. However, in the absence of known mutations on all *ck2* genes, it is the evidence of increased CK2 abundance in a number of solid tumours when compared to their normal counterparts which has fuelled efforts to elucidate its importance in cancer (viz. lung, breast, kidney and colon cancer) (Hanif *et al.*, 2010; O-charoenrat *et al.*, 2004; Staler *et al.*, 1994; Zhu *et al.*, 2010). Consequently, its contribution to cancer is understood in the context of its interaction/s with anti- and pro-oncogenic pathways (Ahmed *et al.*, 2002; Klumpp *et al.*, 2004; Di Maira *et al.*, 2005; Gao & Wang, 2006). One such example is the observation that when CK2 is overexpressed in HEK293T cells, it led to increased PKB activity (Ponce *et al.*, 2010). In another different study, Phosphatase and Tensin homolog (PTEN), an antagonist of PI3K activity, is a CK2 target (Torres & Pulido, 2001. Where this promiscuous kinase phosphorylates PTEN at the C-terminus at a Ser/Thr cluster (5 sites have been found) to promote the stabilisation of PTEN and attenuating its resultant phosphatase (Torres & Pulido, 2001). This is further supported by the loss of these phosphorylations in COS-7 cells treated with CK2 inhibitors (Torres & Pulido, 2001). Given these observations of the known influences by CK2 in the PI3K-PKB signaling axis and the findings in the oesophageal cancer cells used here, like the lack of active PKB (Chapter 2 to 4; Shaw, 2013), a role for CK2 can be speculated to promote the differential dependence of these cells on PKB.

Oesophageal cancers are known for defying most known solid tumour 'norms' and it makes it an important endeavour to clarify the dependence of this cancer on the PI3K-PKB axis to propagate signals from the EGFR; and how CK2 partakes.

5.2 Materials and Methods

5.2.1 Cell Lines and their Maintenance

The oesophageal cancer cells (WHCO1, WHCO3 and SNO), as well as the control cells (A431 and H1299) were cultured as in section **2.2.1** above.

5.2.2 Stimulation of HOSCC Cells Using Exogenous EGF

Cells were cultured to about 70% confluency as in section **2.2.1**, these were then rinsed with 1X PBS to removed cell debris and non-adherent cells. These cultures were then treated with the fresh medium (containing 10% FCS) that was supplemented with 10 ng/ml EGF as done previously (Jones & Veale, 2003). These cells were then incubated in the same tissue culture environment as above for 6 hours. Thereafter, cells were harvested for protein lysates that will be applied in analyses as set out below (**5.2.4**).

5.2.3 Blockade of the Activity of Signalling Intermediates Using Specific Molecular Inhibitors

Cells were cultured, as in section **2.2.1** above, to about 70% confluency. Following this, the culture media was carefully decanted and the adherent monolayer of cells were subjected to the following rinses, by gentle swirling: two 1X PBS rinses, then two rinses with serum-free DMEM/F-12 culture medium and finally one rinse with DMEM/F-12 with 10% FCS. After these rinses, fresh DMEM/F-12 (with 10% FCS) also supplemented with 10 ng/ml EGF as previously optimised for these cancer cells was added to the cultures (Jones & Veale, 2003) and/or with specific molecular inhibitors. These inhibitors specifically targeted CK2 - 4,5,6,7-tetrabromo-1H-benzatriazole (TBB) (Sigma - Cat. No. T0826) at 25mM and 50mM (Appendix B, 2.3); PI3K - LY294002 (Sigma - Cat No. 440204) at 20µM (Appendix B, 2.4) and

p90RSK - BI-D1870 (BioVision - Cat. No. 1924-1) at 10µM (Appendix B, 2.5). These treated cultures were then allowed to incubate for 6 hours under standard tissue culture conditions. It must be noted that aliquots from the same stock of DMEM/F-12 media were used so that any possible stock-stock variations did not affect cell behaviour, so that the behaviour of cellular cultures could only be attributed to the supplementary EGF. These cells were then incubated in the same tissue culture conditions as above for 6 hours. Thereafter, cells were harvested for protein lysates that will be applied in analyses as set out next.

5.2.4 Whole Cell Extraction of Protein from Adherent Cells

The extractions of protein from adherent cells that were stimulated with EGF, treated with pathway inhibitors or corresponding control cultures (standard and solvent controls) were carried out as in section **2.2.2** above.

5.2.5 Determination of Protein Content

The determination of protein content from lysates of cells that were stimulated with EGF, treated with pathway inhibitors or standardly cultured cells was carried out as in section **2.2.3** above.

5.2.6 Polypeptide Resolution using SDS-PAGE and Western Blotting

Lysates from experimental and control samples were then analysed by Western Blots as set out in section **2.2.4** above.

Important Note: In this Chapter, the main focus was to assay the presence of the active PKB (phosphorylated on Serine 473) and inhibited GSK3β (phosphorylated on Serine 09) forms of the proteins in response to multiple challenges (treatments with inhibitors), as well as stimulation with EGF in HOSCC cells. Having demonstrated the absence (the lack of bands on a blot) of active PKB in WHCO3

cells, as well as very faint bands (with inconclusive densitometric analyses) of active PKB in most of the cell lines in previous chapters. The focus here was the physical evidence of the blots, or bands as visualised, without the need for densitometric analyses (which is also a semi-quantitative measure of abundance) ; because the objective was to now provide physical evidence for the presence or absence of the intermediates, whilst also mentioning the size of the band as an indicator of abundance from that sample. Experiments were repeated at least three times and what is presented here are a sample, along with their corresponding controls and the β-actin loading control. The reader is assured that the images are a true reflection of this end-point assay, with regards to protein yields under the different treatment conditions.

5.2.7 Image Capture

Images of X-ray films were captured as per section **2.2.5** above.

5.2.8 Proliferation Assays: The effects of EGF stimulation and/or specific pathway inhibition

Proliferation assays were set out in the same way as in section **4.2.3** were carried out in cultures that were stimulated for 6 hours with EGF and/or specific pathway inhibitors and incubated in the same tissue culture conditions as above (section **5.2.3** for the treatments).

5.2.9 Statistical Analyses of Data

The data in this chapter was also analysed as in section **2.2.7** above.

5.3 Results

It is established herein that the differential abundance of endogenous CK2α within the HOSCC series of cell lines was greater than those of the comparative positive control cell line, the H1299 Non-Small Lung Carcinoma (NSCLC) cells (Table 2.2 in Chapter 2). Under these standard tissue culture conditions, all HOSCC cells contained at least 1.4 times more CK2α than the H1299 cells. Understandably, it is already a widely established fact that there is natural variation abundance of proteins as measured from different tissues. However, these high levels of CK2α in the HOSCC cells potentiate an important role for active CK2 in oesophageal cancer. Pertinently, for these EGFR over-expressing oesophageal cancer cells, it was important to elucidate CK2's role in their proliferation. Ultimately, to elucidate how CK2 influences the PI3K-PKB proliferative signalling axis in oesophageal carcinoma.

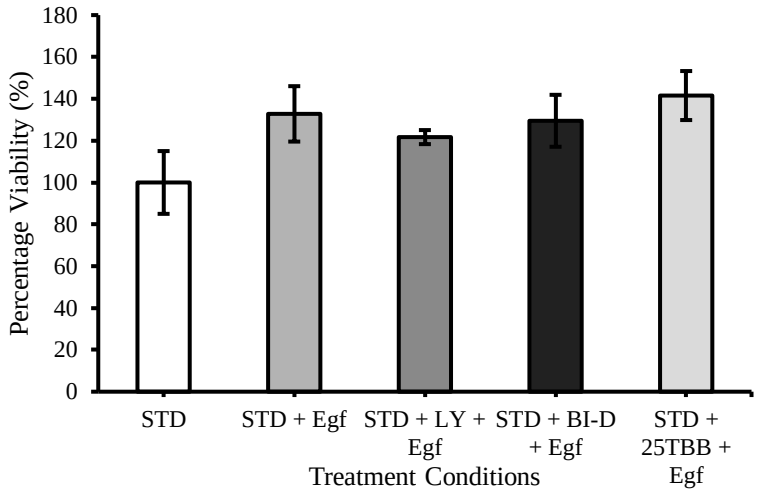
5.3.1 Differing proliferative responses to specific pathway inhibitors suggests varied routes to effect proliferation in oesophageal cancer

Blockade of endogenous CK2 activity by the specific small molecular inhibitor, TBB, was the chosen method as it has been extensively demonstrated as being effective in attenuating the proliferation of epithelial cancer cells (Lee *et al.*, 2010; Miyata & Nishida, 2005; Zhu *et al.*, 2010; Zien *et al.*, 2003). Unexpectedly, blocking the influence of CK2 in EGF stimulated cells, by using TBB did not significantly modify the proliferation of the WHCO1 and WHCO3 cells (Figure 5.1 A, B). However, the SNO oesophageal cancer cells, demonstrated increased proliferative activity in presence of this inhibitor (Figure 5.1 C).

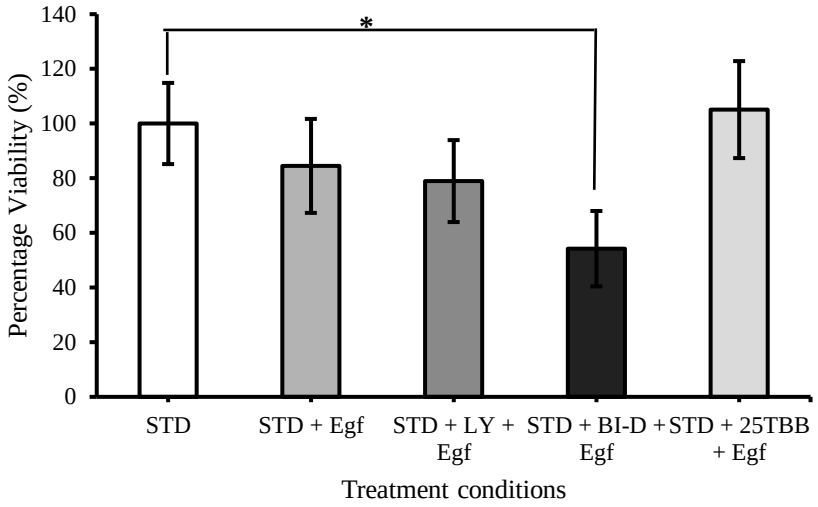
Conversely, the decrease in the proliferation of the A431 cells when CK2 was blocked using TBB was consistent with documented reports in other studies using carcinoma cells (Di Maira *et al.*, 2005; Gao & Wang, 2006) (Figure 5.1 D). It was, however, interesting to note that A431 cells had consistently decreased proliferative activity in the presence of each of the inhibitors used: PI3K (LY294002), RSK (BI-D1870) and CK2 (TBB) (Figure 5.1 D).

The WHCO3 and SNO cells were the only other cells to respond, in opposite ways to RSK blockade in the presence of EGF (Figure 5.1 B and C). Both responses were interesting, with the marked decrease in proliferation in the WHCO3 cells (almost 50% decrease) (Figure 5.1 B). Additionally, although these WHCO3 cells in this particular lane were also exposed to exogenous EGF, this did not sustain their proliferation because they were concurrently treated with the RSK inhibitor. The observations in the WHCO3 cells were different to what the SNO cells demonstrated: there were significant increases in the amount of viable cells in when the cells were treated with the CK2 inhibitor or the RSK inhibitor whilst also being exposed to EGF. These observations are suggestive of disparities in the influence exerted by CK2 (and the other intermediates) in the proliferation of the cancer cells used in this study. Effectively, these cancers cells are differentially dependent on the activity of proliferative signalling pathways.

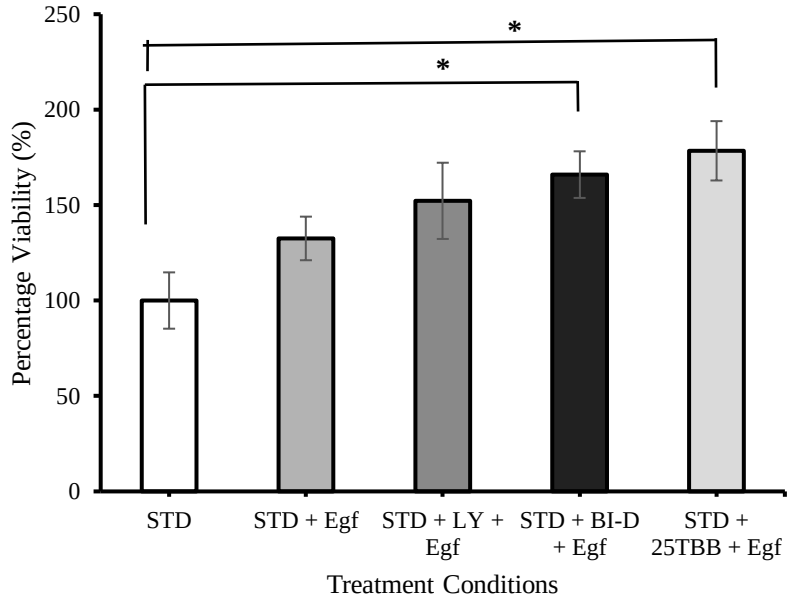
A – WHCO1



B – WHCO3



C – SNO



D – A431

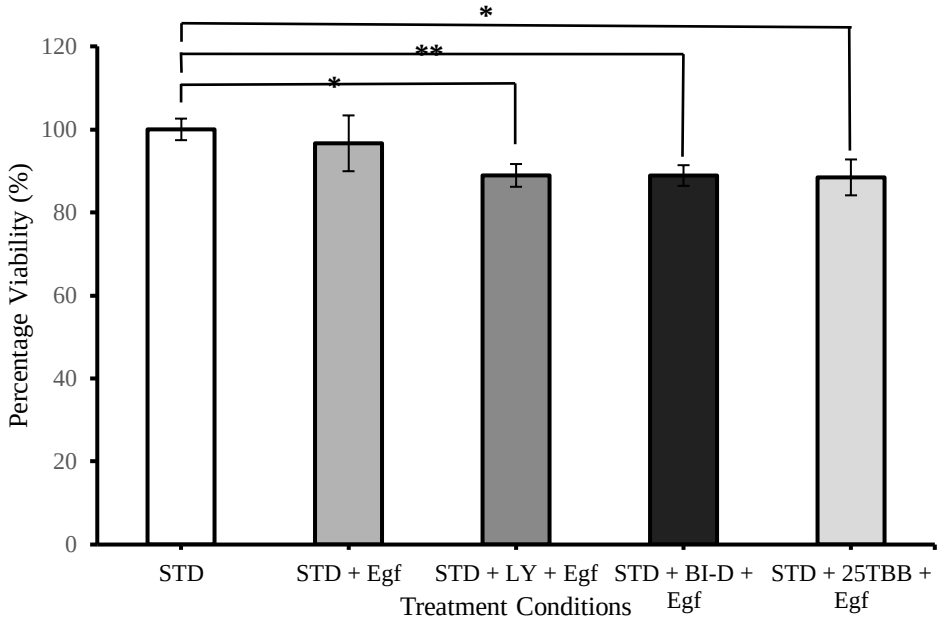


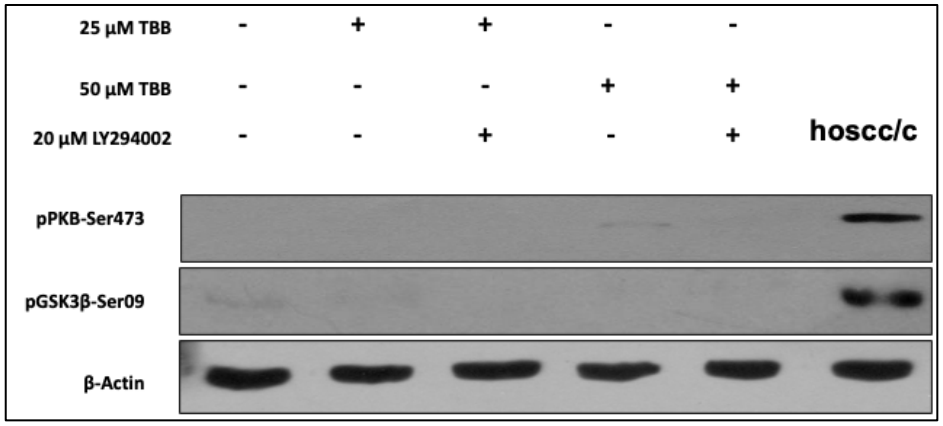
Figure 5.1: EGF stimulated oesophageal cancer cells differentially respond to proliferative pathway inhibitors. Proliferating oesophageal cancer cells which were at about 70% cellular density were exposed to different or combinations of specific inhibitors in standard tissue culture medium (STD). Independent biological replicates ($n \geq 3$) of each cell line were incubated with their respective treatments in a sterile humidified CO_2 incubator (95% air, 5% CO_2) at 37ºC. STD – Standard, for cells exposed to standard tissue culture medium (DMEM/F-12 with 10% FCS); STD+Egf – Standard medium supplemented with 10 ng/ml of exogenous Egf; STD+LY+Egf – Cells cultured in STD medium, supplemented with Egf and 20 μM LY294002; STD+BI-D+Egf – Cells cultured in STD medium, supplemented with Egf and 10 μM BI-D1870; STD+25TBB+Egf – Cells cultured in STD medium, supplemented with Egf and 25 μM TBB. **A,** there were no significant differences in the proliferation of WHCO1. **B,** WHCO3 Cells have a significantly marked decrease in proliferation when treated with a RSK inhibitor and EGF. **C**, the SNO cells demonstrated general increases, with the statistically significant increase being when exposed to EGF concurrently with either a RSK inhibitor or the CK2 inhibitor. **D,** the A431 cells were the only ones to show significant decreases in proliferation in the presence of either of the three inhibitors. ***Note****: A student's t-test was used to compare the proliferation of cells exposed to EGF (with or without FCS) to cells that were cultured standardly. S.E.M. was used for the error bars (n=3) and statistical significance is denoted using an asterisk ($p < 0.05$) versus the respective controls*

5.3.2 Oesophageal Carcinoma Cells are Differentially Dependent on CK2 for their Maintenance of Proliferative Signals

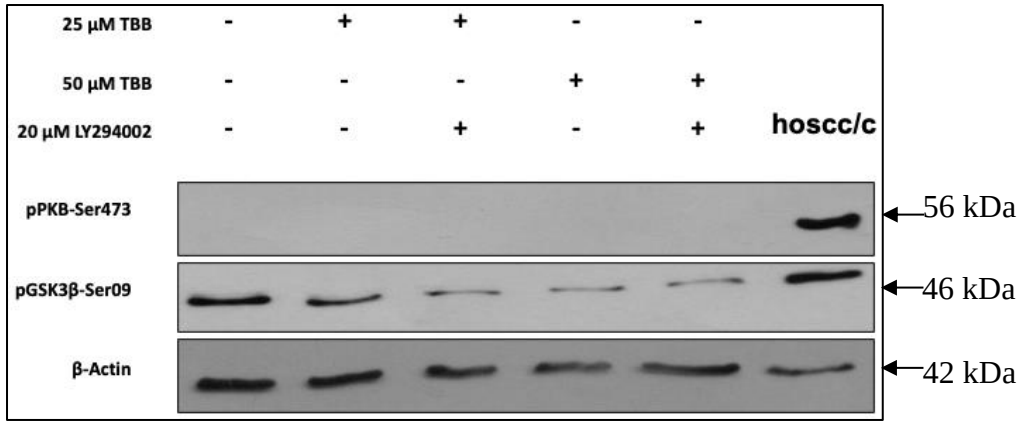
Profiling the activity of the PI3K/PKB pathway and exploring as the influence of CK2 became necessary to understand how these oesophageal cancer could use this kinase for proliferative signalling. The elimination of the influence of active CK2 on proliferative signalling using TBB, modestly impacted the abundance of phospho-PKB (Ser473), as evidenced by a faint band when compared to the corresponding control in the SNO cells (Figure 5.2 C). the other oesophageal cancer cells and the A431 cells, had very little (mostly undetectable) phospho-PKB, and none of the experimental conditions affected this (Figure 5.2 A, B and D). Interestingly, there was an CK2 inhibitor dose-dependent increase in the abundance of phospho-PKB in the SNO oesophageal cancer cells even in the presence of the PI3K inhibitor, LY294002 (Figure 5.2 C). With an almost opposite observation of a dose-dependent decrease in the concentration of phospho-GSK3β in the SNO oesophageal cancer cells (similar to the A431 cells).

Blocking the influence of CK2 by using a specific inhibitor (TBB) in the WHCO3 oesophageal cancer cells decreased the abundance of inhibited GSK3β (pGSK3β-Ser09) when the cells were concurrently exposed to either LY294002 or BI-D1870, as well as at higher TBB concentrations (Figure 5.2 B). However, because it has been established that these cells do not have phospho-PKB (Ser473) (Figure 2.1 and Figure 5.2 B, Table 2.2), the PI3K/PKB signalling axis cannot be the main route for proliferative signalling in this oesophageal cancer cell line. From this, an immediate alternative to GSK3β inhibition in response to growth factors had to be investigated.

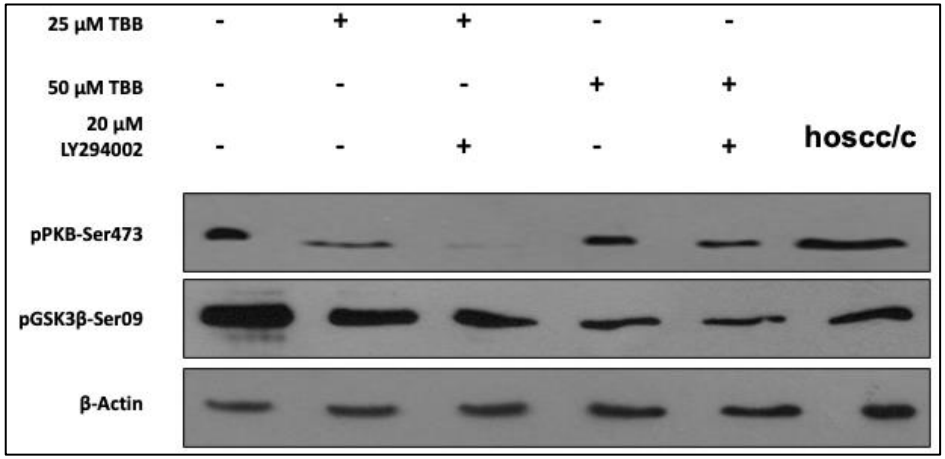
A – WHCO1



B – WHCO3



C – SNO



D – A431

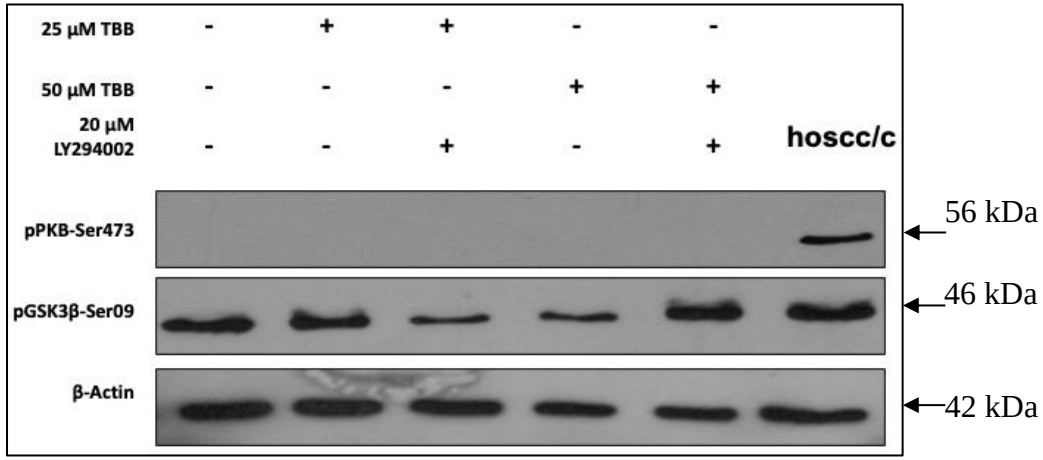
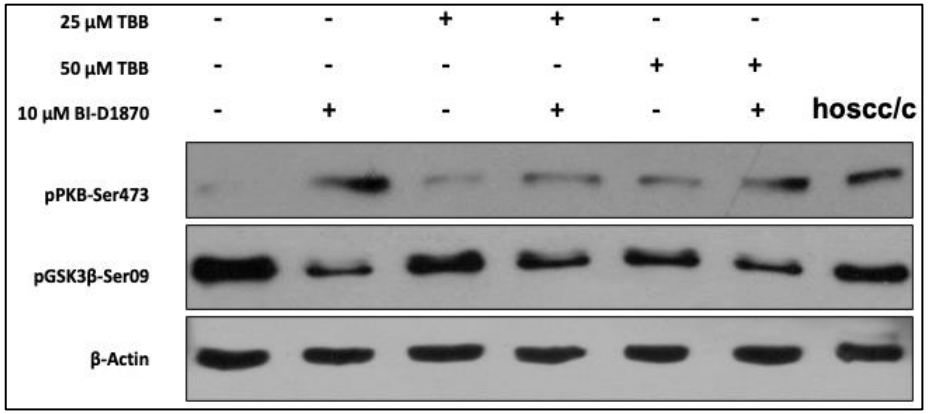


Figure 5.2: Differential responses of the PI3K-PKB signalling axis' activity in oesophageal cancer cells in response to concurrent blockade of CK2 and PI3K. Representative Western Blots from cellular lysates of oesophageal cancer cells (**A** - WHCO1, **B** - WHCO3, and **C** - SNO) and the comparative control cell line (**D** - A431–epidermoid carcinoma), after 6 hours of incubation with the respective inhibitor/s. Concurrent blockade of CK2 function (TBB) with the specific inhibition of PI3K (LY294002) variably affected the abundance of phospho-PKB-Ser473(pPKB-Ser473) abundance, detected at 56 kDa, and phospho-GSK3β-Ser09 (pGSK3β-Ser09), detected at 46kDa . The hoscc/ lane contains a sample that is similar to the oesophageal cancer cells used here, and it is known to contain copious amounts of protein and reacts very well with most known antibodies. Band intensities and size are an indicator of the amount of protein present. A standardized amount of protein was loaded from lysates prepared from cells grown under standard tissue culture conditions as described in the materials and methods section; and a β-Actin (42 kDa) loading control was used.

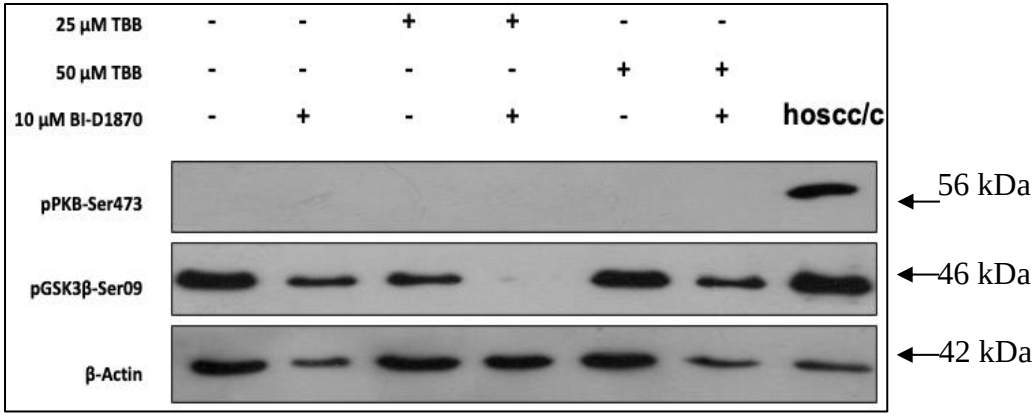
The RSK-class of kinases activated by the MAPK pathway became the closest alternative route, as both the PI3K/PKB and MAPK pathways play major roles in proliferative signalling in a number of cancers. Interruption of RSK activity by a specific inhibitor (BI-D1870) brought about attenuation of the inhibition of GSK3β (decreased pGSK3β-Ser09) in WHCO3 cells (Figure 5.3 B). Moreover, blocking CK2's influence with the concurrent interruption of RSK activity markedly decreased the abundance of phospho-GSK3β (Ser09) as shown by the very faint band on the blot (Figure 5.3 B). These findings point towards an important role for RSK-dependent promotion of proliferative signalling in the WHCO3 cells. Further presenting this as an alternative route for proliferative signalling in oesophageal cancer, as shepherded by CK2.

Conversely, the blockade of CK2 activity concurrent with specific PI3K inhibition in the SNO cells (as well as the WHCO1) deactivated the PI3K/PKB axis, leading to the downstream attenuation of GSK3β inhibition (Figure 5.2 C). Whereas, CK2 blockade concurrent with specific RSK inhibition sustained signalling form the PI3K-PKB axis in the SNO and WHCO1 cells, as they had maintained or increased phospho-PKB (Ser473) and phospho-GSK3β (Ser09) abundance (Figure 5.3 A and C). All these observations support the importance of the PI3K-PKB signalling axis for proliferative signalling especially in the context WHCO1, SNO and A431 cell lines. Whilst helping to define a salvage proliferative pathway for the WHCO3 cells; by considering the role CK2 could play as a lateral influencer to canonical proliferative signalling routes from EGFR.

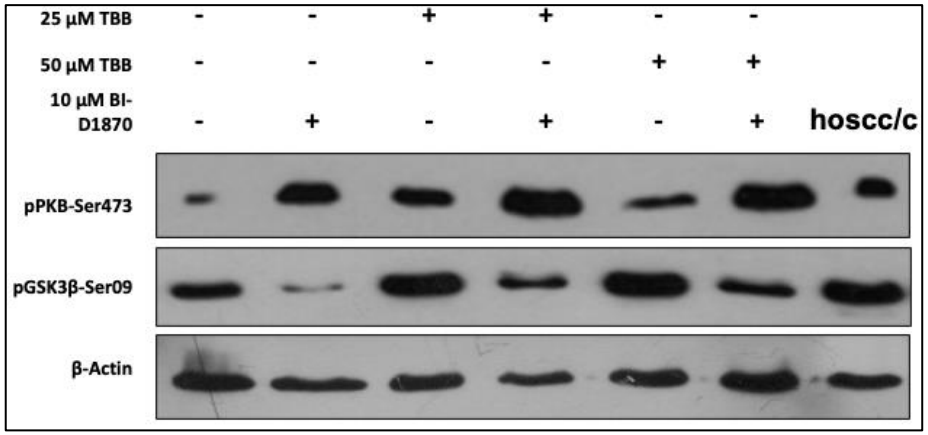
A – WHCO1



B – WHCO3



C – SNO



D – A431

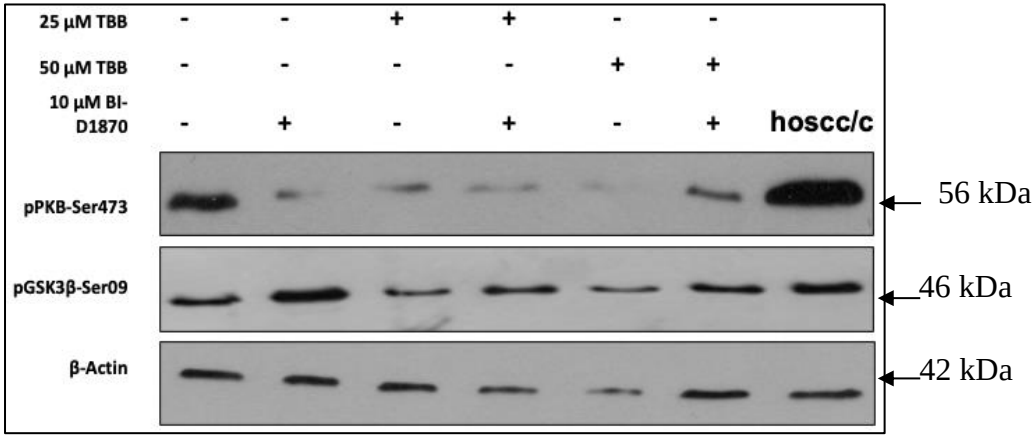


Figure 5.3: RSK's provide an alternative route to effect GSK3β inhibition which is facilitated by CK2 activity in oesophageal cancer cells. Representative Western Blots from cellular lysates of oesophageal cancer cells (**A** - WHCO1, **B** - WHCO3, and **C** - SNO) and the comparative control cell line (**D** - A431– epidermoid carcinoma), after 6 hours of incubation with the respective inhibitor/s. Concurrent blockade of CK2 function (TBB) with the specific inhibition of RSKs (BI-D1870) variably affected the abundance of phospho-PKB-Ser473(pPKB-Ser473) abundance, detected at 56 kDa, and phospho-GSK3β-Ser09 (pGSK3β-Ser09), detected at 46kDa . The hoscc/ lane contains a sample that is similar to the oesophageal cancer cells used here, and it is known to contain copious amounts of protein and reacts very well with most known antibodies. Band intensities and size are an indicator of the amount of protein present. A standardized amount of protein was loaded from lysates prepared from cells grown under standard tissue culture conditions as described in the materials and methods section; and a β-Actin (42 kDa) loading control was used.

5.4 Discussion

The diverse functions of CK2 in tumours are fast growing; they range from anti-apoptotic to pro-survival roles, depending on the signalling intermediate it associates with. Pertinently, this kinase has also been implicated in augmenting the activity of PKB to promote and maintain aberrant signalling in cancer (Di Maira *et al.*, 2005). Additionally, PKB is frequently implicated in the resistance to therapeutic interventions (Ma *et al.*, 2008; Winograd-Katz & Levitzki, 2006). Taken together, it is thus not surprising that CK2 has attracted significant and promising attention as a therapeutic target. Noting these and the evidence of the high abundance of CK2 in HOSCC cells as congruent with literature and the related reports on promoting proliferative signalling as this study has also established (Gowda *et al.*,, 2017). Unexpectedly, these data also reveal a unique CK2 influence, where CK2 shepherds the recruitment of an alternative EGF-responsive pathway in order to maintain proliferative signalling oesophageal cancer cells.

Stimulation of cells by exogenous EGF, of concentrations in the low nM range, has been demonstrated to preferentially stimulate proliferative signalling pathways (Krall *et al.*, 2011).There is, however, always a chance of activating other EGF-responsive pathways downstream EGFR activation. Furthermore, the well-known phenomenon of pathway-crosstalk cannot be overlooked as it is known to provide negative and/ or positive regulatory influences on dominant (or canonical) signalling axes (Gual *et al.*, 2003; Turke *et al.*, 2012). To this end PKB has long been held as the "hub" of EGF-responsive tumourigenic signalling (Datta *et al.*, 1999). Intrusively, an ERK-dependent negative regulatory influence on the PI3K/PKB pathway exists and is well documented; where IRS-1 is phosphorylated by active ERK's to prevent PI3K's docking onto membrane sites of active EGFR's and typically additional to RSK activation (Gual *et al.*, 2003; Smadja-Lamere, 2013). This ERK/RSK relationship impedes on the activation of PI3K and can be utilised by cells to limit inputs to PKB, usually delaying PKB-dependent physiological behaviour.

Accordingly, in these oesophageal carcinoma cells, both the ERK/RSK and PI3K/PKB pathways would have been activated, although the PI3K-PKB signalling axis could be the preferred route of growth factor derived signals (Jones & Veale, 2003; Krall *et al.*,2011). It is possible that cross-talk between these two pathways is more regulatory (perhaps homeostatic). Furthermore, it is probable that CK2 is instrumental in sustaining this relationship, by synergistically augmenting the regulatory interactions. This is supported by the diminishing abundance of active PKB, which is not supported by sustained inhibition of GSK3β when PI3K activity is blocked in some of the oesophageal carcinoma cells (SNO – Figure 5.2 C and Figure 5.3 C; WHCO3 – Figure 5.2 B).

Chapter 6: General Discussion - Defining a role for CK2 in HOSCC

6.1 Synthesis

From very early studies in South African and other African male populations, the accurate diagnoses of oesophageal cancers would be when the diseases are already in the advanced stages and this is compounded by the lack of facilities in rural and other remote regions (Bey *et al*., 1976; Bye *et al.*, 2011; Somdyala *et al*., 2010). The puzzling aetiology of HOSCC, its racialised incidence as well as the elemental characterisations of the molecular mechanisms involved in the progression of this cancer also move it up the list of deadly cancers in black male populations in South Africa and parts of East Africa (Bye *et al.*, 2011; McCabe & Dlamini, 2005). The racial disparities noted in the incidence of HOSCC are not unique to this cancer, as they have been shown in breast cancer as well (Shavers, 2003). Furthermore, elevated EGFR levels in HOSCC and other epithelial cancers have long being held as the main drivers to the resistance of therapeutics even when the targets are downstream effectors (Fichter *et al.*, 2014; Song *et al.*, 2015; Veale & Thornley, 1989; Wang *et al*., 2015). Targeting these receptors directly has been the biggest hurdle, mainly, due to their great abundance. Fortunately a recent study by Song *et al.* in other oesophageal carcinoma cells (these cells are not of African or South African origin) reported that yes-associates protein 1 (YAP1) is a promising target since it is upstream of the overexpression of EGFR. Such advancements introduce a glimmer of hope in the battle against HOSCC. However, these would have to be tested in the context of the geographic and racial disparities that appear to govern the incidence, prognosis and treatment of HOSCC.

All these concerns and potential advancements only increase the urgency to fully elucidate the molecular mechanisms that underpin the progression and survival of

HOSCC. The elevated CK2 levels reported here are just another step towards the full understanding of the molecular interactome that brings about an evasive and aggressive phenotype in oesophageal carcinomas.

The molecular interactions and substrates that this promiscuous kinase has been associated are in excess of 350 and is evidently still growing (Bian *et al.*, 2013). The *ck2* genes for this kinase do not lend themselves to the classical definition of an oncogene, and the associated protein products are also not classically or canonically regulated by any well-defined molecular mechanisms. This means that its influence on cellular events can be understood by knowing its effects on the different molecules it interacts with; and in cancers this could mean enhancing or stabilising some oncogenic pathways. In the HOSCC cells used in this study, stimulation with EGF appears to enhance the abundance of CK2. This is hypothesised to be associated with the cell cycle because these EGFR overexpressing carcinoma cells have been shown in a number of studies to activate EGFR-responsive canonical pathways that terminate at the enhancement of different cell growth and proliferation signals (Krall *et al.*, 2011). However, this unique response to mitogenic stimulation allows for the elevation of CK2 as an important target to understand in the elucidation of molecular mechanisms that underpin HOSCC.

Particularly relevant to this study is the accumulating evidence that points towards CK2 being a "lateral influence" to signals from the PI3K/PKB signalling axis at different levels of its regulation (Salizzato *et al.* 2016). Most relevant, is the phosphorylation of PTEN on Ser370 that leads to a loss of phosphatase activity, favouring PKB activation (Torres and Pulido, 2001; Zhu *et al.*, 2010). Furthermore, the overexpression of CK2 in HEK293 cells has been shown to promote increased PKB activity by phosphorylating it at Ser129 (Di Maira *et al.*, 2005; Ponce *et al.*, 2010). Thus the observed accumulation of active PKB can be further accounted for by this relationship between CK2 and PTEN in HOSCC cells. Most importantly, this observation could explain the increased inhibition of GSK3β in WHCO3 cells

to promote proliferative signalling. At this point, due to the persistently undetectable active PKB in all experimental settings used here, it became very clear that the route employed by WHCO3 cells to inhibit GSK3β is different from that of the other HOSCC cells used in this study. In contrast, the other HOSCC cells only maintain low basal levels of active PKB, but they do increase its abundance in response to EGF stimulation.

The maintenance of low levels of active PKB potentially permits these oesophageal carcinoma cells to variably escape negative regulatory feedback loops (see proposed CK2 influence in Figure 6.1 below) in order to maintain and promote signals which are important for their abnormal proliferation (**Figure 2.1**, **5.2** and **5.3**). The known negative feedback loop in the ERK/RSK axis is possibly restrained by CK2 to allow for PKB-independent proliferative signalling via this alternative pathway (Figure 6.1).

.

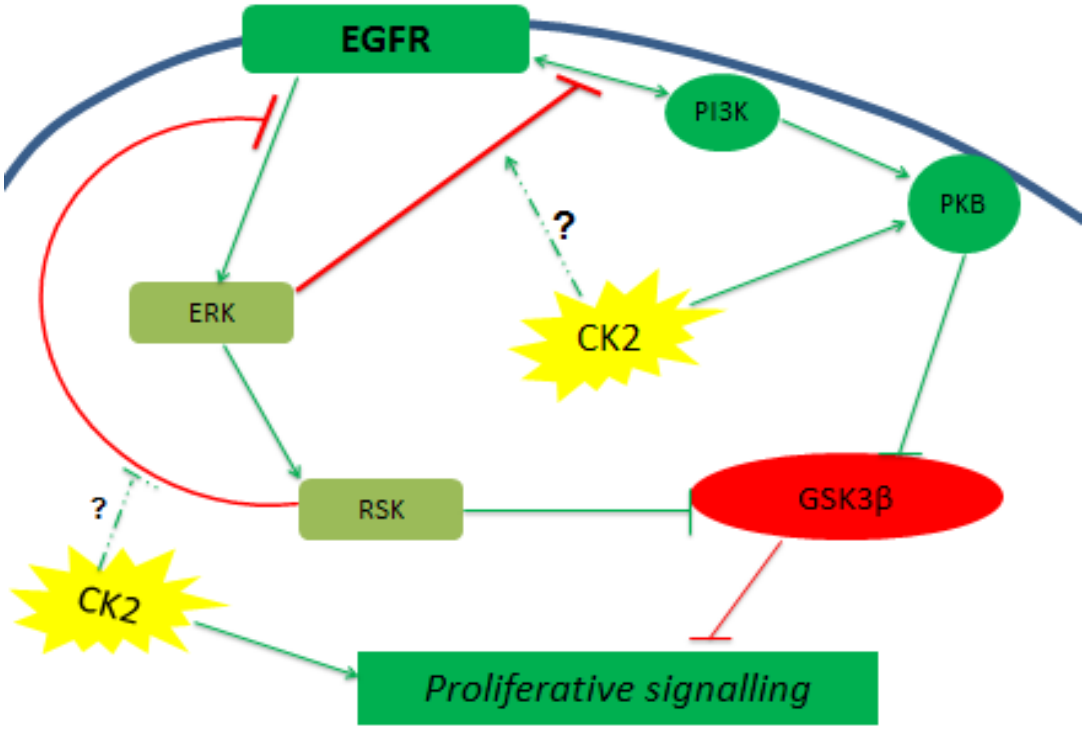


Figure 6.1: A summary of EGFR-derived proliferative signals in oesophageal cancer cells, with the proposed influence of CK2 and the integration of these proliferative signals. Signals from the EGFR - Epidermal Growth Factor Receptor can be transduced via both the PI3K – Phosphatidylinositol-3 Kinase and ERK – signalling routes. Signals transduced via PI3K, canonically, lead to the activation of PKB which would then lead to the inhibition of GSK3β (Glycogen Synthase Kinase 3-β), to promote proliferative signalling. Whereas, when transduced via ERK's, these induce the activation of RSK's; which also lead to the inhibition of GSK3β. Line colours: Pro-proliferative signalling (Green); Anti-proliferative signalling (Red).

In a recent study it was also found that in Src homology-2 domain containing protein tyrosine phosphatase 2 (SHP2)-deficient bone marrow cells, there is enhanced PI3K/PKB activation and this was similar to the blockade of RSKs and ERK (Wang *et al.*, 2018). These not only confirms the existence of the cross-talk (and regulation) between these proliferative signalling pathways, but also support an important regulatory role for CK2. It is also speculated that CK2 chaperones a salvage pathway in instances where cells do not have a sufficiently active

PI3K/PKB axis by preventing the negative feedback loop in the ERK/RSK pathway. More directly, this could also be achieved by promoting signalling to PI3K at the membrane , perhaps in a manner that might involve SHP2 (Wang *et al.*, 2018) as it is an important transducer of signals from soluble factors (like growth factors) at the membrane.

6.2 Conclusions

These and other observations, place CK2 as an important regulator of both the PI3K/PKB and ERK/RSK proliferative pathways in adherent HOSCC cells. This kinase may enhance the resistance to therapeutic interventions that are dependent on PKB, whilst also chaperoning an alternative route in these HOSCC cells.

6.3 Prospects

There is a clear need to further characterise the interactions of CK2 with other EGF responsive pathways; not only as salvage pathways, but also because this could help us identify new therapeutic strategies for oesophageal carcinomas since they demonstrate aberrancies in these oncogenic pathways. This may seem like trivial additions to a growing list of CK2 interactions, but since its influence is predominantly lateral, understanding its role in canonical pathways could support the enhancement of existing targeted therapies as well.

References

Ahmed, K., Gerber, D. A., & Cochet, C. (2002). Joining the cell survival squad: an emerging role for protein kinase CK2. Trends in Cell Biology. **12**: 226–230

Arteaga, C. L. (2003). ErbB-targeted therapeutic approaches in human cancer. Experimental Cell Research. **284**: 122-130

Azemar, M., Schmidt, M., Arlt, F., Kennel, P., Brandt, B., Papadimitriou, A., Groner, B. & Wels, W. (2000). Recombinant antibody toxins specific for ErbB2 and EGF receptor inhibit the in vitro growth of human head and neck cancer cells and cause rapid tumour regression in vivo. International Journal of Cancer. **86**: 269-275

Barkan, D., Green, J.E. & Chambers, A.F. (2010). Extracellular matrix: a gatekeeper in the transition from dormancy to metastatic growth. European Journal of Cancer. **46**:1181-1188

Bey, E., Alexander, J., Whitcutt, J.M., Hunt, J.A. & Gear, J.H.S. (1976). Carcinoma of the esophagus in Africans: Establishment of a continuously growing cell line from a tumour specimen. In Vitro. **12**: 107-114

Bosc, D.G., Lüscher, B. & Litchfield, D.W. (1999). Expression and regulation of protein kinase CK2 during the cell cycle. Molecular and Cellular Biochemistry. **191**: 213-222

Braamhall, S., Noack, N., Wu, M. & Loewenberg, J.R. (1969). A simple colorimetric method for determination of protein. Analytical Biochemistry. **31**: 146-148

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. **72**: 248-254

Brazil, D.P. & Hemmings, B.A. (2001). Ten years of protein kinase B signalling: a hark Akt to follow. Trends in Biochemical Sciences. **26**: 657-664

Brunton, V.G., MacPherson, I.R.J. & Frame, M.C. (2004). Cell adhesion receptors, tyrosine kinases and actin modulators: a complex three-way circuitry. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. **1692**:121-144.

Bye, H., Prescott, N.J., Matejcic, M., Rose, E., Lewis, C.M., Parker, M.I. & Mathew, C.G. (2011). Population-specific genetic associations with oesophageal squamous cell carcinoma in South Africa. Carcinogenesis. **32**: 1855-1861

Clevers, H. (2006). Wnt/beta-catenin signaling in development and disease. Cell. **127**: 469-480

Cooper, C.R., Chay, C.H. & Pienta, K.J. (2002). The role of αvβ3 in prostate cancer progression. Neoplasia. **4**: 191-194

Cozza, G., Bortolato, A. & Moro, S. (2010). How Druggable Is Protein Kinase CK2? Medicinal Research Reviews. **30**: 419-462

D'Amore, C., Borgo, C., Sarno, S. & Salvi, M. (2020). Role of CK2 inhibitor CX-4945 in anti-cancer combination therapy–potential clinical relevance. Cellular Oncology. :1-14

Da Cunha Santos, G., Shepherd, F.A. & Tsao, M.S. (2011). EGFR Mutations and Lung Cancer. Annual Review of Pathology. **6**: 49-69

Dahan, Y.L. (2013). The integrative role of uPAR in outside-in signalling in human oesophageal squamous cell carcinoma cells. (Doctoral dissertation University of the Witwatersrand, Johannesburg, the Republic of South Africa).

Danielpour, D., & Song, K. (2006). Cross-talk between IGF-I and TGF-β signaling pathways. Cytokine & Growth Factor Reviews. **17**: 59-74

Datta, S. R., Brunet, A. & Greenberg, M. E. (1999). Cellular survival: a play in three Akts. Genes & Development. **13**: 2905-2927

Daverey, A., Drain, A.P. & Kidambi, S. (2015). Physical intimacy of breast cancer cells with mesenchymal stem cells elicits trastuzumab resistance through Src activation. Scientific Reports. **5**: 1-13

Delcommenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J., & Dedhar, S. (1998). Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. Proceedings of the National Academy of Sciences. **95**: 11211-11216

De Luca, A., Carotenuto, A., Rachiglio, A., Gallo, M., Maiello, M. R., Aldinucci, D., Pinto, N. & Normanno, N. (2008). The role of the EGFR signaling in tumor microenvironment. Journal of Cellular Physiology. **214**: 559-567

Di Maira, G., Salvi, M., Arrigoni, G., Marin, O., Sarno, S., Brustolon, F., Pinna, L.A. & Ruzzene, M. (2005). Protein kinase CK2 phosphorylates and upregulates Akt/PKB. Cell Death and Differentiation. **12**: 668-677

Dong, L.Q. & Liu, F. (2005). PDK2: the missing piece in the receptor tyrosine kinase signaling pathway puzzle. American Journal of Physiology - Endocrinology and Metabolism. **289**: E187-E196

Doré J.J.E., Yao, D., Edens, M., Garamszegi, N., Sholl, E.L. & Leof, E.B. (2001). Mechanisms of transforming growth factor-βreceptor endocytosis and intracellular sorting differ between fibroblasts and epithelial cells. Molecular Biology of the Cell. **12**: 675-684

Driver, G.A. & Veale, Robin B. (2006). Modulation of integrin-linked kinase (ILK) expression in human oesophageal squamous cell carcinoma cell lines by the EGF and TGFbeta1 growth factors. Cancer Cell International. **6**: 12

Duncan, J.S. & Litchfield, D.W. (2008). Too much of a good thing: the role of protein kinase CK2 in tumorigenesis and prospects for therapeutic inhibition of CK2. Biochimica et Biophysica Acta. **1784**: 33-47

Duncan, J. S., Turowec, J. P., Vilk, G., Li, S. S. C., Gloor, G. B., & Litchfield, D. W. (2010). Regulation of cell proliferation and survival: convergence of protein kinases and caspases. Biochimica et Biophysica Acta. **1804**: 505-10

Ekman, S., Bergqvist, M., Hedlin, C.-H. & Lennartsson, J. (2007). Activation of Growth Factor Receptors in Esophageal Cancer - Implications for Therapy. The Oncologist. **12**: 1165-1177

Enzinger, P.C. & Mayer, R.J. (2003). Esophageal Cancer. The New England Journal of Medicine. **349**: 2241-2252

Fanucchi, S. & Veale, R.B. (2009). Role of p53/FAK association and $p53^{Ser46}$ phosphorylation in staurosporine-mediated apoptosis: Wild type versus mutant p53-R175H. FEBS Letters. **583**: 3557-3562

Fichter, C.D., Timme, S., Braun, J.A., Gudernatsch, V., Schöpflin, A., Bogatyreva, L., Geddert, H., Faller, G., Klimstra, D., Tang, L. & Hauschke, D. (2014). EGFR, HER2 and HER3 dimerization patterns guide targeted inhibition in two histotypes of esophageal cancer. International journal of cancer. **135**: 1517-1530

Franchin, C., Borgo, C., Cesaro, L., Zaramella, S., Vilardell, J., Salvi, M., Arrigoni, G. & Pinna, L.A. (2018). Re-evaluation of protein kinase CK2 pleiotropy: new insights provided by a phosphoproteomics analysis of CK2 knockout cells. Cellular and Molecular Life Sciences. **75**: 2011-2026

Gao, Y. & Wang, H.-Y. (2006). Casein kinase 2 is activated and essential for Wnt/beta-catenin signaling. The Journal of Biological Chemistry. **281**: 18394-18400

Geiger, B., Bershadsky, A., Pankov, R. and Yamada, K.M. (2001). Transmembrane crosstalk between the extracellular matrix and the cytoskeleton. Nature Reviews Molecular Cell Biology. **2**: 793-805

Giepmans, B.N.G. & van Ijzendoorn, S.C.D. (2009). Epithelial cell-cell junctions and plasma membrane domains. Biochimica et Biophysica Acta.**1788**: 820-831

Gilcrease, M.Z. (2007). Integrin signaling in epithelial cells. Cancer Letters. 247: 1-25

Gowda, C., Song, C., Kapadia, M., Payne, J.L., Hu, T., Ding, Y. & Dovat, S. (2017). Regulation of cellular proliferation in acute lymphoblastic leukemia by Casein Kinase II (CK2) and Ikaros. Advances in Biological Regulation. **63**: 71-80

Grasselli, E., Tomati, V., Bernasconi, M. V., Nicolini, C., & Vergani, L. (2004). C-terminal region of protein kinase CK2α: How the structure can affect function and stability of the catalytic subunit. Journal of Cellular Biochemistry. **92**: 270-284

Gual, P., Gremeaux, T., Gonzalez, T., Le Marchand-Brustel, Y. & Tanti, J.-F. (2003). MAP kinases and mTOR mediate insulin-induced phosphorylation of Insulin Receptor Substrate-1 on serine residues 307, 612 and 632. Diabetologia. **46**: 1532-1542

Guerra, B., Boldyreff, B., Sarno, S., Cesaro, L., Issinger, O.G. & Pinna LA. (1999). CK2: a protein kinase in need of control. Pharmacology and Therapeutics. **82**: 303-313

Hanahan, D. & Weinberg, R.A. (2011). Hallmarks of Cancer: The Next Generation. Cell. **144**: 646–674

Hanif, I.M., Hanif, I.M., Shazib, M.A., Ahmad K.A. & Pervaiz, S. (2010). Casein Kinase II: An attractive target for anti-cancer drug design. The International Journal of Biochemistry & Cell Biology. **42**: 1602-1605

Hemmings, B.A. & Restuccia, D.F. (2012). PI3K-PKB/Akt Pathway. Cold Spring Harbor Perspectives in Biology. **4**: a011189

Hennessy, B. T. (2005). Exploiting the PI3K/AKT Pathway for Cancer Drug Discovery. Nature Reviews Drug Discovery. **4**: 988-1004

Holbro, T., Civenni, G. & Hynes, N.E. (2003). The ErbB receptors and their role in cancer progression. Experimental Cell Research. **284**: 99-110

Huyck, L., Ampe, C. &Van Troys, M. (2012). The XTT Cell Proliferation Assay Applied to Cell Layers Embedded in Three-Dimensional Matrix. Assay and Drug Development Technologies. **10**: 382-392

Hwang, S.Y., Chae, J.I., Kwak, A.W., Lee, M.H. & Shim, J.H. (2020). Alternative options for skin cancer therapy via regulation of AKT and related signaling pathways. International Journal of Molecular Sciences. **21**: p.6869

Jeanes, A., Gottardi, C. J., & Yap, A. S. (2008). Cadherins and cancer: how does cadherin dysfunction promote tumor progression?. Oncogene. **27**: 6920-6929

Jones, L.J. & Veale, R.B. (2003). Redistribution of beta-catenin in response to EGF and lithium signalling in human oesophageal squamous carcinoma cell lines. Cancer cell International. **3**: 13

Kandel, E.S. & Hay, N. (1999). The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. Experimental Cell Research. **253**: 210-229

Karunagaran, D., Tzahar, E., Beerli, R.R., Chen, X., Graus-Porta, D., Ratzkin, B.J., Seger, R., Hynes, N.E. &Yarden, Y. (1996). ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. The EMBO Journal. **15**: 254-264

Kawamoto, T., Mendelsohn, J., Le, A., Sato, G. H., Lazar, C. S., & Gill, G. N. (1984). Relation of epidermal growth factor receptor concentration to growth of human epidermoid carcinoma A431 cells. Journal of Biological Chemistry. **259**: 7761-7766

Katz, E. & Streuli, C.H. (2007). The extracellular matrix as an adhesion checkpoint for mammary epithelial function. The International Journal of Biochemistry & Cell Biology. **39**: 715–726

Kishor Roy, N., Bordoloi, D., Monisha, J., Padmavathi, G., Kotoky, J., Golla, R. & B Kunnumakkara, A. (2017). Specific targeting of Akt kinase isoforms: Taking the precise path for prevention and treatment of cancer. Current Drug Targets. **18**: 421-435

Klumpp, S., Mäurer, A., Zhu, Y., Aichele, D., Pinna, L.A. & Krieglstein, J. (2004). Protein kinase CK2 phosphorylates BAD at threonine-117. Neurochemistry International. **45**: 747-752

Krall, J. A., Beyer, E. M., & MacBeath, G. (2011). High- and Low- Affinity Epidermal Growth Factor Receptor-Ligand Interactions Activate Distinct Signalling Pathways. PLoS ONE. **6**: e15945

Laemmli, U.K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature. **227**: 680-685

Lamprecht, S. A., Schwartz, B., Avigdor, A., & Guberman, R. (1990). Growth-related enzyme activities in crypt compartments during rat colon carcinogenesis. Anticancer Research. **10**: 773-778

Lapierre, J.M., Eathiraj, S., Vensel, D., Liu, Y., Bull, C.O., Cornell-Kennon, S., Iimura, S., Kelleher, E.W., Kizer, D.E., Koerner, S. & Makhija, S. (2016). Discovery of 3-(3-(4-(1-Aminocyclobutyl) phenyl)-5-phenyl-3 H-imidazo [4, 5-b] pyridin-2-yl) pyridin-2-amine (ARQ 092): An orally bioavailable, selective, and potent allosteric AKT inhibitor. Journal of Medicinal Chemistry. **59**:6455-6469

Lee, H.-R., Cho, S.J., Park, H.J., Kim, K.H., Rhee, D.K. & Pyo, S. (2010). The inhibitory effect of acrylamide on NCAM expression in human neuroblastoma cells: involvement of CK2/Ikaros signaling pathway. Toxicology in Vitro. **24**: 1946-1952

Legate, K. R., Montañez, E., Kudlacek, O., & Füssler, R. (2006). ILK, PINCH and parvin: the tIPP of integrin signalling. Nature Reviews Molecular Cell Biology. **7**: 20-31

Litchfield, D.W., Lozeman, F.J., Cicirelli, M.F., Harrylock, M., Ericsson, L.H., Piening, C.J. & Krebs, E.G. (1991). Phosphorylation of the beta subunit of casein kinase II in human A431 cells. Identification of the autophosphorylation site and a site phosphorylated by p34cdc2. Journal of Biological Chemistry. **266**: 20380-20389

Litchfield, D.W., Lüscher, B., Lozeman, F.J., Eisenman, R.N. & Krebs, E.G. (1992). Phosphorylation of casein kinase II by p34cdc2 in vitro and at mitosis. Journal of Biological Chemistry. **267**: 13943-13951

Lössner, D., Abou-Ajram, C., Benge, A., & Reuning, U. (2008). Integrin αvβ3 mediates upregulation of epidermal growth-factor receptor expression and activity in human ovarian cancer cells. The international journal of Biochemistry & Cell Biology. **40**: 2746-2761

Ma, S., Lee, T.K., Zheng, B.J., Chan, K.W. & Guan, X.Y. (2008). CD133+ HCC cancer stem cells confer chemoresistance by preferential expression of the Akt/PKB survival pathway. Oncogene. **27**: 1749-1758

Marte, B.M. & Downward, J. (1997). PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. Trends in Biochemical Sciences. **22**: 355-358

Martins, L.R., Lúcio, P., Melao, A., Antunes, I., Cardoso, B.A., Stansfield, R., Bertilaccio, M.T.S., Ghia, P., Drygin, D., Silva, M.G. & Barata, J.T. (2014). Activity of the clinical-stage CK2-specific inhibitor CX-4945 against chronic lymphocytic leukemia. Leukemia. **28**: 179-182

McCabe, M.L. & Dlamini, Z. (2005). The molecular mechanisms of oesophageal cancer. International Immunopharmacology. **5**: 1113-1130

Meggio, F., & Pinna, Lorenzo A. (2003). One-thousand-and-one substrates of protein kinase CK2? The FASEB journal. **17**: 349-68

Mendelsohn, J., & Baselga, J. (2000). The EGF receptor family as targets for cancer therapy. Oncogene. **19**: 6550-6565

Messenger, M.M., Saulnier, R.B., Gilchrist, A.D., Diamond, P., Gorbsky, G.J. & Litchfield, D.W., 2002. Interactions between protein kinase CK2 and Pin1: evidence for phosphorylation-dependent interactions. Journal of Biological Chemistry. **277**: 23054-23064

Mineo C., Gill, G.N. & Anderson, R.G.W. (1999). Regulated migration of epidermal growth factor receptor from caveolae. Journal of Biological Chemistry. **274**: 30636-30643

Miyata, Y., & Nishida, E. (2005). CK2 binds, phosphorylates, and regulates its pivotal substrate Cdc37, an Hsp90-cochaperone. Molecular and Cellular Biochemistry. **274**: 171-179

Niefind, K. & Issinger, O.-G.(2010). Conformational plasticity of the catalytic subunit of protein kinase CK2 and its consequences for regulation and drug design. Biochimica et Biophysica Acta. **1804**: 484-492

O-charoenrat, P., Rusch, V., Talbot, S.G., Sarkaria, I., Viale, A., Socci, N., Ngai, I., Rao, P. & Singh, B. (2004). Casein kinase II alpha subunit and C1-inhibitor are independent predictors of outcome in patients with squamous cell carcinoma of the lung. Clinical Cancer Research. **10**: 5792-5803

Olsten, M.E.K. & Litchfield, D.W. (2004). Order or chaos? An evaluation of the regulation of protein kinase CK2. Biochemistry and Cell Biology. **82**: 681-693

Pickens, A. & Orringer, M.B. (2003). Geographical distribution and racial disparity in oesophageal cancer. The Annals of Thoracic Surgery. **76**: S1367-1369

Pierre F., Chua, P.C., O'Brien, S.E., Siddiqui-Jain, A., Bourbon, P., Haddach, M., Michaux, J., Nagasawa, J., Schwaebe, M.K., Stefan, E., Vialettes, A., Whitten, J.P., Chen, T.K., Darjania, L., Stansfield, R., Anderes, K., Bliesath, J., Drygin, D., Ho, C., Omori, M., Proffitt, C., Streiner, N., Trent, K., Rice, W.G. & Ryckman, D.M.

(2011). Discovery and SAR of 5-(3-chlorophenylamino) benzo[c][2,6] naphthyridine-8-carboxylic acid (CX-4945), the first clinical stage inhibitor of protein kinase CK2 for the treatment of cancer. Journal of Medicinal Chemistry. **54**: 635–654

Rahimi, R. A., & Leof, E. B. (2007). TGF-β signaling: A tale of two responses. Journal of Cellular Biochemistry. **102**: 593-608

Rodríguez, F.,Allende, C.C. & Allende, J.E. (2005). Protein kinase casein kinase 2 holoenzyme produced ectopically in human cells can be exported to the external side of the cellular membrane. Proceedings of the National Academy of Sciences. **102**: 4718-4723

Sarno, S., de Moliner, E., Ruzzene, M., Pagano, M.A., Battistutta, R., Bain, J., Fabbro, D., Schoepfer, J., Elliott, M., Furet, P., Meggio, F., Zanotti, G. & Pinna, L.A. (2003). Biochemical and three-dimensional-structural study of the specific inhibition of protein kinase CK2 by [5-oxo-5,6-dihydroindolo-(1,2-a)quinazolin-7-yl]acetic acid (IQA). The Biochemical Journal. **374**: 639-646

Seldin, D.C., Lou, D.Y., Toselli, P., Landesman-Bollag, E. & Dominguez, I., 2008. Gene targeting of CK2 catalytic subunits. Molecular and Cellular Biochemistry. **316**:141-147

Sepe, M., Festa, L., Tolino, F., Bellucci, L., Sisto, L., Alfano, D., Ragno, P., Calabrò, V., de Franciscis, V., La Mantia, G. & Pollice, A. (2011). A Regulatory Mechanism Involving TBP-1/Tat-Binding Protein 1 and Akt/PKB in the Control of Cell Proliferation. PLoS One. **6**: e22800

Shariati, M. & Meric-Bernstam, F., (2019). Targeting AKT for cancer therapy. Expert Opinion on Investigational Drugs. **28**: 977-988

Shavers, V.L., Harlan, L.C. & Stevens, J.L. (2003). Racial/ethnic variation in clinical presentation, treatment, and survival among breast cancer patients under age 35. Cancer: Interdisciplinary International Journal of the American Cancer Society. **97**: 134-147

Shaw, N. C. (2013). PI3K in Human Oesophageal Squamous Cell Carcinoma Cells: A critical modulator in the PKB signalling pathway (Doctoral dissertation, University of the Witwatersrand, Johannesburg, the Republic of South Africa).

Shen, X. & Kramer, R.H. (2004). Adhesion-mediated squamous cell carcinoma survival through ligand-independent activation of epidermal growth factor receptor. The American Journal of Pathology. **165**: 1315-1329

Siddiqui-Jain, A., Drygin, D., Streiner, N., Chua, P., Pierre, F., O'Brien, S.E., Bliesath, J., Omori, M., Huser, N., Ho, C., Proffitt, C., Schwaebe, M.K., Ryckman, D.M., Rice, W.G. & Anderes, K. (2010). CX-4945, an Orally Bioavailable Selective Inhibitor of Protein Kinase CK2, Inhibits Prosurvival and Angiogenic Signalling and Exhibits Antitumor Efficacy. Cancer Research. **70**: 10288-10298

Smadja-Lamere, N., Shum, M., Deleris, P., Roux, P. P., Abe, J.-I. & Marette, A. (2013). Insulin Activates RSK (p90 Ribosomal S6 Kinase) to Trigger a New Negative Feedback Loop That Regulates Insulin Signaling for Glucose Metabolism. Journal of Biological Chemistry. **288**: 31165-31176

Somdyala, N.I., Bradshaw, D., Gelderblom, W.C. & Parkin, D.M. (2010). Cancer incidence in a rural population of South Africa, 1998-2002. International Journal of Cancer. **127**: 2420-2429

Song, S., Honjo, S., Jin, J., Chang, S.S., Scott, A.W., Chen, Q., Kalhor, N., Correa, A.M., Hofstetter, W.L., Albarracin, C.T. & Wu, T.T. (2015). The Hippo coactivator YAP1 mediates EGFR overexpression and confers chemoresistance in esophageal cancer. Clinical Cancer Research. **21**: 2580-2590

Soria, J.C., Lee, H.Y., Lee, J.I., Wang, L., Issa, J.P., Kemp, B.L., Liu, D.D., Kurie, J.M., Mao, L. and Khuri, F.R. (2002). Lack of PTEN expression in non-small cell lung cancer could be related to promoter methylation. Clinical Cancer Research. **8**:1178-1184

Stalter, G., Siemer, S., Becht, E., Ziegler, M., Remberger, K. & Issinger, O.G. (1994). Asymmetric expression of protein kinase CK2 subunits inhuman kidney tumors. Biochemical and Biophysical Research Communications. **202**:141-147

Stiles, B.L. (2009). PI-3-K and AKT: Onto the mitochondria. Advanced Drug Delivery Reviews. **61**: 1276-1282

Thakkar, H., Chen, X., Tyan, F., Gim, S., Robinson, H., Lee, C., Pandey, S.K., Nwokorie, C., Onwudiwe, N. & Srivastava, R.K. (2001). Pro-survival function of Akt/protein kinase B in prostate cancer cells: relationship with trail resistance. Journal of Biological Chemistry. **276**: 38361-38369

Turke, A. B., Song, Y., Costa, C., Cook, R., Arteaga, C. L., Asara, J. M. & Engelman, J. A. (2012). MEK Inhibition Leads to PI3K/AKT Activation by Relieving a Negative Feedback on ERBB Receptors. Cancer Research. **72**: 3228-3237

Tokuda, E., Fujita, N., Oh-hara, T., Sato, S., Kurata, A., Katayama, R., Itoh, T., Takenawa, T., Miyazono, K. & Tsuruo, T., 2007. Casein kinase 2–interacting protein-1, a novel Akt pleckstrin homology domain-interacting protein, down-regulates PI3K/Akt signaling and suppresses tumor growth in vivo. Cancer Research. **67:**9666-9676

Torres, J. & Pulido, R. (2001). The tumour suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to

proteasome-mediated degradation. The Journal of Biological Chemistry. **276**: 993-998

van Aken, E., De Wever, O., da Rocha, C. A., & Mareel, M. (2001). Defective E-cadherin/catenin complexes in human cancer. Virchows Archiv: an International Journal of Pathology. **439**: 725-751

Varadarajulu, J., Laser, M., Hupp, M., Wu, R., & Hauck, C. R. (2005). Targeting of αv integrins interferes with FAK activation and smooth muscle cell migration and invasion. Biochemical and Biophysical Research Communications. **331**: 404-412

Veale, R. B. & Thornley, A. L. (1989). Increased single class low-affinity EGF receptors expressed by human oesophageal squamous carcinoma cell lines. South African Journal of Science. **85**: 375-379

Voskas, D., Ling, L. S., & Woodgett, J. R. (2010). Does GSK-3 provide a shortcut for PI3K activation of Wnt signalling?. F1000 Biology Reports. **2**: 82

Wang, C.Y., Deng, J.Y., Cai, X.W., Fu, X.L., Li, Y., Zhou, X.Y., Wu, X.H., Hu, X.C., Fan, M., Xiang, J.Q. & Zhang, Y.W. (2015). High EGFR and low p-Akt expression is associated with better outcome after nimotuzumab-containing treatment in esophageal cancer patients: preliminary clinical result and testable hypothesis. Oncotarget. **6**: 18674-18682

Wang, L., Iorio, C., Yan, K., Yang, H., Takeshita, S., Kang, S., Neel, B.G. & Yang, W. (2018). A ERK/RSK-mediated negative feedback loop regulates M-CSF–evoked PI3K/AKT activation in macrophages. The FASEB Journal. **32**: 875-887

Wang, S. & Jones, K.A. (2006). CK2 controls the recruitment of Wnt regulators to target genes in vivo. Current Biology. **16**: 2239-2344

Winograd-Katz, S.E. & Levitzki, A. (2006). Cisplatin induces PKB/Akt activation and p38^{MAPK} phosphorylation of the EGF receptor. Oncogene. **25**: 7381-7390

Wiley H.S. (2003). Trafficking of the ErbB receptors and its influence on signaling. Experimental Cell Research. **284**: 78-88

Wozniak, M. A., Modzelewska, K., Kwong, L., & Keely, P. J. (2004). Focal adhesion regulation of cell behavior. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. **1692**: 103-119

Yun, S.J., Tucker, D.F., Kim, E.K., Kim, M.S., Do, K.H., Ha, J.M., Lee, S.Y., Yun, J., Kim, C.D., Birnbaum, M.J. & Bae, S.S. (2009). Differential regulation of Akt/protein kinase B isoforms during cell cycle progression. FEBS Letters. **583**: 685-690

Zhang, Y. (2013). Epidemiology of esophageal cancer. World Journal of Gastroentorology. **19**: 5598-5606

Zhu, D., Hensel, J., Hilgraf, R., Abbasian, M., Pornillos, O., Deyanat-Yazdi, G., Hua, X.H. & Cox, S. (2010). Inhibition of protein kinase CK2 expression and activity blocks tumor cell growth. Molecular and Cellular Biochemistry. **333**: 159-167

Zień, P., Bretner, M., Zastapiło, K., Szyszka, R. & Shugar, D. (2003). Selectivity of 4, 5, 6, 7-tetrabromobenzimidazole as an ATP-competitive potent inhibitor of protein kinase CK2 from various sources. Biochemical and Biophysical Research Communications. **306**: 129-133

Appendices

Appendix A: Laboratory and Tissue Culture Solutions

1.1. Standard Solutions

1.1.1 Phosphate Buffered Saline (PBS) (1X)

136.9 mM Sodium Chloride

2.680 mM Potassium Chloride

10.10 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 dH_2O

Filter and 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 dH_2O

Autoclave to sterilize

Store at 4 °C

1.1.3 Tris Buffered Saline with Tween (TBS-T)

50.00 mM Tris-HCl (pH 7.8)

147.0 mM Sodium Chloride

2 mM Anhydrous Calcium Chloride

0.1 % Tween-20

Make up to final volume with dH_2O

Store at 4 °C

1.1.4 10% Sodium dodecyl Sulphate (SDS)

10% SDS (w/v)

Make up to final volume with dH_2O

Heat to assist dissolution

Adjust to pH 7.2

Store at room temperature

1.2. Tissue Culture Solutions

1.2.1. DMEM/Hams F12 Medium Solution

Mix DMEM/Hams F12 Medium Solutions (see 1.2.1.1. and 1.2.1.2. below) in a 3:1 ratio

Filter sterilize

Store at 4 °C

1.2.1.1. Dulbecco's Modified Eagles Medium (DMEM)

1.370 %	DMEM
0.370 %	Sodium Bicarbonate
2.000 %	Penicillin (500 U/ml)/Streptomycin (0.5 %) Solution

1.2.1.2. Hams F12 Medium Solution

1.070 %	Hams F12 Medium
0.118 %	Sodium Bicarbonate
2.000 %	Penicillin (500 U/ml)/Streptomycin (0.5 %) Solution

1.2.2. Trypsin/Ethylenediaminetetra-acetic Acid (EDTA)

Mix Trypsin Solution/EDTA (see 1.2.2.1. and 1.2.2.2. below) in a 1:1 ratio

Store at 4 °C (can be frozen at -20 °C for prolonged storage)

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 Solutions

1.3.1. Laemmli Double Lysis Buffer (2X)

123.8 mM Tris-HCl (pH 6.8)

4% SDS

20% Glycerol

10 % β-mercaptoethanol

Make up to final volume with dH_2O

Store at 4 °C

1.4. Protein Determination Solutions

1.4.1. 95 % Ethanol

95% Ethanol (v/v)

Make up to final volume with dH_2O

1.4.2. 7.5 % Trichloroacetic Acid (TCA)

7.5 % TCA (v/v)

Make up to final volume with dH_2O

1.4.3. 0.25 % Coomassie Brilliant Blue Stain

0.25% Coomassie Brilliant Blue G-250 Powder (w/v)

50% Methanol (v/v)

Dissolve, and then add:

10% Glacial Acetic Acid (v/v)

Make up to final volume with dH_2O

1.4.4. Destain Solution

12% Glacial Acetic Acid (v/v)

10% Methanol (v/v)

Make up to final volume with dH_2O

1.4.5. Elution Solution

66% Methanol (v/v)

1% Concentrated Ammonia (v/v)

Make up to final volume with dH_2O

1.4.6. Example of Standard Curve for Protein Determination

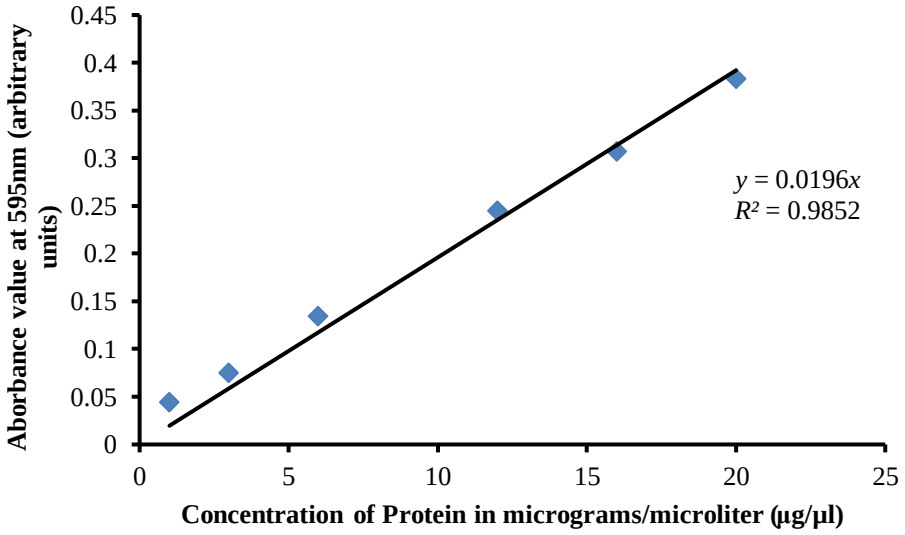


Figure A1: An Example of a Standard Curve used for to Determine the concentration of protein in cell lysates. The absorbance (at 595nm) of the protein-coomassie complexes are plotted against the concentration of protein (in µg/µl) in the BSA standard samples of known concentrations (1, 3, 6, 12, 16 and 20 µg/µl). The equation of this curve (in the form y = mx + c; y = Abs595, x = concentration and c = 0) was then used to determine the concentration of protein from cellular lysates from their absorbance values. The linear regression value is represented by R^2 (a value of 1 being evidence of a perfect score).

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

1.5.1. Buffer Solutions

1.5.1.1. Electrophoresis 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 dH_2O

Store at 4 °C

1.5.1.2. Resolving Gel Buffer Stock

1.8 M Tris Base

Adjust solution to pH 8.8 using 5 N HCl

Make up to final volume with dH_2O

Store at 4 °C

1.5.1.3. Stacking Gel Buffer Stock

0.5 M Tris Base

Adjust solution to pH 6.8 using 5 N HCl

Make up to final volume with dH_2O

Store at 4 °C

1.5.2. SDS-PAGE Working Solutions

1.5.2.1. Resolving Gel

375 mM Tris-HCl – using the resolving gel buffer stock (*1.5.2.2.;* pH 8.8)

10 % or 12% Acrylamide (w/v)

0.1% N,N'–methylenebisacrylamide (w/v)

0.2% SDS (w/v)

Make up to final volume with dH_2O

Just prior to use add:

1.85% Ammonium Persulphate Solution (APS) (v/v to the final volume of the gel mix)

0.26% N',N',N',N'–tetramethylethylene-diamene (TEMED) (v/v to the final volume of the gel mix)

1.5.2.2. Stacking Gel

125 mM Tris-HCl – using the stacking gel buffer stock (*1.5.2.3.;* pH 6.8)

5% Acrylamide (w/v)

0.1% N,N'–methylenebisacrylamide (w/v)

0.2% SDS (w/v)

Make up to final volume with dH_2O

Just prior to use add:

1.92% Ammonium Persulphate Solution (APS) (v/v to the final volume of the gel mix)

0.26% N',N',N',N'–tetramethylethylene-diamene (TEMED) (v/v to the final volume of the gel mix)

1.5.3. SDS Overlay Solution

0.1% SDS (w/v)

Make up to volume with dH_2O

1.5.4. Tracking Dye

0.001% Bromophenol Blue

50.00 % Glycerol

Make up to final volume with dH_2O

Store at – 4 °C

1.5.5. 0.25 % Coomassie Brilliant Blue Stain

As previously described (see Appendix 1, Section 1.4.3)

1.5.6. Destain Solution

10 % Acetic Acid

10 % Methanol

Make up to final volume with dH_2O

1.6. Western Blotting Solutions

1.6.1. Buffers

1.6.1.1. Transfer Buffer

25 mM Tris-HCl (pH 8.3)

20.0% Methanol (v/v)

1.41% Glycine (w/v)

Make up to final volume with dH_2O

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 (v/v)

5.00 % Non-fat Milk Powder (w/v)

Make up to final volume with dH_2O

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 (e.g., PBS, PBS-T, or TBS or TBS-T)

1.6.2. SuperSignal® West Pico Chemiluminescent Substrate Kit

Before use mix the following in a 1:1 ratio:

Luminol Enhancer Solution

Stable Peroxide Buffer

1.6.3. Developer

6.4 M	Metol
0.6 M	Anhydrous Sodium Sulphate
80 mM	Hydroquinone (Quinol)
0.45 mM	Anhydrous Sodium Carbonate
34 mM	Potassium Bromide

Make up to final volume with dH_2O

Store at room temperature in the dark

1.6.4. Fixer

0.8 M	Sodium Thiosulphate
0.2 M	Sodium Metasulphite

Make up to final volume with dH_2O

Store at room temperature in the dark

1.7. Co-Immunoprecipitation Analysis

1.7.1. Radio-immunoprecipitation Assay (RIPA) Buffer

50 mM	Tris-HCl (pH 7.5)

150 mM	NaCl
0.5 %	Deoxycholate (w/v)
0.5 %	Triton X-100 (v/v)
0.05 %	SDS (w//v)
1 mM	PMSF/Aprotinin

Make up to final volume with dH_2O

Store at 4 °C

1.7.1.1. Phenylmethanesulphonylfluoride (PMSF) Stock Solution

34.8 mg PMSF

Make up to 10 ml with Methanol

1.7.1.2. PMSF/Aprotinin Solution

0.1 mM	PMSF Stock Solution
1.00 ml	Trasylol

Make up to 100 ml with PBS (1X)

1.7.2. Immunoprecipitation (IP) Buffer

20 mM	Tris (pH 8.0)
0.5 %	Nonidet P-40 (NP-40) (v/v)
0.9 %	Sodium Chloride (w/v)

Make up to final volume with dH_2O

Appendix B: Proliferative Pathway Manipulation in Tissue Culture

2.1. Stimulation of cells using EGF (Sigma)

2.1.1. EGF Stock

10 µg/ml

2.1.1.1. EGF Working Dilution

Final concentration for treatment is 10 ng/ml, dilute to final volume with treatment tissue culture medium

2.2. MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) Proliferation Assays

50 mg MTT (Sigma)

Make up to final concentration of 5 mg/ml with 10 ml of PBS.

Store at -20 °C.

2.3. Specific Blockade of CK2 (CK2α) using 4,5,6,7-tetrabromo-1H-benzatriazole (TBB) (Sigma – Cat. No. T0826)

25 mM and 50 mM TBB from TBB stock (dissolved in DMSO by Manufacturer), make up to volume with treatment tissue culture medium

2.4. Specific Blockade of PI3K using LY294002 (Sigma – Cat No. 440204)

2.3.1. LY294002 Stock

1 mg/ml

Make up to final volume with 100 % DMSO

2.3.1.1. LY294002 Working Dilution

20 µM LY294002 from the stock (above), make up to volume with treatment tissue culture medium

2.5. Specific Blockade of p90RSK using BI-D1870 (BioVision- Cat. No. 1924-1)

2.5.1. BI-D1870 Stock

1 mg/ml

Make up to volume with 100 % DMSO

2.5.1.1. BI-D1870 Working Dilution

10 µM BI-D1870 from the stock (above), dilute to volume with treatment tissue culture medium

Appendix C: Sample Data (or Analyses) and Sample Statistics

3.1 Protein Sample Blot Data

The blot data here is representative of at 3 biological replicates, and it is presented only as examples of the numerous blots that would have been generated during the study to finally arrive at the data and analyses that were presented in the main part of this Thesis.

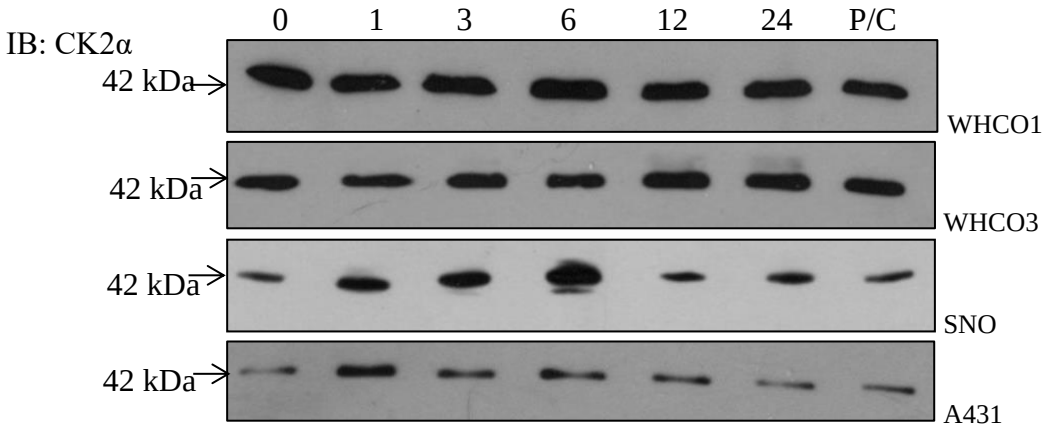


Figure C1: Sample blots of CK2α showing the effect of EGF on protein abundance over a 24-hour period. Oesophageal cancer cells (WHCO1, WHCO3 and SNO) and epidermoid carcinoma cells (A431) cultured under standard conditions were supplemented with 10 ng/ml EGF and incubated for 1, 3, 6, 12 and 24 hours. The presence of bands in the 42 kDa region as demonstrated by Western Blotting confirmed the presence of CK2α in all cell lines under the incubation times noted above. P/C denotes the H1299 cell lysate that was used as a positive control for the detection and subsequent quantification of CK2α. - Densitometric analysis revealed significant increases in CK2α concentration at 1, 3 and 6 hours of exposure to exogenous EGF for the SNO cells, comparable to the significant increases at 1 and 3 hours in the A431 cells. The other HOSCC did not show significant changes in CK2α concentrations. The blots are representative of three independent biological replicates. The bars on the trendline represent the S.E.M and treatments were compared to their respective time zero to determine statistical significance (* symbolising $p < 0.05$; ** symbolising $p < 0.01$)

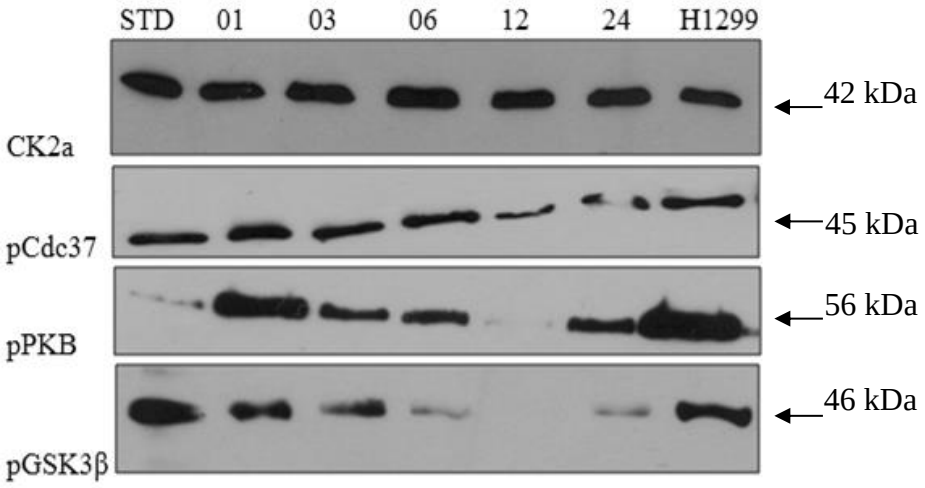


Figure C2: Sample Blot of EGF stimulated WHCO1 HOSCC cells showing the effect of protein abundance over a 24-hour period. WHCO1 cells cultured under standard conditions were supplemented with 10 ng/ml EGF and incubated for 1, 3, 6, 12 and 24 hours. Densitometric analysis (found in the main body of the thesis – Figure 3.2) revealed significant increases in CK2α concentration at 1, 3 and 6 hours of exposure to exogenous EGF for the SNO cells, comparable to the significant increases at 1 and 3 hours in the A431 cells. The other HOSCC did not show significant changes in CK2α concentrations. The blots are representative of three independent biological replicates. The bars on the trendline represent the S.E.M and treatments were compared to their respective time zero to determine statistical significance (* symbolising $p < 0.05$; ** symbolising $p < 0.01$). On the Western Blots that correspond to these figures here: The presence of bands in the 46 kDa region as demonstrated by Western Blotting confirmed the presence of phopho-GSK3β (Ser09) in all cell lines under the incubation times noted above. The presence of bands in the 56 kDa region as demonstrated by Western Blotting confirmed the presence of phospho-PKB (Ser473). P/C denotes the H1299 cell lysate that was used as a positive control for the detection and subsequent quantification of CK2α

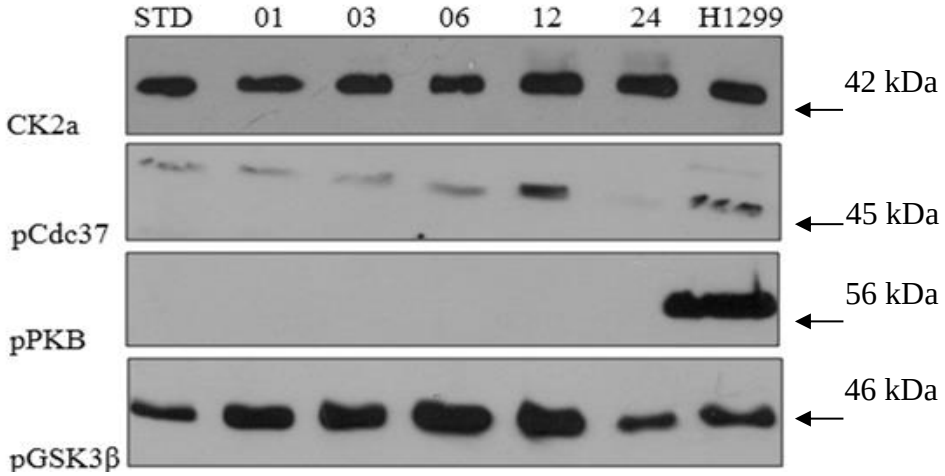


Figure C3: Sample Blot of EGF stimulated WHCO3 HOSCC cells showing the effect of protein abundance over a 24-hour period. WHCO3 cells cultured under standard conditions were supplemented with 10 ng/ml EGF and incubated for 1, 3, 6, 12 and 24 hours. Densitometric analysis (found in the main body of the thesis – Figure 3.2) revealed significant increases in CK2α concentration at 1, 3 and 6 hours of exposure to exogenous EGF for the SNO cells, comparable to the significant increases at 1 and 3 hours in the A431 cells. The other HOSCC did not show significant changes in CK2α concentrations. The blots are representative of three independent biological replicates. The bars on the trendline represent the S.E.M and treatments were compared to their respective time zero to determine statistical significance (* symbolising $p < 0.05$; ** symbolising $p < 0.01$). On the Western Blots that correspond to these figures here: The presence of bands in the 46 kDa region as demonstrated by Western Blotting confirmed the presence of phopho-GSK3β (Ser09) in all cell lines under the incubation times noted above. The presence of bands in the 56 kDa region as demonstrated by Western Blotting confirmed the presence of phospho-PKB (Ser473). P/C denotes the H1299 cell lysate that was used as a positive control for the detection and subsequent quantification of CK2α

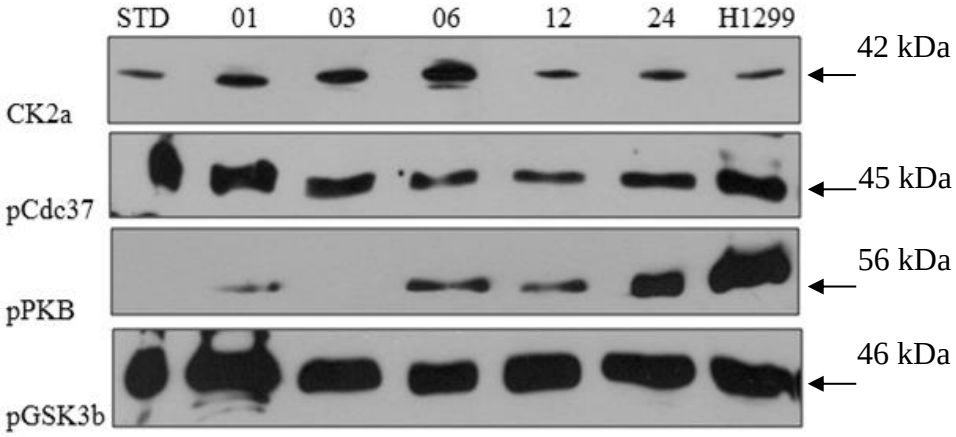


Figure C4: Sample Blot of EGF stimulated SNO HOSCC cells showing the effect of protein abundance over a 24-hour period. SNO cells cultured under standard conditions were supplemented with 10 ng/ml EGF and incubated for 1, 3, 6, 12 and 24 hours. Densitometric analysis (found in the main body of the thesis – Figure 3.2) revealed significant increases in CK2α concentration at 1, 3 and 6 hours of exposure to exogenous EGF for the SNO cells, comparable to the significant increases at 1 and 3 hours in the A431 cells. The other HOSCC did not show significant changes in CK2α concentrations. The blots are representative of three independent biological replicates. The bars on the trendline represent the S.E.M and treatments were compared to their respective time zero to determine statistical significance (* symbolising $p < 0.05$; ** symbolising $p < 0.01$). On the Western Blots that correspond to these figures here: The presence of bands in the 46 kDa region as demonstrated by Western Blotting confirmed the presence of phopho-GSK3β (Ser09) in all cell lines under the incubation times noted above. The presence of bands in the 56 kDa region as demonstrated by Western Blotting confirmed the presence of phospho-PKB (Ser473). P/C denotes the H1299 cell lysate that was used as a positive control for the detection and subsequent quantification of CK2α.

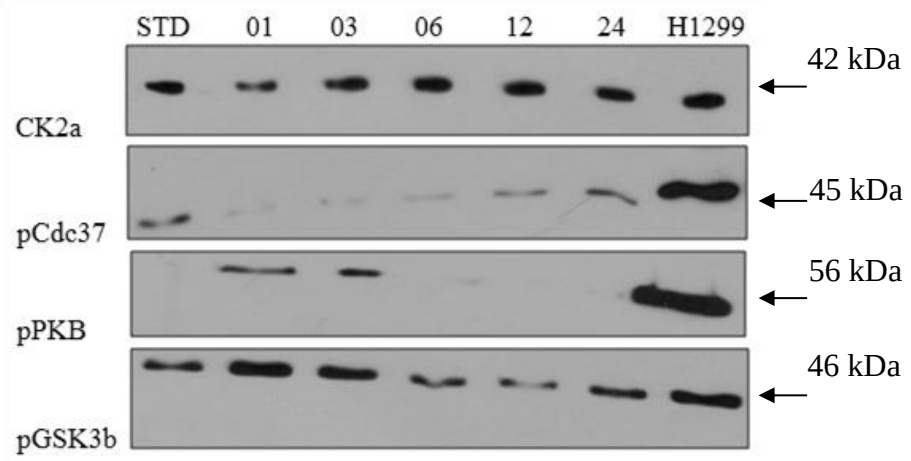


Figure C5: Sample Blot of EGF stimulated A431 cells showing the effect of protein abundance over a 24-hour period. A431 cells cultured under standard conditions were supplemented with 10 ng/ml EGF and incubated for 1, 3, 6, 12 and 24 hours. Densitometric analysis (found in the main body of the thesis – Figure 3.2) revealed significant increases in CK2α concentration at 1, 3 and 6 hours of exposure to exogenous EGF for the SNO cells, comparable to the significant increases at 1 and 3 hours in the A431 cells. The other HOSCC did not show significant changes in CK2α concentrations. The blots are representative of three independent biological replicates. The bars on the trendline represent the S.E.M and treatments were compared to their respective time zero to determine statistical significance (* symbolising $p < 0.05$; ** symbolising $p < 0.01$). On the Western Blots that correspond to these figures here: The presence of bands in the 46 kDa region as demonstrated by Western Blotting confirmed the presence of phopho-GSK3β (Ser09) in all cell lines under the incubation times noted above. The presence of bands in the 56 kDa region as demonstrated by Western Blotting confirmed the presence of phospho-PKB (Ser473). P/C denotes the H1299 cell lysate that was used as a positive control for the detection and subsequent quantification of CK2α.

3.2 Protein Sample Densitometry Analyses Sample Data

The Sample statistical data here is a summary of what was pooled together from a minimum of 3 independent biological replicates.

WHCO1: (Egf Treatments – 24 Hour treatments)

CK2	0	1	3	6	12	24	H1299 ctrl
Density_relative to H1299 ctrl 1	1,74	2,07	2,22	2,49	1,66	1,54	1,00
Density_relative to H1299 ctrl 2	2,09	2,49	1,79	3,29	2,00	0,67	1,00
Density_relative to H1299 ctrl 3	1,65	1,29	2,98	1,90	0,89	1,89	1,00
Avg	1,83	1,95	2,33	2,56	1,52	1,37	1,00
STD Dev	0,23	0,61	0,60	0,70	0,57	0,63	
S.E.M	0,13	0,35	0,35	0,40	0,33	0,36	
T.Test (vs 0)	1,00	0,77	0,28	0,20	0,46	0,33	
T.Test (vs 1)		1,00	0,48	0,32	0,42	0,31	
T.Test (vs 3)			1,00	0,69	0,16	0,13	
T.Test (vs 6)				1,00	0,12	0,09	
T.Test (vs 12)					1,00	0,77	
T.Test (vs 24)							
phospho-cdc37	0,00	1,00	3,00	6,00	12,00	24,00	H1299 ctrl
Density_relative to H1299 ctrl 1	1,01	1,29	1,05	1,16	0,04	0,04	1,00
Density_relative to H1299 ctrl 2	0,38	0,54	0,28	0,32	0,51	0,24	1,00
Density_relative to H1299 ctrl 3	1,27	0,08	0,98	1,29	0,27	0,88	1,00
Avg	0,89	0,64	0,77	0,92	0,27	0,39	1,00
STD Dev	0,62	0,32	0,50	0,68	0,17	0,45	
S.E.M	0,44	0,23	0,35	0,48	0,12	0,32	
T.Test (vs 0)	1,00	0,44	0,76	0,98	0,50	0,68	
T.Test (vs 1)		1,00	0,54	0,49	0,80	0,59	
T.Test (vs 3)			1,00	0,80	0,62	0,90	
T.Test (vs 6)				1,00	0,54	0,72	
T.Test (vs 12)					1,00	0,69	
T.Test (vs 24)							
phospho-PKB	0,00	1,00	3,00	6,00	12,00	24,00	H1299 ctrl
Density_relative to H1299 ctrl 1	0,00	0,98	0,35	0,36	0,01	0,47	1,00
Density_relative to H1299 ctrl 2	0,98	1,81	0,79	0,51	0,29	0,89	1,00
Density_relative to H1299 ctrl 3	0,68	0,88	0,32	0,12	0,56	0,22	1,00
Avg	0,55	1,22	0,48	0,33	0,28	0,53	1,00
STD Dev	0,21	0,66	0,33	0,27	0,19	0,48	
S.E.M	0,15	0,47	0,23	0,19	0,14	0,34	
T.Test (vs 0)	1,00	0,46	0,44	0,18	0,18	0,56	
T.Test (vs 1)		1,00	0,31	0,24	0,28	0,31	
T.Test (vs 3)			1,00	0,52	0,68	1,00	
T.Test (vs 6)				1,00	0,70	0,62	
T.Test (vs 12)					1,00	0,77	

T.Test (vs 24)							
phospho-GSK3β	0,00	1,00	3,00	6,00	12,00	24,00	H1299 ctrl
Density_relative to H1299 ctrl 1	0,07	0,38	0,35	0,23	0,39	0,33	1,00
Density_relative to H1299 ctrl 2	1,89	0,68	1,09	0,60	0,02	0,89	1,00
Density_relative to H1299 ctrl 3	0,26	0,35	0,10	0,13	0,13	0,33	1,00
Avg	0,74	0,47	0,52	0,32	0,18	0,52	1,00
STD Dev	1,16	0,23	0,70	0,33	0,08	0,40	
S.E.M	0,82	0,16	0,50	0,23	0,06	0,28	
T.Test (vs 0)	1,00	0,62	0,68	0,54	0,44	0,67	
T.Test (vs 1)		1,00	0,90	0,66	0,20	0,80	
T.Test (vs 3)			1,00	0,73	0,48	0,98	
T.Test (vs 6)				1,00	0,43	0,57	
T.Test (vs 12)					1,00	0,30	
T.Test (vs 24)							

WHCO3: (Egf Treatments – 24 Hour treatments)

CK2	0	1	3	6	12	24	H1299 ctrl
Density_relative to H1299 ctrl 1	0,99	0,84	0,89	1,22	1,06	1,26	1,00
Density_relative to H1299 ctrl 2	1,55	1,21	1,26	1,40	1,25	1,21	1,00
Density_relative to H1299 ctrl 3	1,63	1,60	1,72	1,92	1,28	1,19	1,00
Avg	1,39	1,22	1,29	1,51	1,20	1,22	1,00
STD Dev	0,35	0,38	0,41	0,36	0,12	0,03	
S.E.M (*100)	0,20	0,22	0,24	0,21	0,07	0,02	
T.Test (vs 0)	1,00	0,60	0,77	0,68	0,45	0,50	
T.Test (vs 1)		1,00	0,83	0,38	0,94	1,00	
T.Test (vs 3)			1,00	0,52	0,74	0,79	
T.Test (vs 6)				1,00	0,27	0,29	
T.Test (vs 12)					1,00	0,78	
T.Test (vs 24)							
phospho-cdc37	0	1	3	6	12	24	H1299 ctrl
Density_relative to H1299 ctrl 1	0,27	0,18	0,19	0,34	0,19	0,06	1,00
Density_relative to H1299 ctrl 2	0,40	0,01	0,30	0,09	0,25	0,31	1,00
Density_relative to H1299 ctrl 3	0,19	0,30	0,12	1,21	1,81	1,98	1,00
Avg	0,29	0,16	0,20	0,55	0,75	0,78	1,00
STD Dev	0,11	0,14	0,09	0,59	0,92	1,05	
S.E.M	0,06	0,08	0,05	0,34	0,53	0,60	
T.Test (vs 0)	1,00	0,30	0,36	0,52	0,48	0,50	
T.Test (vs 1)		1,00	0,71	0,37	0,38	0,41	
T.Test (vs 3)			1,00	0,42	0,41	0,44	
T.Test (vs 6)				1,00	0,77	0,75	
T.Test (vs 12)					1,00	0,97	
T.Test (vs 24)							
phospho-PKB	0	1	3	6	12	24	H1299 ctrl
Density_relative to H1299 ctrl 1	0,00	0,00	0,00	0,00	0,00	0,00	1,00
Density_relative to H1299 ctrl 2	0,00	0,00	0,00	0,00	0,00	0,00	1,00
Density_relative to H1299 ctrl 3	0,00	0,00	0,00	0,00	0,00	0,00	1,00
Avg	0,00	0,00	0,00	0,00	0,00	0,00	1,00
STD Dev	0,00	0,00	0,00	0,00	0,00	0,00	
S.E.M	0,00	0,00	0,00	0,00	0,00	0,00	
T.Test (vs 0)	null	null	null	null	null	null	
T.Test (vs 1)		null	null	null	null	null	
T.Test (vs 3)			null	null	null	null	
T.Test (vs 6)			null	null	null	null	
T.Test (vs 12)			null	null	null	null	
T.Test (vs 24)							
phospho-GSK3β	0	1	3	6	12	24	H1299 ctrl
Density_relative to H1299 ctrl 1	0,75	1,50	1,45	1,96	1,73	0,60	1,00
Density_relative to H1299 ctrl 2	1,21	1,63	1,60	1,28	1,08	0,89	1,00
Density_relative to H1299 ctrl 3	0,25	0,89	0,43	0,98	1,11	0,09	1,00

Avg	0,74	1,34	1,16	1,41	1,31	0,53	1,00
STD Dev	0,48	0,39	0,64	0,50	0,37	0,41	
S.E.M	0,28	0,23	0,37	0,29	0,21	0,23	
T.Test (vs 0)	1,00	0,17	0,42	0,17	0,18	0,59	
T.Test (vs 1)		1,00	0,70	0,86	0,92	0,07	
T.Test (vs 3)			1,00	0,62	0,75	0,23	
T.Test (vs 6)				1,00	0,79	0,08	
T.Test (vs 12)					1,00	0,07	
T.Test (vs 24)							

SNO: (Egf Treatments – 24 Hour treatments)

CK2	0	1	3	6	12	24	H1299 ctrl
Density_relative to H1299 ctrl 1	0,83	2,80	3,16	5,51	1,10	1,54	1,0000
Density_relative to H1299 ctrl 2	1,70	2,11	2,79	4,59	1,69	1,89	1,0000
Density_relative to H1299 ctrl 3	0,70	2,83	2,13	6,87	0,98	1,03	1,0000
Avg	1,08	2,58	2,69	5,65	1,26	1,49	1,0000
STD Dev	0,54	0,41	0,52	1,15	0,38	0,44	
S.E.M	0,31	0,23	0,30	0,66	0,22	0,25	
T.Test (vs 0)	1,00	0,02	0,02	0,01	0,66	0,37	
T.Test (vs 1)		1,00	0,78	0,03	0,01	0,03	
T.Test (vs 3)			1,00	0,03	0,02	0,04	
T.Test (vs 6)				1,00	0,01	0,02	
T.Test (vs 12)					1,00	0,53	
T.Test (vs 24)							
phospho-cdc37	0	1	3	6	12	24	H1299 ctrl
Density_relative to H1299 ctrl 1	0,32	0,91	0,59	0,32	0,24	0,51	1,0000
Density_relative to H1299 ctrl 2	0,29	0,89	0,08	0,91	0,23	0,10	1,0000
Density_relative to H1299 ctrl 3	0,89	0,37	0,49	1,03	0,40	0,09	1,0000
Avg	0,50	0,72	0,39	0,75	0,29	0,23	1,0000
STD Dev	0,34	0,31	0,27	0,38	0,09	0,24	
S.E.M	0,20	0,18	0,16	0,22	0,05	0,14	
T.Test (vs 0)	1,00	0,45	0,68	0,43	0,40	0,34	
T.Test (vs 1)		1,00	0,23	0,92	0,12	0,10	
T.Test (vs 3)			1,00	0,25	0,61	0,51	
T.Test (vs 6)				1,00	0,16	0,13	
T.Test (vs 12)					1,00	0,74	
T.Test (vs 24)							
phospho-PKB	0,00	1,00	3,00	6,00	12,00	24,00	H1299 ctrl
Density_relative to H1299 ctrl 1	0,00	0,06	0,00	0,18	0,14	0,42	1,0000
Density_relative to H1299 ctrl 2	0,00	0,02	0,03	0,10	0,29	0,98	1,0000
Density_relative to H1299 ctrl 3	0,00	0,09	0,00	0,29	0,10	0,22	1,0000
Avg	0,00	0,06	0,01	0,19	0,18	0,54	100,0000
STD Dev	0,00	0,04	0,02	0,10	0,10	0,40	
S.E.M	0,00	0,02	0,01	0,06	0,06	0,23	
T.Test (vs 0)	1,00	0,11	0,44	0,08	0,09	0,14	
T.Test (vs 1)		1,00	0,14	0,13	0,16	0,17	
T.Test (vs 3)			1,00	0,08	0,10	0,15	
T.Test (vs 6)				1,00	0,87	0,26	
T.Test (vs 12)					1,00	0,25	
T.Test (vs 24)							
phospho-GSK3β	0	1	3	6	12	24	H1299 ctrl
Density_relative to H1299 ctrl 1	0,23	1,07	0,92	0,90	1,07	1,00	1,0000
Density_relative to H1299 ctrl 2	0,13	0,37	0,19	0,78	0,90	0,59	1,0000
Density_relative to H1299 ctrl 3	0,50	0,67	0,50	0,73	0,91	0,79	1,0000

Avg	0,29	0,71	0,54	0,80	0,96	0,79	1,0000
STD Dev	0,19	0,35	0,37	0,09	0,10	0,21	
S.E.M	0,11	0,20	0,21	0,05	0,06	0,12	
T.Test (vs 0)	1,00	0,17	0,37	0,03	0,01	0,04	
T.Test (vs 1)		1,00	0,60	0,68	0,34	0,73	
T.Test (vs 3)			1,00	0,34	0,18	0,37	
T.Test (vs 6)				1,00	0,11	0,93	
T.Test (vs 12)					1,00	0,29	
T.Test (vs 24)							

A431: (Egf Treatments – 24 Hour treatments)

CK2	0	1	3	6	12	24	H1299 ctrl
Density_relative to H1299 ctrl 1	1,61	2,24	2,62	1,27	1,24	1,88	1,0000
Density_relative to H1299 ctrl 2	1,50	2,28	2,27	0,89	0,63	0,93	1,0000
Density_relative to H1299 ctrl 3	1,97	2,82	2,97	1,89	1,75	1,76	1,0000
Avg	1,69	2,44	2,62	1,35	1,21	1,52	1,0000
STD Dev	0,25	0,32	0,35	0,51	0,56	0,52	
S.E.M	0,14	0,19	0,20	0,29	0,32	0,30	
T.Test (vs 0)	1,00	0,04	0,02	0,37	0,27	0,64	
T.Test (vs 1)		1,00	0,56	0,04	0,04	0,07	
T.Test (vs 3)			1,00	0,03	0,03	0,05	
T.Test (vs 6)				1,00	0,76	0,70	
T.Test (vs 12)					1,00	0,51	
T.Test (vs 24)							
phospho-cdc37	0	1	3	6	12	24	H1299 ctrl
Density_relative to H1299 ctrl 1	0,12	0,02	0,02	0,02	0,05	0,10	1,0000
Density_relative to H1299 ctrl 2	0,00	0,08	0,02	0,07	0,06	0,17	1,0000
Density_relative to H1299 ctrl 3	0,58	0,07	0,04	0,01	0,00	0,05	1,0000
Avg	0,23	0,06	0,03	0,03	0,04	0,10	1,0000
STD Dev	0,30	0,03	0,01	0,03	0,03	0,06	
S.E.M	0,18	0,02	0,01	0,02	0,02	0,04	
T.Test (vs 0)	1,00	0,42	0,36	0,37	0,38	0,54	
T.Test (vs 1)		1,00	0,26	0,43	0,50	0,32	
T.Test (vs 3)			1,00	0,74	0,59	0,15	
T.Test (vs 6)				1,00	0,88	0,17	
T.Test (vs 12)					1,00	0,19	
T.Test (vs 24)							
phospho-PKB	0	1	3	6	12	24	H1299 ctrl
Density_relative to H1299 ctrl 1	0,00	0,00	0,00	0,00	0,00	0,00	1,0000
Density_relative to H1299 ctrl 2	0,18	0,01	0,09	0,91	0,09	0,79	1,0000
Density_relative to H1299 ctrl 3	0,00	0,28	0,00	0,01	0,00	0,09	1,0000
Avg	0,06	0,10	0,03	0,31	0,03	0,29	1,0000
STD Dev	0,10	0,16	0,05	0,52	0,05	0,43	
S.E.M	0,06	0,09	0,03	0,30	0,03	0,25	
T.Test (vs 0)	1,00	0,76	0,68	0,50	0,69	0,45	
T.Test (vs 1)		1,00	0,55	0,56	0,56	0,52	
T.Test (vs 3)			1,00	0,46	0,98	0,40	
T.Test (vs 6)				1,00	0,46	0,98	
T.Test (vs 12)					1,00	0,40	
T.Test (vs 24)							
phospho-GSK3β	0	1	3	6	12	24	H1299 ctrl
Density_relative to H1299 ctrl 1	0,40	1,00	0,71	0,28	0,16	0,43	1,0000
Density_relative to H1299 ctrl 2	0,61	0,27	0,60	0,19	0,30	0,89	1,0000
Density_relative to H1299 ctrl 3	0,12	1,68	0,39	0,52	0,38	0,27	1,0000

Avg	0,38	0,98	0,57	0,33	0,28	0,53	1,0000
STD Dev	0,24	0,71	0,16	0,17	0,11	0,33	
S.E.M	0,14	0,41	0,09	0,10	0,06	0,19	
T.Test (vs 0)	1,00	0,27	0,33	0,81	0,58	0,55	
T.Test (vs 1)		1,00	0,42	0,25	0,22	0,39	
T.Test (vs 3)			1,00	0,16	0,07	0,87	
T.Test (vs 6)				1,00	0,69	0,42	
T.Test (vs 12)					1,00	0,32	
T.Test (vs 24)							

3.3 Proliferation Assays Data

The Sample statistical data here is a summary of what was pooled together from a minimum of 3 independent biological replicates which were all treated in the same way and incubated in the same way. Each replicate would have also been an average from three wells of the 96-well plates that were used during this part of the experiment. Statistically significant differences are noted by the red fonts in the different tables. This table was used to generate the proliferation graphs that were presented in the main body of this Thesis (Chapter 4 and 5).

A431:

Sample	Rltv to control (%)	STD Dev	S.E.M	T-Test (samples compared to STD)	EGF T.Test	25TBB T.Test	50TBB T.Test	10BI-D T.Test	20LY T.Test
STD	100	0.036718	2.119895	1					
STD + Egf	96.64581392	0.095065	5.488572	0.541980448	1				
SF + Egf	90.03726873	0.131808	7.609916	0.245420306	0.44076526				
STD + 25TBB	106.224766	0.031496	1.818426	0.053530416		1			
STD + 50TBB	97.16269577	0.070833	4.089554	0.504711382		0.096269257	1		
STD + BI-D	100.7453747	0.02882	1.663911	0.752337506				1	
STD + 25TBB + BI-D	97.7683127	0.047169	2.723316	0.474470469		0.041045581		0.328377377	
STD + LY	84.80633568	0.145249	8.385929	0.149407123					1
STD + 25TBB + LY	94.29655264	0.029961	1.729823	0.06526489		0.004343657			0.29792587
STD + 50TBB + LY	99.82696659	0.128936	7.444127	0.980297869			0.40346741		0.17663883
STD + 50TBB + BI-D	105.3374151	0.077781	4.490659	0.283257127				0.337886075	
STD + LY + Egf	88.91255158	0.038753	2.237379	0.011645679	0.21960425				0.61380979
STD + BI-D + Egf	88.88371268	0.035213	2.033002	0.009750711	0.21835235			0.005807526	
STD + 25TBB + Egf	88.43560051	0.061126	3.529137	0.035794043	0.20947269	0.011928124			
STD + 0.5% DMSO	93.53564932	0.081158	4.685641	0.227502395					
BLANK									

WHCO1:

Sample	Relative to control (%)	STD Dev	S.E.M	T-Test (samples compared to STD)	EGF T.Test	25TBB T.Test	50TBB T.Test	10BI-D T.Test	20LY T.Test
STD	100	0.212118	12.24663	1					
STD + Egf	132.7881918	0.187246	10.81067	0.162862872	1				
SF + Egf	126.0584035	0.240702	13.89693	0.296962113	0.76147888				
STD + 25TBB	137.2194593	0.026115	1.507738	0.119861749		1			
STD + 50TBB	155.6044376	0.163409	9.434451	0.040518157		0.236107696	1		
STD + BI-D	128.8096149	0.154878	8.941857	0.186665276				1	
STD + 25TBB + BI-D	137.8474879	0.141163	8.150072	0.103127963		0.953969385		0.55859939	
STD + LY	135.6669217	0.039112	2.258152	0.616466773					1
STD + 25TBB + LY	136.9548585	0.056925	3.286589	0.114956125		0.95436677			0.79806247
STD + 50TBB + LY	137.7996685	0.078028	4.504976	0.105512771			0.924906325		0.74221881
STD + 50TBB + BI-D	152.7512114	0.092971	5.3677	0.049705517				0.137108172	
STD + LY + Egf	121.6877072	0.047471	2.740711	0.267207078	0.47605752				0.02988269
STD + BI-D + Egf	129.4822749	0.175505	10.13276	0.190947898	0.85824588			0.96817127	
STD + 25TBB + Egf	141.5455241	0.165655	9.564087	0.088446306	0.63219484	0.738035686			
STD + 0.1% DMSO	138.5647794	0.161814	9.34232	0.104170111					
BLANK									

WHCO3:

Sample	Rltv to control (%	STD Dev	S.E.M	T-Test (samples compared to STD)	EGF T.Test	25TBB T.Test	50TBB T.Test	10BI-D T.Test	20LY T.Test
STD	99.99999997	0.332112	13.55843	1					
STD + Egf	84.49451355	0.384375	15.69204	0.504937048	1				
SF + Egf	122.5367002	0.173398	7.078954	0.211910104	0.0803089				
STD + 25TBB	91.98428476	0.413499	16.88105	0.739241481		1			
STD + 50TBB	112.8675485	0.349055	14.25011	0.558323427		0.40262974	1		
STD + BI-D	112.966163	0.416396	16.99928	0.593657603				1	
STD + 25TBB + BI-D	116.790762	0.332421	13.57103	0.436660007		0.315114073		0.874103501	
STD + LY	101.9262699	0.316121	12.9056	0.232140774					1
STD + 25TBB + LY	107.7724661	0.313014	12.77876	0.707484663		0.506846475			0.77182022
STD + 50TBB + LY	150.5744458	0.263606	10.76167	0.02309321			0.025303574		0.02370273
STD + 50TBB + BI-D	122.018974	0.331288	13.52478	0.312199667				0.708146864	
STD + LY + Egf	78.9392301	0.335155	13.68263	0.335347742	0.8099937				0.28440233
STD + BI-D + Egf	54.21000087	0.308339	12.58787	0.045064932	0.1950276			0.029579669	
STD + 25TBB + Egf	105.085221	0.396781	16.19851	0.828278886	0.4178043	0.615498945			
STD + 0.1% DMSO	130.5622768	0.449765	18.36158	0.245838861					
BLANK									

SNO:

Sample	Relative to control (%)	STD Dev	S.E.M	T-Test (samples compared to STD)	EGF T.Test	25TBB T.Test	50TBB T.Test	10BI-D T.Test	20LY T.Test
STD	100.0030461	0.208615	12.0443812	1					
STD + Egf	132.5230741	0.161543	9.326672623	0.133580374	1				
SF + Egf	147.5859758	0.267263	15.43042473	0.099624658	0.5051704				
STD + 25TBB	200.4386366	0.133426	7.703328285	0.005563436		1			
STD + 50TBB	184.1847147	0.263169	15.19405317	0.019640358		0.457266019	1		
STD + BI-D	173.9650918	0.218191	12.59724397	0.01931117				1	
STD + 25TBB + BI-D	140.2053063	0.270103	15.59440379	0.146941078		0.055462819		0.209916773	
STD + LY	188.4096378	0.118463	6.839483953	0.009201866					1
STD + 25TBB + LY	158.3508483	0.237905	13.73547664	0.047147937		0.092940501			0.1804128
STD + 50TBB + LY	165.5670292	0.047328	2.732475475	0.034347464			0.043553926		0.0808794
STD + 50TBB + BI-D	179.0033202	0.179482	10.36237889	0.011888558				0.796720233	
STD + LY + Egf	152.2282129	0.282331	16.30036968	0.088427669	0.4140353				0.1756286
STD + BI-D + Egf	165.9630205	0.172654	9.968194234	0.020869511	0.0941264			0.680437077	
STD + 25TBB + Egf	178.4276097	0.219761	12.68790267	0.016143532	0.0651171	0.269510089			
STD + 0.5% DMSO	142.2705535	0.239707	13.83950411	0.109973406					
BLANK									