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The effect of Src inhibition on ROS-triggered signalling in human oesophageal squamous carcinoma cells

Embeth Houston-McMillan

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

Johannesburg, 2017

I. Declaration

I, Embeth Houston-McMillan, declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.

Embeth Houston-McMillan

Signed this 5th day of June in Johannesburg

II. Abstract

Reactive oxygen species (ROS) have been specifically highlighted as instigators of aberrant pro-survival and proliferative signal transduction pathways in recent years. A possibility that ROS stimulates the Src-Protein Kinase B (PKB)-Glycogen Synthase Kinase (GSK)3-β pathway, a non-canonical pro-survival and anti-apoptotic pathway, was identified and investigated. Specifically, Human Oesophageal Squamous Cell Carcinoma (HOSCC) cells were exposed to oxidative conditions, Src inhibition, and a combination of the two to determine the role that Src plays in the phosphorylation of PKB and GSK3-β in terms of ROS stimulation. Oxidative conditions led to a significant increase in pSrc and pPKB in only one of the 5 HOSCC cell lines being studied; however the abundances of pGSK3-β increased significantly in two of these cells lines and decreased significantly in two of the others. This indicates that oxidative conditions lead to different downstream effects in the various HOSCC cell lines, which are most likely achieved via the activation of various pathways as a result of crosstalk. Src inhibition led to a decrease in pPKB levels across all HOSCC cell lines displaying detectable levels of pPKB, however the abundance of pGSK3-β increased in these cell lines, indicating again that other pathways are at play with respect to the activation of GSK3-β in HOSCC cells. This is due to the fact that GSK3-β is a downstream effector of PKB, and should thus decrease in abundance as pPKB does. Oxidative conditions coupled with Src inhibition resulted in an increase in the abundance of pPKB and pGSK3-β in four of the five HOSCC cell lines. These results indicate that Src inhibition under oxidative conditions may lead to cell survival and proliferation via the activation of pPKB, a pivotal pro-survival protein, and subsequent inactivation of pGSK3-β, a known pro-apoptotic protein. Thus, although more than one pathway is likely to be involved in the phosphorylation of PKB and GSK3-β in HOSCC in terms of ROS, it appears as though inactive Src in HOSCC cells undergoing oxidative stress could be used as a testable marker for HOSCC – a devastating disease affecting numerous South Africans.

III. List of Associated Presentations

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VIII. List of Abbreviations and Symbols

µl – microliter
µM- micromolar
APS – Ammonium persulphate
BSA – Bovine Serum Albumin
CO_2 – Carbon Dioxide
DMEM – Dulbecco's Modified Eagle's Medium
DMSO - Dimethylsulfoxide
EDTA – Ethylene Diamine Tetraacetic Acid
EGFR – Epidermal Growth Factor Receptor
ERK – Extracellular Regulated Kinase
FCS – Foetal Calf Serum
GSK3-β – Glycogen Synthase Kinase 3 Beta

H_2O_2 – Hydrogen Peroxide
HOSCC – Human Oesophageal Squamous Cell Carcinoma
HRP – Horse radish peroxidase
MAPK – Mitogen-Activated Protein Kinase
MEK - MAPK/ERK Kinase
Min – minutes
ml – millilitre
mM – millimolar
mTORC1 - Mammalian Target of Rapamycin Complex 1
Na_3VO_4 – Sodium Orthovanadate
NaF – Sodium Fluoride
PAGE – Polyacrylamide Gel Electrophoresis
PBS – Phosphate Buffer Saline
PDK – phosphoinositide-dependent kinase
pGSK3-β – phosphorylated Glycogen Synthase Kinase 3 Beta
PI3K - Phosphoinositide-3-Kinase
PIP_2 – phosphatidylinositol 4,5-bisphosphate
PIP_3 – phosphatidylinositol 3,4,5-triphosphate
PKB – Protein Kinase B
PMSF - Phenylmethane Sulfonyl Fluoride
pPKB – phosphorylated Protein Kinase B
pSrc – Phosphorylated Src
PTEN – Phosphatase and Tensin homologue
ROS – Reactive Oxygen Species
RPM – Revolutions per minute
RTK – Receptor Tyrosine Kinase
SDS – Sodium Dodecyl Sulphate
SEM – Standard Error Mean
SFK – Src Family Kinase
TBS – Tris Buffer Saline
TCA – Trichloracetic acid
TEMED – N,N,N',N'-Tetramethylethylenediamine
WHCO – Wits Human Carcinoma of the Oesophagus
ρM – Picomolar

1. Introduction

1.1. Cell Signalling

In order to maintain a state of homeostasis, it is essential for every cell to be able to monitor its environment and respond appropriately (Kültz, 2005). Monitoring and responding to the environment generally entails reacting to stimuli. Stimuli can originate from the external environment, neighbouring cells, or the cell itself (autocrine signalling). Stimuli, such as growth factors, hormones, and – of relatively new-found importance – reactive oxygen species are usually detected by receptor proteins on the cell surface (Son *et al*., 2011). The receptor proteins transfer the signal from the external environment into the cytoplasm by triggering a signal transduction cascade involving many proteins; usually kinases and phosphatases (Jans, 1994).

Protein kinases are physiological catalysts, or enzymes, that facilitate the phosphorylation of their specific substrates (Walsh *et al*., 1971). Phosphate groups are negatively charged molecules that have the ability to bind to amino acids such as Serine, Threonine and Tyrosine (Taylor *et al*, 1995). Because phosphate groups are negatively charged, phosphorylation of a target molecule can change its structure and thus convert it into its active or inactive conformation (Westheimer, 1987). Protein phosphatases play the opposite role of dephosphorylating their substrates at specific sites (Chen *et al*., 1992), thus also changing the shape of the substrate and activating or inactivating it. In most cases, the kinase or phosphatase phosphorylates, or dephosphorylates, and activates or inactivates more than one protein, leading to the activation of more than one pathway (Jans, 1994). This phenomenon is known as cross-talk.

Cross-talk is described as a complex interaction between cell signalling pathways, for example between the MAPK and PI3K/PKB pathway (Mendoza & Blenis, 2011). There are various points of cross-talk between these two pathways, for instance they can both be activated by the same receptor protein and they can cross-activate, and cross-inactivate, one another (Mendoza & Blenis, 2011). The fact that there are many examples of cross-talk between just two pathways emphasises the complexity of cross-talk, as the possibilities of cross-talk between all known pathways are seemingly endless.

The aim of signal transduction pathways is ultimately to elicit a response from the cell. This is achieved by enabling specific proteins to enter the nucleus, where they can act as, or activate, transcription factors (Silver, 1991). Activation of transcription factors leads to the synthesis of proteins responsible for the desired response, which is mostly cell proliferation, survival or apoptosis (Silver, 1991). Thus, errors in cell signalling, no matter the origin, often result in the transformation of normal cells to cancerous cells (Thompson, 1995).

It is therefore essential to better understand cell signalling and its involvement in cancer to more effectively treat this disease. This unfortunately becomes complicated due to the fact that different cell types behave differently. In fact, samples from the same type of tumour

acquired from different people may behave differently in terms of signalling. Many specimens of the same sub-type of cancer must therefore be analysed in order to identify the different types of signalling within the same type of cancer. This will help to identify targets for potential therapeutics.

Analysing these signal transduction pathways includes pathway manipulation where stimuli and components of the pathway of interest must be investigated. For the purpose of this research, the stimulus being investigated was reactive oxygen species (ROS) and the pathway being manipulated was the Src/PKB/GSK3-β pathway.

1.2. Reactive Oxygen Species in Cell Signalling

Cell signalling begins with a stimulus (Kültz, 2005). These stimuli, such as hormones and growth factors, have long been studied with respect to diseases associated with cell signalling. However, ROS, a relatively new-found stimulus, has emerged as a signalling molecule of great importance in terms of cell signalling-related diseases such as cancer (Pelicano *et al*., 2004).

Reactive oxygen species such as hydrogen peroxide (H_2O_2) are produced as by-products of cellular metabolism in aerobic organisms. These ROS play an essential role in signal transduction, where they act as stimuli and secondary messengers (Nishikawa, 2008). Although ROS are necessary for cellular function, high concentrations - which lead to oxidative stress – can damage or even kill cells by reacting with lipids, proteins and nucleic acids (Sosa *et al*., 2013).

Slightly elevated levels of ROS can, however, have different effects on cells as various cell types have a range of capacities by which they respond to ROS. Because cancer cells tend to produce higher levels of ROS when compared to their non-transformed counter parts (Dang, 2012), they have to adapt to an oxidative environment in order to survive. Indeed, cancer cells not only produce high levels of ROS; they tend to also accumulate ROS. They thus exist in a consistently oxidative environment (Grek & Tew, 2010).

ROS tend to be particularly involved in the initiation and preservation of pathways involved in cell survival and proliferation (Dahan *et al.*, 2009). The oxidative environment which cancer cells create, and exist in, is therefore conducive to the distinguishing trait of uncontrolled proliferation and prolonged survival. Thus, increased levels of ROS are thought to be one of the main mechanisms by which the cancerous phenotype of transformed cells is maintained (Gibellini *et al.*, 2010).

The involvement of ROS in cell signalling pathways in human oesophageal squamous cell carcinomas (HOSCC) are relatively unknown. Thus, HOSCC was used as the model for this investigation as squamous cell carcinomas are of particular interest in terms of ROS-related signalling. This is because Maehata *et al.* (2010) observed that ROS stimulate tumour progression as well as angiogenesis in head and neck squamous cell carcinomas. ROS have also been identified as key players in epithelial-mesenchymal transition (EMT) in skin

squamous cell carcinomas (Lam *et al.*, 2013) and mammary epithelial cells (Cichon & Radisky, 2014).

ROS are therefore important targets for cancer research, particularly in terms of squamous cell carcinomas, as they are highly involved in both the transformation and maintenance of the cancerous phenotype. Thus, in order to better understand the influence of ROS on cell signalling in HOSCC cells, it is essential to gain a superior understanding of the pathways triggered by ROS signalling.

1.3. ROS-triggered Signalling

The undeniable involvement of ROS in the stimulation of proliferation and survival pathways in cancer cells (Groeger *et al.*, 2009) underscores the urgency to identify a component in a signal transduction pathway that is crucial to these processes. Intensive investigations have been undertaken over many years to characterise a pathway involved in proliferation and cell survival in terms of malignancy. This pathway is known as the mitogen-activated protein kinase (MAPK) pathway, or the Ras-Raf-MEK-ERK pathway.

1.3.1. ROS signalling triggers the MAPK pathway

In terms of oncogenesis, the MAPK pathway has often been identified as a component of the origin and/or maintenance of the transformed phenotype in many types of cancers (Son *et al.*, 2011). The MAPK pathway consists of a family of protein serine/threonine kinases that play a major role in transducing extracellular signals to the nucleus, stimulating cell growth and proliferation (Son *et al.*, 2011). This pathway is canonically activated by the Epidermal Growth Factor Receptor (EGFR) (Wu *et al.*, 1993), however it is also affected by ROS (McCubrey *et al.*, 2006).

Just below the surface of the cell, Ras proteins are activated via Receptor Tyrosine Kinases (RTKs) such as EGFR that have been stimulated by their ligands, or by ROS in a ligand-independent manner (McCubrey *et al.*, 2006). Ras proteins form part of the family of small Guanosinetriphosphatases (GTPases). Active Ras goes on to activate Raf in the canonical MAPK pathway, however Ras has other known downstream effectors such as Phosphoinositide-3-Kinase (PI3K) (Rajalingam *et al.*, 2007) which will be discussed in section 1.3.3.

Raf goes on to activate the MAPK/ERK Kinase (MEK), which leads to the activation of Extracellular Regulated Kinase (ERK) (McCubrey *et al.*, 2006). ERK is associated with the

circumvention of apoptosis and the stimulation of proliferation (McCubrey *et al*., 2006). There are also receptor-independent ROS-mediated mechanisms whereby ERK becomes active (McCubrey *et al*., 2006), yet inhibition of ERK and other components of the MAPK pathway has little effect on tumours with hyper-activated Ras and EGFR proteins (Ishii *et al.*, 2013). This hints at the activation of other pro-survival and proliferative pathways by Ras and EGFR.

Thus, although the MAPK pathway (and its association with ROS) is well known, it has provided little promise with respect to therapeutic intervention (Rajalingam *et al*., 2007). It is therefore essential to examine another, equally relevant pathway related to ROS signalling and cancer, such as the Protein Kinase B (PKB) pathway.

1.3.2. The PKB pathway: a noteworthy contributor to transformation

PKB, also known as Akt, is central to the activation of many proteins involved in pro-survival and proliferative pathways as well as the inactivation of proteins involved in pro-apoptotic pathways (see Figure 1.1). PKB becomes active in response to many stimuli such as growth factors and ROS, however, the canonical pathway by which it is activated is via receptor tyrosine kinases (RTKs) on the cell surface (Hemmings and Restuccia, 2012).

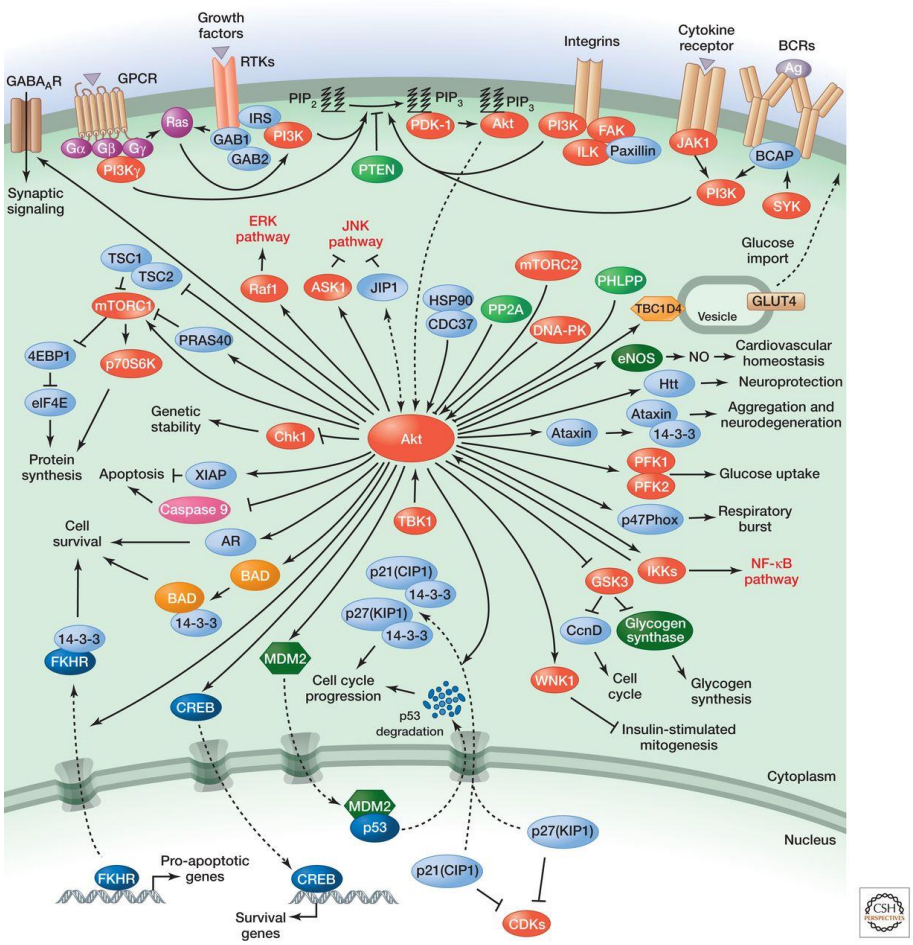


Figure 1.1 Multiple Signla Transduction Pathways Activated by PKB (Akt)

PKB is central to the activation of multiple signal transduction pathways. These include pro-survival pathways, proliferative pathways and anti-apoptotic pathways. The activation of PKB thus presumably results in cell survival and proliferation. Its aberrant activation often results in the transformation of cells and the maintenance of the cancerous phenotype. Figure from Hemmings and Restuccia (2012).

Growth factors bind to and activate these RTKs, which recruit Phosphoinositide-3-Kinase (PI3K) to the cell surface, resulting in its activation. PI3K is localised to the membrane by the RTK which leads to the subsequent activation of its 110 kDa catalytic subunit (Clerkin *et al.*, 2008). The catalytic subunit of PI3K then phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate the secondary messenger, phosphatidylinositol 3,4,5-triphosphate (PIP_3). PIP_3 recruits phosphoinositide-dependent kinase (PDK) 1, to the plasma membrane (Ziemba *et al.*, 2013) where it partially activates the co-recruited PKB by phosphorylating it at Thr308. PKB becomes fully activated (pPKB) when phosphorylated at Ser473 by PDK 2 (see Figure 1.2) (Blanco-Aparicio *et al.*, 2007).

The activation of PKB is negatively regulated by the protein tyrosine phosphatase Phosphate and Tensin Homolog (PTEN), which dephosphorylates PIP_3 (see Figure 1.2), thus inhibiting the activation of PDK 1 (Hemmings and Restuccia, 2012). The inactivation of PTEN thus leads to an increase in the abundance of pPKB, which has been associated with enhanced cell survival, proliferation and invasion (Shukla *et al.*, 2007). Interestingly, H_2O_2, a reactive oxygen species, oxidises and inactivates PTEN and other protein tyrosine phosphatases (Leslie *et al.*, 2003). The mechanism of activation of PKB by ROS will be discussed in section 1.3.4.

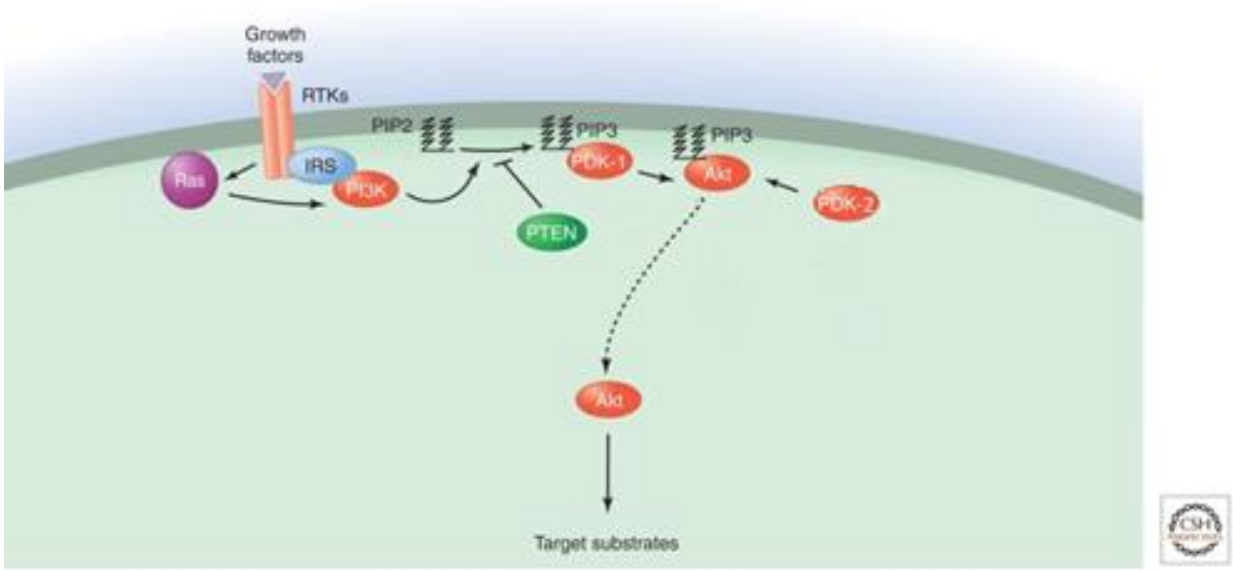


Figure 1.2: Canonical Activation Pathway of PKB (Akt)

PKB becomes canonically activated via the ligand-dependent activation of RTKs and the subsequent activation of PI3K. This leads to a signal transduction cascade that results in the phosphorylation of PKB at Thr308 by PDK1 and Ser473 by PDK2. This process is negatively regulated by PTEN, which inhibits the activation of PDK1 by dephosphorylating PIP_3 to generate PIP_2. Figure adapted from Hemmings and Restuccia (2012).

Because pPKB plays a central role in the activation of many pathways involved in cell survival and proliferation, it is essential that the PKB pathway be studied with as equal an intensity as the MAPK pathway. This is especially true considering the lack of progress the MAPK pathway has produced in terms of therapeutic targets.

1.3.3. Cross-talk between the MAPK and PKB pathways

As mentioned previously, active Ras has the ability to activate PI3K which will ultimately activate PKB. There are, however, other mechanisms by which the MAPK and PI3K/PKB pathways affect each other. Indeed, depending on the upstream signal, these pathways can cross-inhibit or cross-activate one another, as well as converge (Mendoza & Blenis, 2011).

An example of the cross-inhibition between these pathways is the inactivation of PKB by MEK (Yu *et al*., 2002). Conversely, PKB negatively regulates Raf by phosphorylating sites that inhibit its activation (Guan *et al*., 2000). Thus, in untransformed cells, the MAPK and PI3K/PKB pathways regulate each other to mediate growth and proliferative signalling (See Figure 1.3). Therefore, when one pathway is inhibited, the other often becomes hyper-activated, or, when one pathway is activated the other may become inactive.

These pathways also cross-activate one another, where MAPK signalling can activate mammalian target of rapamycin complex 1 (mTORC1) – a downstream effector of pPKB. However, as this activation is PI3K and PKB independent (Mendoza & Blenis, 2011), this particular instance of cross-talk between the pathways is not of interest for the purpose of this research. The PI3K/PKB and MAPK pathway can also act in unison to phosphorylate downstream proteins involved in survival and proliferation of the cell (Mendoza & Blenis, 2011). One such protein is the glycogen synthase kinase 3 β (GSK3- β), which will be discussed in section 1.3.5.

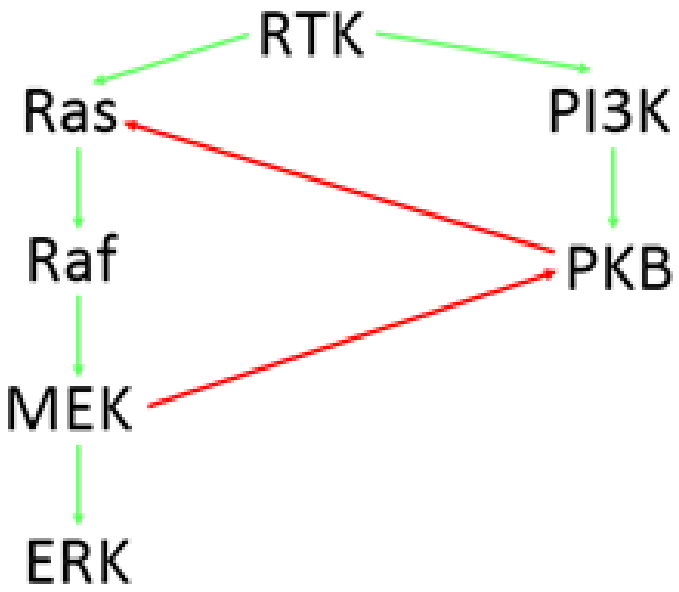


Figure 1.3: Cross-inhibition between the PI3K/PKB and MAPK pathways

Although both the MAPK and PI3K/PKB pathways may become activated by the same receptor tyrosine kinase (RTK), they often have inhibitory effects on each other to prevent hyper-activation of a mutual downstream target. Green arrows indicate activating phosphorylations, while red arrows indicate inhibition.

1.3.4. ROS-dependent activation of PKB

As mentioned briefly in section 1.3.2, the activation of PKB in cells exposed to oxidative conditions has been found to be due PTEN oxidation and inactivation in various cell types (Leslie *et al*., 2003; Silva *et al*., 2008; Zhang *et al*., 2007). Previous laboratory data, however, indicate that this does not appear to be the case in HOSCC cells (Shaw, 2011). This implies that there may be a non-canonical mechanism by which PKB becomes activated in response to oxidative conditions in HOSCC. It is thus necessary to investigate alternative mechanisms by which PKB becomes active in the presence of ROS, other than via PTEN inactivation or Ras activation. This is due to the apparent irrelevance of PTEN inactivation via ROS in HOSCC cells, as well as the lack of a solution to carcinogenesis provided by MAPK pathway manipulation. The next candidate most likely to have a major influence on the PKB pathway at this level, in terms of ROS, is the non-receptor tyrosine kinase Src.

Src is a proto-oncogene that forms part of the Src family kinases (SFKs), a family of Serine/Threonine kinases with conserved homology. These kinases have been implicated in cell signalling pathways involved in survival, proliferation, morphology, motility and invasion (Parsons and Parsons, 2004). Src is inactive when phosphorylated at Tyr527 and must be dephosphorylated at this site in order for activation to occur. Once Src has been dephosphorylated at Tyr527, it adopts an active, "open" conformation. This allows Tyr416 to become auto-phosphorylated, thus fully activating the protein (pY416 Src) (Giannoni *et al.*, 2005). The abundance of Src phosphorylated at Y416 increases when cells are exposed to exogenous H_2O_2 (Zhougang & Schnellmann, 2004).

Active Src has many substrates such as adaptor molecules, cytoskeletal proteins, other kinases, as well as nucleotide-binding proteins (Parsons and Parsons, 2004). These substrates include EGFR, Ras and PI3K (Arcaro *et al.*, 2007), all of which play a role in the phosphorylation of PKB. pSrc itself may also interact directly with PKB (see Figure 1.4), phosphorylating it at numerous tyrosine residues which allows for the activating phosphorylation at Thr308 by PDK1 (Vojtechová *et al.*, 2008). It thus appears that pSrc may play a major role in the activation of PKB via various pathways, all of which can be activated by ROS. In addition to this, there is a positive correlation between the abundance of pY416 Src and invasiveness in HOSCC cells (Chen *et al.*, 2010).

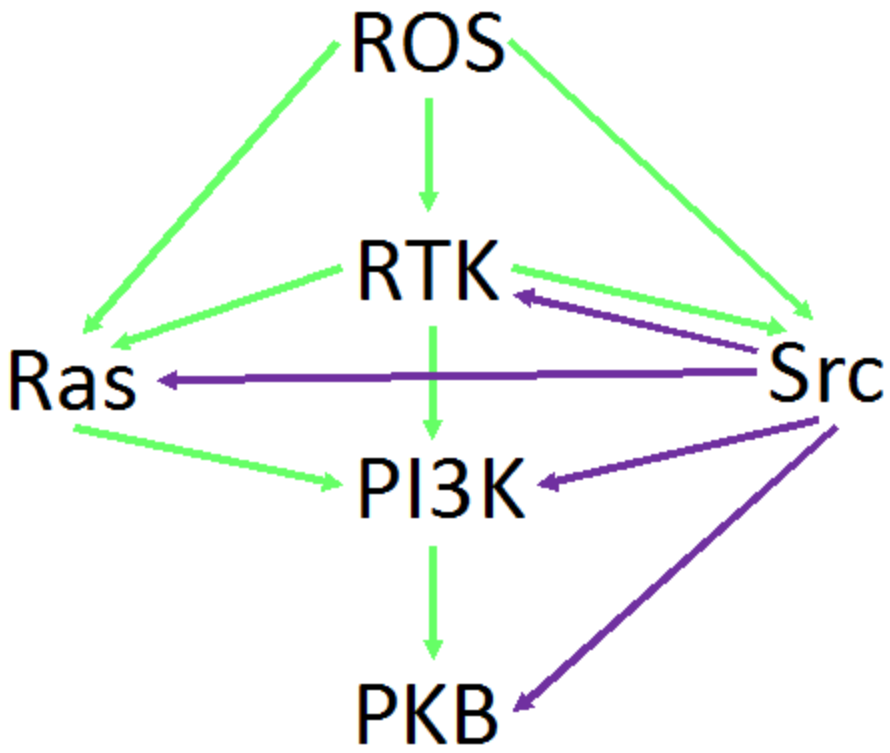


Figure 1.4: Proposed Mechanism of PKB Activation by ROS-induced Src Activation

Sub-lethal levels of ROS lead to an increase in the abundance of active (phosphorylated Tyr416) Src. It has been found that H_2O_2 stimulates the activation of PKB. This activation may be mediated by Src, which plays a role in the activation of EGFR, Ras as well as PI3K. Green arrows represent activating phosphorylations, purple arrows represent activating phosphorylations by Src.

Examining Src, and not Ras or PI3K individually, is essential, as Penuel and Martin (1999) found that inhibition of the individual pathways in chicken embryo fibroblasts did not block transformation by v-Src. In contrast, simultaneous inhibition of these pathways essentially prevented transformation. This indicates that both pathways are involved in the transformation activities of Src. It is therefore imperative to investigate the activation of Src in response to oxidative conditions in HOSCC cells. This will determine whether the alternate pathway by which PKB becomes phosphorylated in response to oxidative conditions is, in fact, related to the abundance of pSrc within the cell.

1.3.5. ROS signalling and PKB inactivate GSK3-β

Although the activation of PKB points to increased cell survival and proliferation, as well as a decline in apoptosis, it is not enough to assume that this is the case. Thus, a downstream effector of pPKB must also be monitored in response to pathway stimulation and manipulation.

Glycogen Synthase Kinase 3-β (GSK3-β) is a Serine/Threonine kinase involved in pro-apoptotic signalling (Yoshino, 2015), where it degrades the pro-survival protein β-catenin and cell cycle progression-related Cyclin D1 when active (Hardt & Sadoshima, 2002). A lack of these proteins, due to active GSK3-β, thus presumably results in a lower rate of cell survival and proliferation. Indeed, active GSK3-β has been associated with apoptosis, while inactive GSK3-β phosphorylated at Ser9 (pGSK3-β) is associated with cell survival (Paweletz *et al*., 2001). It has been long known that GSK3-β is inactivated by PKB when it becomes phosphorylated on the Ser9 residue (Cross *et al*., 1995). An increase in phosphorylation of GSK3- β on the Ser9 residue is thus associated with an increase in pPKB abundance.

GSK3-β has recently been found to play a role in the reaction of the cell to oxidative stress, where it becomes phosphorylated at the "inactivating" Ser9 residue in response to oxidative conditions (Feng *et al*., 2013). However, although this phosphorylation is assumed to be linked to PKB, little is known about the mechanism of the phosphorylation of GSK3-β in response to ROS stimulation. Thus, this study will also seek to discover whether the inactivation of GSK3-β in response to ROS is pPKB and/or pSrc related.

GSK3- β in particular is of interest to this study because of its contribution to tumourigenicity in squamous cell carcinomas such as that of the cutaneous (Ma *et al*., 2007). Thus, it can be assumed that GSK3-β will play a similar role in the squamous cells of oesophageal carcinomas.

1.4. Oesophageal Squamous Cell Carcinoma

Oesophageal cancer is the 8th most common cancer in the world and causes the 6th most cancer-related deaths (Ferlay *et al.*, 2013). It is thus associated with an extremely poor prognosis with a mortality to incidence ratio of 0.88 (Ferlay *et al.*, 2013). It is, however, one of the least studied subtypes of cancer. It is for this reason that HOSCC cells were used as a model for this investigation.

Squamous cells in particular, and not adenocarcinomas, were examined because HOSCC mainly affects residents of developing countries such as South Africa, where black males are highly susceptible. Approximately 6% of South African males are affected by this disease (Ferlay *et al.*, 2013). It is thus essential that this type of cancer be understood in order to attempt to better treat this disease that affects so many South Africans.

Previous laboratory data indicate that PKB becomes activated in HOSCC cells in the presence of exogenous H_2O_2. This process does not, however, appear to occur due to inactivation of protein tyrosine phosphatases or EGFR stimulation (Shaw, 2011). It is therefore of interest to examine the possibility that the activation of PKB by H_2O_2 in HOSCC cells is mediated by Src. Src is particularly relevant because a decrease in Srcasm (Src-activating and signalling molecule), a molecule that inhibits Src activity, was observed in Chinese HOSCC cells (Qi *et al.*, 2010). This indicates that the activity of Src may be

increased in HOSCC cells, possibly leading to increased levels of pPKB and pGSK3-β, and thus hyper-proliferation and extended cell survival.

It is thus essential that the relationship between ROS, Src, and the phosphorylation status of PKB and GSK3-β be examined. This investigation was therefore performed in an attempt to better understand and perhaps identify a target for potential treatments of oesophageal squamous cell carcinomas – a devastating tumour that affects numerous South Africans.

1.5. Aim and Objectives

The connection between elevated levels of pSrc and squamous cell carcinomas, as well as its involvement in the activation of PKB and GSK3-β, has made it an interesting target for research in terms of HOSCC. Not only Src itself, but also pPKB and pGSK3-β, have been implicated in the formation of many tumours, including squamous cell carcinomas. These particular proteins of interest have all been recorded as being affected by reactive oxygen species (ROS). Thus, the aim of this investigation was to determine the role of Src in the phosphorylation of PKB and GSK3-β under oxidative conditions in HOSCC, in which the role of ROS and the Src-PKB pathway is relatively unknown.

The objectives were as follows:

- To determine the impact of an oxidative environment on the abundance of pSrc, pPKB and pGSK3-β in HOSCC cells. This was performed in order to establish whether Src plays a role in the phosphorylation of PKB in response to ROS stimulation. The effectiveness of the PKB activation was determined by examining the abundance of pGSK3-β, a downstream effector of pPKB.
- To determine the role of Src in the phosphorylation of PKB and pGSK3-β by inhibiting Src. The abundances of pPKB and pGSK3-β were investigated in response to Src inhibition to identify the role of Src in their phosphorylation status under standard tissue culture conditions.
- To determine the role of Src in the phosphorylation of PKB and pGSK3-β in response to ROS stimulation. The abundance of pPKB and pGSK3-β were investigated in response to Src inhibition in an oxidative environment. This was performed to determine the role of Src in the phosphorylation of PKB and GSK3-β in cells undergoing oxidative stress.

2. Methods and Materials

2.1. Tissue Culture

Five HOSCC cell lines derived from moderately differentiated and metastatic tumours, as well as two control cell lines, were obtained from the School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg. The HOSCC cell lines included the Wits Human Carcinoma of the Oesophagus WHCO1, WHCO3, WHCO5 and WHCO6 cell lines (Veale and Thornley, 1989) as well as the SNO cell line (Bey *et al*., 1976). H1299 non-small cell lung carcinoma cells (Giaccone *et al*., 1992) were used as a positive control cell line for pPKB and pGSK3-β detection. This is due to the high levels of pPKB and pGSK3- β present in H1299 cells as a result of a methylated PTEN promoter (Soria *et al*., 2002). A431 epidermoid carcinoma cells (Giard *et al*., 1973) were used as a positive control for the detection of pSrc, and as a comparative epithelial cell line with high EGFR expression (Shimizu *et al*., 1984). These control cell lines were also used due to "normal" human oesophageal squamous cells being unavailable for comparison (Jankowski *et al*., 1995).

All cell lines were cultured as a monolayer in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (3:1) (see appendix A 1.1 – 1.3) supplemented with 10% Foetal Calf Serum (FCS) (Highveld Biological) and were maintained in a humidified 37 ^{o}C incubator with an established atmosphere of 5% CO_2. These conditions mimic physiological conditions. Cells were sub-cultured upon reaching 80% confluency to avoid potential changes in signalling due to contact inhibition. This was performed by discarding the medium, rinsing the cells with sterilized 1 x Phosphate Buffer Saline (PBS) (see appendix A 1.4) and adding Trypsin (Gibco BRL)/ Ethylene Diaminetetra Acetic Acid (EDTA) (BDH Laboratory reagents) (see appendix A 1.5). Cells were incubated in Trypsin/EDTA at 37 ^{o}C for 5 minutes to facilitate detachment. A fraction of the detached cell-Trypsin/EDTA mixture was transferred to a new dish, where medium containing 10% FCS was then added to the cells. Tissue culture then continued as stated above. All studies were routinely performed on cells approaching 80% confluency due to there being no detectable contact inhibition at this density.

2.1.1. ROS stimulation through exposure to Hydrogen peroxide (H_2O_2)

Cells approaching a confluency of 80 % were exposed to oxidative conditions. The cells were rinsed twice with 1 x PBS. They were then incubated in medium supplemented with 5% FCS containing 1 mM hydrogen peroxide (Unilab®/SAARCHEM (Pty) Ltd) for 1 hour.

2.1.2. Src inhibition

Cells approaching 80 % confluency were rinsed twice with 1 x PBS. They were then incubated for 1 hour in medium supplemented with 5 % FCS to which 10 nM KX2-391 (KX-01) (Selleckchem) in dimethylsulphoxide (DMSO) was added.

2.1.3. Src inhibition under oxidative conditions

Cells approaching 80 % confluency were rinsed twice with 1 x PBS. They were then incubated for 1 hour in medium supplemented with 5 % FCS containing 1 mM H_2O_2, to which 10 nM KX-01 in DMSO was added.

2.2. Whole Cell Protein Extraction

In order to extract all protein from the cells, untreated cells at 80 % confluency or cells treated as detailed above were rinsed twice with 1 x PBS. The cells were then rinsed with 1 x PBS containing a protease inhibitor; 1mM phenylmethane sulfonyl fluoride (PMSF) in methanol, as well as phosphatase inhibitors; 5 mM Sodium Fluoride (NaF) and 10 mM Sodium Orthovanadate (Na_3VO_4). This was to avoid protein degradation and to maintain the phosphorylation status of the proteins. Cells were scraped off the dish into the PBS containing protease and phosphatase inhibitors. This solution was transferred to a 1.5 ml Eppendorf microfuge tube and centrifuged at 12 000 RPM for 30 seconds. The supernatant was discarded and the cells were resuspended in 2x Laemmli lysis buffer (Laemmli, 1970) (see appendix A 2.1). The lysate was boiled for 5 minutes and centrifuged at 4 ºC for 15 minutes at 12 000 RPM.

2.3. Protein Determination

The protein content in each whole cell protein extraction was determined via a Bradford assay with the modifications proposed by Bramhall *et al.* (1969) to reduce the effects of SDS on the measurement. Whatmann filter paper was rinsed in dH_2O for 20 minutes and was then dehydrated by rinsing it in 95% ethanol, 100% ethanol and 100% acetone for 5 minutes each. This was performed to reduce any loose fibres that would otherwise come off in later steps and possibly lead to the rinsing off of stain. The filter paper was left to dry. Volumes of 1, 3, 6, 12, 16, and 20 µl of 1 mg/ml Bovine Serum Albumin (BSA) in 2x Laemmli lysis buffer, as well as a known volume of the whole cell protein extracts, were dotted onto the filter paper and left to dry. The protein content was then fixed onto the filter paper by incubation in 7.5 % trichloracetic acid (TCA) for 45 min. The filter paper was rinsed with Coomassie Brilliant Blue stain (see appendix A 3.1) for 5 minutes, after which it was stained for 1 hour. The filter paper was then destained (see appendix A 3.2) for 1 hour, leaving stained protein-dye spots. The spots were placed into separate test tubes, into which 5 ml of elution solution (see appendix A 3.3) was added, and left overnight in the dark. The absorbance of each sample was then determined using an Abotta SV-1100 spectrophotometer at a wavelength of 595 nm. The BSA readings were used to create a standard curve (see appendix B Figure B1), to which the readings of the protein extracts were compared to obtain the concentration of protein present in each respective sample.

2.4. SDS PAGE and Western Immunoblotting

The protein extracts prepared as described in 2.2 were separated via a discontinuous Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) prepared according to Laemmli (1970) (see appendix A 4.1 and 4.2). Equal amounts of protein were loaded onto the

gel. Equal loading was confirmed later by analysing β-actin as a loading control. The proteins were resolved according to weight by applying a constant current of 21 mA using the Mighty Small ™ Electrophoresis Unit, which was filled with running buffer (see appendix A 4.3). Resolved proteins were visualised (see appendix B Figure B2) by staining the gel with Coomassie Brilliant Blue (see appendix A 3.1) and destaining (see appendix A 4.4).

Western Immunoblotting was performed as described by Towbin *et al.* (1979). Protein from unstained gels was transferred onto a nitrocellulose membrane (Nitrobind, MSI USA) by applying a current of 400 mA for 2 hours using a BioRad Trans-Blot ™ filled with Transfer Buffer (see appendix A 5.1). The nitrocellulose membrane was rinsed with Tris Buffer Saline (TBS) (see appendix A 5.2). Non-specific binding sites were blocked by incubating the membrane in a blocking solution (see appendix A 5.3) at room temperature for 1 hour. The membrane was rinsed three times in TBS with 0.1 % Tween-20 (TBS-T) for 5 minutes and then incubated with the appropriate primary antibody (see appendix B table B1) at 4 ^{o}C overnight. The membrane was then rinsed three times in TBS-T for 5 minutes per rinse to eliminate any unbound antibody. The membrane was thereafter incubated in the appropriate dilution of horse radish peroxidase (HRP)-linked secondary antibody (see appendix B table B1) for 1 hour. Unbound antibody was eliminated once more by rinsing the membrane in TBS-T three times for 5 minutes per rinse. The detergent was then removed by rinsing the membrane in TBS (with no Tween-20) for 5 minutes. This was followed by a 5 minute incubation in the dark in a 1:1 ratio of luminol and peroxide (SuperSignal®, West Pico). The reaction was then contained by sealing the membrane in clear cling wrap (Versafilm®), which was exposed to CL-XPosure™ X-Ray film (Pierce) for 10 minutes in the dark. The X-Ray film was immediately submerged in Developer (see appendix A 5.4), rinsed in water, submerged in Fixer (see appendix A 5.5) and again rinsed in water.

2.5. Semi-Quantification of Western Blots

The intensities of the visible bands present on the X-Ray film subsequent to Western Blotting are indicative of the abundance of a particular protein within the specific protein extract. Those bands were digitalised with a Hewlett Packard ScanJet G3110 desktop scanner and semi-quantified using Matlab Generator Densitometry Programme. Densitometry was standardised between different Western blots generated from extracts from the same cell line by dividing by the untreated control, which is represented as a relative percentage to the standard. The standard in this case was the intensity of the particular protein being examined in a whole cell extract from an untreated control cell line (H1299 for pPKB and pGSK3-β and A431 for pSrc).

2.6. Statistical Analysis

Statistical significance was determined by means of Student's T-test, where a p-value of < 0.05 indicates statistical significance. Error bars represent the standard error mean (SEM) of the biological replicates performed.

3. Results

3.1. HOSCC cells express differential levels of pSrc, pPKB and pGSK3-β

To detect the relative abundances of pSrc, pPKB, pGSK3-β and Actin, the whole cell protein contents extracted from untreated HOSCC and control cell lines, grown to 80 % confluency under standard conditions, were separated by SDS PAGE and underwent immunoblotting (as described in the methods and materials section 2.4). Unless otherwise stated, pGSK-β indicates the inactivating phosphorylation at the Ser9 residue.

It should be noted that the changes in the phosphorylation status of proteins in response to stimuli is at a ρM level. Thus, detecting these small changes can be challenging. Therefore, a seemingly small and insignificant change in a phospho-site may have an impact on the cell in terms of signalling.

Detecting these proteins in the untreated cells under standard tissue culture conditions served to confirm the presence or absence of the specific phospho-sites on the proteins of interest. The inclusion of the Western blot (See section 2.4) detecting Actin serves to demonstrate accurate loading of equal amounts of protein, as cytoskeletal protein expression remains unchanged across different cell lines (See Figure 3.1 D). The cell lines A431 and H1299 were used as comparative controls for pSrc, and pPKB and pGSK3-β respectively as they contain high levels of these phospho-proteins. This was necessary due to the unavailability of untransformed oesophageal squamous cells to be used as a comparison.

All the HOSCC cell lines as well as the A431 control cell line, but not H1299 cells, expressed detectable levels of pSrc (See Figures 3.1 A). The untreated WHCO3 cell line did not express detectable levels of pPKB (See Figures 3.1 B), while the SNO cells did not display detectable levels of pGSK3-β (See Figures 3.1 C). Performing densitometry on these Western blots allowed for the semi-quantification of the intensity of the bands present on the blots (See Figure 3.2). The densitometric values confirmed that there are indeed differences in the amounts of the proteins phosphorylated at specific sites across the different cell lines.

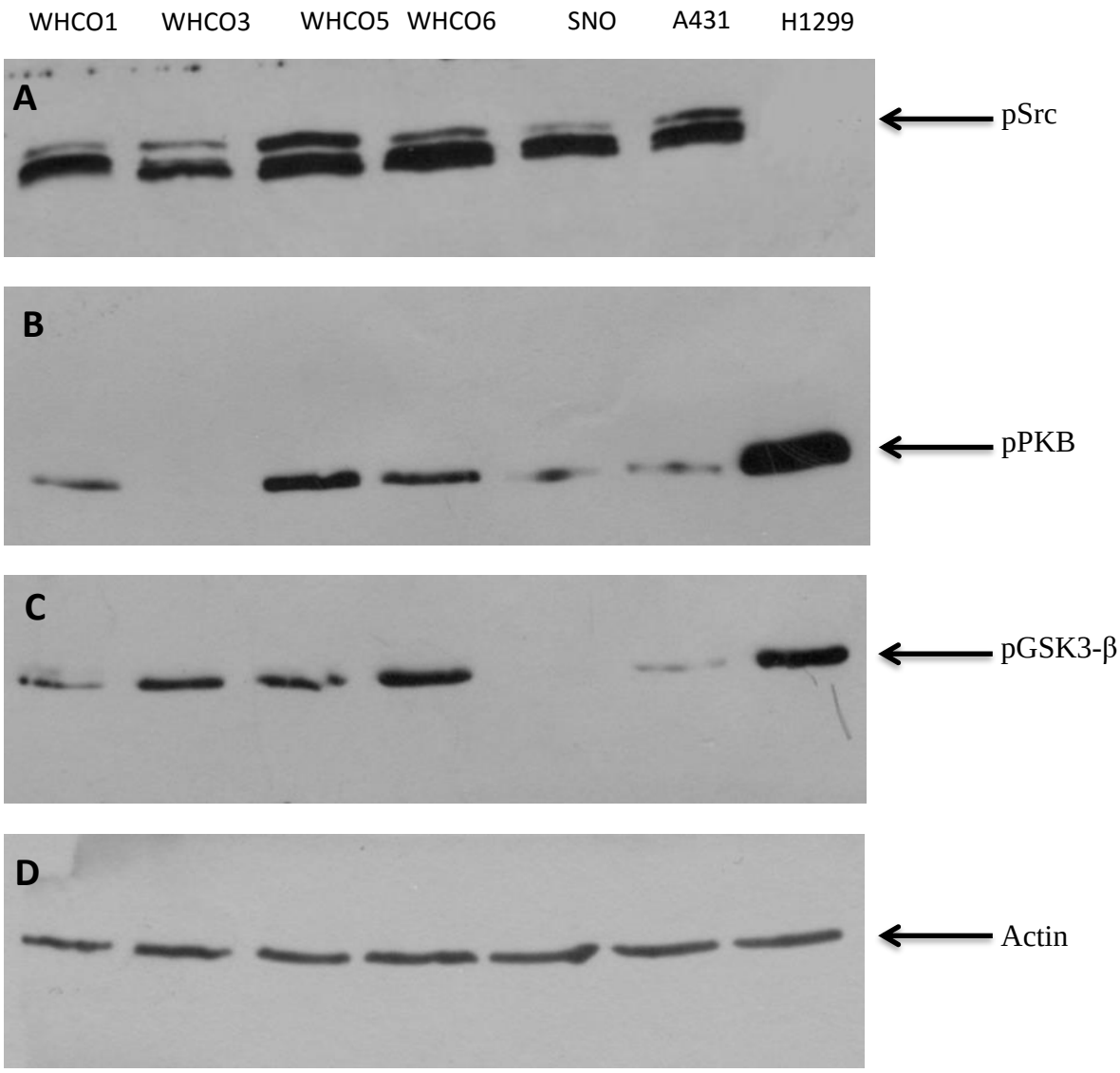


Figure 3.1: Differential Abundance of pSrc, pPKB and pGSK3-β in HOSCC and control cells

The relative abundances of A) pSrc, B) pPKB, C) pGSK3- β and D) Actin across the 5 untreated HOSCC cell lines (WHCO1, WHCO3, WHCO5, WHCO6 and SNO) as well as the untreated A431 and H1299 control cell lines detected by Western Blotting. Actin (D) was used as a control to ensure that loading was accurate and of equal amounts.

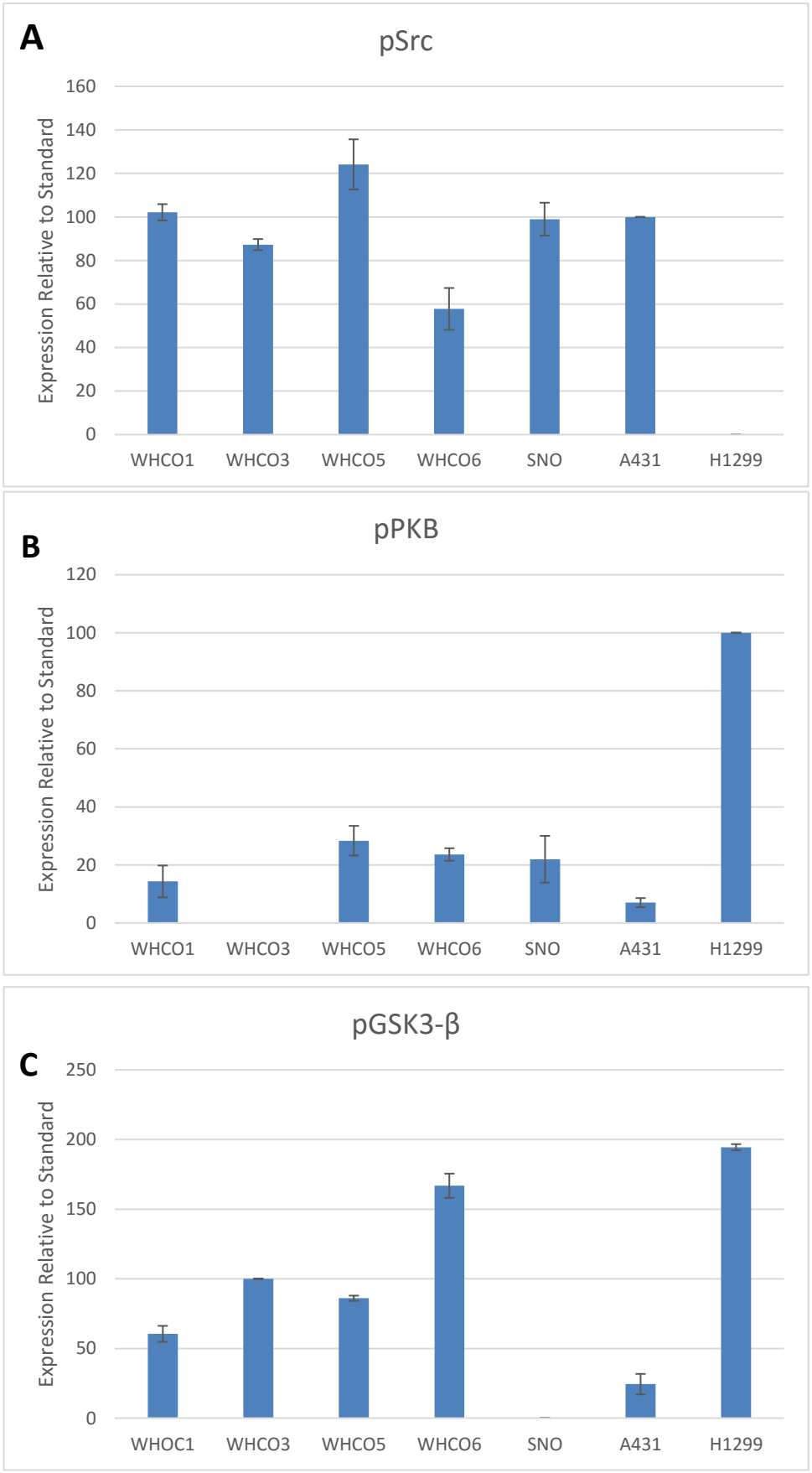


Figure 3.2: Different abundances pSrc, pPKB and pGSK3-β in HOSCC and control cell lines reflected semi-quantitatively (n=3).

Representation of the mean intensities of the bands present on Western blots in figures 3.1. A) depicts the mean densitometric values obtained from Western blots detecting pSrc in the untreated cell lines, while B) and C) represent those on the Western blots detecting pPKB and pGSK3-β respectively. Error bars represent the standard error mean (SEM) of the replicates.

3.2. HOSCC cells respond dissimilarly to ROS stimulation

The effect of ROS stimulation on the Src/PKB/GSK3- β pathway was analysed to determine whether oxidative conditions have an effect on the phosphorylation status of these proteins, and thus presumably cell survival, proliferation and ultimately carcinogenesis. This was performed by exposing HOSCC and control cells to 1 mM H_2O_2 for 1 hour. This particular concentration of H_2O_2 was used because the addition of micromillimolar levels of H_2O_2 has been found to be more effective at causing DNA lesions than millimolar concentrations. This could have a profound impact on the results (Nakamura, 2003). High levels of H_2O_2 have been found to be lethal to mammalian cells, whereas 1 mM has been used frequently to induce oxidative stress in cells (Janero, 1993; Kobayashi, 2005; Miguel, 2009) and has previously been used to induce oxidative stress in HOSCC cells in our laboratory (Shaw, 2011). H_2O_2 was also used as the representative ROS because Forman *et al.* (2010) found that, of all the ROS, H_2O_2 satisfies the requirements of being a secondary messenger best.

The cells were exposed to oxidative conditions for 1 hour because a titration performed previously determined that the cells are most responsive in terms of changes in phosphorylation after 1 hour. The oxidation of proteins is also thought to be reversible. Thus, allowing the cells to remain in an oxidative environment for too long risks missing the full effect of the oxidative conditions on the cells. There is also a risk of the antioxidants within the cells neutralising the ROS if the treatment is applied for too long.

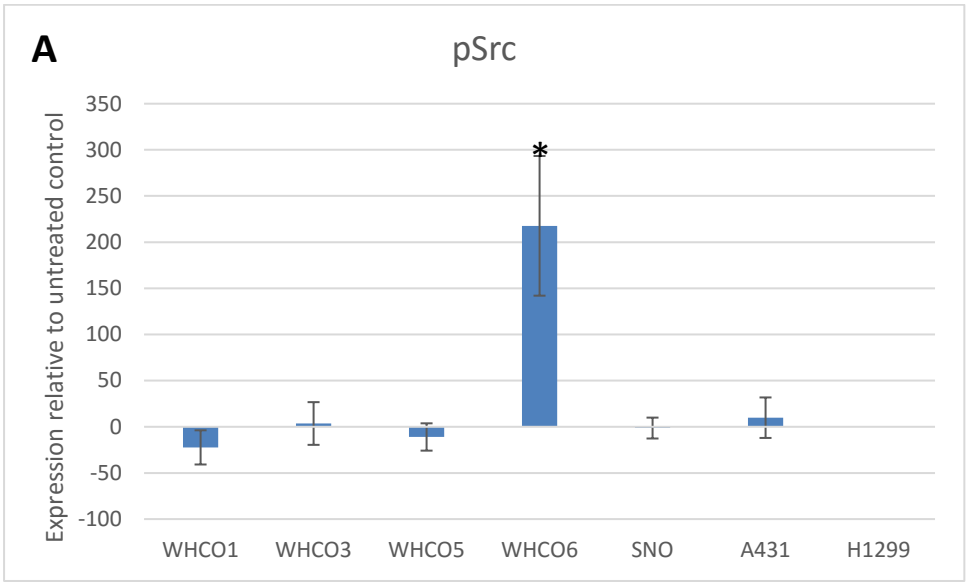
The densitometric analysis of biological replicates depicting the response of HOSCC and control cells to ROS stimulation are displayed as bar graphs below (see Figure 3.3). The bars represent the mean change in expression relative to the untreated control, while the error bars represent the standard error mean (SEM) of the replicates. An asterix above the bar indicates that the change in phosphorylation of a signalling intermediate is statistically significant, with a p-value less than 0.05.

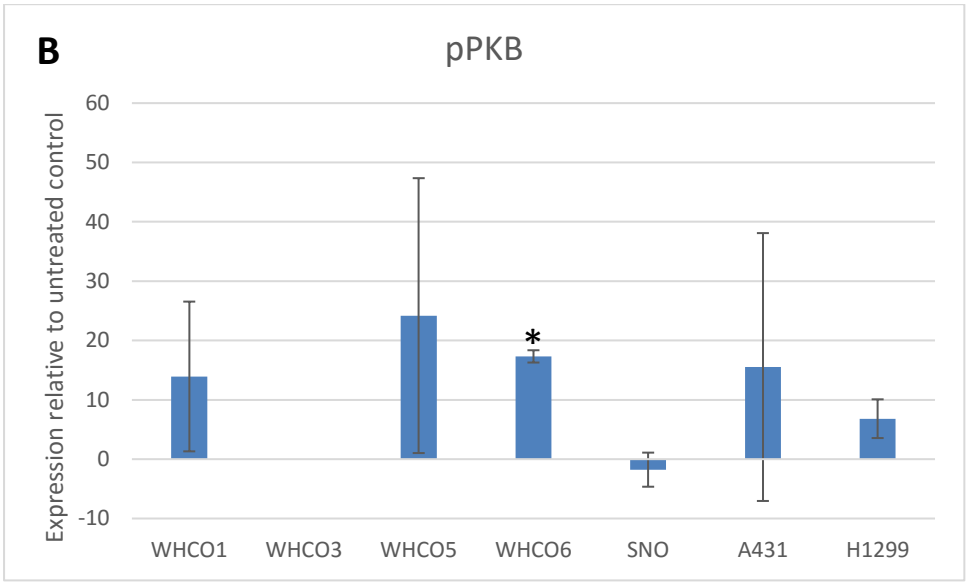
The abundance of pSrc in all cells lines was calculated relative to the abundance in A431 cells, while pPKB and pGSK3-β levels were calculated relative to the abundance in H1299 cells. These cell lines were thus used as loading controls for the respective proteins, allowing for the comparison of Western blots performed separately by controlling for day-to-day variation. This control for variation was practiced over all experiments performed.

Although the conditions under which the cells were cultured were attempted to be kept as standard as possible, some variation (displayed as error bars) did occur. Because ROS levels can potentially be altered by stress, small differences in conditions can result in seemingly large variations in the abundance of sensitive phosphorylation sites. This is particularly true of proteins more sensitive to oxidative conditions.

With the exception of WHCO6 cells which responded with a significant increase in the abundance of pSrc, all HOSCC and control cell lines displayed unchanged levels of pSrc in response to ROS stimulation (See Figure 3.3). ROS stimulation appeared to increase the abundance of pPKB in all cells lines other than SNO. However, due to variation, the only increase that can be said to be significant was again in the WHCO6 cell line.

Interestingly, the abundance of pGSK3-β decreased significantly in WHCO6 and WHCO3 cells in response to ROS stimulation. WHCO1 and SNO cells responded to ROS stimulation with a significant increase in pGSK3-β.





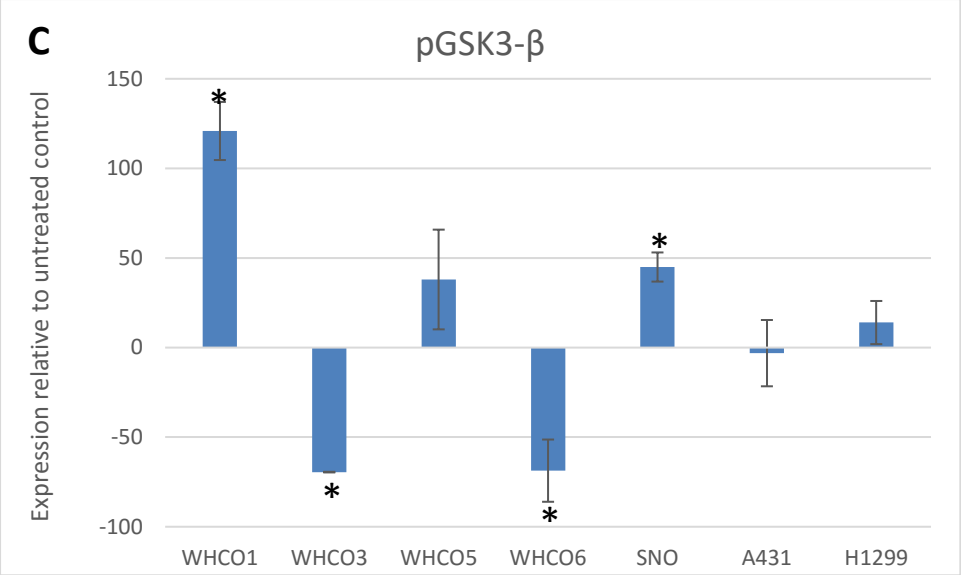


Figure 3.3: Changes in the abundances of pSrc, pPKB and pGSK3-β in HOSCC and control cell lines responding to ROS stimulation (n = 3).

Representation of the mean changes in intensities (compared to untreated controls) obtained from biological replicates of HOSCC and control cells stimulated with ROS (1 mM H_2O_2 for 1 hour). Semi quantitative intensities originate from densitometric analysis of Western blots detecting A) pSrc, B) pPKB and C) pGSK3-β in the above-mentioned cells. Error bars represent the SEM of the replicates, while an asterix represents statistical significance ($p < 0.05$). See Table B2-4 for means, SEMs and p-values.

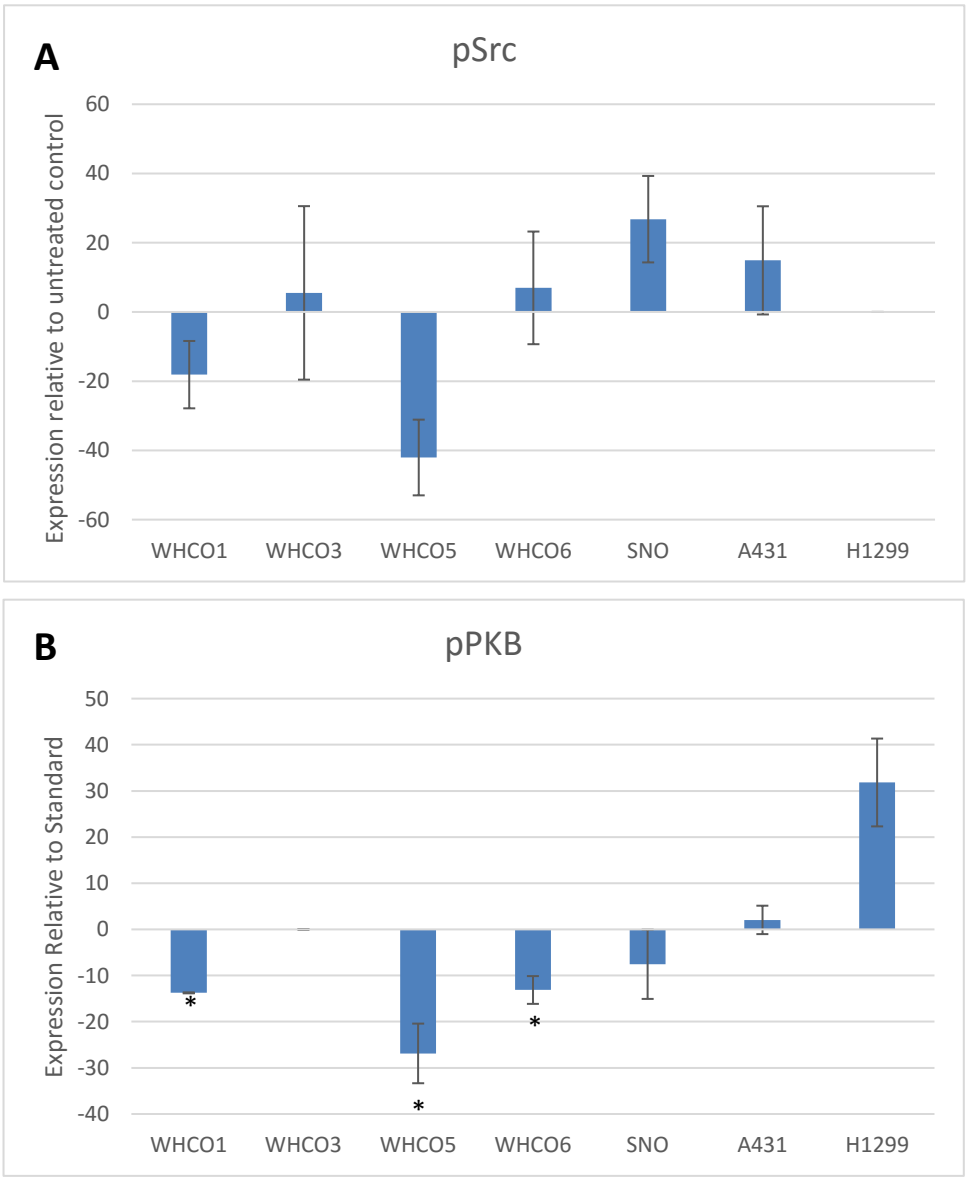
3.3. pPKB decreases, while pGSK3-β increases, in response to Src inhibition

The experiments involving ROS stimulation indicate that oxidative conditions do not have an effect on pSrc levels in all HOSCC cell lines besides WHCO6 (See Figure 3.3A). They also indicate that oxidative stress may lead to an increase the abundance of pPKB, however this increase cannot be said to be significant in most cell lines (See Figure 3.3B), and had varying effects on the abundance of pGSK3-β (See Figure 3.3C). The effect of Src on these signalling intermediates was thus determined under standard tissue culture conditions – in the absence of ROS – in order to establish the relationship between pSrc and the activation status of PKB and GSK3-β. This was achieved by inhibiting Src with 10 nM KX-01, a specific inhibitor that targets the substrate pocket of Src. This concentration is consistent with the IC50 values (9 – 60 nM) provided by the distributers.

Because of the nature of this inhibitor, the abundance of pSrc was not expected to change in response to being treated with KX-01 (See Figure 3.4 A). This is because KX-01 binds to the substrate-binding pocket but does not prevent the protein from being phosphorylated. In fact, in order for the inhibitor to work, Src must be phosphorylated at Y416 so that it is in its "open" conformation which allows for KX-01 to bind to the active site. The downstream signalling intermediates affected by Src should, however, be altered in response to the inhibition of Src.

The changes in the abundance of phosphates on the sensitive phosphorylation sites of the proteins of interest are again displayed as a mean of biological replicates performed. Inhibition of Src resulted in a significant decrease in the abundance of pPKB in the WHOC1, WHCO5 and WHCO6 cell lines. The levels of pPKB in the SNO and A431 cells remained mostly unchanged in response to Src inhibition, while interestingly the H1299 cell line displayed an increase, albeit insignificant, despite displaying undetectable levels of pSrc (See Figure 3.4 B).

Although the levels of pPKB generally decreased in response to Src inhibition, the significant changes in pGSK3-β are represented as increases in WHCO5, SNO and H1299 cells. The levels of pGSK3-β decreased significantly in WHCO3 cells despite them containing undetectable levels of pPKB, whereas the levels in WHCO1, WHCO6 and A431 cell lines appear to be unchanged (See Figure 3.4 C).



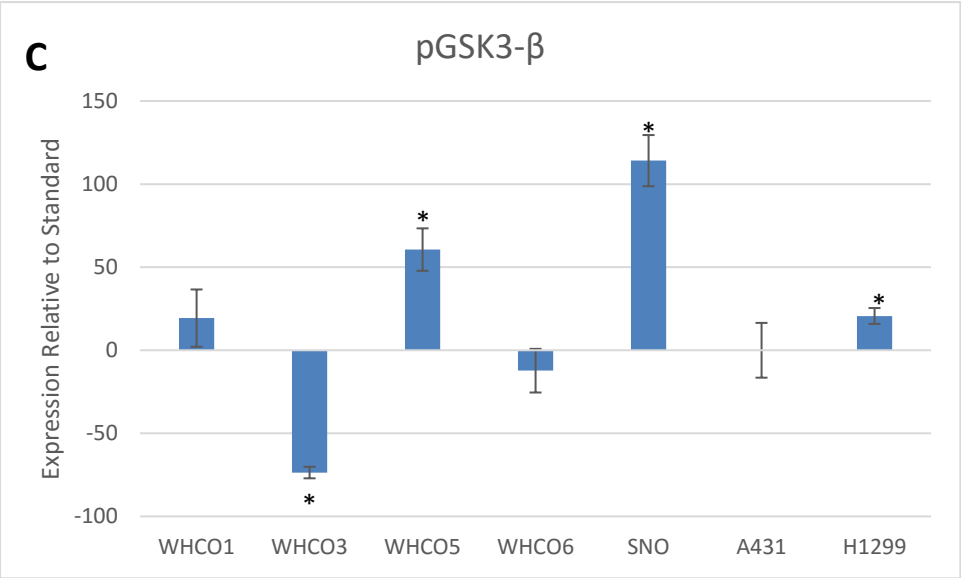


Figure 3.4: Variations in the abundances of pSrc, pPKB and pGSK3-β in HOSCC and control cell lines responding to Src inhibition (n = 3).

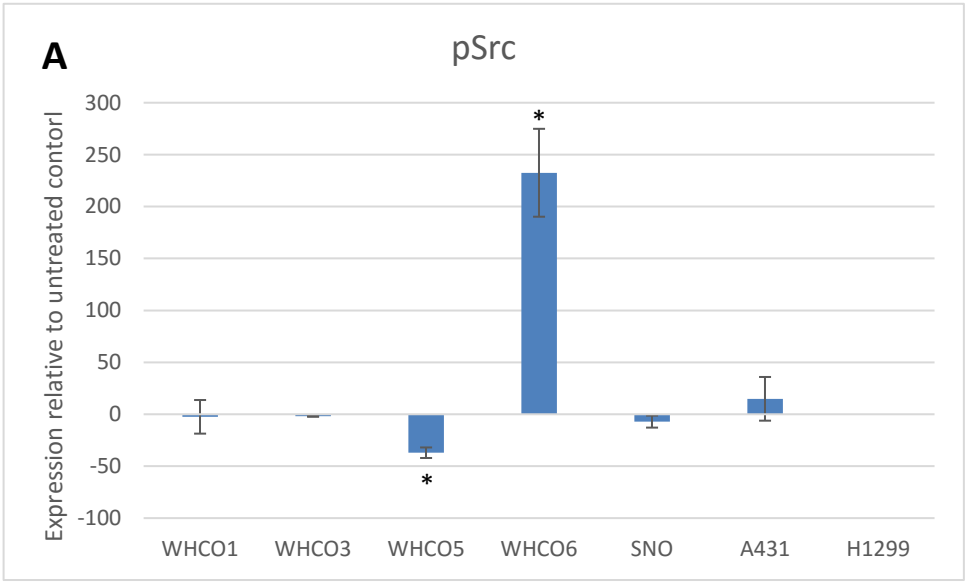
Changes in the abundances (compared to untreated controls) of A) pSrc, B) pPKB and C) pGSK3-β in HOSCC and control cell lines responding to Src inhibition (10 nM KX-01 for 1 hour). Bars of the graphs represent the mean intensities of Western blots detecting the appropriate proteins in biological replicates, while error bars represent the SEM. An asterix indicates statistical significance ($p > 0.05$). See Table B5-7 for means, SEMs and p-values.

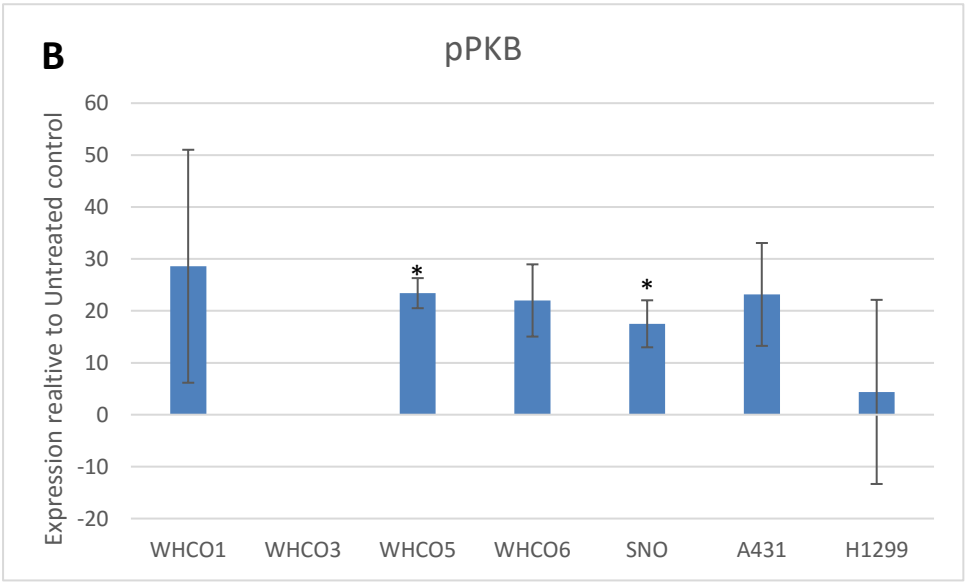
3.4. Src inhibition under oxidative conditions increases the abundances of both pPKB and pGSK3-β

Both oxidative stress as well as Src inhibition had an effect on the levels of pGSK3-β and pPKB (although less notably in terms of oxidative stress). The role of Src in the activation of PKB and inactivation of GSK3-β under conditions of oxidative stress thus needed to be determined in order to better understand the effects of ROS on signalling intermediates implicated in carcinogenesis. This was achieved by stimulating the cells with ROS, while simultaneously inhibiting Src.

A combination of Src inhibition and oxidative stress did not have a dramatic effect on most cells with respect to pSrc abundance. There was, however, a major and significant increase in pSrc abundance in WHCO6 cells and a less noteworthy but significant decrease in WHCO5 cells (See Figure 3.5 A). These results highlight the fact that the Src inhibitor does not have an effect on the phosphorylation of Src itself, as the resultant increase in pSrc in WHCO6 cells is highly similar to that of ROS stimulation alone. The small but significant decrease in pSrc abundance in WHCO5 cells indicates that the decrease in response to oxidative conditions alone may have been significant had the results been refined.

All cell lines with detectable levels of pPKB responded to Src inhibition coupled with ROS stimulation with an increase in the abundance of pPKB (See Figure 3.5 B). These increases were significant in the WHCO5 and SNO cell lines. Other than WHCO3 cells which displayed an insignificant decrease, the abundance of pGSK3-β in the cells increased in response to a combination of ROS stimulation and Src inhibition (See Figure 3.5 C). The increases were significant in WHCO5, WHCO6, A431 and H1299 cells.





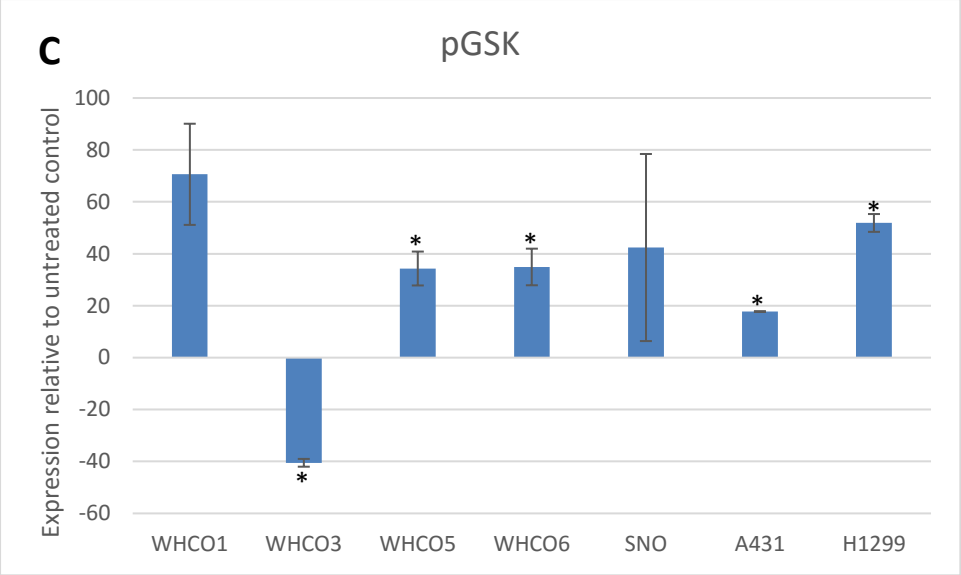


Figure 3.5: Changes in Abundance of pSrc, pPKB and pGSK3-β in HOSCC and control cells responding to Src inhibition under oxidative conditions (n = 3).

Changes in abundance of A) pSrc, B) pPKB and C) pGSK3-β from the untreated controls depicted as mean densitometric intensities. Results were obtained from Western blots performed on biological replicates of HOSCC and control cell lines responding to Src inhibition under oxidative conditions. These intensities are semi-quantitative representations of the bands present on the Western blots. Error bars represent the SEM of the replicates and an asterix symbolises statistical significance ($p > 0.05$). See Table B8-10 for means, SEMs and p-values.

4. Discussion

The role of ROS as a cellular stressor has received a great deal of attention in recent years. An increase in ROS at sub-lethal doses may induce several cellular processes that have an influence on critical events such as cell survival and proliferation. The possibility that ROS stimulation of the Src-PKB-GSK3-β pathway has a significant input in this regard was investigated. Specifically, HOSCC cells were exposed to oxidative conditions, Src inhibition, and a combination of the two to determine the possible role played by Src in the phosphorylation of PKB and GSK3-β in terms of ROS stimulation. It is imperative that detailed knowledge of these signalling pathways, and their aberrant counterparts, in response to critical stressors is acquired. This is necessary in order to obtain superior knowledge of the signal transduction pathways that enable the prolonged survival and hyper proliferation of cells. Moreover, such information is crucial if a testable marker for HOSCC is to be identified

4.1. ROS stimulation affects HOSCC cells differentially in terms of pGSK3-β

ROS stimulation did not have a significant effect on the abundance of pSrc and pPKB in the HOSCC and control cell lines other than WHCO6, which displayed a significant increase in both intermediates. Despite this, the levels of pGSK3-β increased insignificantly in WHCO5, significantly in WHCO1 and SNO, and decreased significantly in WHCO3 and WHCO6 cell

lines in response to ROS stimulation. The levels appeared to be unchanged in the A431 and H1299 control cell lines.

The behaviour of the signalling intermediates in WHCO6 cells in response to oxidative stress indicates that ROS activate Src, which in turn activates PKB. The decrease in the inhibitory phosphorylation of GSK3-β is however contradictory to the "pro-survival" responses in the above mentioned intermediates, as it is well known that pPKB phosphorylates GSK3- β at the Ser 9 residue to avoid apoptosis (Cross *et al*., 1995).

This decrease in the abundance of GSKS3-β on the Ser 9 residue may be due to the activation of the SFK Fyn. As with Src, Fyn becomes activated upon stimulation with H_2O_2 (Frossi *et al*., 2007). However, unlike Src, Fyn has been reported to activate GSK3-β, and therefore presumably decrease the phosphorylation on Ser 9 (Medina & Wandosell, 2011). Thus, WHCO6 cells may contain higher abundances of Fyn than the other HOSCC cell lines, however it is almost impossible to determine whether this is true as Src and Fyn have the same molecular mass.

ROS stimulate more than one pathway corresponding to PKB and GSK3-β. Thus, despite the lack of response by pSrc and pPKB, there was an increase in the abundance of pGSK3-β in WHCO1, WHCO5 and SNO cells responding to oxidative conditions. This increase could be due to the MAPK pathway. The MAPK pathway has been instigated in the phosphorylation of both PKB and GSK3-β. However, the MAPK pathway also has a negative regulatory effect on the activation status of PKB (Yu *et al*., 2002), as mentioned in section 1.3.3. ERK, a member of the MAPK pathway, is known to regulate the PKB pathway tightly by inactivating PKB when the pathway appears to be signalling too strongly (Hayashi *et al*., 2008). Thus, by activating the MAPK pathway, the cell may essentially be by-passing the Src-PKB pathway to phosphorylate GSK3-β (See Figure 4.1). In addition to this, pPKB inactivates Ras (Mendoza & Blenis, 2011). Thus, by inactivating PKB, the MAPK pathway may be further activating itself and therefore phosphorylating GSK3-β at the Ser 9 residue independently of Src and PKB.

The fact that the levels of pPKB did not decrease may also implicate yet another pathway that is activated via ROS. The Wnt pathway, although less documented, is also known to become activated by oxidative conditions (Korswagen, 2006). The main role of the Wnt pathway is to increase survival by allowing for the accumulation of β-catenin in the cell. It does so by phosphorylating GSK3-β at the ser 9 residue, as active GSK3-β targets β-catenin for destruction (Hardt & Sadoshima, 2002). There is also evidence of crosstalk between the Wnt and PKB pathway, where Wnt has been found to activate PKB (Fukumoto *et al*., 2001).

Thus, although pSrc and pPKB do not become phosphorylated in the majority of HOSCC cells in response to ROS stimulation, pGSK3- β may become phosphorylated by two pathways: one of which inhibits PKB phosphorylation while the other actives PKB. Therefore, the levels of pPKB remain unchanged in WHCO1, WHCO5 and SNO cells while the levels of pGSK3- β increase (See Figure 4.1).

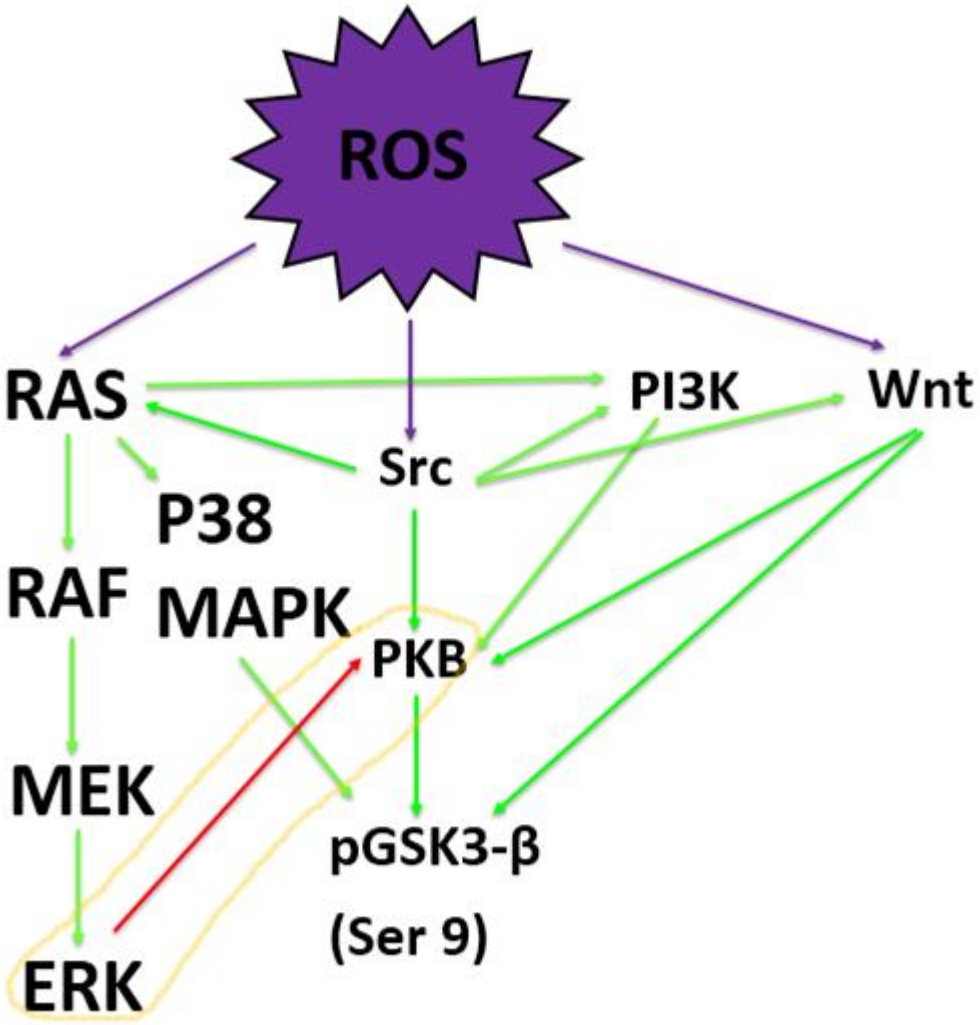


Figure 4.1: Proposed pathway by which the abundances of pSrc and pPKB remain unchanged, while that pGSK3-β increases in WHCO1, WHCO5 and SNO cells

It is proposed that ROS stimulate the MAPK and Wnt pathways in the WHCO1, WHCO5 and SNO cell lines. ROS do not appear to activate Src or PKB in these cell lines, however the abundances of pGSK3-β increased. It is therefore thought that the MAPK pathway inhibits PKB, while the Wnt pathway activates it – resulting in the levels of pPKB being unchanged. On the other hand, both of these pathways inhibit GSK3- β by phosphorylating it on the Ser 9 residue. The abundance of pGSK3-β thus increases independent of pSrc and pPKB. Green arrows depict an increase in the phosphorylation of the respective proteins, while the red arrow indicates inhibition of PKB.

4.2. pSrc plays a PKB-independent role in the activation status of GSK3-β

The abundance of pSrc did not change significantly in any of the cell lines in response to Src inhibition. This was expected due to the nature of the inhibitor, as explained previously. Src inhibition did, however, result in a significant decrease in pPKB levels in three of the four cell lines that contain detectable levels of pPKB. The abundance of pPKB appeared to increase in the H1299 cell line, however this increase was insignificant. The decrease in the levels of pPKB in response to Src inhibition confirms that Src does indeed play a role in the

activation of PKB in HOSCC cells under standard tissue culture conditions, as the abundance of active PKB in these cells decreased significantly when Src was no longer active.

The significant increase in pGSK3-β in WHCO5 and SNO cells and the insignificant increase in WHCO1 cells indicates that the activation status of pGSK3-β is influenced by Src inhibition in a PKB-independent manner. This is due to the fact that pGSK3-β is a downstream effector of PKB and should thus decrease as the levels of pPKB decrease.

This again points to the influence of other pathways in the inactivation of GSK3-β in HOSCC cells. By inhibiting the activities of pSrc and thus decreasing the abundance of pPKB in these three cell lines, other pathways may have been activated in an attempt to avoid apoptosis, as pSrc is considered to be pivotal in cell survival. The most likely pathway to be activated is again the MAPK pathway, as the p38 MAPK is known to respond to many types of cellular stress (Zhang & Liu, 2002). In addition to this, as described previously, the PKB pathway has an inhibitory effect on the MAPK pathway. Thus, in the absence of active PKB, the MAPK pathway may become hyper-activated.

The activation of the p38 MAPK via the MAPK pathway should result in an increase in pGSK3-β (Zhang & Liu, 2002). This pathway will also activate ERK, which will lead to the inactivation of pPKB (Zhang & Liu, 2002). The inhibition of pSrc may have also led to a decrease in activity in PI3K and the Wnt pathway, which would further decrease the abundance of pPKB within the WHCO1, WHCO5 and SNO cells (See Figure 4.2).

WHCO6 cells also displayed a significant decrease in pPKB levels in response to Src inhibition, however the levels of pGSK3- β did not change significantly. This could be due to the inhibition of Fyn by KX01 as the abundance of pGSK3- β was unchanged, whereas a decrease was noticed when Fyn was presumably activated by ROS. This highlights the lack of symmetry between the levels of pPKB and pGSK3- β in WHCO6 cells and implies that GSK3-β is phosphorylated independently of pPKB in this cell line.

Interestingly, there was an increase in the levels of pPKB and a slight yet significant increase in pGSK3-β in H1299 cells in response to Src inhibition. This was unexpected, seeing as H1299 cells do not display detectable levels of active Src. There is however a possibility that the inhibitor affects the abundance of pSrc at a level below what is detectable by the Western blotting technique applied in aid of this study.

WHCO3 cells displayed a significant decrease in the abundance of pGSK3- β in response to Src inhibition. WHCO3s are however known within the laboratory to behave differently to the other cell lines, often for reasons that have yet to be discovered (Nerwich, 2013; Shaw, 2011; Fanucchi, 2011).

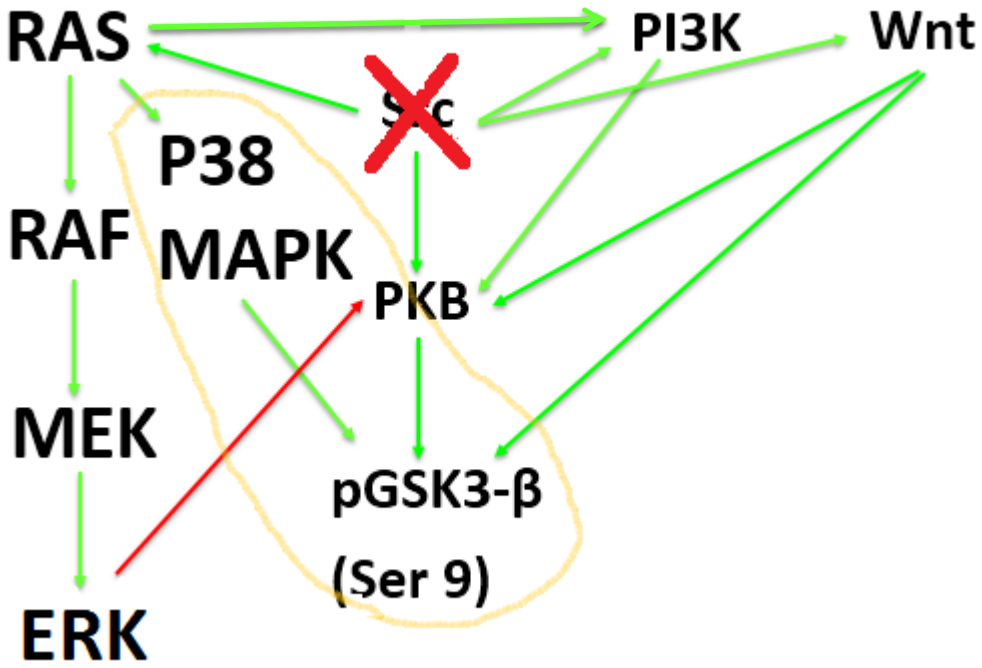


Figure 4.2: Proposed pathway by which the abundance of pGSK3- β increases in response to Src inhibition, despite a decrease in pPKB abundance in WHCO1, WHCO5 and SNO cells
It is proposed that the inactivation of Src incites a stress response in the WHCO1, WHCO5 and SNO cells, which leads to the activation of the MAPK pathway. The MAPK pathway has the potential to decrease pPKB abundance while increasing pGSK3-β abundance via the p38 MAPK. The lack of Src activity may also decrease the activation of PI3K and the Wnt pathway, which will further decrease the abundance of pPKB within the cells. Green arrows depict an increase in the phosphorylation of the respective proteins, while the red arrow indicates inhibition of PKB. The red cross is indicative of Src inhibition.

4.3. pSrc may inhibit pPKB activation in response to oxidative stress

The levels of pSrc after exposure to oxidative conditions coupled with Src inhibition are not dissimilar to those exposed to oxidative conditions only. This underscores the fact that the Src inhibitor does not alter the phosphorylation status of Src. However, a slight yet significant decrease in pSrc abundance was observed in WHCO5 cells, which displayed an insignificant decrease in pSrc levels in response to ROS stimulation only. This indicates that oxidative conditions alone may result in a significant decrease in pSrc in WHCO5 cells if the results were to be refined.

Src inhibition under oxidative conditions resulted in an increase in pPKB across all cell lines that display detectable levels of pPKB. The increases were significant in the case of WHCO5 and SNO cells. Although the increase in pPKB abundance in WHOC1 cells remains insignificant, there was a visible increase in the levels when compared to those obtained from exposure to oxidative stress alone. The same can be said for the A431 cell line.

The abundance of pPKB in WHCO5 cells did appear to decrease when compared to ROS stimulation only, however the error bar also decreased to the point that difference from the control became significant. This implies that the levels of pPKB may in fact be higher in response to Src inhibition under oxidative conditions when compared to the response to oxidative conditions alone, as the results are far less erratic. The levels of pPKB in WHCO6 and H1299 cells did not change very much when the Src inhibitor was applied in conjunction with the oxidative conditions compared to oxidative conditions alone. However, in SNO cells, the difference was visible and significantly higher.

The results indicate that active Src may actually inhibit the phosphorylation of PKB in response to oxidative conditions in WHCO1, WHCO5, SNO and A431 cells. Conversely, inactivating Src does not appear to have an effect on the activation status of pPKB in WHCO6 and H1299 cells undergoing oxidative stress. The abundance of pGSK3-β in WHCO6 cells did, however, increase under oxidative conditions coupled with Src inhibition, whereas a decrease was observed under oxidative conditions only. This indicates that Src may play a role in the activation (decline in inhibitory phosphorylation) of GSK3-β under oxidative conditions in WHCO6 and possibly H1299 cells. This falls in line with the notion that Fyn plays a role in GSK3-β activation in these cells because, if Fyn is inhibited by KX01, it cannot activate GSK3-β. This will allow pPKB to phosphorylate GSK3- β at Ser9.

In the case of WHCO1, WHCO5, SNO and perhaps A431 cells, the evidence again points to the MAPK pathway. ERK activation, which can inhibit PKB, has been found to be Src-dependent (Lee, 2006), particularly under oxidative conditions (Aikawa, 1997). Inhibition of Src thus allows for the activation of PKB by disabling the activation of ERK. Thus, ROS could be activating the MAPK, Wnt and PI3K (through RAS) pathways, which all lead to the phosphorylation of PKB and GSK3- β (see Figure 4.3).

In order to confirm whether these experiments are in fact activating the above-mentioned pathways, a reliable way to isolate the Src-PKB-GSK3-β pathway and thus determine the effects of ROS on this pathway in a more secluded manner must be found. Because the A431 skin epidermoid carcinoma cells responded similarly to four of the five HOSCC cell lines with respect to Src inhibition under oxidative conditions, it would also be useful to determine whether the observed reaction applies to other forms of squamous cell carcinomas.

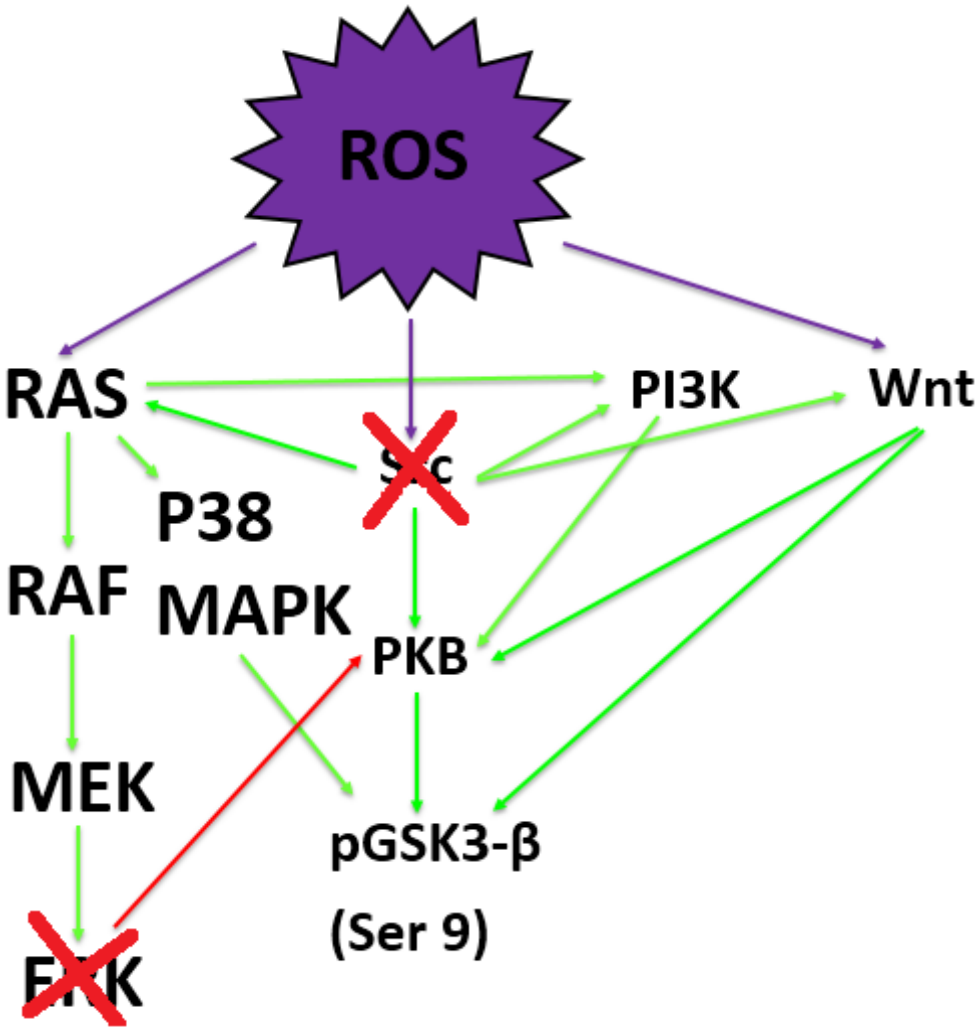


Figure 4.3: Proposed mechanism by which pPKB and pGSK3- β become activated in WHCO1, WHCO5 and SNO cells responding to Src inhibition under oxidative conditions
It is proposed that, in the absence of Src, ERK does not become activated under oxidative conditions. This allows for the activation of PKB via the MAPK, Wnt and PI3K (via RAS) pathways by ROS. These pathways, as well as pPKB, all phosphorylate GSK3- β at the Ser 9 residue. Thus, WHCO1, WHCO5 and SNO cells display an increase in the abundance of both pPKB and pGSK3- β in response to Src inhibition coupled with ROS stimulation. Green arrows depict an increase in the phosphorylation of the respective proteins, while the red arrow indicates inhibition of PKB. The red crosses are indicative of inhibition. Src is inhibited by KX-01, while ERK activation under oxidative conditions is Src-dependent.

5. Conclusion

It appears that, in three of the five HOSCC cell lines (WHCO1, WHCO5 and SNO), Src inhibits the phosphorylation of PKB, but not GSK3-β, under oxidative conditions. This is likely to occur via activation of the MAPK pathway which can play a negative regulatory role in the PKB pathway by inhibiting PKB activation (Yu *et al*., 2002). The MAPK pathway still has the capacity to phosphorylate GSK3- β at the Ser 9 residue, even in the absence of pPKB,

while other pathways stimulated by ROS, such as the Wnt pathway, can also phosphorylate GSK3-β.

The behaviour of the WHCO6 and WHCO3 cell lines deviates from the pattern observed in the other HOSCC cells. It has been shown previously that WHCO3 cells often behave differently to the other HOSCC cell lines (Nerwich, 2013; Shaw, 2011; Fanucchi, 2011), although the reason for this has yet to be elucidated. The behaviour of WHCO6 cells, however, may be explained by the activation of Fyn, a SFK that is also activated by ROS stimulation yet plays a role in the activation of pGSK3-β (resulting in a decline in the abundance of the Ser 9 phosphorylation).

The control cell lines A431 and H1299 also behave differently as the reactions of A431 appeared to be similar to the WHCO1, WHCO5 and SNO cell lines, whereas that of H1299 was sometimes similar to WHCO6. It is unsurprising that these cell lines behave differently as A431 cells were derived from epidermoid carcinoma, while H1299 cells were derived from non-small cell lung carcinoma. The alignment of the behaviours of the respective HOSCC cell lines with these control cell lines is also not entirely surprising, as they are both squamous cell carcinomas. However, the fact that three of the five cell lines behave similarly to A431s, one behaves similarly to H1299s, and one does not behave similarly to any of the above-mentioned cell lines highlights the differences between HOSCC cell lines derived from different patients.

The differences in these cell lines also emphasises the fact that cross-talk plays a major role in the transformation and maintenance of cancer cells. It is clear that further research must be undertaken in order to understand the intricacies involved in the cross-talk between the MAPK, Src/PKB/GSK3-β and Wnt pathways. The comprehension of these complicated pathways is essential for the ultimate treatment of this devastating disease.

Thus, the need to identify a variety of testable markers for HOSCC is underscored. Although the cell lines investigated in this study are all moderately differentiated carcinomas, the path to becoming a tumour can vary from person to person. With more testable markers available, the treatment of this disease that affects so many South Africans may become easier and more successful.

I believe that inactive Src (phosphorylated at Y527) in HOSCC cells with elevated ROS levels could potentially be one of these markers, as Src inhibition under ROS stimulation led to an increase in pPKB and a decrease in GSK3-β activation in four out of five HOSCC cell lines. This is most noteworthy, as an increase in inactive GSK3-β has been found to significantly increase cell growth (Wang *et al*., 2008), while an increase in pPKB has been found to enhance cell proliferation in HOSCCs (Gen *et al*., 2013). It should also be noted that similar behaviour was observed in A431 cells. Thus, inactive Src present in HOSCC cells, and possibly other squamous cell carcinomas, undergoing oxidative stress may be a contributing factor to the carcinogenesis of these cells.

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7. Appendices

Appendix A

1. Tissue Culture Solutions

1.1. DMEM medium

1.37 % Dulbecco‘s Modified Eagle‘s Medium (DMEM)
0.37 % Sodium bicarbonate
2 % Penicillin/Streptomycin solution (Penicillin: 500 U/ml; Streptomycin: 0.5 %)
Made up to final volume with dH_2O.

1.2. Ham’s F12 medium

1.07% Hams F12 medium
0.118 % Sodium bicarbonate
2 % Penicillin/Streptomycin solution
Made up to final volume with dH_2O

1.3. Mix

3 Volumes DMEM medium solutions: 1 volume Hams F12 medium solution
Filter sterilized
Stored at 4 ^{o}C.

1.4. 1 x PBS

136.9 mM NaCl
2.86 mM KCL
10.1 mM $Na_2HPO_4.12H_2O$
1.76 mM KH_2PO_4
pH adjusted to 7.2-7.3, made up to 1 L with dH_2O.
Solution sterilized for 20 minutes at 1511bq and store at 4 ^oC

1.5. Trypsin:EDTA solution

0.02 % EDTA in 500 ml PBS
0.1 % Trypsin in 1 L PBS
EDTA:Trypsin mixed (1:1) to obtain a final concentration of 0.01 % EDTA and 0.05 % Trypsin
Stored at 4 ^oC.

2. Whole cell protein extraction solutions

2.1. 2x Laemmli lysis buffer

4 % SDS
20 % Glycerol
10 % β-mercaptoethanol
Solution made up to a final volume with 0.0625 M Tris-HCL buffer, pH 6.8.

3. Protein determination solutions

3.1. Coomassie Brilliant Blue Stain

0.25 % Coomassie blue powder
50 % Methanol
10 % Acetic Acid
Made up to final volume with dH_2O. Stored at room temperature

3.2. Destain

10 % Glacial acetic acid
12 % Methanol
Made up to final volume with dH_2O

3.3. Elution Solution

66 % Methanol
33% dH_2O
1 % Ammonia

4. SDS PAGE solutions

4.1. Stacking gel

5 % acrylamide
0.1 % NN'-methylenebisacrylamide
125 mM Tris-HCl, pH 6.8
0.2 % SDS
Made up to final volume with dH_2O
1 mM Ammonium persulphate (APS) and 0.25 % N,N,N',N'-Tetramethylethylenediamine (TEMED) added for gel polymerisation

4.2. Separating gel

10 % acrylamide
% NN'-methylenebisacrylamide
375 mM Tris-HCl, pH 8.8
0.2% SDS
Made up to final volume with dH_2O

4.3. Running Buffer

3.74 mM SDS
2.5 mM Tris-HCl pH 8.3
192.5 mM Glycine
Made up to final volume with dH_2O

4.4. Gel Destain Solution

10 % Acetic Acid
10 % Methanol
Made up to final volume with dH_2O

5. Western Blotting Solutions

5.1. Transfer Buffer

25 mM Tris-HCl, 9H 8.3
1.41 % Glycine
20 % Methanol
Made up to final volume with dH_2O

5.2. Tris Buffer Saline

50 mM Tris-HCl (pH 7.8)
150 mM NaCl
2 mM $CaCl_2.2H_2O$
Made up to final volume with dH_2O

5.3. Blocking Solution

5 % Fat Free milk powder
Made up to final volume with TBS with 0.1 % Tween-20

5.4. Developer

6.4 M Metol
0.6 M Sodium Sulphite (Anhydrous)
80 mM Hydroquinine
0.45 mM Sodium Carbonate (Anhydrous)
34 mM Potassium Bromide
Made up to final volume with dH_2O. Stored in the dark

5.5. Fixer

0.8 M Sodium Thiosulphate
0.2 M Sodium Metasulphite
Made up to final volume with dH_2O. Stored in the dark

Appendix B

1. Representative standard curve for Protein Determination

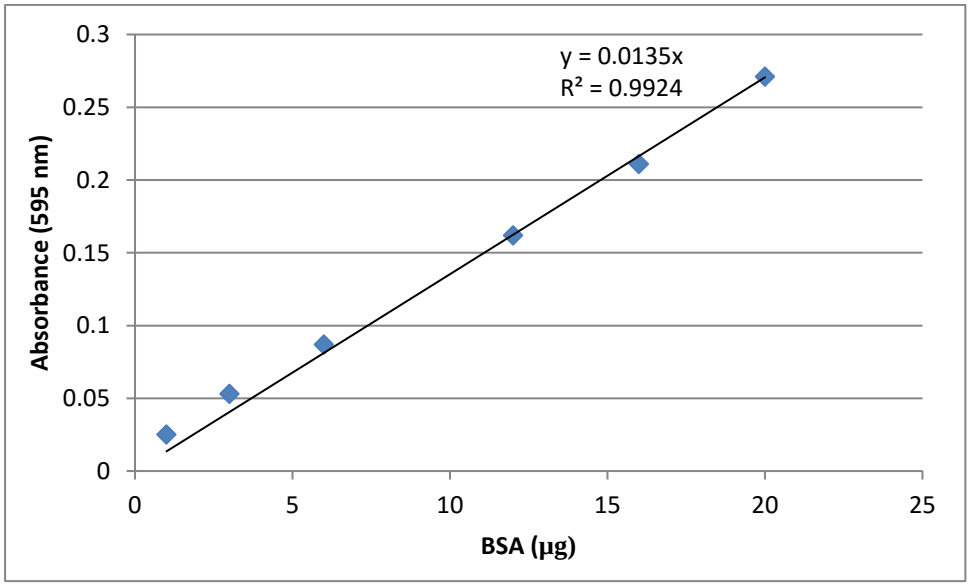


Figure B1: Standard Curve for Protein Determination

Absorbance readings at 595 nm for 1, 3, 6, 12, 16 and 20 µg BSA. The readings were used to construct a standard curve for the determination of protein concentration of various whole cell protein extractions. The respective protein concentrations were determined from the equation of the standard curve y=0.0135x in this case. The R^2 value represents linear regression, where a value of 1 represents a perfect fit between the line of best fit and the data.

2. Representation of 10 % SDS PAGE-resolved protein extracts

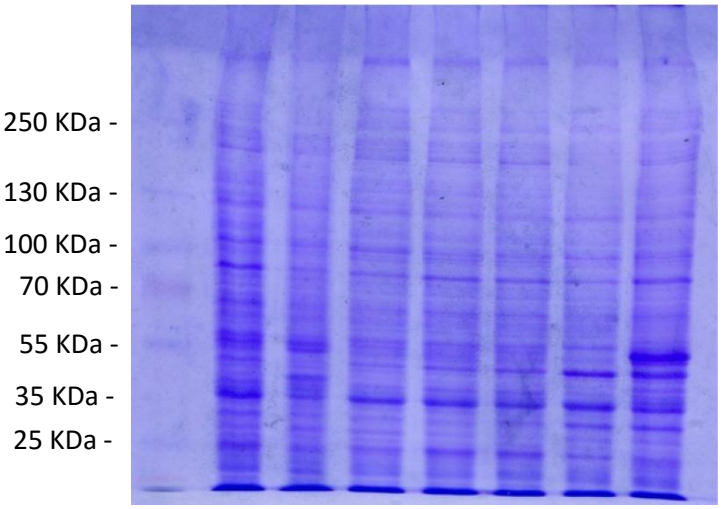


Figure B2: Resolution of Whole Cell Extracts

Representative SDS PAGE gel comprised of 10 % acrylamide, prepared according to Laemmli (1970). 40 µg of whole cell protein extracts as well as 1 µl of molecular weight marker were loaded. The protein samples were separated by applying a constant current of 21 mA. The gel was stained with Coomassie Brilliant Blue and destained to enable visualisation of the bands present on the gel. The gel depicts proteins varying in mass from approximately 250 kDa to 25 kDa.

3. Antibody dilutions and incubation conditions for Western Blot analysis

Table B1: Antibody dilutions and incubation conditions

Primary antibody	**Dilution**	**Buffer**	**Secondary antibody**	**Dilution**	**Buffer and temperature**
Actin	1:2 500	2.5 % Fat-Free milk powder in PBS	Goat-anti-rabbit HRP-conjugate	1:10 000	PBS at 30 ºC in the dark
pPKB	1:1 000	5 % BSA in TBS-T	Goat-anti-rabbit HRP-conjugate	1:2 000	5 % milk powder in TBS-T at 30 ºC in the dark
pSrc	1:1 000	2.5 % BSA in TBS-T	Goat-anti-rabbit HRP-conjugate	1:3 000	2.5 % milk powder in TBS-T at 30 ºC in the dark
pGSK3-β	1:1 000	2.5 % BSA in TBS-T	Goat-anti-rabbit HRP-conjugate	1:3 000	2.5 % milk powder in TBS-T at room temperature in the dark

*All antibodies obtained from Sigma-Aldrich, MO USA

4. Statistical analyses of data

Table B2 Statistical analysis of data representing change in pSrc abundance in comparison to untreated control in response to ROS stimulation.

Cell line:	Mean – untreated control	SEM	Significant	P-value
WHCO1	-22.34	18.383	No	0.3463
WHCO3	3.56	23.137	No	0.8918
WHCO5	-11.05	14.799	No	0.4966
WHCO6	217.68	75.655	Yes	0.0451
SNO	-1.39	11.315	No	0.9081
A431	9.84	21.946	No	0.6978
H1299	No detectable expression			

Table B3 Statistical analysis of data representing change in pPKB abundance in comparison to untreated control in response to ROS stimulation.

Cell line	Mean – untreated control	SEM	Significant	P-value
WHCO1	13.935	12.618	No	0.3845
WHCO3	No detectable expression			
WHCO5	124.190	23.160	No	0.3552
WHCO6	17.322	1.036	Yes	0.0102
SNO	-1.770	2.874	No	0.5730
A431	15.526	22.571	No	0.5626
H1299	6.819	3.263	No	0.1718

Table B4 Statistical analysis of data representing change in pGSK3-β abundance in comparison to untreated control in response to ROS stimulation.

Cell line	Mean – untreated control	SEM	Significant	P-value
WHCO1	120.88	16.22	Yes	0.0175
WHCO3	-69.51	0.18	Yes	< 0.0001
WHCO5	37.10	27.83	No	0.2438
WHCO6	-68.69	17.37	Yes	0.0167
SNO	44.96	8.125	Yes	0.0311
A431	-3.11	18.50	No	0.8821
H1299	13.97	12.04048	No	0.3104

Table B5: Statistical analysis of data representing change in pSrc abundance in comparison to untreated control in response to Src inhibition.

Cell line	Mean – untreated control	SEM	Significant	P-value

WHCO1	-18.1171	9.73	No	
WHCO3	5.5	25.062	No	0.891
WHCO5	-42.05	10.933	No	0.3086
WHCO6	6.95	16.273	No	0.7430
SNO	26.80	12.485	No	0.0984
A431	14.9	15.624	No	0.8475
H1299	No detectable expression			

Table B6: Statistical analysis of data representing change in pPKB abundance in comparison to untreated control in response to Src inhibition.

Cell line	Mean – untreated control	SEM	Significant	P-value
WHCO1	-13.752	0.10	Yes	0.0048
WHCO3	No detectable expression			
WHCO5	-26.88	6.46	Yes	0.0141
WHCO6	-13.13	3.01	Yes	0.0358
SNO	-7.555	7.51	No	0.4342
A431	2.053	3.08	No	0.8373
H1299	31.83	9.51	No	0.3854

Table B7: Statistical analysis of data representing change in pGSK3-β abundance in comparison to untreated control in response to Src inhibition.

Cell line	Mean – untreated control	SEM	Significant	P-value
WHCO1	19.34	17.25	No	0.3789
WHCO3	-73.60	3.47	Yes	0.0299
WHCO5	60.60	12.77	Yes	0.0416
WHCO6	-12.29	13.14	No	0.4026
SNO	114.12	15.39	Yes	0.0018
A431	-0.018	16.49	No	0.9992
H1299	20.64	4.77	Yes	0.0124

Table B8: Statistical analysis of data representing change in pSrc abundance in comparison to untreated control in response to Src inhibition under oxidative conditions.

Cell line	Mean – untreated control	SEM	Significant	P-value
WHCO1	-2.45	16.19	No	0.8937
WHCO3	-1.89477	0.5442	No	0.0735
WHCO5	-37.03	5.075	Yes	0.0183
WHCO6	232.56	42.32	Yes	0.0384
SNO	-7.057	5.79	No	0.2258
A431	14.88978	21.03805	No	0.5525
H1299	No detectable expression			

Table B9: Statistical analysis of data representing change in pPKB abundance in comparison to untreated control in response to Src inhibition under oxidative conditions.

Cell line	Mean – untreated control	SEM	Significant	P-value
WHCO1	28.5914	22.43278	No	0.3305
WHCO3	No detectable expression			
WHCO5	23.41	2.899	Yes	0.0062
WHCO6	22	6.956	No	0.0871
SNO	17.5057	4.528026	Yes	0.0083
A431	23.16124	9.904842	No	0.5626
H1299	4.39	17.73	No	0.3418

Table B10: Statistical analysis of data representing change in pGSK3-β abundance in comparison to untreated control in response to Src inhibition under oxidative conditions.

Cell line	Mean – untreated control	SEM	Significant	P-value
WHCO1	70.61	19.48	No	0.0684
WHCO3	-40.51	1.50	Yes	0.0014
WHCO5	34.33	6.53	Yes	0.0150
WHCO6	34.93	7.05	Yes	0.0384
SNO	42.41	36.03	No	0.3044
A431	17.74	0.01	Yes	> 0.0001
H1299	51.88	3.43	Yes	0.0001

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