β-catenin interaction with, and regulation of, integrin-linked kinase-1 in human oesophageal squamous cell carcinoma

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Declaration

I declare that this dissertation is my own unaided work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted for any other degree in any other University.

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Bronwyn Mary Thomson

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Abstract

Integrin-linked kinase-1(ILK-1), an ankyrin repeat containing serine-threonine protein kinase, is highly overexpressed and aberrantly localised in several invasive carcinomas. The promoter region of the ILK-1 gene was examined by Melchoir *et al* (2002) and featured two T-cell factor-1 (Tcf-1) binding sites. Tcf transcription factors require β-catenin binding for activation of gene expression. The aim of this study was to determine whether these Tcf-1 sites interact with β-catenin and if so, are they essential to activate transcription of ILK-1. The role of β-catenin in the nucleus at a regulatory level and its influence on the protein levels of ILK-1 in moderately differentiated human oesophageal squamous cell carcinoma (HOSCC) cell lines were also examined. β-catenin and ILK-1 are key components in signal transduction pathways that when dysregulated lead to uncontrolled cell proliferation, one of the main hallmarks of cancer. This study shows that β-catenin and ILK-1 are expressed and localised in HOSCC cell lines as demonstrated by Western blotting. Evidence from co-immunoprecipitation assays indicates that β-catenin and Tcf-1 complex in the nucleus of a carcinoma derived from stratified epithelia such as the HOSCC cell lines under investigation. Electrophoretic mobility shift assays showed that the β-catenin/Tcf-1 complex binds to the two proposed binding sites in the ILK-1 promoter region. Therefore, it was reasonable to question whether this bound complex influenced ILK-1 expression levels. In order to increase expression of β-catenin within the HOSCC, the WHCO6 cell line was stably transfected with the pEGSH-β-catenin-pERV3 construct and stimulated with 1 μM and 2 μM of ponA respectively. However, when β-catenin levels were increased in a HOSCC cell line, it did not considerably affect ILK-1 expression levels. The tight regulatory mechanisms known to control β-catenin could possibly have masked the increase in β-catenin levels. Also the various degradation pathways of β-catenin have not been examined extensively within these cells lines and could possibly provide an explanation of the lack of a visually substantial increase of β-catenin and/or ILK-1. These results show that β-catenin and Tcf-1 complex in the nucleus and bind to the documented ILK-1 binding sites in the promoter region. This interaction does not influence the expression levels of ILK-1 in HOSCC cell lines. However, results presented in this study identify oesophageal squamous cell carcinoma as a prime candidate for β-catenin, Tcf-1 and ILK-1 specific immunotherapy.

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If we examine the accomplishments of man in his most advanced endeavours, in theory and in practice, we find that the cell has done all this long before him, with greater resourcefulness and much greater efficiency.

Albert Claude

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List of Abbreviations and Symbols

α	alpha
Abs	absorbance
AP-1	activator protein-1
APC	adenomatous polyposis coli
APS	ammonium persulphate
ATP	adenosine triphosphate
β	beta
Bis	*N'*,*N'*,-methylenebis-acrylamide
bp	base pair
BSA	bovine serum albumin
β-TrCP	beta–transduction–repeat–containing protein
BWS	Beckwith Wiedemann syndrome
Ca^{2+}	Calcium
^{o}C	degrees Celcius
C–terminal	carboxy–terminal
CBP	CREB binding protein
C-clamp	cysteine clamp
cdc	cell division cycle
cDNA	complementary deoxyribonucleic acid
CH	calponin homology
CH-ILKBP	calponin homology domain containing ILK-binding protein
CHO	Chinese hamster ovary
CKII	casein kinase II

cm	centimetre(s)
CMV	cyclomegalovirus
CT	cytoplasmic tail
CtBP	carboxy-terminal binding protein
CTD	c-terminal domain
dH_2O	distilled water
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dsh	dishevelled
E-cadherin	epithelial–cadherin
ECM	extracellular matrix
EcR	ecdysone receptor
EDTA	ethylenediaminetetra–acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
E/GRE	ecydysone/glucocorticoid responsive element recognition
EMSA	electrophoretic mobility shift assay
EtBr	ethidium bromide
FAK	focal adhesion kinase
FCS	fetal calf serum
fmol	femtomol
F-box	forkhead
E2F1	E2 Transcription factor-1
frz	frizzled

γ	gamma
x g	gravitational units
g	gram(s)
G418	G418 sulphate
GFP	green fluorescent protein
Grg	Groucho
GSK3β	glycogen synthase kinase 3β
GTP	guanosine triphosphate
HCl	hydrochloric acid
HMG	high mobility group
HOSCC	human oesophageal squamous cell carcinoma
HRP	horse radish peroxidase
IB	immunoblotting
Ig(s)	immunoglobulin(s)
ILK-1	intergrin linked kinase-1
IP	immunoprecipitation
IPTG	isopropyl β-D-thiogalactopyranoside
JNK	c–Jun NH_2 -terminal kinase
Kb	kilobase
kDa	kilodalton
LEF1	lymphoid enhancer factor1
LiCl	lithium chloride
lipofection	liposome-mediated transfection
LOH	loss of heterozygosity

Lys	lysine
M	molar
mA	milliampere(s)
MAPK	mitogen-activated protein kinase
mHSP	minimal heat shock promoter
M-cadherin	mesenchymal cadherin
MDCK	Madin-Darby canine kidney
ml	millilitre(s)
mM	millimolar
MMP13	matrix metalloproteinase 13
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
N-cadherin	neural-cadherin
NF-κB	nuclear factor-kappa B
NLS	nuclear localisation signal
nm	nano metre(s)
N-terminal	amine-terminal
ORF	open reading frame
OSCC	oesophageal squamous cell carcinoma
P	phosphate
PAGE	polyacrylamide gel electrophoresis
Pax5	paired box 5
PBS	phosphate buffered saline
PINCH	particularly interesting cysteine-and histidine-rich protein

PIP_3	phosphatidlinositol-3,4,5-P_3
PI3K	phosphoinositide 3–kinase
PKB	protein kinase B
PKC	protein kinase C
p53	tumour protein 53
ponA	ponasterone A
PPIase	peptidyl-prolyl *cis-trans* isomerase
R^2	linear regression
RAR	retinoic acid receptors
RelA	reticuloendotheliosis A
RNA	ribonucleic acid
RxR	retinoid-x-receptors
SCC	squamous cell carcinoma
SDS	sodium dodecyl sulphate
Siah1	seven in absentia homolog 1
Smad	mothers against decapentaplegic homolog
S9WT	Sox9WT
SOX	SRY-related HMG box
SRY	sex-determining region Y
STE	NaCl Tris EDTA
SV40	simian virus 40
TBE	tris boric acid EDTA
TCA	tri-chloroacetic acid
Tcf	T-cell factor

TE	tris EDTA
TEMED	*N*', *N*', *N*', *N*'-tetramethylene-diamine
Tris	tris(hydroxymethyl)aminomethane
TR	thyroid hormone receptor
tWHCO6	transfected WHCO6
UV	ultraviolet
µ	micro
µg	microgram(s)
µl	microlitre(s)
V	volt(s)
VP16	virion protein 16
WHCO	Wits human carcinoma of the oesophagus
Wnt	wingless type
WRE	wnt response elements

Chapter One: General introduction and literature review

1.1 Cancer

Problems of cellular growth control are not simple to approach, since they encompass a potentially lethal interplay of developmental, proliferative and differentiative themes, any one of which, if altered may contribute to the cancerous process (Boynton *et al*., 1982; Lepourcelet *et al*., 2004). Cancer is viewed as a complex group of diseases affecting a wide range of cells and tissues that is characterised by uncontrolled growth and dissemination of abnormal cells (Klug and Cummings, 2000; reviewed by Knudson, 2002). In virtually all cancers, mutations arise in somatic cells and are not passed on to future generations through the germ cells. However, about 1% of all cancer cases are due to germ-line mutations of various genes that are transmitted to offspring and are responsible for susceptibility to cancer (Jemal *et al*., 2009; Klug and Cummings, 2000). Other inherited internal factors may also contribute towards a cancerous state, such as abnormalities in hormones, immune conditions and metabolism. Alternatively excessive exposure to external factors such as tobacco, various chemicals, radiation and infectious organisms, can also contribute towards the onset of cancer (Jemel *et al*., 2009). Approximately 562 340 people will die in the United States of America in 2009 from cancer, or complications developing during the course of this disease, and 1 479 350 new cases are expected to be diagnosed during the same year, of which more than 1 million cases are basal and squamous cell skin cancers (Jemal *et al*., 2009). 11 million oesophageal cancers will be diagnosed world wide in 2009 of which the majority of cases (80-85%) will occur in developing countries, where it is the forth most common cancer in men (Jemal et al., 2009). The most current statistics at the time of writing this dissertation suggest that in South Africa, one in four men, and one in seven women will get cancer during their lives (http://www.health24.co.za). It has also been determined that oesophageal cancer is the most prevalent cancer in black South African men, whereas it is the third most prevalent cancer in black South African women (http://www.health24.co.za). This is considered a significant disease that requires in depth investigation.

Uncontrolled cell proliferation is one of the central hallmarks of cancer, tumourgensis begins when a stem cell/partially differentiated stem cell acquires damaged genes in a "gatekeeping pathway" that provides it with a selective growth advantage; "a cell's ability to divide continuously in the absence of persistent stimulation by a triggering carcinogenic agent, refers to tumourgenesis" (Sandal *et al*., 2002; Vogelstein and Knizler, 2004; Welch *et al*., 2000). No single genetic defect causes cancer, only when there is a combination of several defective genes can an invasive cancer develop, of which there are three possible gene types responsible for tumourgenesis: stability genes, oncogenes and tumour suppressor genes (reviewed by Vogelstein and Knizler, 2004). Thus it is evident that tumour formation is a result of uncontrolled proliferation, but proliferation does not necessarily always contribute to tumour cell invasion. Not all tumours turn invasive or malignant and are termed benign tumours and so it is incorrect to say uncontrolled cell proliferation results in tumour cell invasion (reviewed by Vogelstein and Knizler, 2004). A post-tumourgenic event however, is the ability of tumour cells to leave the primary tumour mass and invade distant tissues. In general the mechanisms of tumour invasion are poorly understood, although the three most likely possibilities are that it occurs as a result of mechanical pressure; release of lytic enzymes (proteases or collagenases); and/or increased motility of individual cells via changes of adhesion (Franks *et al*., 1998; Leber and Efferth, 2009). A small percentage of these invasive tumour cells have the ability to successfully colonise distant tissues via a process termed metastasis (Welch *et al*., 2000).

1.2 Invasion

Cancer deaths do not primarily result from primary neoplasias, but rather from secondary metastases that have drastically changed both phenotypically and biochemically from the primary neoplasm to an invasive cancer tissue (Leber and Efferth, 2009). Welch and colleagues (2000) described metastasis as the progressive growth of cells at a site that is discontinuous from the primary neoplasm (This definition does not incorporate the route of spread of the metastatic cells). The cells can disperse via the lymphatics, blood vasculature or within body cavities (Franks *et al*., 1998). A subset of invasive tumour cells within a primary tumour mass, have

acquired the ability to complete a multistep metastatic cascade (Vogelstein and Kinzler, 2004; Welch *et al*., 2000). The metastatic cascade has been divided into five steps for the sake of clarity. Each consecutive step must be fulfilled for successful invasion and metastasis to occur. Disruption of one of these steps will result in an unsuccessful metastatic process.

Neoplastic cells must acquire a motile phenotype and escape the primary neoplasm (either as single cells or small clumps of neoplastic cells and platelets/lymphocytes) and degrade the extracellular matrix, migrate to distant sites and arrest in the capillaries of distant organs (Leber and Efferth, 2009; reviewed by Franks *et al*., 1998; Mareel and Leroy, 2002; Wu *et al*., 2008). These cells must then proliferate and tumour formation commences i.e. mitosis occurs. Once a tumour is beyond the size of about 2 mm in diameter, nutritional requirements increase and diffusion, which is the current process of attaining these nutrients, is no longer sufficient, new blood vessels are derived from pre-existing capillaries in a process termed angiogenesis (Franks *et al*., 1998; Wu *et al*., 2008).

It is clear that adhesive events are an integral component of the metastatic phenotype and that certain types of cell adhesion when disrupted, promote metastasis, whereas other types inhibit the metastatic process (Brodt, 1996; Franks *et al*., 1998; Vogelstein and Knizler, 2004). It is critical to recognize that invasion and adhesion do not amount to the metastatic propensity of the tumour cell, yet both invasion and adhesion are necessary, but not sufficient for metastasis. Cells that are efficient at either or both, yet lack the ability to complete any other step of the above mentioned metastatic cascade, are non-metastatic (Welch *et al*., 2000).

The literature review thus far has revealed the ambit and complexity of this disease, and it is for to this reason that this review attempts to highlight certain regulatory mechanisms that are crucial for adhesion and communication, that when disrupted play a pivotal role in cancer progression.

1.3 Cell adhesion

The disruption of cellular adhesive events underlies invasive phenomena. Cell adhesion is crucial for establishing epithelial cell polarity, organisation and differentiation, which subsequently leads to the proper development of the epithelium (Wodarz, 2002). Signals can be transmitted via ligands which bind appropriate cell adhesion receptors on the cell surface. However some signals depend on direct contact between either neighbouring cells or the extracellular matrix (ECM) (Ben-Ze'ev, 1997). Cell-cell and cell-matrix interactions are tightly regulated processes, which result in the regulation of cell shape, cell polarity, tissue architecture, and the expression of growth factor receptors, thereby affecting cell proliferation (Brodt, 1996; Wodarz, 2002). Research has shown that transmembrane structural linkages such as adhesion receptors are involved in signal transduction. These cell surface adhesion receptors are classified into four main families, namely, selectins, immunoglobulins, cadherins and integrins (Ben-Ze'ev, 1997; Hood and Cheresh, 2002). All four types of adhesion receptors are co-expressed on most cells and the overall contribution of adhesion is the net effect of the individual contributions of each of these groups (Fashena and Thomas, 2000).

1.3.1 Cell adhesion receptors

The above mentioned cell surface receptors will now be discussed in detail, with special emphasis placed on the cadherins and integrins and their regulatory role in cell adhesion, as they are the predominant adhesion receptors for epithelial cells. The following brief description of the structure and expression of the selectins and immunoglobulins, serves only as a contextual comparison, with the cadherins and integrins detail that follows later.

1.3.1.1 Selectins:

Selectins are calcium dependent, integral membrane proteins composed of an amine-terminal (N-terminal) C-type lectin domain, epidermal growth factor (EGF) domain and a cytoplasmic tail (CT) (Brodt, 1996; Kannagi *et al*., 2004). Adhesion assays

have established that the lectin and EGF domains are involved in ligand binding (Brodt, 1996). Selectins are expressed on the surface of leukocytes, platelets and cells lining the internal surface of the blood and lymph vasculature. There are three members of the selectin family; P-selectin, which is the largest selectin and is expressed on activated platelets and endothelial cells (reviewed by Kannagi *et al*., 2004; Petruzzelli *et al*., 1999); L- selectin which is constitutively expressed on most leukocytes and is the smallest sized selectin of the vascular selectins, and finally, the E-selectins which are expressed on activated endothelium with chemically or cytokine-induced inflammation (Kannagi *et al*., 2004). Selectins act as "hooks" that recruit leukocytes to the site of infection. Endothelial cells express selectins in response to cytokines from leukocytes present at the site of an infection (Kannagi *et al*., 2004). Once the leukocyte is recruited it is rolled along the endothelium from selectin to selectin until it reaches the edge of the endothelial cell, and via other adhesion receptors is transferred to the site of infection (Brodt, 1996). The shifting of leukocytes is a slow downstream movement along the endothelium via momentary reversible, adhesive reactions (Petruzzelli *et al*., 1999).

1.3.1.2 Immunoglobulins:

Immunoglobulins (Ig(s)) are plasma proteins capable of undergoing homophilic and heterophilic interactions with a variety of ligands, and function in a wide range of physiological processes, including embryonic and neural development, inflammation and wound healing, and oncogenesis (Aricescu and Jones, 2007; Brodt, 1996). Igs act either as plasma membrane bound antigen receptors on the surface of B-cells or as antibodies free in cellular fluids, and function in eliminating antigenic determining factors (Aricescu and Jones, 2007; Prescott *et al*., 1999). Antibodies bind foreign molecules such as toxins, viruses and other pathogens and inhibit these molecules from entering host cells where it can illicit its effect. There are five major classes of Igs: IgA, IgD, IgE, IgG and IgM (Prescott *et al*., 1999). Cell-cell adhesion mediated by Igs can be a fairly complicated process. The presence of internal repeats in most Igs implies the presence of multiple contact sites along the length of the molecule. These sites may vary in their adhesive strength, thus providing a mechanism for fine-tuning the adhesive interaction (Aricescu and Jones, 2007; Brodt, 1996).

The role of cadherins in cell-cell interactions as well as integrins role in cell-ECM adhesion will now be discussed in detail. Each adhesion receptor and its consequences will be discussed separately for the sake of clarity, but it should be noted that cells do express both receptors and that a degree of interplay or crosstalk among these molecules has been found.

1.3.2 Cell-cell adhesion and the role of cadherins

Cell-cell adhesion regulates diverse cellular functions including cell proliferation, migration and apoptosis, processes that are important in embryo development, normal physiology, and cancer progression (Beherens, 1999; Wadham *et al*., 2003). Therefore adhesion is a dynamic process for which interactions between cell adhesion molecules and the cytoskeleton must be continually modified (Lickert *et al*., 2000). Cadherins (Calcium (Ca^{2+}) dependent adhesion proteins) and intracellular effectors called catenins are important in developing cell-cell contacts in an array of tissue types and various species (Lickert *et al*., 2000). Different cadherins are associated with different cell types, e.g. Neural-cadherin (N-cadherin) (neural tissue); Mesenchymal-cadherin (M-cadherin) (mesenchymal tissue); epithelial-cadherin (E-cadherin) (epithelial tissue) (Ben-Ze'ev, 1997; Cavallaro *et al*., 2005; Giannis, 2000; Ozawa *et al*., 1998). Classical cadherins are highly conserved glycoproteins that form clusters at points of cell-cell contact in the membrane, forming adherens junctions between cells via homophilic interactions (Cavallaro *et al*., 2005; Roura *et al*., 1999). These integral transmembrane glycoproteins are composed of five extracellular domains, each of which contains approximately 110 amino acid residues, a single transmembrane domain and two cytoplasmic domains (Ozawa *et al*., 1998). The extracellular domains contain two conserved acidic amino acid motifs that bind Ca^{2+} (Ozawa *et al*., 1998).

The attachment of E-cadherin to the cytoskeleton is mediated by a set of proteins referred to as catenins, of which there are three, namely alpha (α)-, beta (β)- and gamma (γ)-catenin (Cavallaro *et al*., 2005; Ozawa *et al*., 1998). The catenins mediate the interaction between the cadherins and the microfilaments of the cytoskeleton (Cavallaro *et al*., 2005). Consistent with the role of the catenins in linking cadherins

to the cytoskeleton, a loss of the cadherin function has been correlated with alterations in catenins (Cavallaro *et al*., 2005; Kitahara *et al*., 2008; Ozawa *et al*., 1998; Roura *et al*., 1999). It has been shown that cell-cell adhesion can be restored by transfecting cells lacking α- or β-catenin with the respective complementary deoxyribonucleic acid (cDNA) (Cavallaro *et al*., 2005; Ozawa *et al*., 1998). β-catenin binds directly to the cytoplasmic domain of E-cadherin and the N-terminal of α-catenin binds to β-catenin. α-catenin mediates the interaction between the cadherin-catenin complex and the actin cytoskeleton through its association with α-actinin and actin filaments (Cavallaro *et al*., 2005; Ozawa *et al*., 1998). An essential catenin in an adherens junction is β-catenin. β-catenin is a 94 kilodalton (kDa) protein belonging to the armadillo repeat protein family, a class of proteins characterized by a repeating motif originally identified in the product of the *Drosophila* segment polarity gene *armadillo* (Graham *et al*., 2000). It is directly bound to the cytoplasmic domain of E-cadherin, which contains a 30-amino acid region that is highly conserved among all cadherins and is essential for binding β-catenin (Cavallaro *et al*., 2005; Lickert *et al*., 2000). This region of E-cadherin bears a serine cluster that is phosphorylated by casein kinase II (CKII) and glycogen synthase kinase-3β (GSK-3β) (Lickert *et al*., 2000). Phosphorylation of these sites results in increased binding of β-catenin to E-cadherin *in vitro*, yet Lickert and colleagues (2000) have shown that, preventing phosphorylation at this site reduces E-cadherin-mediated cell-cell adhesion in transfected mouse NIH3T3 fibroblasts (Lickert *et al*., 2000).

The β-catenin central core region, consisting of thirteen armadillo repeat sequences, binds E-cadherin (Graham, 2000; Lickert *et al*., 2000). Since cells have to regulate the strength of their adhesiveness, interactions between cell adhesion molecules and the cytoskeleton are continually modified (Ozawa *et al*., 1998). Tyrosine phosphorylation of β-catenin decreases cell-cell adhesion, whereas ectopic expression of tyrosine phosphatases strengthens it, but the exact underlying mechanisms are poorly understood (Licket *et al*., 2000; Roura *et al*., 1999). Thus both E-cadherin and β-catenin are considered major target sites for post-translational modifications, namely phosphorylation and dephosphorylation events (Lickert *et al*., 2000; Ozawa *et al*., 1998). Therefore phosphorylation of E-cadherin appears to be a crucial mechanism by which cell-cell adhesion is modulated (Lickert *et al*., 2000). Treatment of adenocarcinoma cells with growth factors, results in the accumulation of

phosphorylated tyrosine residues on β-catenin, accompanied by a loss of cadherin-mediated adhesion. This suggests that the degree of tyrosine phosphorylation of β-catenins is a critical parameter of controlling cadherin function, by regulating its association with the cytoplasm (Cavallaro and Christofori, 2004; Ozawa *et al*., 1998; Roura *et al*., 1999). Roura and colleagues (1999) demonstrated that the tyrosine residue at position 654 is phosphorylated in conditions in which adherens junctions are disrupted, and that binding of β-catenin to E-cadherin *in vivo* is controlled by phosphorylation of β-catenin tyrosine-654 (Roura *et al*., 1999).

1.3.3 Cell-ECM attachment and the role of integrin receptors

Cell anchorage or adhesion to the ECM generates transmembrane signals that impact cellular activities and these cellular activities are triggered by integrin-mediated cell-ECM interactions (Wu, 2001). Upon adhesion to the ECM many integrins as well as a particular group of cytoskeletal and signalling proteins are recruited to cell matrix contact sites where they link the actin cytoskeleton to the ECM and mediate signal transduction between the extracellular and intracellular environments (Cavallaro and Christofori, 2004; Wu, 2001). Integrins are heterodimeric transmembrane receptors composed of two subunits, α and β, and each αβ combination has its own binding specificity and signalling properties (Ben-Ze'ev, 1997). The CTs of integrins are short and devoid of enzymatic features (Ben-Ze'ev, 1997; Giancotti and Ruoslahti, 1999).

The action of integrins is directly related to its clustering at the focal adhesion sites, allowing a cytoskeletal membrane anchor complex to assemble around the integrin cluster (Li *et al*., 1999). Focal adhesion complexes are comprised of integrins that are physically connected to the cytoskeleton through stable and transient interactions (such as actin stress fibres and microtubules) (Li *et al*., 1999). In addition, integrins and associated proteins talin, viniculin and actinin, connect the CT of integrin with actin to form a molecular bridge from the ECM to the intracellular environment (Hehlgans *et al*., 2007). These bridges allow integrin to directly mediate mechanical stresses across the cell surface and into the cytoplasm. Protein kinases are also recruited into focal adhesions in response to cell adhesion, which lead to increased

protein phosphorylation of focal adhesion components such as focal adhesion kinase (FAK) and Src (Hood and Cheresh, 2002; Li *et al*., 1999). Thus integrins can coordinate changes in cytoskeletal structure as a result of mechanical stresses which may also lead to the activation of several signalling molecules (Giancotti and Ruoslahti, 1999; Hynes, 2002; Li *et al*., 1999).

It is important to note that it has been shown that integrins mediate signalling bi-directionally i.e. "outside in" and "inside out" (Hynes, 2002). "Outside in" signalling requires a ligand to bind to the integrin to illicit a signalling pathway within the cell (Wu, 1999). Whereas "inside out" signalling alters the binding activity of the external domain of the cell due to internal cell events. This arises by the clustering of integrins and is followed by the binding of an extracellular effector which requires a ligand to bind to the integrin to illicit a signalling pathway within the cell (Giancotti and Ruoslahti, 1999; Hynes, 2002).

This review will discuss "outside in" signalling events that arise by the clustering of integrins triggered by the binding of an extracellular effector. Examples of integrin linked signals are increases in pH or Ca^{2+}, the activation of tyrosine kinase such as FAK that leads to the stimulation of the rac/rho pathway, the activation of the serine/threonine kinases such as mitogen-activated protein kinase (MAPK) and c-Jun NH_2-terminal kinase (JNK), as well as the activation of phosphatidylinositol 3-kinase (PI3K) and integrin-linked kinase (ILK) (Beherens, 2000; Giancotti and Ruoslahti, 1999; Hehlgans *et al*., 2007; Schwartz, 1997). The integrins are attached to linker or adapter proteins such as PI3K, ILK or FAK and these proteins link integrins to, and mediate, three main pathways (Giancotti and Ruoslahti, 1999). The three main signal pathways are i) the rac and rho pathway, ii) the MAPK pathway and iii) the PI3K pathway.

All the adhesion receptors have been described and it is now critical to examine the other aspect of cell adhesion, namely the signal transduction pathways that are activated in response to adhesion events. In particular, the integrin-ILK/PI3K pathway will be examined with emphasis placed on two of the proteins involved in this pathway, namely ILK and β-catenin.

1.4 Integrin-ILK-1/PI3K signal transduction pathway:

The key molecules involved in this pathway are ILK-1, protein kinase B (PKB or Akt) and GSK-3β, β-catenin, p44/42 MAPKs and the myosin light chain. Upstream events that regulate ILK-1 activity include cell-ECM interactions and stimulation, which are probably mediated by PI3K (Hehlgans *et al*., 2007; Wu, 2001). A description of ILK-1's structure and function is necessary before examining the PI3K pathway. ILK-1 is a serine-threonine kinase that interacts with the cytoplasmic domains of β-integrin and also binds with high affinity to particularly interesting cysteine-and histidine-rich protein (PINCH), a focal adhesion protein (Hehlgans *et al*., 2007; Li *et al*., 1999; Wu, 2001). ILK-1 also interacts with an actin-binding focal adhesion protein containing two calponin homology (CH) domains (calponin homology domain containing ILK-binding protein (CH-ILKBP)). CH-ILKBP interacts with ILK's carboxy-terminus (C-terminus) and the PINCH-ILK-CH-ILKBP complex provides an important connection between transmembrane receptors such as integrins and the actin filaments (Hehlgans *et al*., 2007; Li *et al*., 1999; Wu, 2001). ILK is not only a scaffold protein but it also participates in the PI3K pathway which will now be examined in detail.

It is important to take into account that both ILK-1 and PI3K are in direct contact with the integrin as shown in figure 1.0. This is because ILK-1 can either be directly activated by ligand-integrin interactions or activated via PI3K (Giancotti and Ruoslahti, 1999; Novak and Dedhar, 1999). Ligand integrin engagement activates PI3K, which mediates the production of phosphatidlinositol-3,4,5-P_3 (PIP_3), which activates ILK that is bound to the integrin β-subunit (Novak and Dedhar, 1999). GSK-3β inactivation is accomplished via one of two routes; either ILK-1 inhibits GSK-3β directly by phosphorylation, or inhibition occurs by first phosphorylating and activating PKB (phosphorylation of PKB occurs at serine (ser)-473), which in turn phosphorylates GSK-3β at the ser-9 residue (Li *et al*., 1999; Novak and Dedhar, 1999; Wu, 1999). This results in the accumulation of free β-catenin that is able to translocate to the nucleus. GSK-3β activities will be discussed later in relation to β-catenin regulation in the cytoplasm. This pathway is depicted in figure 1.0, page 18. ILK-1 plays an integral role in adhesion as well as in influencing cellular events or

outcomes via β-catenin, such as cell proliferation and regulating cell survival, and therefore can be significant in the progression of cancer (Li *et al*., 1999). Over expression of ILK-1 in epithelial cells disrupts cell-extracellular matrix as well as cell-cell interactions, suppresses anoikis (suspension induced apoptosis) and promotes anchorage-independent cell cycle progression (Novak *et al*., 1998).

From this discussion it is evident that β-catenin plays an important role, not only in adhesion-cadherin complexes, but also in integrin mediated signalling, therefore further examination of this molecule is warranted.

1.5 Dual role of β-catenin

As mentioned above β-catenin is a critical component of adherens junctions as it links the actin cytoskeleton to the cytoplasmic component of E-cadherin via α-catenin (Shtutman *et al*., 1999). The mediating role of β-catenin within the adherens junction is critical to suppress a cell from acquiring an invasive state. In addition, cytoplasmic accumulation of β-catenin induces a translocation of this molecule into the nucleus. Here β-catenin is involved with other transcription factors, namely, lymphoid enhancer factor 1(LEF1) or T-cell factors (Tcf's) (of which there are three Tcf1, Tcf3 and Tcf4), to induce specific gene expression (Hurlstone and Clevers, 2002; Sadot *et al*., 1998). By playing this dual role – a structural one within the junction and a regulatory one outside the adherens junction – changes in cell adhesion as well as junction formation affect transmembrane signalling and gene expression (Sadot *et al*., 1998). It has been demonstrated in *Xenopus* that the signalling activity of β-catenin is independent of cadherin-based adhesion. On the other hand, this signalling is strongly affected by the levels of cadherin, because over expression of cadherin blocks β-catenin signalling in *Xenopus*, indicating the negative regulatory role of cadherin for β-catenin (Sadot *et al*., 1998). Therefore the concentration of β-catenin in the cytoplasm and nucleus is regulated by a number of mechanisms of which the PI3K pathway is one. An alternative pathway, able to regulate the concentration of β-catenin, is the wingless type (Wnt) signaling pathway. The Wnt pathway has an adhesion role in that it inactivates GSK-3β, therefore it will also be necessary to

investigate the role of this protein (GSK-3β), which will be discussed in relation to the Wnt signaling pathway.

1.6 Regulation of β-catenin in the cytoplasm with respect to the Wnt signaling pathway

β-catenin is regarded as existing in three different subcellular forms: membrane bound, cytosolic and nuclear. β-catenin in the cytoplasm exists in a soluble monomeric state, where it may subsequently be degraded or translocated to the nucleus (Wong and Pignatelli, 2002). Catabolism of β-catenin is mediated on a scaffold protein adenomatous polyposis coli (APC). APC is a large polypeptide of 2843 amino acids, which brings β-catenin into proximity with axin and GSK-3β (Novak and Dedhar, 1999). GSK-3β binds axin and phosphorylates it. This complex then binds APC which is in turn phosphorylated by GSK-3β. β-catenin binds to the phosphorylated APC, interacts with axin, and is phosphorylated by GSK-3β (Novak and Dedhar, 1999). GSK-3β phosphorylates the N-terminal domain of β-catenin which contains four conserved serine/threonine residues. Phosphorylated β-catenin is recognized by the forkhead (F-box) protein, β–transduction–repeat–containing protein (βTrCP), that links with an ubiquitin ligase complex to degrade β-catenin; refer to figure 1.0 (Hurlstone and Clevers, 2002; Novak and Dedhar, 1999). Alternatively unphosphorylated β-catenin can be recognized by the F-box protein Ebi resulting in the destruction of β-catenin in response to tumour protein 53 (p53) mediated stress signals (Hurlstone and Clevers, 2002). As already mentioned the Wnt and integrin-ILK-1 signalling mediate β-catenin translocation from the cytoplasm into the nucleus and are antagonists of the action of GSK-3β. The Wnt pathway is a highly conserved signalling pathway found in organisms ranging from invertebrates to mammals (Graham *et al*., 2000; Valenta *et al*., 2003). This pathway is critical in embryonic development, cellular proliferation and differentiation and in particular, stabilizing the cytoplasmic β-catenin concentration (Levy *et al*., 2002).

β-catenin is translocated to the nucleus via the Wnt pathway, which is activated by proteins of the Wnt family (secreted glycoproteins), that bind to the Frizzled (frz) family of seven transmembrane-span receptors and a proteoglycan coreceptor

(Fashena and Thomas, 2000; Valenta *et al*., 2003). This interaction activates the proteins disheveled (dsh), a positive regulator of the Wnt pathway, and protein kinase C (PKC) (Fashena and Thomas, 2000; Novak and Dedhar, 1999). Activation of dsh involves hyperphosphorylation of the protein and includes phosphorylation by CKII (Novak and Dedhar, 1999). Dsh and PKC inactivate GSK3-β, which allows for the accumulation of β-catenin in the cytoplasm, and this results in the protein being translocated into the nucleus, see figure 1.0 (Novak and Dedhar, 1999). Before examining the translocation and effects of β-catenin in the nucleus it is important to look at the integration and possible influence between the above mentioned pathways. This is necessary since fitting in with the Wnt pathway is the integrin-ILK-1 pathway. Both pathways inactivate GSK-3β and result in an increase in free cytoplasmic β-catenin.

1.7 Is there crosstalk between the Wnt and integrin-ILK-1 pathways?

The adhesion receptors and their relevant pathways that were discussed above (integrins and cadherins) were dealt with individually for clarity of their functional roles; however the degree of "crosstalk" between these molecules and the activated signaling cascades should be considered. Crosstalk is accompanied by the perception that numerous pathways interacting and regulating the same molecules, provide additional levels of control of cell signaling to further reduce chances of cellular disruption (Beherens, 2000; Novak *et al*., 1998). Although associations between these molecules are not fully understood, the concept of crosstalk between adhesion receptors over recent years has begun to gain a considerable amount of interest.

Novak *et al*. (1998) have shown that over expression of ILK-1 in intestinal epithelia results in an invasive phenotype concomitant with a down-regulation of E-cadherin expression, translocation of β-catenin to the nucleus, and activation of gene expression. They also found that LEF-1 protein levels increased during ILK-1 over expression. These results demonstrate that oncogenic properties of ILK-1 involve activation of the LEF-1/β-catenin signalling pathway, and this also suggests that ILK-mediated crosstalk occurs between cell-ECM interactions and cell-cell adhesion as well as components of the Wnt signalling pathway (Novak *et al*., 1998). Another

example of crosstalk was shown by Tan *et al*. (2001). They demonstrated that the integrin-ILK-1 pathway can also activate LEF-1/β-catenin mediated gene transcription and down regulate E-cadherin expression. They also found that over expression of ILK-1 caused a stimulation of expression of the snail transcription factor, yet gene expression of snail is not regulated by Tcf/β-catenin (Tan *et al*., 2001). In addition, it was also shown that inhibition of ILK-1 resulted in substantial inhibition of Tcf mediated gene transcription and the inhibition of transcription and expression of cyclin D1, which is regulated by Tcf. Inhibition of ILK-1 also resulted in decreased levels of free cytoplasmic β-catenin (Tan *et al*., 2001).

These findings have triggered the question as to whether crosstalk occurs between these discussed signal transduction pathways, extended to the control of transcription, with specific reference to β-catenin and ILK-1. With this consideration in mind it is necessary to examine β-catenin activities at a nuclear level as well as look at the genes and promoter regions involved in expressing both β-catenin and ILK-1, in order to determine whether β-catenin has a possible regulatory influence on ILK-1. Once again this will be dealt with separately for the sake of clarity.

1.8 Translocation of β-catenin from the cytoplasm into the nucleus

β-catenin was first observed to enter the nucleus in embryonic *Xenopus* cells. The mechanism by which β-catenin entered the nucleus was unclear (Funayama *et al*., 1995). There are two proposed models of β-catenin nuclear translocation that have been proposed. Firstly, it was shown that β-catenin binds to LEF/Tcf in the cytoplasm and then translocates to the nucleus via the nuclear localisation signals (NLS) of LEF/Tcf (Beherens *et al*., 1996). Beherens and colleagues (1996) showed that LEF-1 and β-catenin form a complex that results in co-localisation of this complex to the nucleus in Neuro 2A cells (cells that lack endogenous cadherins). Beherens *et al*. (1996) showed that successful nuclear translocation of this complex is dependent on both the β-catenin armadillo repeats 1-7 and the LEF-1 N-terminal domain as well as the high mobility group (HMG) domain that contains the NLS (Beherens *et al*., 1996). However it has also been observed that mutant β-catenin forms lacking LEF/Tcf binding sites are able to localise to the nucleus. Thus the

second proposal was that β-catenin can naturally enter the nucleus via its armadillo repeats, which are also found in nuclear importin molecules (Novak and Dedhar, 1999; Giannini *et al*., 2000). The translocation of β-catenin in this manner is similar to that of importin β, which is a subunit of the NLS receptor complex that are collectively known as importins. Importins are 60 kDa proteins composed primarily of hydrophobic armadillo repeat motifs (Prieve *et al*., 1998). β-catenin docks to the nuclear envelope through its armadillo domain by binding directly to the nuclear pores, it then translocates into the nucleus in an energy dependent manner, although this process can be inhibited by non-hydrolysable guanosine triphosphate (GTP) analogs, and by a dominant-negative mutant form of the Ran GTPase (Fagotto *et al*., 1998).

Because β-catenin translocates to the nucleus via a similar mechanism to that of β-importin, Giannini and colleagues (1996) raised the question of whether β-catenin is an importin for a subset of nuclear proteins. They showed that β-catenin acts as an importin and translocates to the nucleus in complex with green fluorescent protein (GFP), and also carries α-catenin into the nucleoplasm when in complex with Tcf, yet uncomplexed β-catenin is sequestered by α-catenin in the cytoplasm. It has been suggested that α-catenin exhibits a negative regulatory role on β-catenin within the nucleus (Giannini *et al*., 1996).

1.9 β-catenin in the nucleus

Once β-catenin has entered the nucleus the interaction between β-catenin and LEF-1/Tcf transcription factors plays a pivotal role in the Wnt signalling pathway (Brodt *et al*., 1996). In the nucleus β-catenin displaces Groucho (Grg) or carboxy-terminal binding protein (CtBP) which are corepressors bound to LEF/Tcf transcription factors (Hurlstone and Clevers, 2002). β-catenin, together with Pontin52 (a deoxyribonucleic acid (DNA)-dependent ATPase with helicase activity) and a TATA-box binding protein, stimulates the formation of the β-catenin/LEF-1/Tcf complexes (Novak and Dedhar, 1999; Song *et al*., 2003). In the absence of β-catenin, LEF-1/Tcf repress rather than activate gene targets through the recruitment of the abovementioned co-repressors. β-catenin can therefore be viewed as a docking molecule that recruits

essential co-activators to transcribe target gene promoters (Hurlstone and Clevers, 2002).

The gene targets of β-catenin/LEF-1/Tcf expression have been found to have several LEF/Tcf binding sites in their promoters, and have been identified in the gene promoters of *c-myc, cyclin D1, cyclin A, cell division cycle* (*cdc*) *2* and *cdc25* to name but a few. In humans, *c-myc* was the first target gene of the β-catenin signalling pathway to be discovered (Levy *et al*., 2002; Wong and Pignatelli, 2002). The relationship between β-catenin and *c-myc* was demonstrated by He *et al*. (1998). The expression of a mutant β-catenin resistant to APC binding increased *c-myc* gene expression via Tcf-4 binding to the *c-myc* promoter (He *et al*., 1998).

Shtutman and colleagues (1999) have shown that cyclin D1 expression always, and only, takes place in association with nuclear β-catenin expression, demonstrating the critical role of β-catenin for gene expression. Like the cyclin D1 protein, the gastrointestinal hormone gastrin is thought to have a driving role in cell proliferation, and up regulation of the *gastrin* gene expression, which has Tcf-4 binding sites on the promoter region and is mediated by nuclear β-catenin (Wong and Pignatelli, 2002). Due to the fact that there are in order of 50 genes to date that are recognised to be regulated/influenced by β-catenin, it would be senseless to describe every one, as this is not the focus of the review (Wong and Pignatelli, 2002). With regards to β-catenin in the nucleus and the above mentioned regulation hypothesises it would be more beneficial at this stage to examine the gene and promoter region that expresses β-catenin as well as the gene expressing ILK-1.

1.10 The β-catenin and ILK-1 genes

To date β-catenin studies have predominantly focused on the β-catenin protein, stabilization and subcellular localisation including trafficking during adherens junction remodelling, and the corresponding activation of downstream β-catenin/LEF-1/Tcf target genes; however, there is only sparse information on the regulation of β-catenin expression at the transcriptional level (Li *et al.*, 2004). The human β-catenin gene *CTNNB*1 is located on chromosome three at position 3p22-p21.3. Li *et al.*

(2004) characterised the promoter region of this gene. The promoter was found to contain several putative binding sites for AP1 and one Tcf/LEF-1 site; the presence of these sites suggests a possible feedback mechanism for augmenting β-catenin expression under certain circumstances (Li *et al*., 2004). For instance, mutations that constitutively over express β-catenin via protein stabilisation would allow for the potential for increased messenger ribonucleic acid (mRNA) levels as seen in various colorectal carcinomas (Li *et al*., 2004). The identification of numerous bound transcription factors (E2 transcription factor-1 (E2F1), nuclear factor-kappa B (NFκB), MEF1, paired box 5 (Pax5), ISRE2, mothers against decapentaplegic homolog (Smad) 3/4, GATA and ZIC) to the promoter of the rat β-catenin gene *Ctnnb1* was observed by Li *et al*. (2004), of which the strong binding of E2F1 and NFκB are noteworthy, as E2F1 is regulated by cyclin D1, a downstream target of β-catenin/LEF-1/Tcf signalling; whereas NFκB has been implicated in crosstalk between the Wnt and NFκB pathways (Li *et al*., 2004; Novak *et al*., 1998; Shtutman *et al*., 1998). A component of NFκB, p65 has been found to suppress β-catenin/LEF/Tcf signalling. A direct interaction between β-catenin and p65 in colorectal cancer cells has been proposed in response to aspirin treatment (Li *et al*., 2004; Sharma *et al*., 2002; Song *et al*., 2003). Sharma *et al*. (2002) has proposed a link between the Wnt, PI3K and androgen receptor pathways in prostate cell lines; both NFκB and β-catenin protein levels were high.

The ILK-1 gene has been mapped on human metaphase chromosomes to the distal end of the short arm of chromosome 11 at the interface of the band 11p15.5 and 11p15.4 (Melchior *et al*., 2002). The 11p15 locus harbours well-defined translocations associated with Beckwith Wiedemann syndrome (BWS). Also regional loss of heterozygosity (LOH) suggests that it is this region of chromosome 11 that is strongly associated with tumourgenesis. The ILK-1 gene promoter has been shown by Melchior *et al*. (2002) to feature the typical characteristics of housekeeping gene promoters, such as lack of TATA and CAAT boxes, a high GC content, presence of CpG islands and multiple transcription initiation start sites. The promoter activity of ILK occurs within 349 base pairs (bp) immediately upstream of exon 1 (Melchior *et al*., 2002). Three elements (Inr1, Inr2, Inr3) that resemble the consensus sequence for initiator elements (Py Py ANT/APy Py) were identified within the promoter as well as various *cis*-acting transcription factor binding sites, such as Sp1, LBP1, JCV and Tcf1

(Melchior *et al*., 2002). The Tcf1 sites, of which there are two, are of particular interest; as it is known that Tcf transcription factors require β-catenin binding for activation of gene expression (Hurlstone and Clevers, 2002). This raises an interesting question as to whether these Tcf1 sites that interact with β-catenin, are essential to activate transcription; furthermore why are there two Tcf1 sites within the promoter region? Do these Tcf1 sites act synergistically to enhance transcription or are both sites required to activate transcription? Therefore to what extent is ILK-1 gene expression regulated by β-catenin, and how does it consequently effect the protein levels of ILK-1?

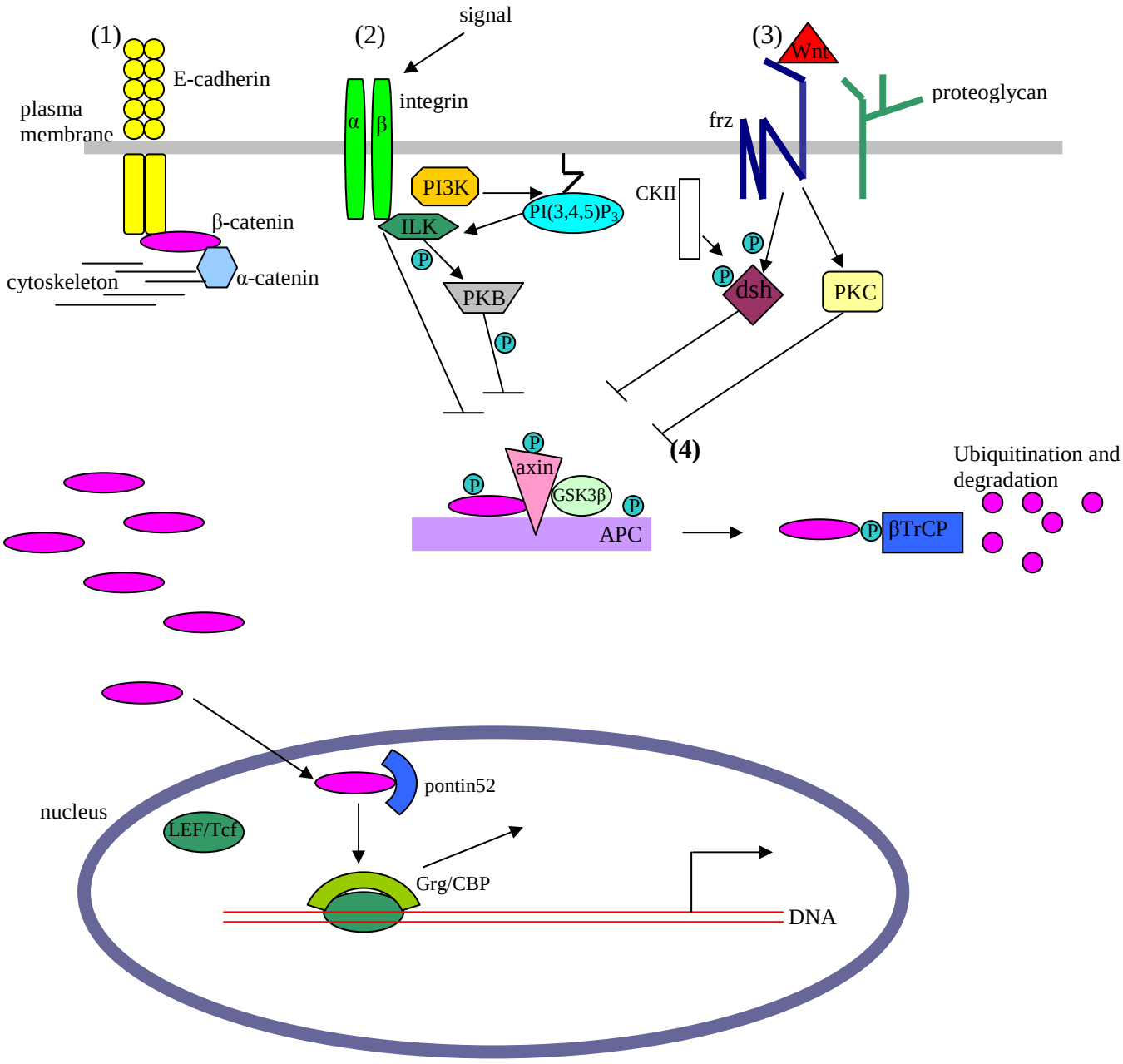


Figure 1.0: Diagram depicting the various events and interactions involved in cell adhesion and signal transduction.

(1) β-catenin bound to E-cadherin in an adherens junction. (2) αβ-integrin receptor and its association with ILK-1 and PI3K, and upon ligand activation the resultant signal transduction pathway. (3) Wnt signal transduction pathway. Both pathways allow β-catenin translocation into the nucleus and target gene activation occurs. (4) The degradation complex that inhibits β-catenin translocation into the nucleus. Free cytoplasmic β-catenin translocates into the nucleus and binds with LEF/Tcf to activate target gene expression (adapted from Novak and Dedhar, 1999).

1.11 β-catenin and ILK-1's role in tumourgenesis and pathogenesis

Thus far the literature review has shown that both β-catenin and ILK-1 play a pivotal role in cell adhesion and regulation with the possibility of a degree of crosstalk, contributing to additional levels of control of these processes. It is now relevant to investigate whether β-catenin and ILK-1 directly contribute to cancer progression.

Increased ILK-1 expression and activity have been correlated with malignancy in several human tumour types, including breast, brain and colon carcinomas (Kawasoe *et al*., 2000). Novak and colleagues (1998) showed that modest over expression of ILK in intestinal and mammary cells resulted in an invasive phenotype concomitant with a down-regulation of E-cadherin expression, nuclear translocation of β-catenin, and β-catenin/LEF-1 complex formation and subsequent transcription. It was also found that LEF-1 protein expression was constitutively up-regulated during ILK-1 over expression. Therefore Novak *et al*. (1998) demonstrated that the oncogenic properties of ILK-1 involve activation of the β-catenin/LEF-1 signalling pathway. It was also suggested that ILK-1-mediated crosstalk occurred between cell-ECM and cell-cell adhesion and components of the Wnt signalling pathway (Novak *et al*., 1998).

With respect to β-catenin and cancer progression, studies have shown that a deletion in β-catenin that abolishes its binding to α-catenin, was shown to diminish E-cadherin function (Sadot *et al*., 1998). Moreover mutations of the β-catenin gene or the APC gene, result in the failure of degradation complex assembly, and subsequently lead to increased transcriptional activity of the β-catenin/LEF-1/Tcf complex, a process that plays an important role in the development of most colorectal carcinomas and melanomas (Ben-Ze'ev, 1997; Mann *et al*., 1999; Sadot *et al*., 1998). Also, mutations of β-catenin itself in its GSK-3β phosphorylation sites render resistance to proteolytic degradation and are reported to occur in half of sporadic colorectal cancer cells with wild-type APC (Yamada *et al*., 2000). Thus, accumulation of β-catenin is a common phenomenon in the majority of colorectal cancers (Yamada *et al*., 2000).
Although the majority of studies involving β-catenin and ILK-1 irregularities and their associations have been studied in colorectal carcinomas, for the purpose of this

study, with respect to the effects of β-catenin regulation on ILK-1, human oesophageal squamous cell carcinoma (HOSCC) cell lines will be utilized. The reasons for this decision will become apparent in the following text.

1.12 Human oesophageal squamous cell carcinoma

Among the various types of digestive tract cancers, squamous cell carcinoma (SCC) of the oesophagus has the poorest prognosis, with the overall five year survival rate of patients with metastatic HOSCC being <10% (Shiozaki *et al*., 2001; Stoner and Gupta, 2001). The reason for this is that prognosis of cancer is strongly dependent on the organ of the primary site, and so the anatomic location can therefore make diagnosis or surgical treatment difficult (Shiozaki *et al*., 2001). In addition, the early occurrence of micrometastases contributes to the difficulty of treating the disease with either chemo-or radiotherapeutic techniques (Zampieri and Veale, 2001). More than 90% of oesophageal cancers world wide are SCC's and nearly 50% of all HOSCC's involve the middle third of the oesophagus (Gore *et al*., 1997).

The incidence of HOSCC geographical distribution is significant in certain parts of China, Iran, Uruguay, Italy, France and South Africa, particularly in the Transkei region, where up to 50% of all registered cancers are HOSCC (Gore *et al*., 1997; Stoner and Gupta, 2001). The prevalence of HOSCC in endemic areas has been attributed to environmental rather than hereditary factors, nutrition being the predominant factor, as most diets are vitamin A, C and E, riboflavin and protein deficient (Gore *et al*., 1997; Stoner and Gupta, 2001). A significant characteristic of these endemic regions is low rainfall pertaining to changes in vegetation, soil types and farming practises (Gore *et al*., 1997). Other predominant causes of HOSCC are excessive use of tobacco and alcohol, as well as salt-pickled/cured and mouldy foods. These factors have been implicated in the pathogenesis of this disease.

In South Africa studies have shown that a significantly high rate of oesophageal cancer deaths occur among the Xhosa population in both females and males whereas among the Zulus, the mortality rate is significantly lower (McGlashan, 1988). McGlashan (1988) has also shown that HOSCC is significantly higher in males

compared to females and the risk of acquiring HOSCC increases with age. Thus it is clear that at a nationwide level HOSCC is a principle cancer among South African blacks and therefore justifies any attempt to understand the events involved in its development. No recent statistics concerning HOSCC for South Africa are available at present.

With respect to β-catenin and HOSCC it has already been shown by Jones and Veale (2003) that β-catenin's activities were skewed towards cell-cell adhesion as the cytoplasmic and nuclear concentration of β-catenin were low. However, when Jones and Veale induced the Wnt pathway and activated EGF signalling, a β-catenin shift from the membrane into the cytoplasm was induced (Jones and Veale, 2003). Therefore although β-catenin's adherent role is intact in HOSCC's the stability of free β-catenin contributes to a reduction in cell-cell adhesion within HOSCC's.

1.13 Aims

1. To examine the role of β-catenin in the nucleus at a regulatory level and its influence on the protein levels of ILK-1 in moderately differentiated HOSCC cell lines.

2. To determine whether Tcf-1 exists in the cell lines under study and whether it interacts with β-catenin in the nucleus.

Chapter Two: Tcf-1/β-catenin complex in human oesophageal squamous cell carcinoma cell lines

2.1 Introduction

Tcfs bind to Wnt target genes through their high mobility DNA-binding motif's, complex with β-catenin and position the potent regulatory domain within β-catenin to activate transcription (Hurlstone and Clevers, 2002) (reviewed by Waterman., 2004). Therefore, the nuclear accumulation of β-catenin and the formation of the complex between β-catenin and LEF/Tcf are critical in the activation of Wnt target genes (Schohl and Fagotto, 2002) (reviewed by Hoppler and Kavanagh, 2007).

The mammalian LEF/Tcf family of transcription factors is comprised of four different proteins that form a subgroup of the high mobility group (HMG) box-containing superfamily of transcription factors (Hurlstone and Clevers, 2002; Waterman, 2004). These transcription factors contain four defined functional domains that will be discussed separately. All LEF/Tcfs possess an 88 amino acid region in close proximity to the carboxy terminus that contains both a HMG box motif and nuclear localisation signal (Waterman, 2004). A trademark of the HMG box motif is that this particular motif binds to and bends the minor groove of the DNA double helix (90^{o}-130^{o}) (Giese *et al*., 1992; Waterman 2004). This HMG box motif recognises a specific core consensus sequence YCTTTGWW, and is the most highly conserved feature of the transcription factor family (between 93-99% amino acid sequence identity) (Love *et al*., 1995; van de Wetering and Clevers, 1992; van de Wetering *et al*., 1997; Waterman, 2004). Multiple C-termini (generated from alternative splicing) is another feature of the LEF/Tcfs that is worth noting because within the multiple C-terminus is the E-tail domain which is necessary for repression and/or activation of a select subset of Wnt target genes (Rahmani, *et al*., 2005; Waterman, 2004). Atcha *et al.* (2003) have found that the E-tail may contribute to selective promoter activity. It was established by Atcha and colleagues (2003) that the LEF-1 promoter is selectively activated by specific LEF/Tcf isoforms (Tcf-1E and Tcf-

4E) that contain a complete E-tail domain (Hovanes *et al*., 2001; Roel *et al*., 2003). Interestingly, the carboxy-terminal binding protein (CtBP) binding elements found in the E-tail of Tcf-3 and Tcf-4 are absent in Tcf-1 (Molenaar *et al*., 1996; Roel *et al*., 2003; Waterman, 2004). Instead, Tcf-1 carries a CRARF or CR motif (33 amino acid region required for transcription activation) (Atcha *et al*., 2003; Rahmani *et al*., 2005; Waterman, 2004). It is this alternative splice feature of the LEF/Tcf family that distinguishes one member from another (Clevers and van de Wetering, 1997; Rahmani *et al*., 2005; Waterman, 2004). An additional defined feature of the LEF/Tcf family is the transcription repression domain that is located in the central region of the LEF/Tcf proteins; the overall function of this domain is conserved for the protein family, however the primary sequences of this domain are not (Atcha *et al*., 2003; Hurlstone and Clevers, 2002). LEF/Tcf cannot independently activate or repress target genes, they are actually architectural proteins that bend DNA to maximise productive interactions between DNA-bound proteins in a larger enhancesosome or repressing complex, hence recruitment of co-activators or repressors are necessary (Graham *et al*., 2000; Huber *et al*., 1997; Hurlstone and Clevers, 2002). LEF/Tcfs function as transcription repressors in the absence of β-catenin binding, however, this co-repressor activity is initiated by the recruitment of co-repressors such as Grg/TLE, via the central domain (Billin *et al*., 2000; Brantjies *et al*., 2001) (reviewed by Willert and Jones, 2006). These co-repressors are histone-binding proteins and additionally interact with a histone deacetylase-1 (Billin *et al*., 2000; Brantjies *et al*., 2001). Finally the β-catenin binding domain is found at the extreme N-terminus, corresponding to approximately 60 amino acids, and is a highly conserved region of the LEF/Tcf family. Interestingly β-catenin does not have a DNA-binding domain however it does contain a potent transcription activation domain (Beherens *et al*., 1996; Graham *et al*., 2000; Nelson and Nusse, 2004). In contrast LEF/Tcfs are able to bind and bend DNA, but they do not have a strong transcription activation domain (Atcha *et al*., 2003; van Beest *et al*., 2000) (reviewed by Willert and Jones, 2006). Therefore, a combination of the two proteins results in a potent transcription regulatory complex (Hurlstone and Clevers, 2002; Waterman *et al*., 2004).

Binding is proposed to be a multi-step interaction (Beherens *et al*., 1996; Graham *et al*., 2000). LEF/Tcf bind β-catenin in its large central core which consists of twelve armadillo repeats, and each repeat consists of three α helices (Graham *et al*., 2000; Hurlstone and Clevers, 2002). The twelve repeats form a superhelix that is analogous to double stranded DNA and creates a long positively charged groove (Graham *et al*., 2000). The binding domain of Tcf (extended modular structure) snakes its way in an anti-parallel fashion along the β-catenin groove (Hurlstone and Clevers, 2002). Between the first fifty amino acids of LEF/Tcf and armadillo repeats 3-10 of β-catenin is where the actual binding of these two proteins occurs (Hurlstone and Clevers, 2002). Two amino acids namely lysine (Lys) 312 and Lys 435 of β-catenin form particularly strong and specific anchors that fasten the two molecules together (Hurlstone and Clevers, 2002).

To fully appreciate the intricacies of LEF/Tcf functioning, particularly in relation to transformation events, it must be clarified that isoforms of these proteins exist (Clevers and van de Wetering, 1997; Hurlstone and Clevers, 2002; Rahmani *et al*., 2005; Valenta *et al*., 2003; van de Wetering and Clevers, 1992) (reviewed by Waterman, 2004 and Willert and Jones, 2006). These distinguishing factors are firstly alternative splicing in the C-terminus (Molenaar *et al*., 1996; van de Wetering *et al*., 1996) (reviewed by Waterman, 2004 and Willert and Jones, 2006). Different termini were first discovered for Tcf-1, but they have since been identified in all family members (Hovanes *et al*., 2000; Molenaar *et al*., 1996; van de Wetering *et al*., 1996). There are seven identified human Tcf-1 isoforms that are named Tcf-1A to Tcf-1 G and the molecular weight of these isoforms range from between 55.2 kDa (Tcf-1E) to 28.3 kDa (Tcf-1D) (van de Wetering *et al*., 1996) (reviewed by Waterman, 2004 and Willert and Jones, 2006). Secondly, due to alternative promoter usage; both the *TCF7* (encoding Tcf-1 protein) and *LEF1* genes contain two different promoters for transcription (van de Wetering *et al*., 1996). The original/first promoter encodes a complete mRNA sequence that results in a fully functional protein (Li *et al*., 2006; van de Wetering *et al*., 1996). However, a second promoter exists in the second intron and produces a truncated protein that lacks a β-catenin binding domain (Li *et al*., 2006). Yet they do have functional HMG-box domains and nuclear localisation signals and so reside in the nucleus, bind to Wnt response

elements (WRE), and recruit co-repressors (Hurlstone and Clevers, 2002). However, they cannot mediate Wnt signals and therefore act as a block, because if the truncated forms exceed the levels of full length forms they could effectively suppress Wnt signalling by occupying all regulatory sites (Hurlstone and Clevers, 2002; Li *et al*., 2006; van de Wetering *et al*., 1996; Willinger *et al*., 2006). These truncated forms are called natural dominant negatives. Therefore with respect to normal and cancerous tissue, the relative amounts of full-length and dominant negative proteins may be very important for normal cell differentiation and tumour progression (Li *et al*., 2006; van de Wetering *et al*., 1996; Willinger *et al*., 2006) (reviewed by Waterman, 2004). For the purpose of this study intact/full length Tcf-1 will be examined in depth.

A number of signalling pathways have been discovered to date that respond to a variety of environmental changes by evoking cellular responses (Hoppler and Kavanagh, 2007; Masui *et al*., 2002). Of late a number of these pathways were found to exhibit cross talk (Bagnato and Rosano, 2007; Labbe *et al*., 2000; Masui *et al.*, 2002). It has been established that cross talk plays an important role in modifying signal effects, which increases the complexity of signal cascades. In the case of Wnt/β-catenin signalling, several examples of cross talk have been reported (Bagnato and Rosano, 2007; Medici *et al*., 2006). For instance the signalling pathway that involves NF-κB plays a pivotal role in the immune response, inflammation and apoptosis, however the Wnt/β-catenin pathway plays an important role in development and oncogenesis (Masui *et al*., 2002). Masui and colleagues (2002) showed that β-catenin/Tcf-dependent transcription was specifically suppressed by reticuloendotheliosis A (RelA), a component of NF-κB. This suppression was not shown to be dependent on the *trans*-acting transcriptional ability of RelA nor did RelA affect the nuclear import of β-catenin or the DNA binding ability of the β-catenin/Tcf complex (Masui *et al*., 2002). Therefore Masui *et al* (2002) suggests that only after the binding of the β-catenin/Tcf complex with target DNA, does NF-κB modify this signalling pathway.

Another example of cross talk involving ILK-1 and β-catenin was shown by Novak and colleagues (1998). They showed that over expression of ILK-1 resulted in an invasive

phenotype concomitant with a down-regulation of E-cadherin expression, translocation of β-catenin to the nucleus, and activation of gene expression. With the phenomena of cross talk in mind it is noteworthy to look at whether there is cross talk between both the integrin/ILK-1 pathway and Wnt signalling. Melchior and colleagues (2002) have characterised the promoter region of ILK-1 and it was found to have two Tcf-1 binding sites. This finding has triggered the question of whether cross talk occurs between the integrin-ILK-1 and Wnt pathways, extended to the control of transcription with reference to β-catenin and ILK-1. Therefore it is necessary to study β-catenin and Tcf-1 at a nuclear level in order to determine whether the β-catenin/Tcf complex has a possible regulatory influence on ILK-1.

This study examined and compared (individually) the expression of ILK-1, β-catenin and Tcf-1 in five cell lines derived from moderately differentiated human squamous cell carcinomas of the oesophagus. It was necessary to first determine whether these three proteins were expressed in oesophageal squamous cell carcinomas (OSCCs). Furthermore the association between Tcf-1 and β-catenin in the nucleus was examined with respect to Tcf-1 binding sites that have previously been said to be found in the promoter sequence of ILK-1.

2.2 Objectives

2.2.1 Determine the expression of β-catenin and Tcf-1 in HOSCC cell lines.

2.2.2 Consider the complexing of β-catenin and Tcf-1 in the nucleus.

2.2.3 Determine ILK-1 expression in HOSCC cell lines.

2.2.4 Examine whether the β-catenin/Tcf-1 complex binds Tcf-1-DNA binding sites, with specific reference to the two Tcf-1 binding sites found in the ILK-1 promoter region.

2.3 Methods and materials

2.3.1 Tissue culture

Five HOSCC cell lines, Wits human carcinoma of the oesophagus (WHCO) WHCO1, WHCO3, WHCO5, WHCO6 (Veale and Thornley, 1989), and SNO, derived from moderately differentiated tumour resections were obtained from the Cell Biology Laboratory, School of Molecular Cell Biology, University of the Witwatersrand. The cell lines were maintained in Dulbecco's Modified Eagle medium (DMEM) (appendix 5.0) supplemented with Hams F12 (3:1) (appendix 5.0) and 10% foetal calf serum (FCS). The cell lines were maintained at 37 degrees Celcius (^{o}C) in a 5% CO_2 atmosphere.

2.3.2 Total cellular protein extraction

Cell lines were grown to 80% confluence in 10 centimetres (cm) Nunc™ tissue culture dishes. To determine the presence of Tcf-1, β-catenin and ILK-1 in the cell lines, total cellular protein extractions were performed on the above mentioned cell lines. The cultures were washed three times in sterile phosphate buffered saline (PBS, appendix 5.0). The cells were then scraped from the culture dishes with a rubber policeman in a small amount (1 ml) of PBS and transferred into a sterile eppendorf tube. The culture was centrifuged for 5 minutes at 1 145 g in a TOMY HF-120-desktop centrifuge. The supernatant was removed and the pellet was suspended in a small amount of Laemmli lysis buffer (Laemmli, 1970; appendix 5.1). Thereafter the samples were boiled for 5 minutes and stored at -20^{o}C.

2.3.3 Nuclear extractions

CelLytic™NuCLEAR EXTRACTION KIT

Nuclear extractions were obtained using the SIGMA CelLytic™ NuCLEAR™ EXTRACTION KIT (catalogue number N-XTRACT-1KT). This kit utilised the

principle where the harvested cells were allowed to swell in a hypotonic buffer (appendix 5.5). Their membranes were then disrupted, the cytoplasmic fractions were removed and the nuclear proteins were released from the nuclei by a high salt buffer (appendix 5.5). Fractions were stored at -20^{o}C.

Nuclear extractions (Wen *et al*., 2003)

The cell cultures were grown to 80% confluence in Nunc™ cell culture dishes and washed three times in PBS at 4^{o}C. Nuclear preparation buffer (1.5 ml, appendix 5.5) was added to the cells and left for 2 hours at 4^{o}C. The cells were then scraped with a rubber policeman in the nuclear preparation buffer and the cell membranes were disrupted with a dounce homogeniser (40-50 strokes). The homogenate was transferred to a sterile eppendorf tube and centrifuged in a Selecta-P Medifriger-BL centrifuge at 13 000 x g for 1 hour. The supernatant (cytoplasmic fraction) was transferred to a sterile eppendorf and stored at -20^{o}C. The pellet was rinsed twice with nuclear preparation buffer and then suspended in 20-30 µl of nuclear extraction buffer (appendix 5.5) followed by briefly vortexing the mixture. The mixture was stored at 4^{o}C overnight. The following day the mixture was transferred to a dounce homogeniser and homogenised for 20-30 strokes. The homogenate was then centrifuged for 25 minutes at 13 000 x g at 4^{o}C. The supernatant (nuclear fraction) was transferred to a sterile eppendorf tube and stored at -20^{o}C.

2.3.4 Protein estimation

Protein concentrations of the various fractions were determined utilising the Bradford assay (Bradford, 1976), following the protocol from Bramhall *et al.* (1969). Whatman® glass fibre discs were rinsed in distilled water (dH_2O) for 20 minutes, followed by three consecutive 5 minute washes in 95% alcohol, 100% alcohol and acetone respectively, to dehydrate the fibre discs, which were then air dried. Bovine serum albumin (BSA) was used to construct a standard curve. Protein samples (5 µl) from each respective cell line were dotted onto the discs and again allowed to air dry. Proteins were fixed to the glass

fibre discs, by placing the discs in 2.5% trichloroacetic acid (TCA, appendix 5.2) for 30-40 minutes with gentle agitation. The discs were subsequently immersed in Coomassie blue stain (appendix 5.2) for 5 minutes to rinse off excess TCA. Thereafter the discs were stained in Coomassie blue for 1 hour with gentle agitation. This was followed by a 2 hour destaining period in acetic acid:methanol:dH_2O (10:12:100) solution, until the background levels were completely removed. The destained protein marks were excised from the discs and placed in 5 ml of elution solution (appendix 5.2) and stored in a darkroom overnight. Absorbance (Abs) readings were measured the following day using a Beckman DU®-64 Spectrophotometer (λ=595 nm). The concentration of the total protein was calculated from the BSA standard curve (y = 0.0245x-0.0054, linear regression (R^2) = 0.9837, appendix 5.2, figure 5.0). The graphs were plotted using Microsoft® Excel.

2.3.5 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were resolved on 8% or 10% SDS polyacrylamide gels (appendix 5.3) according to Laemmli (1970) and run at a constant current of 25 milliamperes (mA) with electrophoresis reservoir buffer pH 8.3 (appendix 5.3). The proteins were visualised with Coomassie blue protein stain (appendix 5.3). Equivalent nuclear protein concentrations were made up to equal volumes with Laemmli double lysis buffer (Laemmli, 1970; appendix 5.1) boiled for 5 minutes and centrifuged at 1 145 x g for 1 minute in a TOMY HF-120-desktop centrifuge prior to loading. All protein samples were run with a PageRuler™ Prestained Protein Ladder (Catalogue number SN0671) from Fermentas, to verify specific protein molecular mass.

2.3.6 Antibodies

β-catenin was specifically detected with a polyclonal antibody supplied by Sigma®, SA (catalogue number C2206). The primary antibody used in this study to detect Tcf-1 was a rabbit polyclonal antibody obtained from Santa Cruz Biotechnology, Inc, USA (catalogue number sc-13025). This antibody was raised against amino acids 1-118,

mapping at the conserved N-terminus of Tcf-1 of human origin. ILK-1 was detected by the rabbit polyclonal anti-ILK-1 antibody obtained from Sigma®, SA (catalogue number I1907). In order to detect the presence of the primary antibodies a secondary antibody was used that binds to the heavy and light chains of the IgG antibodies. A polyclonal horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody was used in Western blot experiments (Sigma®, SA; catalogue number A0545).

2.3.7 Antibody binding assay for primary and secondary antibodies

A dot blot was used to determine the functionality of both the primary and secondary antibodies. A whole cell protein extract (2 µl) was dotted onto a piece of nitrocellulose Hybond™-C pure and allowed to air dry for 5 minutes. The nitrocellulose was blocked for 15 minutes in a 5% non-fat milk blocking buffer (Blotto; appendix 5.4). The nitrocellulose was then rinsed 6 times in PBS, followed by incubating the nitrocellulose in polyclonal rabbit anti-β-catenin primary antibody (1:5 000) for 1 hour. To confirm binding of the primary antibody to the secondary antibody, a second nitrocellulose blot was prepared containing 1 µl of air dried rabbit anti-β-catenin and blocked for 15 minutes. The two nitrocellulose membranes were washed 6 times in PBS at 5 minute intervals and incubated in HRP-bound anti-rabbit secondary antibody (1:10 000) for 1 hour. Both nitrocellulose pieces were again washed 6 times with PBS at 5 minute intervals. A third piece of nitrocellulose was prepared that contained air dried HRP-bound secondary antibody (1 µl). This reaction was performed to ensure reactivity of the secondary antibody with the Chemiluminescent Working Solution. Subsequently, all three blots were reacted with Supersignal® West Pico Chemiluminescent Working Solution (appendix 5.4) for 5 minutes. The three membranes were wrapped in polyethylene saran wrap. The blots were exposed to CL-XPosure™ Film autoradiography film (Pierce, USA, catalogue number 34090) for 5 minutes. The film was placed in D19B developer (appendix 5.4) for 5 minutes, briefly rinsed in water (H_2O), placed in fixer (appendix 5.4) for 5 minutes and rinsed again.

2.3.8 Western blots

Samples from cell lines WHCO1, WHCO3, WHCO5, WHCO6, SNO were resolved on polyacrylamide gels, and transferred onto Hybond™-C pure nitrocellulose membrane (0.45μM) (Amersham, USA) in a BIO-RAD Criterion Blotter at 400 mA for 2 hours in chilled Western blot transfer buffer (appendix 5.4). Following transfer, the nitrocellulose membranes were rinsed three times in TBS (appendix 5.4) and stored overnight at 4^{o}C. In order to determine the efficiency of transfer, the SDS-PAGE gel was stained with Coomassie blue for 1 hour and then destained for 1 hour. To prevent non-specific antibody binding each membrane was blocked in blocking buffer for 1 hour, followed by six washes in PBS. The blots were incubated in a rabbit anti-β-catenin (1: 1000) or anti-Tcf-1 (1:500) or anti-ILK-1 (1:1500) primary antibody for 1 hour. The blots were washed with PBS 6 times at 5 minute intervals to remove any residual antibody. The membranes were then incubated in the HRP-bound secondary anti-rabbit antibody (1:10 000) for 1 hour. The incubations were carried out in the dark to prevent photo bleaching of the antibody. Unbound secondary antibody was removed by washing the membrane six times at 5 minute intervals with PBS. Thereafter a 5 minute incubation followed in Supersignal® West Pico Working Solution (appendix 5.4) from the West Pico Supersignal® Chemiluminescent Substrate Kit (Pierce, USA). The blots were sealed in polyethylene saran wrap and exposed to CL-XPosure™ Film autoradiography film (Pierce, USA, catalogue number 34090) for 5 minutes. Films were developed in D19B developer (appendix 5.4) for 5 minutes, briefly rinsed in H_2O, followed by fixing (appendix 5.4) for 5 minutes and then rinsed briefly again. The experiments were repeated three times.

2.3.9 Co-immunoprecipitation

Nuclear fractions of equal amount of protein (150 μg) of all the cell lines were incubated with 3 μl of undiluted rabbit anti-β-catenin overnight at 4^{o}C on rotating apparatus. The following day 600 μl of Protein G on Sepharose® 4B fast flow (Sigma, SA; catalogue number P-3296) beads in 20% ethanol were washed in 900 μl of immunoprecipitation (IP)

buffer (appendix 5.6). The IP buffer was added to the beads and the mixture was gently flicked to mix. The contents were then centrifuged at 12 000 x g in a Sorvall® MC 12 V for 30 seconds and the supernatant was removed. This was repeated three times. 600 µl of IP buffer was added to the beads and of that 100 µl was aliquoted to each nuclear sample. The samples were again incubated overnight rotating at 4°C. Thereafter the samples were centrifuged at 12 000 x g in a Sorvall® MC 12 V centrifuge at room temperature for 15 - 30 seconds. Supernatants were discarded and the pellets of each sample were suspended in 700 µl of IP buffer and centrifuged at 12 000 x g at room temperature for 30 seconds in a Sorvall® MC 12 V centrifuge. The supernatant was discarded and the washing step was repeated once more but with 0.1X IP buffer. The supernatant was again decanted and the pellets were suspended in 40 µl of 1X Laemmli lysis buffer and boiled for 5 minutes. Thereafter the mixture was centrifuged at 12 000 x g for 5 minutes in a Sorvall® MC 12 V centrifuge at room temperature. The supernatant was transferred to a sterile eppendorf tube and stored at -20°C. The product was resolved on a 10% SDS-PAGE gel and transferred to a Hybond™-C pure nitrocellulose membrane in a BIO-RAD Criterion Blotter at 400 mA for 2 hours in chilled Western blot transfer buffer. The blot was probed for Tcf-1 using standard antibody concentrations. Thereafter blots were exposed to Supersignal® West Pico Working Solution from the West Pico Chemiluminescent Substrate Kit (Pierce, USA) for 5 minutes and then sealed in polyethylene saran wrap and exposed as described above in section 2.3.8.

2.3.10 Annealed oligonucleotides

Two 5'-biotinylated oligonucleotides 5'-BioTEG-GCAGGGTCGCCGAGTCCCCGCTCTTCTCCACGTGCACAGCGCTCCT-3' (Tcf-1.1) and 5'-BioTEG-ACCGACCCGGAAACCAAAGCGTGACAGCCAG-3' (Tcf-1.2) and their corresponding complementary sequences 5'-CGTCCCAGCGGCTCAGGGGCGAGAAGAGGTGCACGTGTCGCGAGGA-3' and 5'-TGGCTGGGCCTTTGGTTTCGCACTGTCGGTC-3' respectively were obtained from Operon (the underlined regions indicate the Tcf-1 binding sites determined by Melchior *et al*. (2002)). Tcf-1.1 and Tcf-1.2 are the names designated to each of the double

stranded oligonucleotides throughout the text. The oligonucleotides were dissolved in tris EDTA (TE) pH 8.0 (appendix 5.7). 10 µl of the biotinylated and unlabeled corresponding complementary DNA sequences were alliqotted into a sterile eppendorf and 10 µl of NaCl tris EDTA (STE) buffer (appendix 5.7) was added to the oligonucleotides. The presence of salt is necessary for the sequences to hybridise. The contents were gently mixed and placed in a heating block at 95^{o}C for 10 minutes. The heating block was then turned off and the oligonucleotides were allowed to cool gradually in the heating block. The resulting product was stored at -20^{o}C.

2.3.11 Resolving and detecting double stranded oligonucleotides

A non-denaturing polyacrylamide gel was used to resolve the double-stranded DNA. A 15% acrylamide gel was poured and run in 1X tris boric acid EDTA (TBE) (appendix 5.8) at a low voltage (50 volts (V)) with a H_2O cooling system maintained at a constant temperature of 10^{o}C. The low temperature is necessary to prevent denaturation of small fragments of DNA by heat that is generated by the electric current. Before loading the samples, the wells of the gel were flushed out with 1X TBE and the gel was pre-electrophoresed for 30 minutes. The samples were loaded with agarose gel loading buffer (appendix 5.8) and run for 3 hours. To detect the bands the gel was gently submerged in staining solution (appendix 5.8) for 45 minutes with gentle agitation at room temperature in the dark. The gel was then placed on a piece of saran wrap and viewed on an ultraviolet (UV) transilluminator.

2.3.12 Binding assay of double stranded oligonucleotides

A dot blot of both double stranded oligonucleotides was performed. 2 µl of each DNA fraction was dotted onto a small piece of Hybond-N^{+} nylon membrane and allowed to air dry. The DNA was cross linked using an UV lamp (312 nm) for 15 minutes and the membrane was prepared for detection according to the LightShift® Chemiluminescent EMSA Kit (Pierce, USA). The nylon membrane was wrapped in a piece of saran wrap and exposed according to the description in section 2.3.8

2.3.13 Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) technique is based on the principle that DNA-protein complexes migrate slower than unbound DNA in a native polyacrylamide gel (appendix 5.9), resulting in a "shift" in migration of the labelled DNA band. The LightShift® Chemiluminescent EMSA kit from Pierce, USA (catalogue number 20148) was used to detect binding of protein complexes to the two Tcf-1 DNA-binding sites in the ILK-1 promoter region. The nuclear fraction needed for this analysis was obtained using the above mentioned Wen *et al.* (2003) extraction method (see section 2.3.3). 8 µg of nuclear extract was added to the binding reaction mixture, and 1 µl of undiluted rabbit anti-β-catenin polyclonal antibody (Sigma, SA). This reaction was incubated for 1 hour at 25^{o}C. Thereafter, 1 µl (2 fmol) of labelled biotinylated double stranded oligonucleotides was added to the binding reaction, followed by another 30 minute incubation at 25^{o}C in the dark. The DNA-protein complexes were resolved on a 5% native polyacrylamide gel in (0.5X TBE buffer) at 50 V. The DNA-protein complexes were transferred to a positively charged Hybond-N^{+} nylon membrane (catalogue number RPN1219B) in a BIO-RAD Criterion Blotter at 400 mA for 1 hour in chilled 0.5X TBE. The complexes were then cross-linked to the nylon membrane using a UV transilluminator (312 nm) for 15 minutes. The DNA-protein complexes bound to the nylon membrane were prepared according to the instructions of the LightShift® Chemiluminescent EMSA kit from Pierce, USA. The oligonucleotides were labelled with biotin which has a high affinity for the bacterial protein strepavidin that is conjugated to a reporter enzyme such as horseradish peroxidise. The introduction of chemilumenescent that upon hydrolysis by the enzyme emits UV light provides a very sensitive method of detection. The membrane was then wrapped in saran wrap and exposed as in section 2.3.8.

2.3.14 Densitometry

All Western blots were semi-quantitatively analysed by densitometry using the LabWorks™ Image Acquisition and Analysis Software Version 4.5 program in order to

compare the levels of a specific protein between the various cell lines. The area under the peak for each sample was used as a basis for comparison between samples.

2.3.15 Image capturing

Images were captured on a Hewlard Packard Scanjet 4400c series scanner. DNA-PAGE gel was captured on a BioDoc-It™ System (UVP, Inc, USA).

2.4 Results

SDS-PAGE separation of the total cellular protein and nuclear fractions obtained from the WHCO1, WHCO3, WHCO5, WHCO6 and SNO cell lines (figure 2.0 a and b respectively) yielded a distinct banding pattern across the cell lines for each fraction. Upon further examination of the polypeptide profiles for both SDS-PAGE gels, banding was noted at 94 kDa (β-catenin), in the region of Tcf-1 isoforms known molecular weights, 55 kDa to 22 kDa (as mentioned there are 7 isoforms of Tcf-1) there was a distinct banding pattern, and there were polypeptides of appropriate molecular weight at the 59 kDa region, which is said to be the molecular weight of ILK-1 (Hannigan *et al.*, 1997).

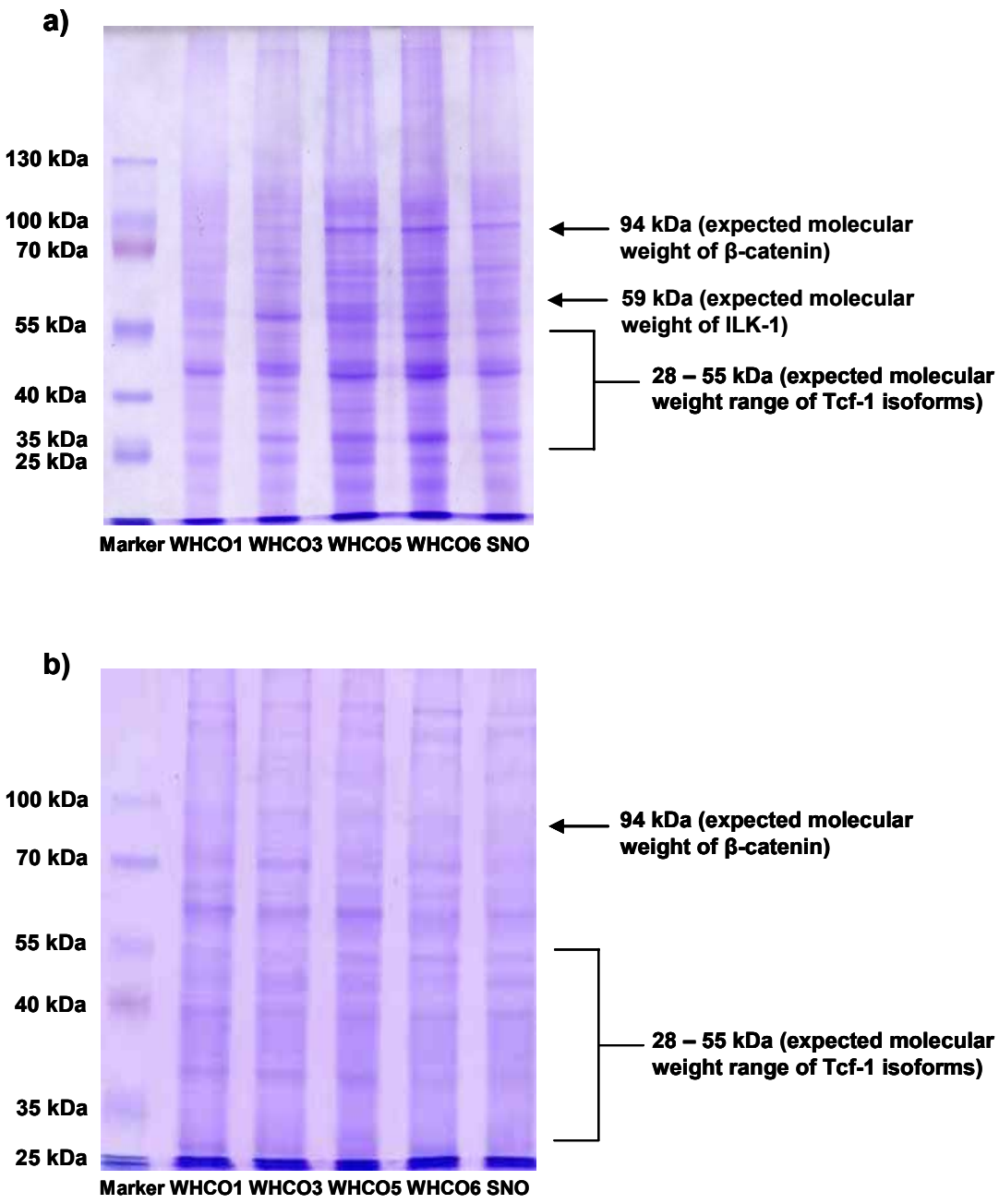
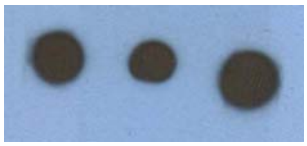


Figure 2.0: SDS-PAGE resolution of total cellular and nuclear protein extracts.

(a) Total cellular extract and (b) nuclear protein extract were obtained from the OSCC cell lines and distinct banding patterns were present for all of the samples separated. Low molecular weight markers were separated, the size of the bands (kDa) produced by the markers are indicated next to the corresponding band.

To confirm that both β-catenin and Tcf-1 were present in the cell lines Western blot analysis was performed. However before proceeding with Western blot analysis, a dot blot procedure was used to assess optimum antibody concentrations and confirm that the reactivity of the antibodies were satisfactory. Dot blots demonstrated that rabbit anti-β-catenin (1: 5 000) was bound by the HRP-bound anti-rabbit secondary antibody (1:10 000) (figure 2.1b) and that the HRP-bound anti-rabbit secondary antibody (1:10 000) (figure 2.1c) reacted with the Supersignal® West Pico Chemiluminescent substrate efficiently. Total protein extract was pre-absorbed onto nitrocellulose and demonstrated a strong reactivity with the β-catenin antibody (figure 2.1a). The HRP-bound secondary antibody (bound to the rabbit anti-β-catenin antibody) reacted with the Supersignal® West Pico Chemiluminescent solution (figure 2.1b). This same procedure was used to determine reactivity of the rabbit anti-Tcf-1 antibody (1:500) and the HRP-bound anti-rabbit secondary antibody (1:5 000) with the Supersignal® West Pico Chemiluminescent substrate. Total protein was also used to validate the reaction with both the Tcf-1 antibody and the secondary antibody.



a **b** **c**

a = rabbit anti-β-catenin antibody bound to β-catenin in total protein extract bound

b = rabbit anti-β-catenin antibody bound to HRP-bound anti-rabbit secondary antibody

C = HRP-bound anti-rabbit secondary antibody

Figure 2.1: Dot blot to determine usage of rabbit anti-β-catenin antibody

Western blot analysis revealed the presence of a polypeptide, specifically recognised by anti-β-catenin across all the cell lines (figure 2.2a). Furthermore, densitometric analysis revealed that, for standardized protein concentrations WHCO6 and SNO showed the

highest levels of β-catenin expression whereas β-catenin expression in the WHCO3 line was shown to be 2 fold less than WHCO6 and SNO (figure 2.2b). The third highest level of β-catenin expression in WHCO5 revealed a 1.8 fold higher level of β-catenin expression compared to WHCO3 and a 1.5 higher level of expression when compared to WHCO1. The levels of β-catenin varied over a range of 50%.

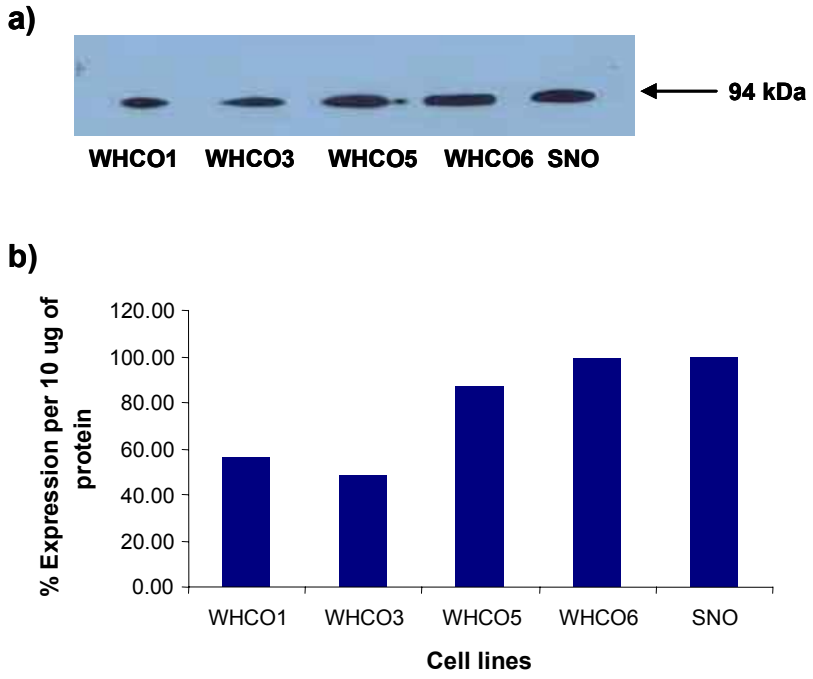


Figure 2.2: β-catenin protein expression in the cell lines from total cellular extracts.

(a) Western blot analysis of β-catenin expression across all the cell lines. (b) Densitometric analysis of β-catenin expression across all five human oesophageal SCC lines under standard culture conditions. Note: The expression levels are representative as a percentage of maximum per 10 µg of protein from total cellular protein fractions.

Analysis of nuclear β-catenin revealed similar results. Per 10 µg of protein, the levels of β-catenin also varied over a range of 50% (figure 2.3b). Similar to total cell protein extract results, WHCO6 showed the highest level of nuclear β-catenin and WHCO1 had 2 fold less β-catenin levels. Since it has been confirmed that β-catenin is expressed in the human oesophageal SCCs it was necessary to determine expression of Tcf-1 in the HOSCC lines.

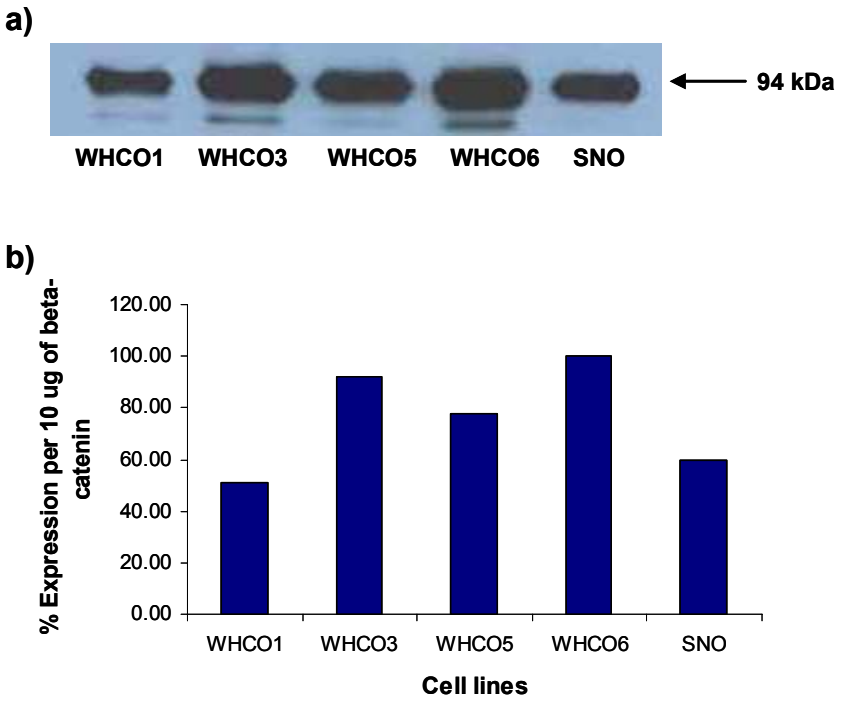


Figure 2.3: β-catenin protein expression in the cell lines from nuclear extracts.

(a) Western blot analysis of β-catenin expression in the nucleus across all the cell lines. An 80 kDa band was also exposed, partial degradation product. (b) Densitometry of β-catenin expression across all five human oesophageal SCC lines under standard culture conditions. Note: The expression levels are representative as a percentage of maximum per 10 µg of protein from nuclear protein fractions.

Western blot analysis using a polyclonal antibody specific for Tcf-1, revealed the presence of a definite Tcf-1 polypeptide (figure 2.4a), specifically recognised by the anti-Tcf-1 antibody at an apparent molecular weight of 30 kDa, this molecular weight is representative of the Tcf-1A/C/G isoforms. An additional polypeptide below the 30 kDa polypeptide band was noted in WHCO1, WHCO3, WHCO5, WHCO6 (figure 2.4a) this polypeptide is likely to be representative of Tcf-1D/F (28.3 kDa and 28.6 kDa respectively). This polypeptide was not noted in WHCO6 (figure 2.4 a and b). Subsequent densitometric analysis showed that the total Tcf-1 expression levels were highest in both WHCO6 and WHCO1. WHCO1, WHCO3, WHCO6 and SNO display

similar but higher levels of total Tcf-1 in comparison to WHCO5. WHCO6 had a 2 fold higher expression of Tcf-1 compared to WHCO5. Across all the cell lines Tcf-1 isoforms of molecular weight 30 kDa were noted, with the highest level found in WHCO6 however no 28 kDa band was noted in this line. The 30 kDa Tcf-1D/F isoforms were observed in WHCO1, WHCO3, WHCO5, SNO lines only, with the highest level of relative expression noted in WHCO5.

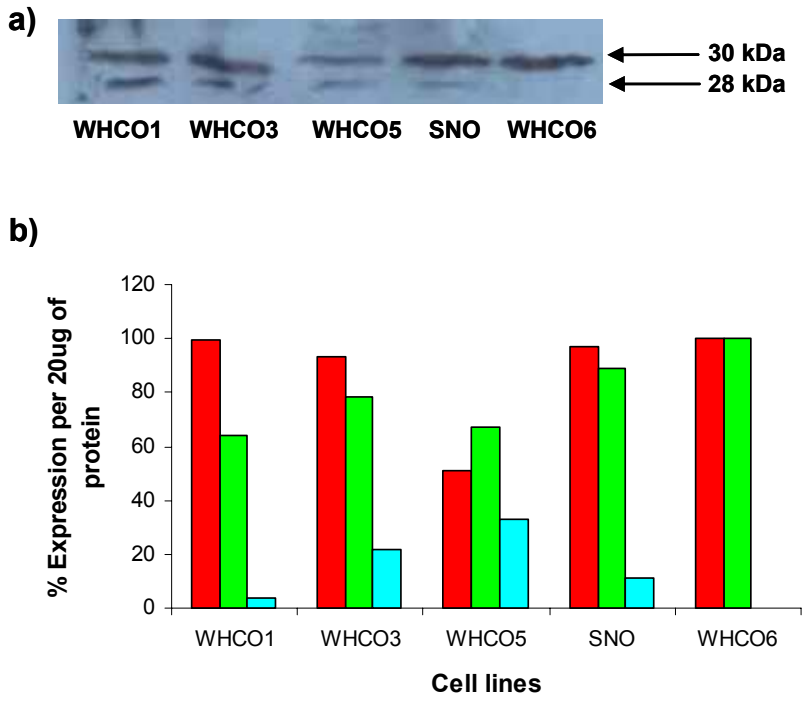


Figure 2.4: Tcf-1 protein expression in cell lines from total cellular extracts.

(a) Western blot analysis of Tcf-1expression across all the cell lines, Tcf-1A/C/G isoforms (30 kDa) and Tcf-1D/F isoforms (28 kDa). (b) Densitometry of Tcf-1 expression across all five human oesophageal SCC lines under standard culture conditions. The red bars indicate the relative percentage for the total cellular Tcf-1 expression, green bars are representative of Tcf-1A/C/G isoforms (30 kDa) and the light blue bars represent the Tcf-1D/F isoforms (28 kDa). Note: The expression levels are representative as a percentage of maximum per 20 µg of protein from total cellular protein fractions.

In order to detect nuclear Tcf-1 an alternative extraction method was used. Although the SIGMA CelLytic™ NuCLEAR™ EXTRACTION KIT yielded nuclear extract that was sufficient for nuclear protein Western detections of β-catenin/Tcf-1 co-

immunoprecipitation by Western blot detection. The abovementioned extraction kit did not however yield sufficient nuclear product to allow satisfactory detection of nuclear Tcf-1 by Western blotting or EMSA. van de Wetering *et al.* (1996) have found that Tcf-1 protein is absent from standard nuclear extracts such as the SIGMA CelLytic™ NuCLEAR™ EXTRACTION KIT. This was determined by both Western blotting and gel retardation (van de Wetering *et al*., 1996). It was however readily visualised when van de Wetering and colleagues (1996) performed Western blot analysis on total cellular protein. This finding was also true for this study (results not shown). With that noted an alternative nuclear extraction protocol of Wen *et al.* (2003) was used to detect nuclear Tcf-1. Western blot analysis of these extracts revealed 3 isoforms (figure 2.5a) Tcf-1E (55 kDa), Tcf-1B (44.9 kDa), Tcf-1A/C/G (30 kDa) within the nucleus (Hovanes *et al*., 2000; van de Wetering *et al*., 1996). The nuclear profile of Tcf-1 isoforms across the HOSCC cell lines is consistent however it is different when compared to the WHCO6 total protein extract control (figure 2.5a and b, lane 6). Densitometric analysis revealed that the highest level of Tcf-1 was found to be in the SNO line. SNO had a 3 fold higher total Tcf-1 (figure 2.5b, red bar) expression relative to WHCO1 which was shown to have the second highest total Tcf-1 expression (figure 2.5b, red bar). Upon examining the different isoforms within the lines, it was noted that in WHCO5, WHCO6, and SNO the Tcf-1A/C/G (30 kDa) isoforms were predominantly present relative to the total Tcf-1 expression for each cell line respectively. Tcf-1A/C/G was found to be expressed highest in the SNO line. The lowest expression of the 30 kDa Tcf-1 isoforms was noted in WHCO1 which had an 18 fold lower expression than the SNO cell line. However, WHCO1 had an 11.6 fold higher expression of Tcf-1B than that of the SNO line, which had the lowest Tcf-1B expression.

Finally, the cell lines showed expression of the Tcf-1E isoform (figure 2.5a and b) however, Tcf-1E expression was not evident in the total cell protein extract and was barely noteworthy in the SNO line total cell protein extract. The highest expression of nuclear Tcf-1E was evident in the WHCO3 line, with approximately 70% of the total Tcf-1 nuclear expression to be that of Tcf-1E. Tcf-1E expression in WHCO3 was 14 fold

higher than the expression of WHCO6 which showed the second lowest Tcf-1E expression across the five lines.

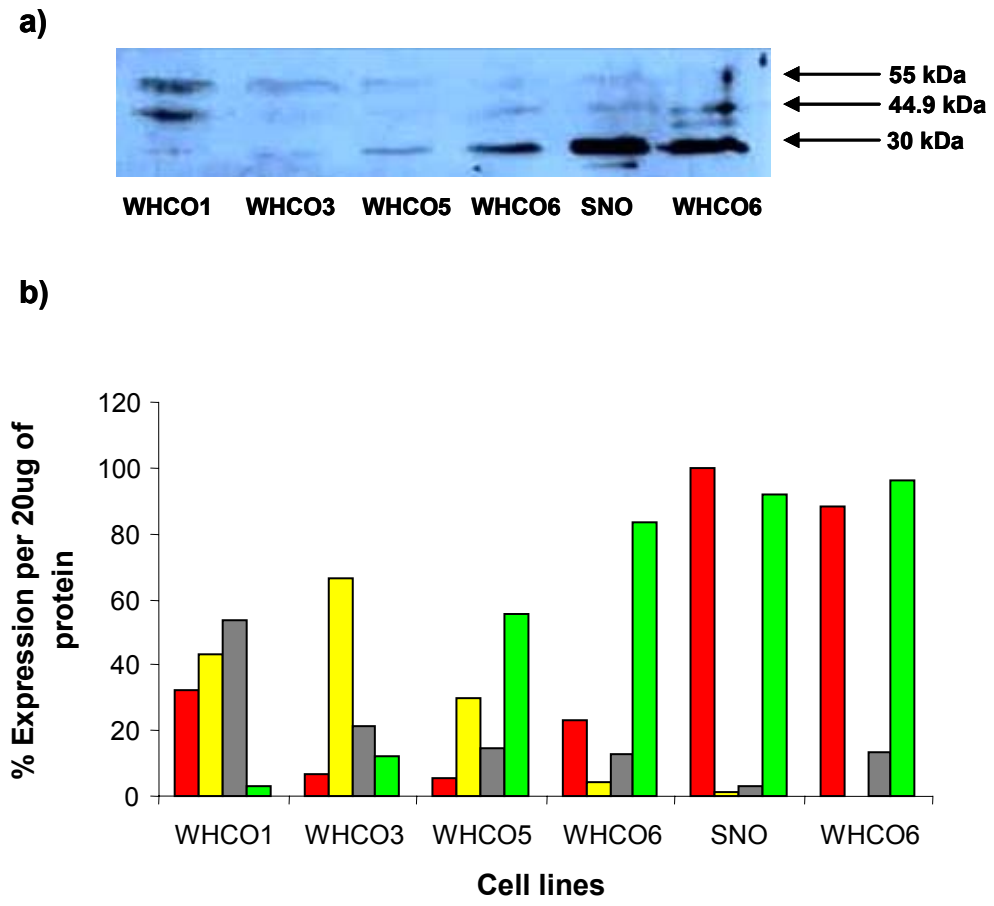


Figure 2.5: Tcf-1 protein expression profile in the cell lines from nuclear extracts.

(a) Western blot analysis of total nuclear Tcf-1 expression across all the cell lines. Arrows indicate different Tcf-1 isoforms, 55 kDa (Tcf-1E), 44.9 kDa (Tcf-1B), 30kDa (Tcf-1A/C/G). Lane 6 = WHCO6 total protein extraction control. (b) Densitometry of total nuclear Tcf-1 expression across all five human oesophageal SCC lines under standard culture conditions (yellow bars = Tcf-1E; grey bars = Tcf-1B; green bars = Tcf-1A/C/G). The red bars indicate the relative percentage for the total cellular Tcf-1 expression. Note: The expression levels are representative as a percentage of maximum per 20 µg of protein from nuclear protein fractions.

β-catenin and Tcf-1 are expressed in all the human oesophageal SCCs under study. Both proteins localise in the nucleus, as previously shown in colorectal squamous cell

carcinoma of intestinal epithelium, mammary epithelia and thymus (Clevers and van de Wetering, 1997; Sancho *et al*., 2003; Waterman., 2004); these proteins function together to induce gene expression. It would be of relevance for this study to determine whether localisation occurs between β-catenin and Tcf-1 in oesophageal SCCs. Co-immunoprecipitation analysis was preformed to confirm that Tcf-1 and β-catenin complex in the nucleus. In all the cell lines analysed, β-catenin and Tcf-1 were indeed shown to co-immunoprecipitate. Two polypeptides at 55 kDa and 30 kDa for Tcf-1 were obtained following protein G sepharose immunoprecipitation and Western blotting (figure 2.6a). All five cell lines indicated that β-catenin predominantly complexed with Tcf-1E, determined by protein densitometry analysis (figure 2.6b) and to lesser degree the 30 kDa polypeptide indicated that β-catenin complexed with Tcf-1 A/C/D/F/G, whose molecular weights range between 28.3 kDa - 30.3 kDa. It is noted that it is not possible from this analysis to determine which Tfc-1 isoform between 28.3 kDa - 30.3 kDa is bound to β-catenin, because of the width of the bands seen in the Western blot (figure 2.6a).

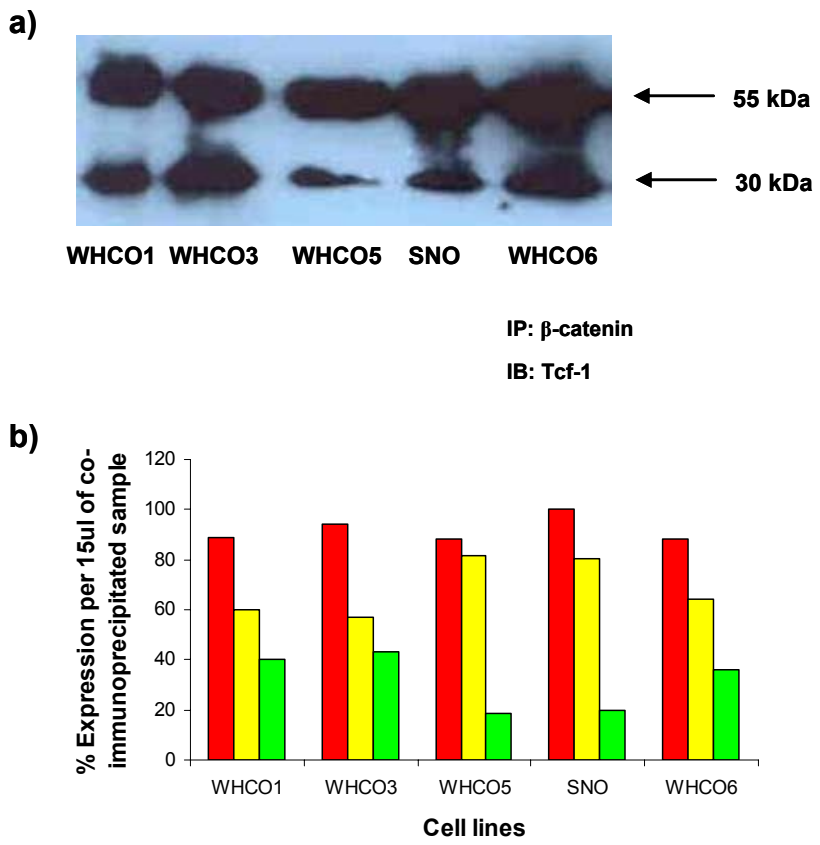


Figure 2.6: β-catenin and Tcf-1 proteins co-immunoprecipitated from nuclear extracts.

(a) Western blot analysis following protein G immunoprecipitation resulted in a band at 55 kDa for the Tcf-1E isoform and a 30 kDa band indicating Tcf-1A/C/G in all the cell lines. Note: A whole cell WHCO6 (20 µg) sample was used as a control. (b) Densitometric analysis of Tcf-1, the relative percentage of Tcf-1 that complexes with β-catenin across the cell lines is shown (red bar = total Tcf-1 content, yellow bar = Tcf-1E, green bar = Tcf-1A/C/G). IP = Immunoprecipitating antibody; IB = Immunoblotting antibody

So far this study has established that HOSCC cells contain β-catenin and Tcf-1 in the nucleus and that these two proteins associate in the nucleus. Therefore, on these grounds analysis could continue along the direction of whether this β-catenin-Tcf-1 complex binds to DNA, specifically within the promoter region of ILK-1, probing the question, of 'cross talk' between the PI3K and Wnt signalling pathways. However the investigation into DNA-protein complexing at these particular transcription factor binding sites requires clarification that ILK-1 is in fact expressed in all the HOSCC cell lines.

For that reason, total cellular protein fractions from all the cell lines were resolved on an 8% SDS-PAGE gel followed by Western blot analysis using the rabbit anti-ILK-1 polyclonal antibody (Sigma, SA). Indeed ILK-1 was expressed in all the cell lines (figure 2.7a) and densitometric analysis indicated that ILK-1 expression under standard conditions is similar across the cell lines (figure 2.7b).

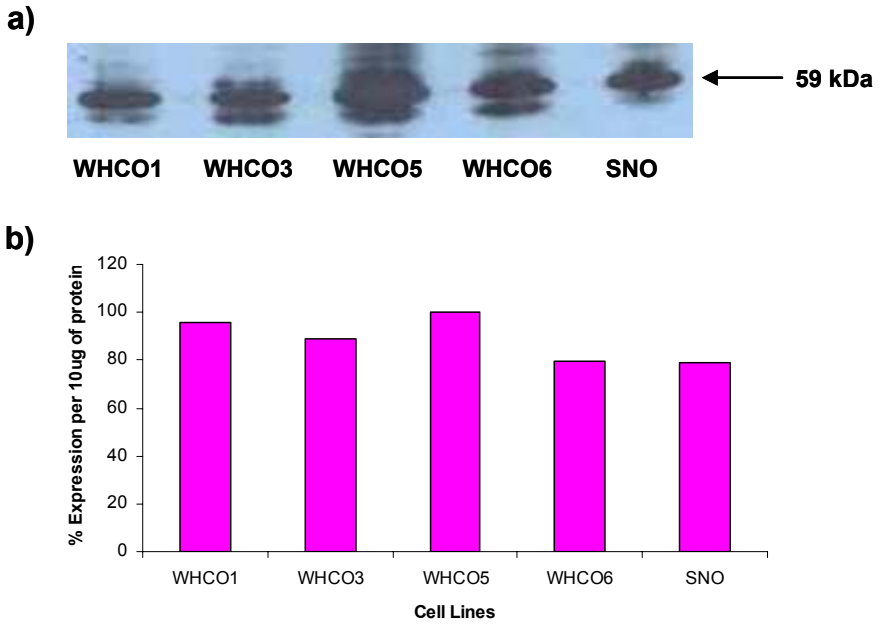


Figure 2.7: ILK-1 protein expression in the cell lines from total cellular extracts.

(a) Western blot analysis of ILK-1 expression across all the cell lines. (b) Densitometry of ILK-1 expression across all five human oesophageal SCC lines under standard culture conditions. Note: The expression levels are representative as a percentage of maximum per 10 µg of protein from total cellular protein fractions and only the upper band was used to determine densitometry and not the doublet.

Expression was the highest in the WHCO5 line and it was noted that this line was 20% higher in expression than the SNO and WHCO6 lines which showed a uniform expression level (figure 2.7b). The second highest ILK-1 expression level was found to be that of WHCO1, followed by WHCO3 that was shown to have a 10% higher

expression level than the WHCO6 and SNO lines. The additional bands noted on the Western blot are thought to be representative of two things; either detergent-resistant ILK-1 complexes or other ILK-related proteins (Huang *et al*., 2000).

To deduce whether β-catenin and Tcf-1 actually complex at the previously mentioned Tcf-1 transcription factor binding sites in the ILK-1 promoter region, gel retardation assay analyses also referred to as EMSA were performed on the WHCO6, SNO and WHCO3 cell lines. Both the WHCO6 and SNO lines were chosen to analyse because these cell lines were shown to have high levels of Tcf-1 in the nuclear region and therefore would allow the greatest possibility of success for the gel retardation assay. WHCO3 was chosen as it was representative of cell lines with low Tcf-1 expression levels.

In order to successfully perform the EMSA analyses it was important to ensure that the oligonucleotides had successfully annealed and that there were not a large proportion of unbound oligonucleotides present in the sample. For each oligonucleotide sample, the goal was to denature the complementary strands to remove any secondary structures and then allow the strands to hybridise. Unbound oligonucleotides could distort the results by binding with protein and migrating an incorrect distance in the PAGE gel. Furthermore, unbound oligonucleotides are not conformationally stable and are more prone to conformational change and so inhibiting protein/DNA binding. Another consideration to note is that Tcf-1 DNA binding conformationally changes the DNA (Graham *et al*., 2000). Proteins containing HMG-domains bind within the minor groove and bend the double helix and the final nine residues of the DNA-binding domain of Tcf-1 swing over the minor groove and back under to make specific contacts with the phosphate backbone in the bent major groove (Graham *et al*., 2000; Prieve *et al*., 1998). Therefore it was sensible to create double stranded oligonucleotides in order to ensure that the Tcf-1/DNA binding in the EMSAs were a 'predictable' uniform component of the reactions, and to allow this reaction to mimic *in vivo* binding as much as possible. The production of double stranded oligonucleotides was confirmed by resolving 10 µl of annealed oligonucleotides on a 15% native-PAGE (appendix 5.9) and staining the PAGE gel with

ethidium bromide (EtBr) (0.5 µg/ml, appendix 5.8) (figure 2.8a). Once this was completed it was necessary to confirm that the biotin tag was still bound to the oligonucleotides resulting in successful detection. The resulting dot blots (figure 2.8b) indicated that the prepared oligonucleotides were suitable for the EMSA experiments.

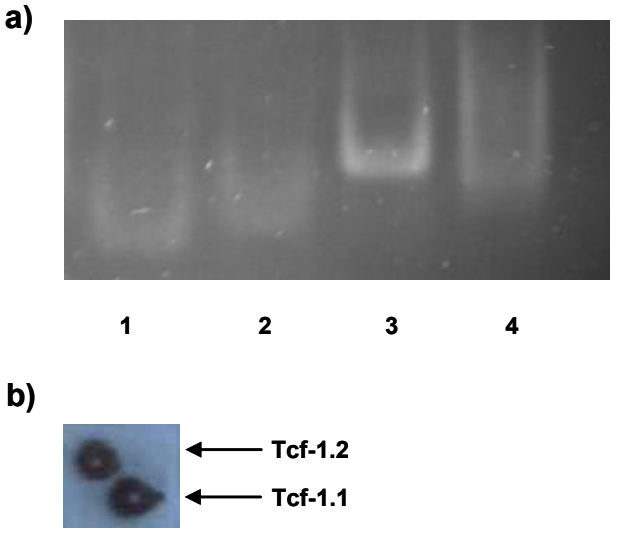


Figure 2.8: Analysis of the functionality of Tcf-1 oligonucleotides required for EMSA.

(a) SDS-PAGE of annealed oligonucleotides for Tcf-1.2 (lane 3) and .Tcf-1.1(lane 4). Lane 1 = single stranded biotinylated oligonucleotide, lane 2 = single stranded complementary oligonucleotide. (b) Dot blot of double stranded oligonucleotides TCF-1.2 and Tcf-1.1 to confirm the functionality of the biotin tag after annealing.

The EMSA analyses have shown that both Tcf-1 and β-catenin do actually bind to the two Tcf-1 DNA binding sites in three cell lines (WHCO3 figure 2.9 lanes 2 and 4, WHCO6 figure 2.10 lanes 2 and 4, SNO; figure 2.11 lane 2 and 4). This provides evidence and confirms that the two Tcf-1 sites in the ILK-1 promoter region are *bona fide* Tcf-1 binding sites.

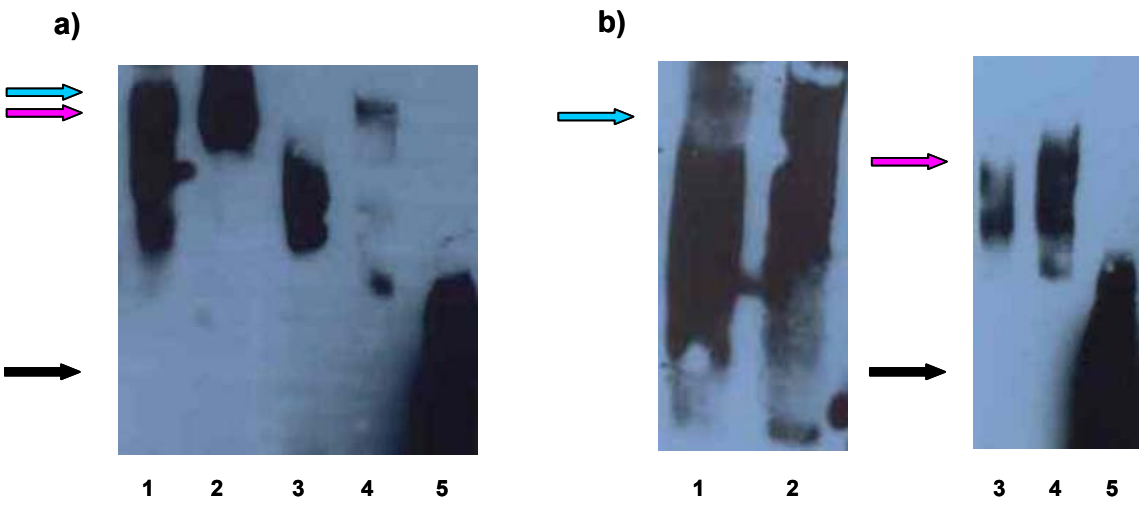


Figure 2.9: β-catenin/Tcf-1 protein complex in the WHCO3 cell line.

Binding to two different Tcf-1 DNA-binding sites occurred in the ILK-1 promoter region of the WHCO3 cell line. (a) EMSA with the anti-β-catenin antibody was performed. The light blue arrow indicates the supershift (lane 2) of the complex with the Tcf-1.1 oligonucleotide. Lane 1 depicts the DNA-protein complex shift (a large amount of protein drag is noted). The purple arrow indicates the supershifted β-catenin/Tcf-1 complex in lane 4 and lane 3 depicts the shifted β-catenin/Tcf-1 complex, both are bound to theTcf-1.2 oligonucleotide. (b) EMSA with the anti-Tcf-1 antibody was performed to ensure that the complex definitely included Tcf-1. Lane 2 = supershifted β-catenin/Tcf-1 complex (light blue arrow) bound to Tcf-1.1 oligonucleotide, lane 1= shifted DNA-protein complex bound to Tcf-1.1 oligonucleotide. Lane 4 = supershifted β-catenin/Tcf-1 complex (purple arrow) bound to Tcf-1.2 oligonucleotide, lane 3 = shifted DNA-protein complex bound to Tcf-1.2 oligonucleotide. The black arrow in both (a) and (b) indicates the negative control in lanes 5 (free probe; no nuclear extract was added to the control binding reaction).

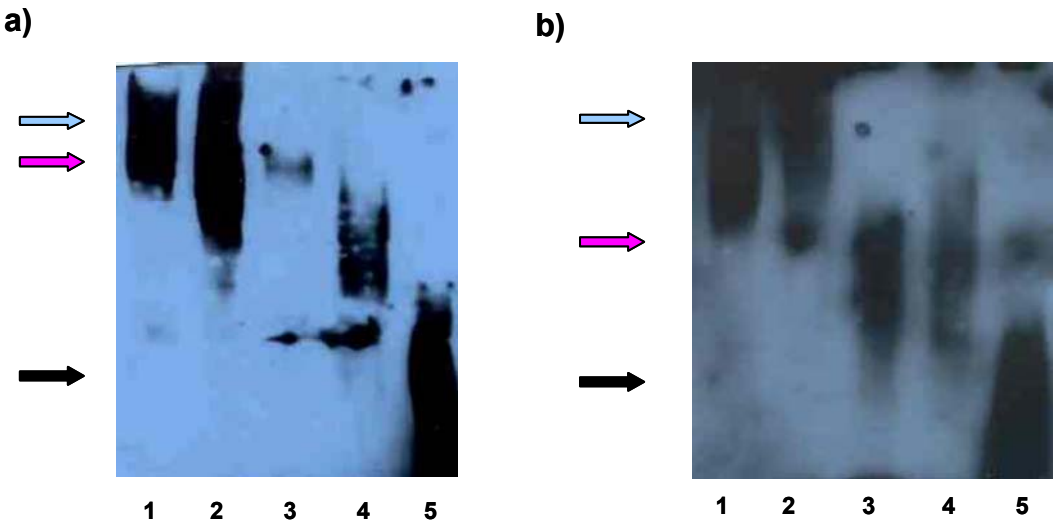


Figure 2.10: β-catenin/Tcf-1 protein complex in the WHCO6 cell line.

Binding to two different Tcf-1 DNA-binding sites occurred in the ILK-1 promoter region of the WHCO6 cell line. (a) EMSA with the anti-β-catenin antibody was performed. The light blue arrow indicates the supershift (lane 1) of the complex with the Tcf-1.1 oligonucleotide. Lane 2 depicts the DNA-protein complex shift (a large amount of protein drag is noted). The purple arrow indicates the supershifted β-catenin/Tcf-1 complex in lane 3 and lane 4 depicts the shifted β-catenin/Tcf-1 complex, both are bound to theTcf-1.2 oligonucleotide. (b) EMSA with the anti-Tcf-1 antibody was performed to ensure that the complex definitely included Tcf-1. Lane 2 = supershifted β-catenin/Tcf-1 complex (light blue arrow) bound to Tcf-1.1 oligonucleotide, lane 1= shifted DNA-protein complex bound to Tcf-1.1 oligonucleotide. Lane 4 = supershifted β-catenin/Tcf-1 complex (purple arrow) bound to Tcf-1.2 oligonucleotide, lane 3 = shifted DNA-protein complex bound to Tcf-1.2 oligonucleotide. The black arrow in both (a) and (b) indicates the negative control in lanes 5 (free probe; no nuclear extract was added to the control binding reaction).

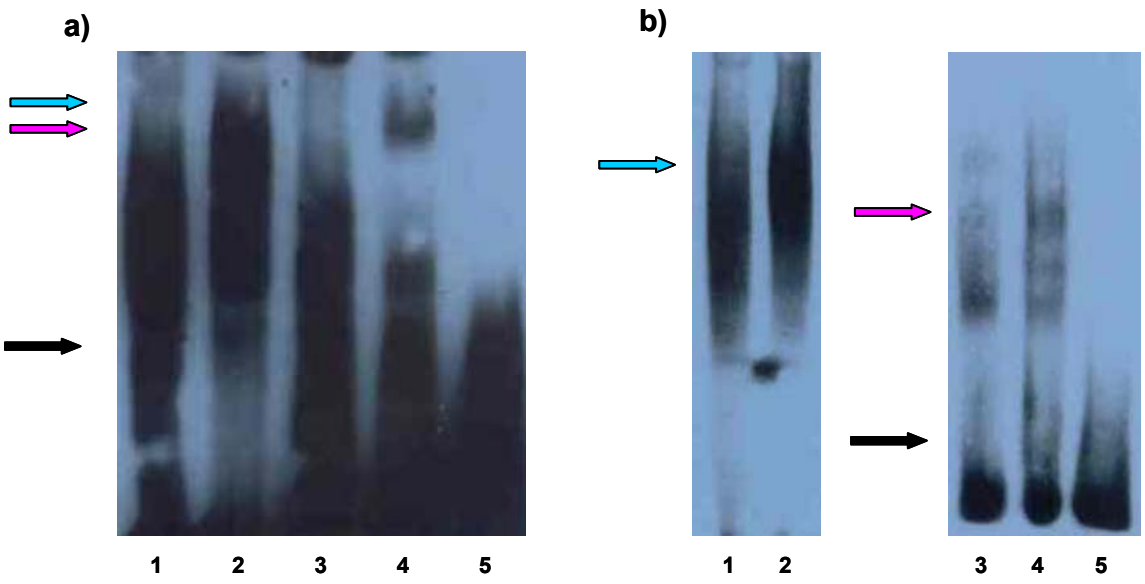


Figure 2.11: β-catenin/Tcf-1 protein complex in SNO cell line.

Binding to two different Tcf-1 DNA-binding sites occurred in the ILK-1 promoter region of the SNO cell line. (a) EMSA with the anti-β-catenin antibody was performed. The light blue arrow indicates the supershift (lane 2) of the complex with the Tcf-1.1 oligonucleotide. Lane 1 depicts the DNA-protein complex shift (a large amount of protein drag is noted). The purple arrow indicates the supershifted β-catenin/Tcf-1 complex in lane 4 and lane 3 depicts the shifted β-catenin/Tcf-1 complex, both are bound to theTcf-1.2 oligonucleotide. (b) EMSA with the anti-Tcf-1 antibody was performed to ensure that the complex definitely included Tcf-1. Lane 2 = supershifted β-catenin/Tcf-1 complex (light blue arrow) bound to Tcf-1.1 oligonucleotide, lane 1= shifted DNA-protein complex bound to Tcf-1.1 oligonucleotide. Lane 4 = supershifted β-catenin/Tcf-1 complex (purple arrow) bound to Tcf-1.2 oligonucleotide, lane 3 = shifted DNA-protein complex bound to Tcf-1.2 oligonucleotide. The black arrow in both (a) and (b) indicates the negative control in lanes 5 (free probe; no nuclear extract was added to the control binding reaction).

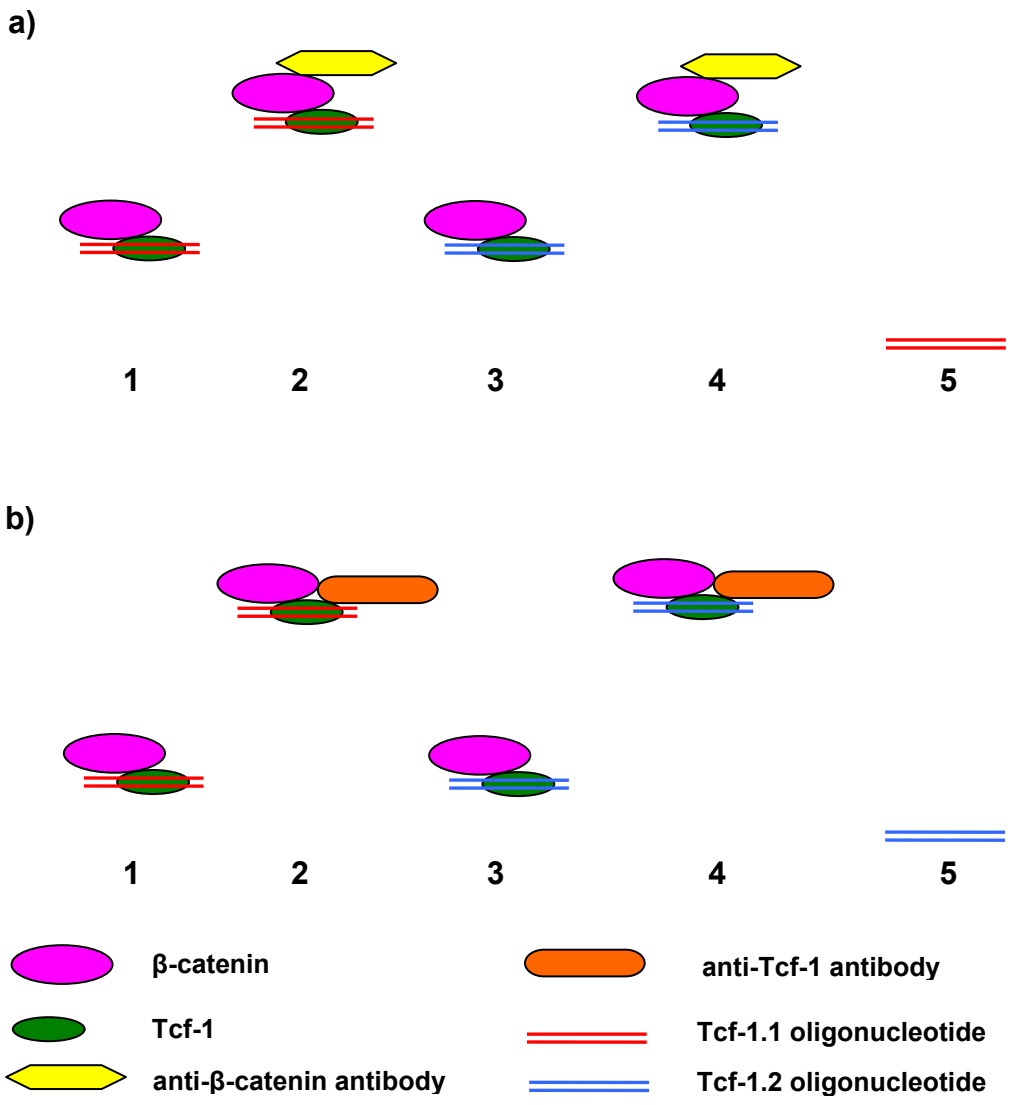


Figure 2.12: Diagram depicting the binding of two different Tcf-1/DNA-binding sites by a β-catenin/Tcf-1 protein complex.

Binding to two different Tcf-1 DNA-binding sites occurred in the ILK-1 promoter region. (a) EMSA with the anti-β-catenin antibody was performed. Lane two shows the supershift of the complex with the Tcf-1.1 oligonucleotide. Lane 1 depicts the DNA-protein complex shift. Lane 4 depicts the supershifted β-catenin/Tcf-1 complex and lane 3 shows the shifted β-catenin/Tcf-1 complex, both are bound to the Tcf-1.2 oligonucleotide. (b) EMSA with the anti-Tcf-1 antibody. Lane 2 = supershifted β-catenin/Tcf-1 complex bound to Tcf-1.1 oligonucleotide, lane 1 = shifted DNA-protein complex bound to Tcf-1.1 oligonucleotide. Lane 4 = supershifted β-catenin/Tcf-1 complex bound to Tcf-1.2 oligonucleotide, lane 3 = shifted DNA-protein complex bound to Tcf-1.2 oligonucleotide. The negative control in lanes 5 (free probe; no nuclear extract was added to the control binding reaction).

The EMSAs showed two major shifted bands in comparison to the negative control (free probe). The lower of the two bands (figure 2.9a, lane 1 and 3), was possibly shifted by the binding of the Tcf-1/β-catenin complex to the labelled oligonucleotide however, a change in relative mobility does not identify the bound complex. In order to confirm this speculation, specific rabbit anti-β-catenin polyclonal antibody was added to a binding reaction and the result indicated that in fact β-catenin was bound to the DNA- protein complex (figure 2.9a, lane 2 (Tcf-1.1) and 4 (Tcf-1.2)), concurrently another separate EMSA was performed, where anti-Tcf-1 antibody was added to the binding reaction to ensure that it was actually Tcf-1 binding to the double stranded oligonucleotides complexed with β-catenin. This would corroborate the literature that consistently states that β-catenin complexes with Tcf-1 and together contribute to activate gene expression. WHCO6 cell line also showed a super shift for both β-catenin and Tcf-1 (figure 2.10a, lane 1 and 3 and figure 2.10b, lane 2 and 4), confirming that the proteins complex in the nucleus at the transcription factor binding sites specifically found in the promoter region of ILK-1. It is also worth mentioning that the same observation was noted for the SNO cell line (figure 2.11a, lane 2 and 4 and figure 2.11b, lane 2 and 4). Thus, all the cell lines showed a supershift (upper band) for both β-catenin and Tcf-1 confirming that the proteins complexed at the transcription factor binding sites. The shifts were assessed from the predominantly dense portion of each band. Refer to figure 2.12, a diagrammatic representation of the DNA-protein complexing to clarify the EMSA results.

2.5 Discussion

The regulation of gene expression is critically important to ensure that correct protein levels within the cell are maintained. It is here that both gene activation and repression are regulated by a conglomerate of transcription factors, co-activators and/or co-repressors. These regulatory events are particularly pertinent to tumourgenesis since many cancers are known to express dysregulated protein levels due to incorrect gene-regulation; examples to date being colon, breast, melanoma, hepatocellular and prostate carcinomas to name but a few (Mann *et al*., 1999; Waterman *et al*., 2004).

This study focuses on a key transcription factor Tcf-1 and essential co-activator β-catenin, which are known to be essential not only during normal cellular processes, but also important in the development of malignancies by cells. β-catenin and Tcf-1 have been shown to bind with high specificity and affinity to each other. Furthermore, it is known that Tcf-1 binds with high affinity to the minor groove of the DNA double helix (Graham *et al*., 2000). Therefore, it is correct to state that Tcf-1 and members of the Tcf family are functionally neutral on their own and their role in gene regulation is to serve as a docking site for an array of interaction partners, and so promoting the formation of different transcription factor complexes with distinct properties (Love *et al*., 2002; Sukhdeo *et al*., 2007). This discussion will revolve around the Tcf-1/β-catenin interaction at two Tcf-1 binding sites within the ILK-1 promoter sequence of human oesophageal SCCs.

Western blot analysis confirmed β-catenin expression across all five oesophageal lines as well as β-catenin's presence within the nucleus. Although it has been stated that the primary role of β-catenin is regulation of the strength of adhesion, via linking the cadherin plasma membrane bound proteins to the cytoskeleton (Behrens, 1999; Hülsken *et al*., 1994), in figure 2.3 it is shown (using the Western blot technique) that under standard cell conditions there is a noteworthy presence of β-catenin within the nucleus. This upholds the literature that states that β-catenin translocates to the nucleus, binds to Tcf/LEF-1 and influences gene expression (Prieve *et al*., 1998; reviewed in van Noort *et al*., 2002). It has also been shown that high levels of β-catenin protein in the nucleus are

detected in > 85 % of sporadic colon cancers (Wong and Pignatelli, 2002). This study has revealed that under standard culture conditions, the nuclear β-catenin is present across five cell lines and the presence of β-catenin across the WHCO1 and SNO lines were relatively similar and slightly less when compared to WHCO3, WHCO5 and WHCO6 cell lines, which exhibited higher β-catenin levels (figure 2.3). This could be indicative of dysregulated β-catenin expression in the latter lines based on them being of the same pathological grading, because under standard culture conditions low expression levels within the nucleus would be expected. In normal cells it has been stated that there is very little β-catenin in the cytoplasm or nucleus because of its rapid degradation by the proteosome (Aberle *et al*., 1997). A comparison to normal epithelial oesophageal cells would be needed to draw further conclusions, however due to difficulties in obtaining normal oesophageal epithelium, no comparison could be deduced. This study still provides evidence that the β-catenin protein is expressed in oesophageal SCC cell lines.

Tcf-1 was identified in both the total cellular fraction (Laemmli, 1970) and the nuclear fraction (Wen *et al*., 2003). The anti-Tcf-1 polyclonal antibody recognises multiple bands ranging from 28-55 kDa; this concurs with previous studies, which have shown multiple Tcf-1 bands to be of similar size range (Mizushima *et al*., 2002; van de Wetering *et al*., 1996). Western blotting of fractions prepared from the five oesophageal SCCs revealed noticeable differences in abundance between the different isoforms within each cell line (figure 2.4 and 2.5). The predominant isoforms were the 30 kDa Tcf-1A/C/G isoforms across all the cell lines, furthermore the Tcf-1D/E isoforms showed substantially less expression. This result was not consistent with van de Wetering and colleagues (1996) who found no noteworthy differences in abundance between the individual Tcf-1 isoforms. van de Wetering *et al.* (1996) reasoned that neither alternative splicing nor promoter activities of Tcf-1 appeared to be regulated in a stage-specific fashion during cell differentiation. If this is so it can also be reasonable to assume that expression of Tcf-1 isoforms can differ in dynamic cultures in moderately differentiated HOSCCs. Hence, the results obtained for the oesophageal SCC lines here, clearly show that the isoforms are not expressed in similar abundance, yet the total Tcf-1 expression abundance profile across the cell lines was similar. Another observation was that no Tcf-

1E or Tcf-1B isoforms were observed in any of the total cell fractions, yet detection of these isoforms was noted in the nuclear fraction (figure 2.5). Variations in the observation of different Tcf-1 isoforms in different fractions of the same cell lines is not unlikely as van de Wetering *et al.* (1996) found that the Tcf-1 protein was absent from standard nuclear fractions, as determined by gel retardation and Western blotting. van de Wetering and colleagues (1996) concluded that the Tcf-1 proteins are intimately associated noncovalently with components of the nuclear matrix. However, use of a nuclear extraction method from Wen *et al.* (2003) allowed us to overcome this problem, and so it was possible to conclusively determine the presence of Tcf-1 in the nucleus of human oesophageal SCCs (figure 2.5) and examine the different isoforms within the nucleus across the five cell lines. The presence of Tcf-1 in the nuclei of OSCC cell lines (figure 2.5a and b) were of particular importance as literature to date seldom investigates the presence of Tcf-1 in this cell type. Furthermore, the literature rarely shows Western blots of Tcf-1 therefore comparisons and analyses have been difficult to confirm. Examination of the nuclear fractions, show considerable differences among the Tcf-1 isoforms, it is also important to consider that the expression profile across the lines of total Tcf-1 abundance varies considerably. In this respect WHCO3 and WHCO5 expression is relatively similar, and WHCO1 and WHCO6 were similar, yet SNO exhibited considerably higher Tcf-1 expression levels. The inconsistencies in Tcf-1 protein expression levels argue against the possibility of considering Tcf-1 as a possible biological/molecular marker for moderately differentiated oesophageal SCCs. Nevertheless, it is important to take into account that protein densitometry is a semi-quantitative technique and so a more quantitative analysis of Tcf-1 might corroborate these data. Investigation into a biological marker for this particular grade of HOSCC cell lines was not the intrinsic aim of this project and so was not investigated further. However these data provide evidence that Tcf-1 is expressed in oesophageal SCC cell lines. Due to the fact that β-catenin and various isoforms of Tcf-1 have been confirmed to reside in the nucleus of HOSCCs investigation into the complexing of these two proteins proved to be the next logical question to answer in this investigation.

Co-immunoprecipitation studies revealed specific complexing of β-catenin with two Tcf-1 isoforms, Tcf-1E and to a consistently lesser degree Tcf-1A/C/G (figure 2.6). This was a significant result as it has been reported that Tcf-1 expression in normal thymus tissue shows that the predominant forms are truncated polypeptides missing the complete intact β-catenin binding domain, known as dominant negative isoforms (van de Wetering *et al*., 1996; Waterman, 2004). Therefore, in this respect, it can be said that under normal conditions Tcf-1 acts as a Wnt-linked tumour suppressor. Given this evidence that Tcf-1 has tumour suppressive functions, it allows for the possibility that Wnt-linked tumours may progress in part by negating expression of dominant negative isoforms and upregulating the expression of full-length isoforms (Waterman, 2004; Willert *et al*., 2006). This evidence allows consideration that Tcf-1E and Tcf-1A/C/G isoforms have intact β-catenin binding domains while Tcf-1B does not and thus acts as a tumour suppressor (Atcha *et al*., 2003; Hovanes *et al*., 2000; van de Wetering *et al*., 1996). Tcf-1D/F will not be considered here as they were not identified within the nuclear fraction (figure 2.5). The Tcf-1E isoform contains an intact E-tail in the alternative C-terminal that has been suggested to enable better and more stable interactions with β-catenin, however this contradicts evidence that Tcfs without an E-tail efficiently co-immunoprecipitate with β-catenin in COS-7 cells (Prieve *et al*., 1996., Waterman, 2004). The data in this study suggests that Tcf-1E and Tcf-1A/C/G have intact β-catenin binding domains and interact with β-catenin in human oesophageal SCC cell lines. However, further studies need to be undertaken to examine whether these isoforms are the initial contributors to the acquisition of the tumourgenic state in HOSCC cell lines. β-catenin binds efficiently with Tcf-1 in the HOSCC cell lines used in this study. According to Melchior's *et al.* (2002) examination of the ILK-1 promoter region there are two Tcf-1 binding sites within the ILK-1 promoter region. The promoter activity of ILK-1 occurs within 349 bp immediately upstream of exon 1 (Melchior *et al*., 2002) and exhibits various *cis*-acting transcription factor binding sites, such Sp1, LBP1, JCV and Tcf-1 (Melchior *et al*., 2002). With this in mind ILK-1 expression was investigated in the HOSCC cell lines.

As shown in figure 2.7, ILK-1 expression is similar for WHCO6 and SNO whereas WHCO1, WHCO3, WHCO5 show slightly higher levels of expression (figure 2.7b), with WHCO5 having the highest ILK-1 expression levels. Thus these results show that ILK-1 is expressed within moderately differentiated oesophageal SCC cell lines. This corroborates the results of Driver and Veale (2006) where ILK-1 expression was also observed in moderately differentiated HOSCC lines with a similar ILK-1 expression profile across the cell lines.

We have thus far shown β-catenin, Tcf-1 and ILK-1 expression in moderately differentiated oesophageal carcinoma cell lines. Furthermore we have shown that β-catenin and Tcf-1E and to a lesser degree Tcf-1A/C/G complex in the nucleus. The next logical step in gaining an understanding as to whether there is cross talk between the ILK-1/PI3K and Wnt pathways would be to examine the ILK-1 Tcf-1 promoter binding sites. As stated previously in section 2.2.4 a primary objective of this study was to examine whether the β-catenin/Tcf-1 complex binds Tcf-1-DNA binding sites, with specific reference to the two Tcf-1 binding sites found in the ILK-1 promoter region (these DNA sites were determined by Melchior *et al.* (2002)). For this we used an EMSA to verify whether binding actually occurred.

Three cell lines (WHCO3, WHCO6, SNO; figures 2.9, 2.10, 2.11 respectively) were used as a representative of whether binding actually occurs between the β-catenin /Tcf-1 complex and the specific DNA binding sites in moderately differentiated HOSCCs. The WHCO3 cell line exhibited a recognisable shift (DNA-protein complex) in the binding reaction for both the Tcf-1.1 and Tcf-1.2 oligonucleotides. However, to ensure that the protein complex definitely included Tcf-1 and β-catenin, EMSAs with anti-β-catenin antibody were performed, it was important to clarify and confirm that the oligonucleotides did actually bind β-catenin and furthermore show that Tcf-1 is involved in the complex. To ensure that Tcf-1 was involved in the DNA-protein complex further EMSAs were performed with anti-Tcf-1 antibody. Both binding reactions (anti-β-catenin and anti-Tcf-1 antibodies) for WHCO3 showed a small but noticeable supershift (figure 2.9a, lane 2 and 4 and figure 2.9b, lane 2 and 4). The same was also noted for the

WHCO6 and SNO cell lines (refer to results section 2.3, figure 2.10a, lane 1 and 3 and figure 2.10b, lane 2 and 4 and figure 2.11a, lane 2 and 4 and figure 2.11b, lane 2 and 4). Mizushima *et al.* (2002) found that EMSAs in oesophageal SCC cell lines contained Tcf-specific DNA binding activities with Tcf-4 and demonstrated barely detectable β-catenin as part of the DNA-Tcf-4 complex, this corroborates the current findings of small yet noticeable supershifts in the oesophageal cell lines WHCO3, WHCO6 and SNO. However, there was a distinctly noticeable supershift with the Tcf-1.2 oligonucleotide for all three cell lines with both the β-catenin and Tcf-1 antibodies. In figure 2.11b there are multiple bands in lane 4 (Tcf-1 supershift with Tcf-1.2 oligonucleotide), this could be due to the known isoforms that are expressed in the nuclear region of HOSCC cell lines.
It was interesting to note that Tcf-1.1 (figure 2.9a, 2.10a and 2.11a) did not migrate in the PAGE as far as Tcf-1.2 (figure 2.9b, 2.10b and 2.11b) in all the cell lines. This was probably due to the fact that the Tcf-1.1 oligonucleotide is 15 bp longer that Tcf-1.2 therefore retarding migration of the complex. Another possibility could be due to the exact position of the protein binding site on the oligonucleotides. DNA-protein complexing causes the pliable DNA helix to bend (Crothers, 1987). The bend adds to the overall curvature of the complex and bent helices migrate slower than straight helices in electrophoretic gels as their end-to-end distance is smaller. Crothers *et al*., 1987 has shown that isomers with a DNA bend near the end results in a larger end-to end distance and greater gel mobility than if the bend occurs in the middle of the isomer (Sidorova *et al*., 2005).

Another noticeable aspect about the supershift was that the actual supershift was somewhat less obvious in some cases, this was probably due to protein drag. This is not uncommon and has been observed in the various literatures (Masui *et al*., 2002; Mizushima *et al*., 2002; Tago *et al*., 2000; Takahashi *et al*., 2002; Valenta *et al*., 2003; van Beest *et al*., 2000; van de Wetering *et al*., 1996). Reducing the protein amount resulted in no supershift (probably due to the low Tcf-1 expression in the nucleus noted in the Tcf-1 nuclear densitometric analysis) and so the problem was not solved via changing protein concentrations. The literature also recommends using high concentrations of probe (concentrations considered high for EMSA reactions) as this

helps drive the formation of complexes and helps increase the detection of a shift (Stead *et al*., 2006). Due to the pore size of the gel (5% PAGE) and the large volume of binding reaction sample (20 µl of binding reaction) that had to enter the gel, the 'caging effect' was not entirely effective upon commencing electrophoresis. The low salt concentrations in the gel and the gel matrix provides a 'caging effect' where if the complex dissociates the gel matrix helps to 'localise' the concentration of the complex components, these remain high, prompting reassociation (Foulds *et al*., 1998; Stead *et al*., 2006). These attributes help stabilize even weak complexes (Crothers, 1987; Stead, *et al*., 2006). Furthermore a noticeable volume of the DNA-protein complex was observed at the bottom of the well. The equilibrium between free DNA and protein-DNA complex can become severely unstable during the time from when the sample is loaded to when it actually enters the gel (Sidorova *et al*., 2005). The ability to resolve DNA-protein complexes depends largely upon the stability of the complex during the brief time (approximately one minute) when the complex is migrating into the gel. When the DNA-protein complex enters the gel, protein complexes are quickly resolved from free DNA, in effect freezing the equilibrium between bound and free DNA, therefore minimising electrophoretic dead time is important (Stead *et al*., 2006). Sidorova *et al.* (2005) found that the time during which the sample is in the well the local pH, concentrations of salt, DNA and protein would equilibrate with the running buffer and so change significantly. Sequence-specific DNA-protein interactions are transient and so are stabilised by the low ionic strength of the electrophoresis buffer that is used (0.5X TBE). Furthermore, as described above, stabilisation of the complex can occur by the caging effects of the gel matrix. These EMSAs clearly show that β-catenin and Tcf-1 do bind to oligonucleotides that contain the Tcf-1 binding sites in the ILK-1 promoter region determined by Melchior *et al.* (2002).

The results of the EMSAs support the model that because β-catenin associates with Tcf-1 at the proposed binding sites, there is the activation and/or increase in the gene expression of ILK-1. At this point in the investigation there is an increasing possibility that cross talk at a regulatory level between the PI3K/ILK-1 and Wnt pathways exists in moderately differentiated HOSCC cell lines.

Chapter Three: Stable transfection and stimulation of exogenous β-catenin in the WHCO6 cell line and its effects on ILK-1 protein levels

3.1 Introduction

Experimental genetic manipulation is fast becoming a common and informative way to investigate the activity of eukaryotic gene regulatory elements and components of signalling pathways (Zhao *et al*., 2004). In particular overexpression of various gene products can help define gene function, find a certain signal transduction pathway or component thereof, or determine post-translational modifications of proteins (Hovannes *et al*., 2001). Hence, it can be stated that gene transfer is critical in the advance of knowledge of gene regulation, post-translational events, recombinant protein effects and gene therapy and the list is still growing (Hovannes *et al*., 2001; Makrides, 1999).

An increasing understanding of biological processes now allows the design of vector components to meet specific objectives (Zhao *et al*., 2004). Thus, gene expression in a tissue-selective or ubiquitous manner can be accomplished by taking into deliberation the following factors and selecting as appropriate (Makrides, 1999):

- promoter/enhancer elements
- stabilization of labile mRNAs may be effected through removal of 3' untranslated regions or fusion to heterologous stabilizing sequences
- protein targeting to selected tissues is carried out using specific signal sequences
- fusion moieties effect the detection, enhanced yield, surface expression, extension of half-life and facile purification of recombinant proteins
- modifying of the codon of heterologous genes enhances protein production from poorly translated transcripts (Makrides, 1999).

Efficient gene expression in mammalian cells depends on a variety of factors such as:

- transcriptional and translational control elements
- ribonucleic acid (RNA) processing

- gene copy number
- mRNA stability
- the chromosomal site of gene integration
- potential toxicity of recombinant proteins to the host cell
- the effect of the agent used to induce recombinant gene transcription on the host cell
- the genetic properties of the host (Makrides, 1999).

Hence, the choice of an expression system for production of recombinant proteins depends on many factors, including cell growth characteristics, expression levels, intracellular and extracellular expression, posttranslational modifications and biological activity of the protein of interest, as well as regulatory issues and economic considerations in the production of therapeutic proteins (Datar *et al*., 1993; Goeddel, 1990; Marino, 1989).

Recombinant DNA technology is dependent on the successful ligation of a gene into a suitable vector, transporting/transfecting the vector-gene construct into a host cell that in turn allows for successful expression of the desired gene (Bondyk, 1995; Klug and Cummings, 2000). To date transient and stable heterologous gene expression in cultured cell lines are the best methods to define or determine gene function and protein function *in vivo* (Ozdemir, 1998). In addition, the method lends itself to the rapid testing of vector functionality as well as optimization of different combinations of promoters and other elements in expression vectors (Ozdemir, 1998). Transient transfections result in a high yield of gene expression within a short period (8 - 72 hours) which is subsequently suppressed (Makrides, 1999). However this type of transfection remains a controversial issue, although this transfer method directs plasmid DNA to the cytoplasm it is not certain what percentage of plasmids enter the nucleus (this could allow for the genes to become transcriptionally activated) (Sambrook and Russell, 2001). Defined parameters are needed with this technique to reach a satisfactory degree of reproducibility, as transient transfected gene expression can be over simplified and so allow for misinterpretations.

Stable transfections involve integrating the exogenous gene into the host cell's genome and the vector is maintained within the cell in the generations following

transfection (Sambrook and Russell, 2001). The cells are usually co-transfected with a separate plasmid that acts as a selectable marker and gives drug resistance to select for positive integrants, thereby determining if a cell is stably transfected (Davies and Jimenez, 1980; Sambrook and Russell, 2001). One of the most frequently used marker genes is the aminoglycoside phosphotransferase gene which confers resistance to the geneticin-selective antibiotic (G418 sulphate (G418)) (Davies and Jimenez, 1980). It synthesizes the aminoglucoside-acetylating enzyme, APH that inactivates G418 through phosphorylation (Davies and Jimenez, 1980). It is important prior to transfection that the optimal concentration for the particular cell type in a particular culture condition is determined (Sambrook and Russell, 2001).

Various gene delivery methods have been employed in order to successfully transfect animal cells, such as electroporation, calcium-phosphate, particle bombardment and cationic liposome-mediated transfection (lipofection) (Sambrook and Russell, 2001). Lipofection is considered the most efficient method for gene transfer both *in vivo* and *in vitro*. The principle involves positively charged cationic liposomes forming complexes with negatively charged DNA molecules, allowing fusion to the cellular membrane, thus successfully delivering the gene of interest (Davies and Jimenez, 1980; Sambrook and Russell, 2001). It can be said that transfection is an important technique used to enhance our understanding of the function of a desired protein.

With reference to β-catenin, Zhao *et al*. (2004) showed that the introduction of a degradation-resistant β-catenin S37A stimulates the Wnt pathway resulting in cell proliferation. β-catenin suppression was examined by Roh *et al*. (2001), where antisense oligonucleotides were used to down-regulate β-catenin suppression *in vivo* in human colon cancer cells. As a result it was found that proliferation, anchorage-independent growth and cellular invasiveness was inhibited in these tumour cells (Roh *et al*., 2001). Giannini *et a*l. (2000) studied β-catenin protein-protein interactions in living cells by fusing β-catenin to a green fluorescent protein (GFP). The GFP-β-catenin was identified in both the cell junctions and the nucleus (Giannini *et al*., 2000). These examples give an indication as to how gene regulation experiments have advanced our knowledge in understanding cellular activities. With specific reference to this study, stable transfection of the β-catenin gene was

performed in order to examine the possible influence of β-catenin's gene regulatory role on ILK-1 protein expression.

Both single and double vector systems can be used in mammalian cells for gene regulation experiments. Some of the commonly used single vector systems include pMAMneo and pCI-neo mammalian expression vectors (Bondyk, 1995). Both the pCI-neo and the pMAMneo single vector systems carry simian virus 40 (SV40) splicing and polyadenylation sites that provide RNA processing in mammalian cells, while the *E. coli* neomycin resistance gene, driven by the SV40 early promoter, allows selection of transformants growing in media containing G418 (Bondyk, 1995). The pCI-neo vector carries a human cytomegalovirus (CMV) immediate-early enhancer/promoter region to promote constitutive expression of cloned DNA inserts in mammalian cells and the pMAMneo vector contains an enhancer linked to the dexamethasone-inducible promoter (Bondyk, 1995). Acton *et al*. (1993) used the pMAMneo expression vector to show the potential for functional specialization of the two different light chain subunits of clathrin, LC_a and LC_b (these light chains are involved in the regulation of coated vesicle disassembly and other aspects of clathrin cycling within the cell). To investigate the possibility of exclusive roles for LC_a and LC_b, expression was manipulated using the pMAMneo vector with a dexamethasone-inducible mouse mammary tumour promoter and the neomycin resistance gene (Acton *et al*., 1993). However, there was no evidence of change of clathrin distribution. Because the promoter was induced with dexamethasone there is a possibility that the cells were naturally dexamethasone responsive, and so the control would mask any overexpression response (Acton *et al*., 1993). Dexamethasone is a synthetic glucocorticoid that binds to specific nuclear steroid receptors and interferes with NF-κB activation and apoptotic pathways (Acton *et al*., 1993; No *et al*., 1996). It is important to state that the HOSCC cell lines that were used for this study have been previously shown to be naturally responsive to dexamethasone (unpublished results, Cell Biology Laboratory) and therefore dexamethasone was not considered for this study.

LacR/O-based systems have been used in mammalian cells, however, these systems are limited since the inducer isopropyl β-D-thiogalactopyranoside (IPTG), despite its rapid uptake by mammalian cells, acts slowly and inefficiently, resulting in moderate

induction (Sambrook and Russell, 2001). An alternative to IPTG inducible vectors are vectors containing tetracycline-responsive promoters. However, the disadvantages for tetracycline inducible systems are that continuous use of the antibiotic interferes with quick and precise inductions when a precise on-off switch is essential (No *et al*., 1996; Sambrook and Russell, 2001). No *et al*. (1996) showed that ecdysone regulatory systems exhibit a lower basal activity and higher inducibility in comparison to tetracycline regulatory systems. Advantages for ecydysteroid inducible systems include its lipophilic nature for efficient penetrance into tissues, short half-life, precise and potent inductions and expedite clearance and they are non-toxic or teratogenic (No *et al*., 1996). After careful consideration an ecydysteroid inducible system was used for this particular study due to the above mentioned advantages. Although there is considerable choice for gene regulatory systems in mammalian cells, it is important to consider the type of host cell, since it may have a significant impact on gene expression levels (Sambrook and Russell, 2001). For example, myeloma cells have been mainly used for high-level production of monoclonal antibodies. However, amplifiable expression systems using Chinese hamster ovary (CHO) cells have been widely used for the successful production of proteins of therapeutic interest (Pei and Yi, 1998). Furthermore, a well-differentiated epithelial cell line Madin-Darby canine kidney (MDCK), was shown to be capable of producing large amounts of protein when transfected with a plasmid containing a CMV promoter, controlling the expression of matrix metalloproteinase 13 (MMP13) (Pei and Yi, 1998). The yield of MMP13 was 10 mg/L of conditioned medium, an amount that rival yields obtained from CHO amplification systems (Pei and Yi, 1998). The authors concluded that the high yield could be attributed partially to the properties of MDCK cells, since CHO cells transfected with the same vector yielded much less protein (Pei and Yi, 1998).

An investigator's choice of vector depends on the experimental goal and is an important parameter for a successful outcome. On the other hand the cells which are to be transfected with a foreign gene must be identifiable with an antibiotic resistance gene. Therefore, after careful consideration, a double ecydysone-receptor vector regulatory system was used to induce exogenous β-catenin expression in the WHCO6 cell line. This was required for the ultimate aim of the study to investigate β-catenin's influence on ILK-1 expression in HOSCC.

3.2 Objectives

3.2.1 Stably transfect the WHCO6 HOSSC cell line with the pERV3 receptor vector and pEGSH-β-catenin construct respectively.

3.2.2 Determine that β-catenin is still fully functional within the transfected cell line.

3.2.3 Stimulate expression of β-catenin in the transfected cell line with ponasterone A (ponA).

3.2.4 Examine the level of β-catenin expression within the cell.

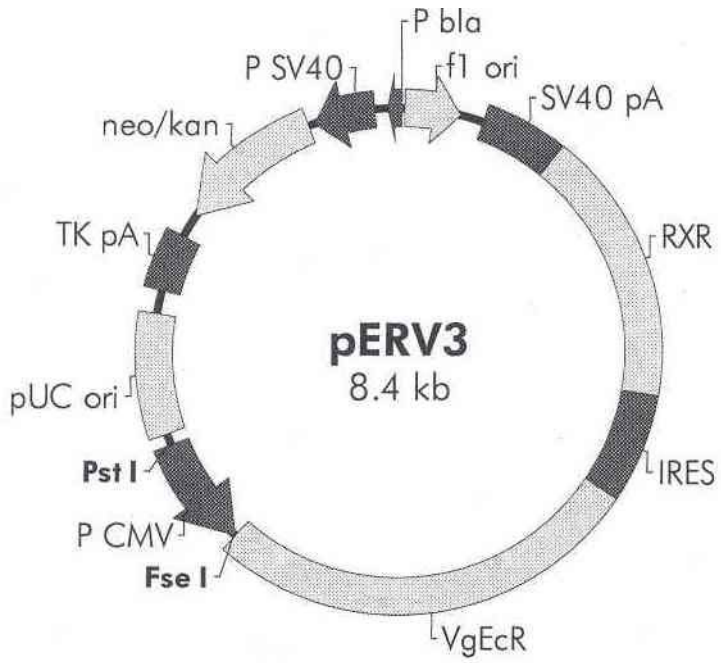
3.2.5 Determine if overexpression of β-catenin affects ILK-1 protein levels.

3.3 Methods and materials

3.3.1 Tissue culture

Tissue culture preformed as previously described. See chapter two.

3.3.2 Plasmids used for transfection procedure



f1 origin 24-330
SV40 polyA 901-463
RXR receptor ORF 902-2333
Internal ribosome entry site 2334-2903
neomycin / kanamycin resistance ORF 7121-7915
FseI 5187
***bla* promoter** 8362-5
Pst I 5802
pUC origin 5869-6536
HSV-TK polyA 6668-6998
CMV promoter 5188-5802
VgEcR receptor ORF 2904-5187
SV40 promoter 7950-8288

Figure 3.0: Schematic diagram of the pERV3 vector.

This vector was transfected in the WHCO6 oesophageal squamous cell carcinoma cell line. Complete Control® Inducible Mammalian Expression System Plasmids (direct citation from the manual)

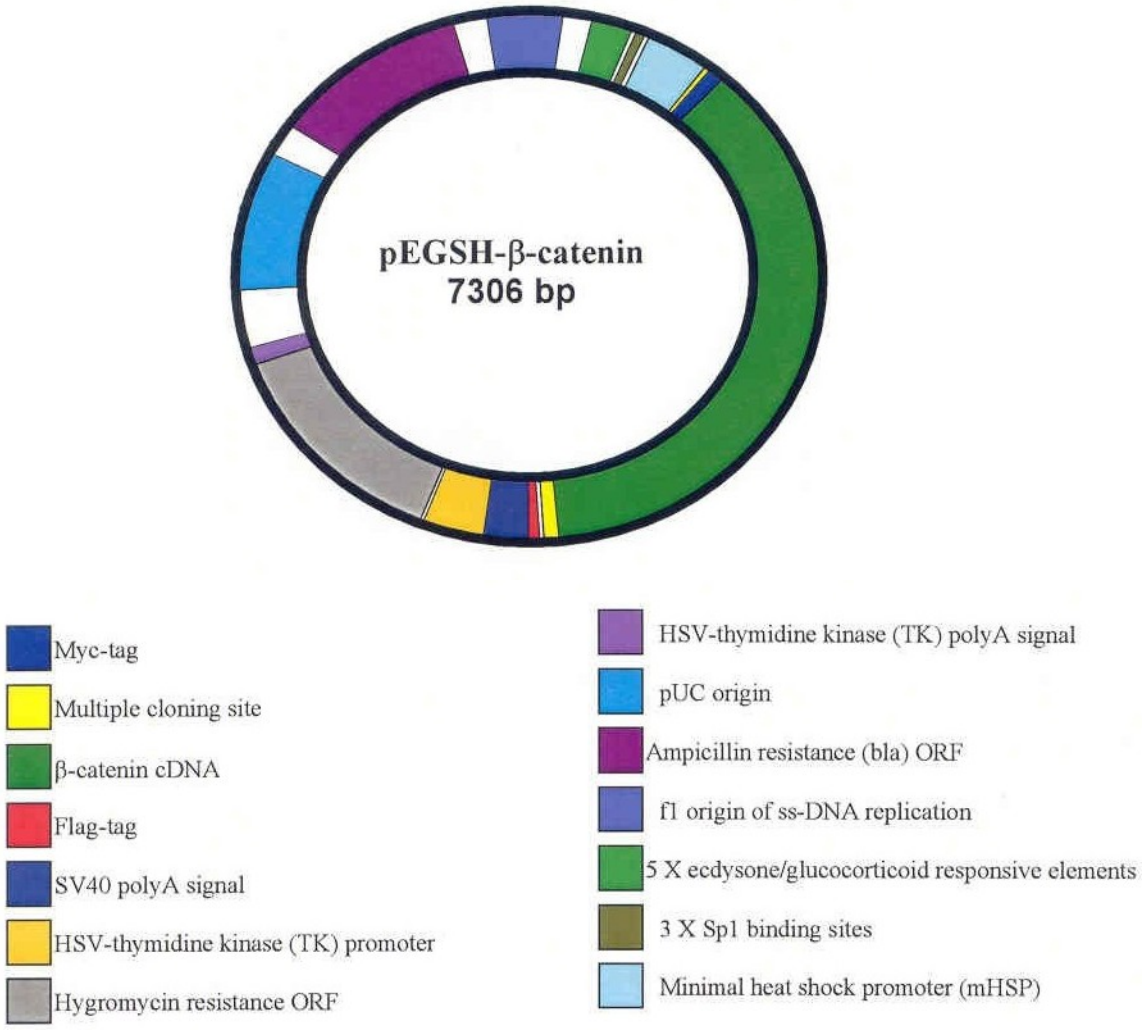


Figure 3.1: Schematic diagram of the pEGSH-β-catenin vector.

This construct was transfected in the WHCO6 oesophageal squamous cell carcinoma cell line (Dahan, 2005).

3.3.3 Transfection of pERV3 receptor vector into WHCO6 cells

One day prior to the transfection procedure, 3 x 10^6 WHCO6 cells were counted using a haemocytometer and cultured onto 6 cm Nunc™ tissue culture dishes, so that 50% confluency was achieved on the day of transfection. This allowed for higher transfection efficiencies. The cells were maintained in 5 ml of DMEM supplemented with Hams F12 (3:1) and 10% FCS and maintained at 37^{o}C in a 5% CO_2 atmosphere. The pERV3 plasmid was transfected into the WHCO6 cell line according to the protocol of the FuGENE6 transfection kit (Roche). Serum free tissue culture medium (86 µl) was transferred into an eppendorf tube and 12 µl of FuGENE6 transfection

reagent was added. Furthermore 2 µl of pERV3 plasmid (0.99 µg/µl) (figure 3.0) was added, and the mixture was set aside for 30 minutes. Following the incubation, the mixture was added drop wise to the 5 ml of tissue culture medium covering the WHCO6 cells. For the control, 12 µl of FuGENE6 was added to 88 µl of tissue culture medium and incubated for 30 minutes, followed by the addition of the mixture in a drop wise fashion to a separate dish of WHCO6 cells. After a 24 hour incubation period the WHCO6 cells were passaged. Although the DNA-lipofectamine complex is not considered to be hazardous to the cells during long incubations, the literature still recommends changing the culture medium 24 hours after transfection. After a 48 hour incubation period from the time of transfection, the tranformants were selected for by adding 50 µl of G418 (20 mg/ml) to the tissue medium. After 10 days the volume of G418 (20 mg/ml) was increased to 100 µl and after another 10 days it was again increased to 150 µl. The pre-determined toxicity of the WHCO6 cell line was found to be 600 µg/ml. Once there were no cells left on the control dish, the remaining viable cells were designated WHCO6-pERV3.

3.3.4 Transfection of the pEGSH-β-catenin plasmid into WHCO6-pERV3 cells

One day prior to the transfection procedure, 3 x 10^6 WHCO6-pERV3cells were counted using a haemocytometer and cultured onto 6 cm Nunc™ tissue culture dishes. The cells were maintained in 5 ml of DMEM supplemented with Hams F12 (3:1) and 10% FCS and maintained at 37^{o}C in a 5% CO_2 atmosphere. Again 50% confluency was needed in order to attain high transfection efficiencies. The pEGSH-β-catenin plasmid (figure 3.1) was transfected into the WHCO6-pERV3 cell line according to the protocol of FuGENE6. Serum free tissue culture medium (86 µl) was transferred into an eppendorf tube and 12 µl of FuGENE6 transfection reagent was added. Furthermore 2 µl of pEGSH-β-catenin plasmid (1.37 µg/µl) was added, and the mixture was allowed to incubate for 30 minutes. Following the incubation, the mixture was added drop wise to the 5 ml of tissue culture medium covering the WHCO6-pERV3 cells. For the control, 12 µl of FuGENE6 was added to 88 µl of tissue culture medium in a sterile eppendorf tube and incubated for 30 minutes, followed by the addition of the mixture in a drop wise fashion to a separate dish of WHCO6-pERV3 cells. After a 24 hour incubation period the WHCO6-pERV3 cells were passaged and 48 hours after transfection the transformants were selected for by

adding 150 µl of G418 (20 mg/ml) and 5 µl of hygromycin B (20 mg/ml) to the tissue medium. Once there were no viable cells left on the control dish, the cells remaining on the experimental dish were designated transfected WHCO6 (tWHCO6). After 10 days the concentration of hygromycin B was increased to 60 mg/ml and after another 10 days the concentration was again increased to 70 mg/ml.

3.3.5 Total cellular protein extraction

This procedure was performed as previously described in chapter two.

3.3.6 Nuclear protein extraction

Nuclear extracts were obtained using the SIGMA CelLytic™ NuCLEAR™ EXTRACTION KIT (Sigma, SA). The principle of the extraction method is described in chapter two.

3.3.7 Protein estimation

This procedure was carried out as in chapter two.

3.3.8 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein (10 µg) from both the total cellular and nuclear extracts, were separated on a 15% SDS-PAGE. The gel was prepared and electrophoresis was conducted according to the procedure detailed in chapter two.

3.3.9 Western blots

The antibodies described in chapter two, section 2.3.6 were also used for the Western analyses in this chapter. The Western blot procedure was performed according to the description in chapter two.

3.3.10 Co-immunoprecipitation

Co-immunoprecipitation was carried out according to the procedure detailed in chapter two.

3.3.11 Treatment of tWHCO6 cells with ponA

PonA (appendix 5.10, figure 5.1) is an analogue of the insect hormone ecdysone and can activate transcription in mammalian cells that harbour the gene for the *Drosophila melanogaster* ecdysone receptor (EcR) and a promoter containing a binding site for the EcR. Treatment with ponA is advantageous as it has no known measurable effects on mammalian physiology, a short *in vivo* half-life, and its lipophilic nature allows for effective penetration of all tissue types (No *et al*., 1996).

The tWHCO6 cells were treated with 1μM of ponA solution (Stratagene; 1 mg of ponA was dissolved in 1 ml of 100% ethanol) for 8 hours, tWHCO6 cells with the addition of 100% ethanol (no ponA) were also incubated for 8 hours and constituted a control. Following the incubation the cells were harvested. The procedure was repeated with a treatment of 2 μM ponA solution and an 8 hour incubation.

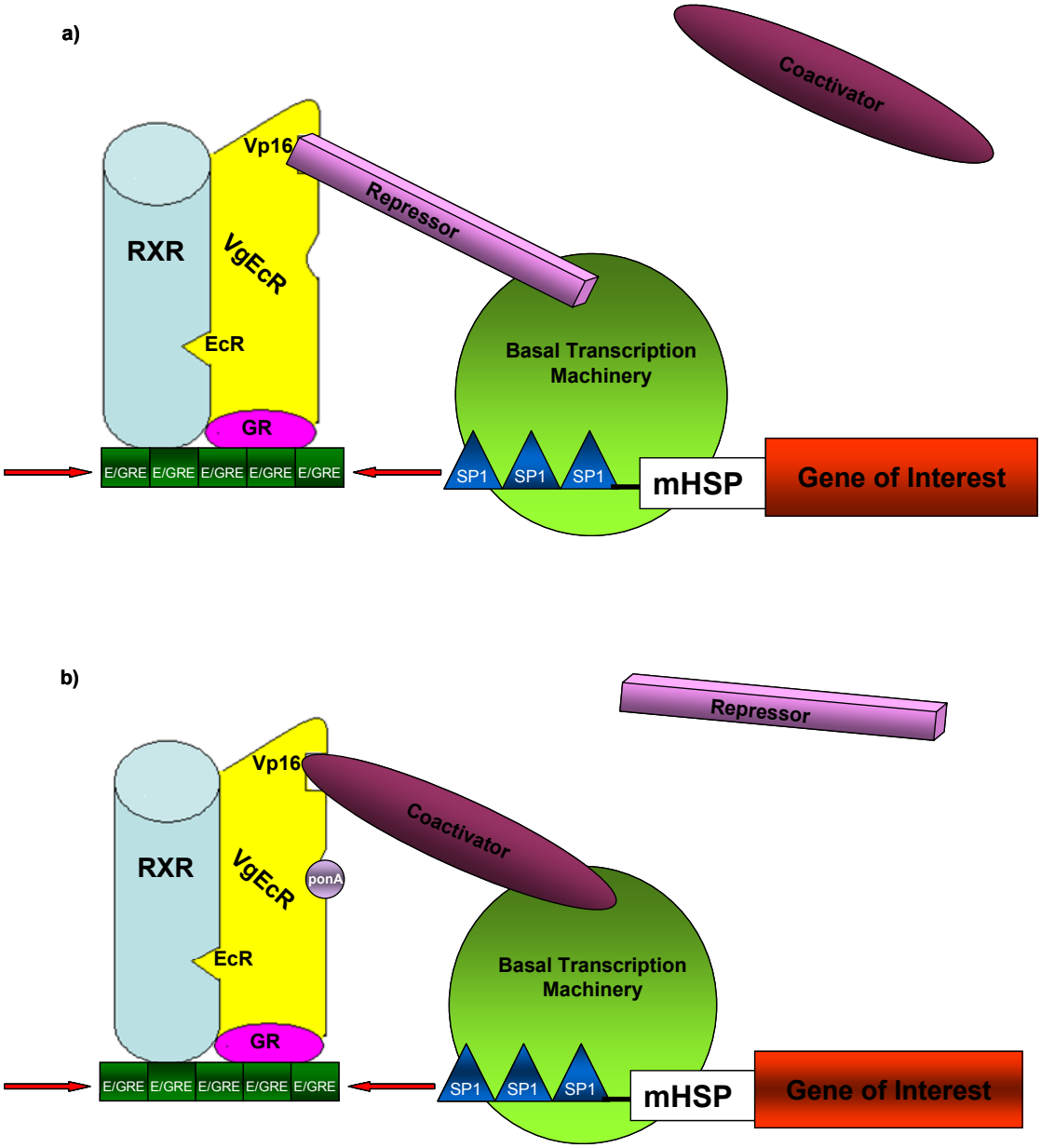


Figure 3.2: Activation of gene expression via ponA (Complete Control® Inducible Mammalian Expression System).

VgEcR, a synthetic receptor, is comprised of the DNA-binding glucocorticoid receptor (GR), the ligand-binding and dimerisation domain of the Drosophila ecdysone receptor (EcR), and the transcription activation domain of herpes simplex virus (HSV)/virion protein 16 (VP16). This synthetic receptor (VgEcR) and retinoid-X-receptor (RXR) bind as a heterodimer to multiple copies of the ecdysone/glucocorticoid-responsive element recognition (E/GRE) sequence. This sequence is upstream of a minimal promoter (composed of three SP1 binding sites and a minimal heat shock promoter (mHSP)). The basal transcriptional machinery is bound to the minimal promoter. (a) In the absence of ponA, the hetrodimer binds to repressors and transcription of an expression cassette is inhibited. (b) In the presence of ponA; ponA binds to the receptor and the receptor complex (hertrodimer) binds to activators that result in transcription activation of the gene of interest. This diagram is based on the Complete Control® Inducible Mammalian Expression System.

3.3.12 Densitometry

All Western blots were semi-quantitatively analysed by densitometry using the LabWorks™ Image Acquisition and Analysis Software Version 4.5 program in order to compare the levels of a specific protein between the various cell lines. The area under the peak for each sample was used as a basis for comparison between samples.

3.3.13 Image capturing

Images were captured on a Hewlard Packard Scanjet 4400c series scanner.

3.4 Results

The WHCO6 cell line was stably transfected with the pERV3 receptor vector containing an expression cassette that constitutively expresses the VgEcR and RXR proteins. pERV3 also contains a neomycin-resistance gene for selection of mammalian cell transfectants with the antibiotic G418. Vector delivery was accomplished using the FuGENE6 delivery system and it appeared not to affect the cells adversely as the control culture had standard epithelial cell morphology and growth rate until the G418 antibiotic was added to the cells. During the first 10 day period of G418 treatment, cells lost their epithelial squamous morphology, they became more rounded and granular and the percentage of attached cells decreased rapidly. By day 12 no control cells (cells that were not transfected with pERV3, but treated with G418) remained attached to the culture substrate. At this point however, the percentage of attached transfected cells was +/- 50%. Following this procedure the WHCO6-pERV3 (transfected cells) were again transfected with pEGSH-β-catenin and treated with a combination of G418 and hygromycin B antibiotics as described in sections 3.3.3 and 3.3.4 of this chapter. Once again epithelial cell morphology and survival of the control cells were compared with that of the cells transfected with the vector and construct (pERV3 and pEGSH-β-catenin). The successfully transfected WHCO6 cells (pERV3 and pEGSH-β-catenin WHCO6) retained original epithelial morphology in the presence of G418 and hygromycin B. This is indicative of a successful transfection. The successfully transfected WHCO6 cells are referred to as tWHCO6 throughout the following text.

SDS-PAGE separation of the total cellular protein and nuclear fractions obtained from tWHCO6, and WHCO6 cell lines (figure 3.3) yielded distinct banding patterns. The extensive molecular weight range of the total cellular protein-extract polypeptide profile extended over a greater distance when compared to that of the nuclear extract. This is expected as a greater ‘variety’ of proteins is expected from the total cell extract when compared to the polypeptide profile of the nuclear extract. The nuclear extract polypeptide profile was enriched from approximately 100 kDa to 25 kDa when separated by a 15% SDS-PAGE, which is a noticeably smaller molecular weight range when compared to the total cellular protein extract. This expected difference

between the two extracts is indicative of the success of the extraction procedures. Importantly, the polypeptide profiles for both the nuclear total protein extractions featured polypeptides of the appropriate molecular weight to that of β-catenin (94 kDa) (figure 3.3). The uniformity of staining in each lane (WHCO6 and tWHCO6) is a good visual indicator that the protein estimation was accurate and successful.

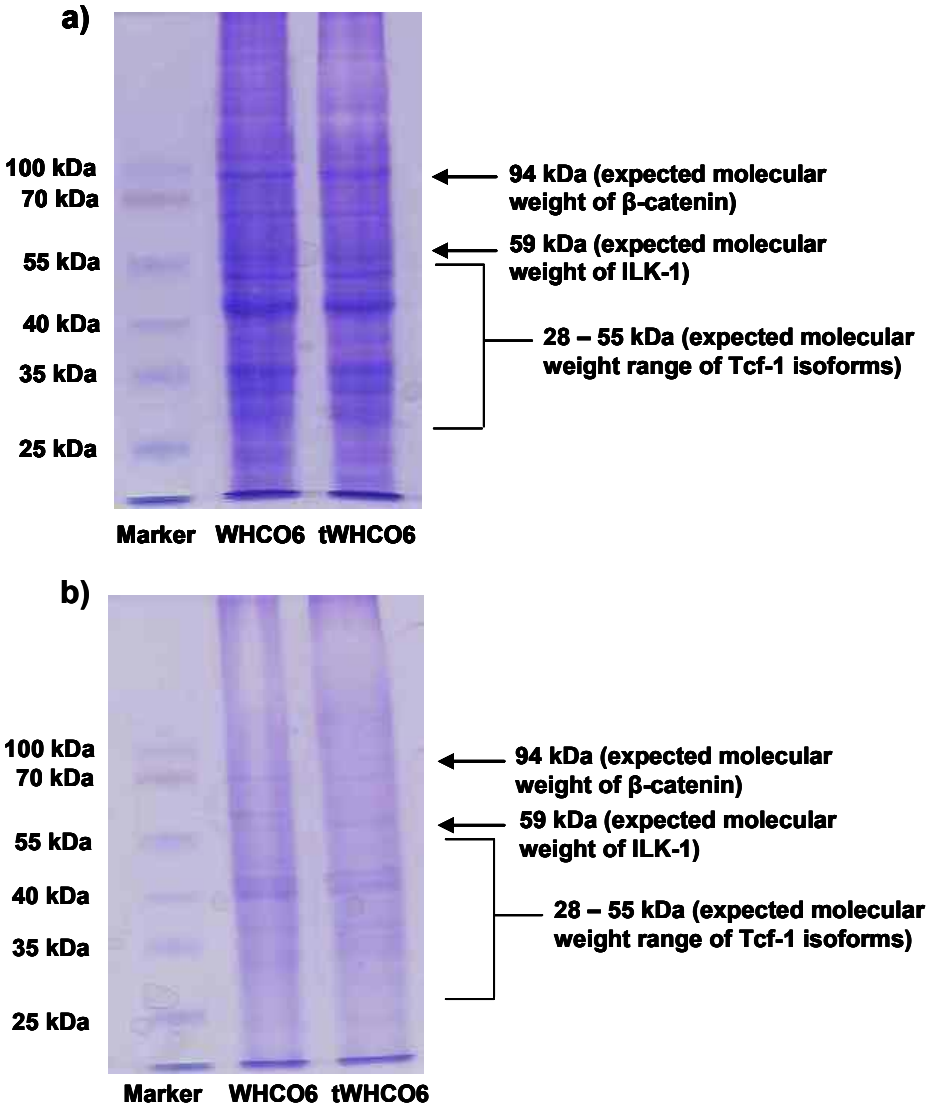


Figure 3.3: Total cellular and nuclear protein extracts.

(a) Total cellular protein-extracts and (b) nuclear protein-extracts from the WHCO6 cell line and tWHCO6 cell line were resolved by a SDS-PAGE (15%). The presence of distinct bands in all the lanes for both protein extracts indicates that the extraction procedure was successful.

The presence of β-catenin in both the total cell and nuclear extracts were confirmed by performing Western blots using the specific anti-β-catenin antibody described previously (chapter two, sections 2.3.6 - 2.3.8). The data presented below (figure 3.4a and b) show that both the total cellular and nuclear protein extraction methods were successful for identifying β-catenin and therefore were used in all subsequent analyses. However, it was important to determine whether β-catenin continued to localise with Tcf-1 in the tWHCO6 cell line. Co-immunoprecipitation was used to establish this (figure 3.4c). Protein G sepharose immunoprecipitation with anti-β-catenin, followed by Western blotting with anti-Tcf-1 antibody, presented two bands (55 kDa and 30 kDa respectively) that were Tcf-1 reactive. Protein densitometric analysis confirmed as detailed in chapter two section 2.3.14, that β-catenin predominately complexed with Tcf-1E (55 kDa) and to a lesser degree Tcf-1A/C/D/F/G (whose molecular weights range between 28.3 kDa - 30.3 kDa) in the tWHCO6 cell line (figure 3.4c).

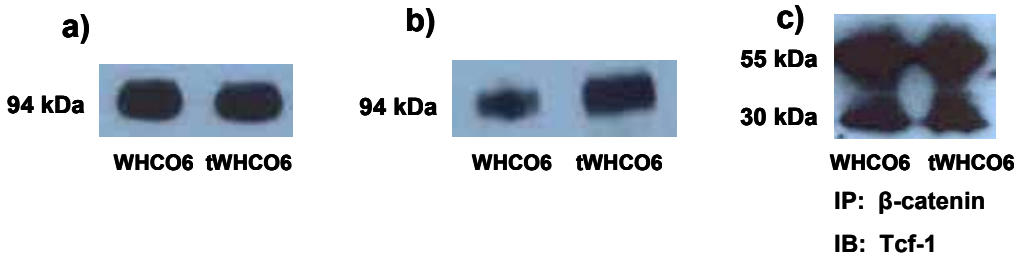


Figure 3.4: Presence of β-catenin in WHCO6 and tWHCO6 cell lines.

(a) and (b) Western blot analyses confirming the presence of β-catenin in the total cellular and nuclear protein-extracts. (c) Western blot analysis of β-catenin immunoprecipitates indicates the presence of a Tcf-1/β-catenin complex. IP = Immunoprecipitating antibody; IB = Immunoblotting antibody.

Therefore on these grounds it is clear that the HOSCC cell line, WHCO6 expresses the pEGSH-β-catenin construct that expresses β-catenin. Furthermore, β-catenin and Tcf-1 localise in the nucleus. It is known, that a cytoplasmic accumulation of β-catenin results in the translocation of the protein into the nucleus (the processes and mechanisms of these reactions are discussed in detail in chapter one). This therefore begs the question, whether an increase of β-catenin results in its accumulation in the nucleus of the tWHCO6 cells. In addition to this it can be investigated whether an

increased level of β-catenin could affect the protein level of ILK-1. And if ILK-1 levels are affected due to the nuclear accumulation of β-catenin is this ILK-1 increase via the Tcf-1 binding sites in the promoter region of ILK-1. The results of the EMSAs (chapter two, section 2.4) lend support to the possibility that the association between β-catenin and Tcf-1, at the proposed binding sites, activates and/or increases the gene expression of ILK-1. Therefore, we attempted to overexpress β-catenin in the tWHCO6 cell line and investigate the consequences of an increased β-catenin level on ILK-1's protein level.

In order to potentially achieve an increase in β-catenin in tWHCO6 cells, it was necessary to activate the expression cassette in the tWHCO6 cell line and examine the effect of exogenous β-catenin expression with respect to ILK-1 levels in these cells. Although the above seems relatively simple and achievable, there are various processes that normally control β-catenin levels within the cell that had to be considered when trying to overexpress β-catenin. These regulatory processes occur at both a cytoplasmic/post-transcriptional level (GSK-3β, APC and the Wnt signalling pathway, as mentioned in chapter one) and at a transcriptional level (NF-κB and activator protein-1 (AP-1)). With these influences in mind, β-catenin was stimulated in the tWHCO6 cell line through exposure to ponA.

The tWHCO6 line stably contains pERV3 and pEGSH-β-catenin (according to the previously mentioned antibiotic resistant criteria) and this cassette was stimulated with ponA. The tWHCO6 cells were treated separately, with both 1 μM and 2 μM of ponA for an 8 hour treatment respectively. The optimum time period for this treatment for the HOSCC cell lines has been previously determined by Dahan (2005). Concomitantly, tWHCO6 cells with the addition of 100% ethanol (no ponA), treated for an 8 hour period constituted a control. The presence of β-catenin was confirmed in the cell extract of ponA treated vs. non-treated tWHCO6 cells. Western blot analysis confirmed the presence of β-catenin in the cellular extract of the total cell population following ponA (1 μM and 2 μM) stimulation (figures 3.5a and 3.7a). Uniform loading of protein concentration of cell extracts permitted comparative densitometric analyses. At this time it should be noted why the stable transfection method was used instead of the transient method, the reason being, was to prevent the need to reproduce the result multiple times as well as prevent the possibility of bias.

There was a 1.3 fold increase of β-catenin following ponA stimulation (1 μM) (figure 3.5b). This was not considered to be a substantial increase in β-catenin. Nuclear extract following ponA simulation (1 μM) was analysed via densitometric analysis (figure 3.6b) and a similar increase of 1.4 fold of β-catenin was noted. Following these results a Western blot analysis for ILK-1 from total cellular extract was performed (figure 3.8a) and densitometric analysis (figure 3.8b) showed an increase in the concentration of ILK-1 in 1μM ponA stimulated tWHCO6 cells. Densitometric analysis showed this to be a 1.2 fold increase in ILK-1 expression. To further examine this increase, the concentration of ponA was increased to 2 μM to determine if this would result in increased β-catenin levels (figure 3.7). However, densitometric analysis (figure 3.7b) revealed that an increase in ponA (2 μM) concentration did not actually yield an increase in β-catenin. Stimulation with 2 μM ponA caused a 0.4 fold decrease in β-catenin levels when compared to β-catenin levels of 1 μM ponA stimulated cells and a 0.2 fold decrease compared to the control cells (figure 3.7b). See table 3.1 which outlines the respective fold increase/decrease of β-catenin due to stimulation with ponA. This result showed that an increase in ponA concentration would not have the desired effect, that is, an accumulation of β-catenin within the cytoplasm that would result in β-catenin translocation to the nucleus and binding to Tcf-1 in order to gauge β-catenin's influence on ILK-1 levels in HOSCC cells. It is likely that a considerable increase in β-catenin expression upon ponA stimulation was not observed due to the tight intrinsic regulation of β-catenin.

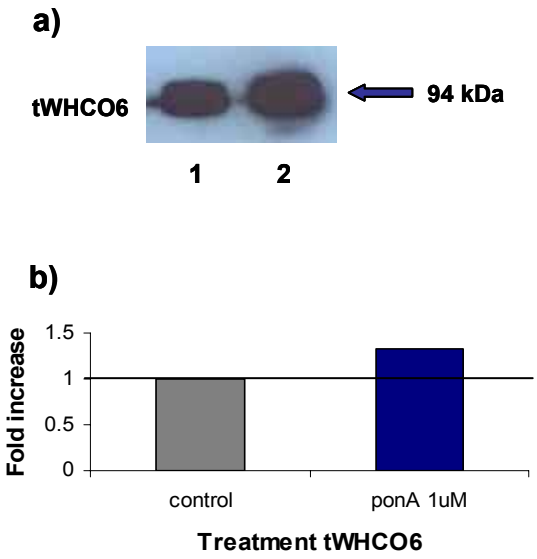


Figure 3.5: β-catenin is expressed in tWHCO6 cells stimulated with ponA (1 μM).

(a) Western blot analysis confirming the presence of β-catenin in both non-treated (lane 1) and ponA treated tWHCO6 cells (lane 2) in total cellular extract. The dark blue arrow indicates the β-catenin bands. (b) Densitometry of β-catenin expression, comparing the fold difference of β-catenin expression between treated and non treated tWHCO6 cells.

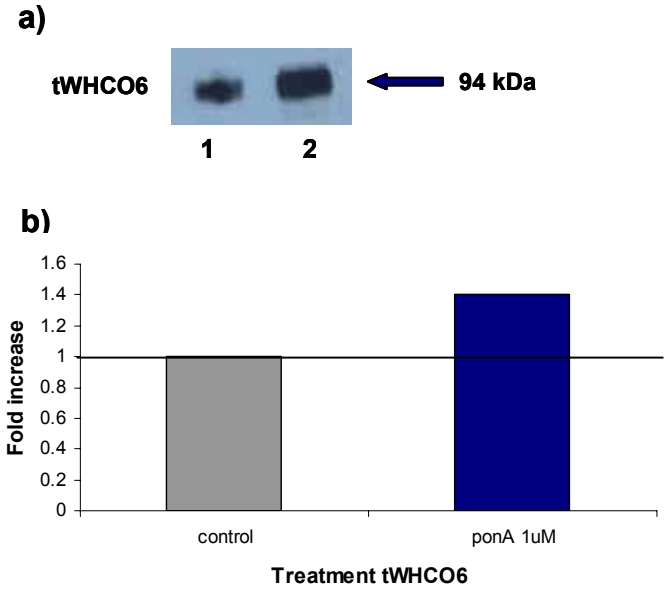


Figure 3.6: Presence of β-catenin in nuclear extracts of tWHCO6 cells stimulated with ponA (1 μM).

(a) Western blot analysis confirming the presence of β-catenin in both non-treated (lane 1) and ponA (1 μM) treated nuclear extracts of tWHCO6 cells (lane 2). The dark blue arrow indicates the β-catenin bands. (b) Densitometry of β-catenin expression, comparing the fold difference of nuclear β-catenin levels between treated and non treated tWHCO6 cells.

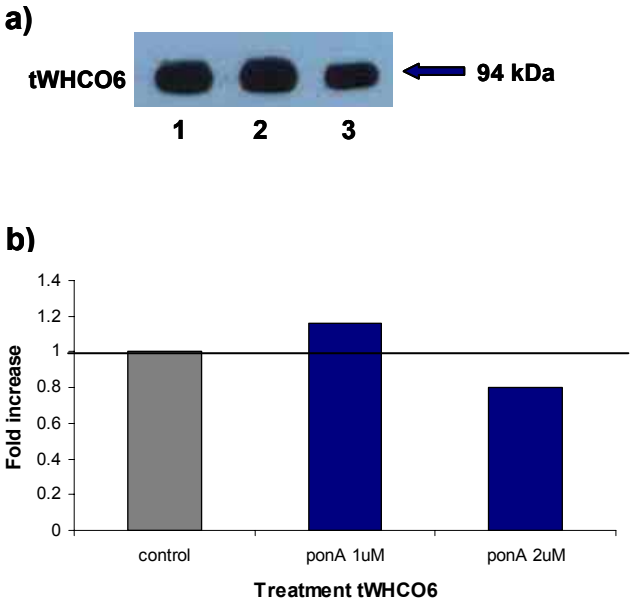


Figure 3.7: β-catenin expression in tWHCO6 cells stimulated with ponA (1 μM and 2 μM).

(a) Western blot analysis confirmed the presence of β-catenin in total cellular extract in non-treated (lane 1) tWHCO6 cells and lane 2 and 3 shows ponA treated tWHCO6 cells 1 μM and 2 μM respectively. The dark blue arrow indicates the β-catenin bands. (b) Densitometry of β-catenin expression, comparing the fold difference of β-catenin expression in non-treated and treated tWHCO6 cells as well as the fold difference of β-catenin expression between 1 μM and 2 μM ponA treated tWHCO6 cells.

Table 3.1: Respective fold increase/decrease of β-catenin due to stimulation of tWHCO6 cells with ponA.

tWHCO6 cells			
	Control	**1 μM ponA**	**2 μM ponA**
β-catenin fold increase/decrease	**1**	**1.3**	**0.8**

*** The above tabulated data is a summary of figures 3.5 and 3.7.**

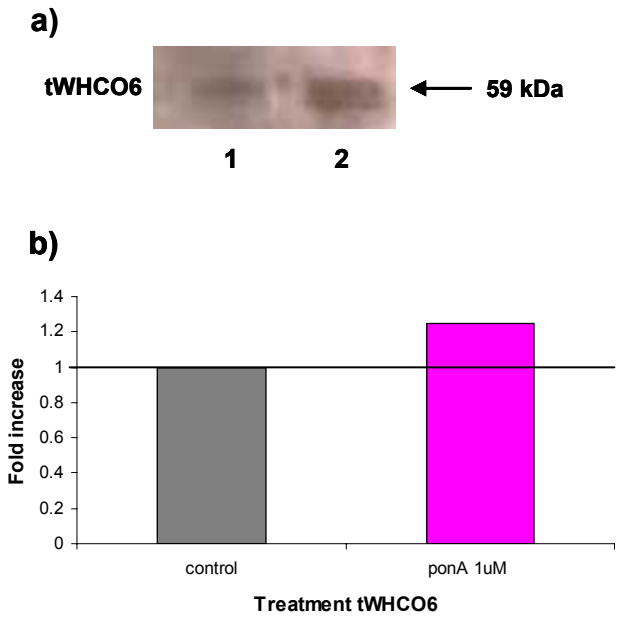


Figure 3.8: ILK-1 expression in tWHCO6 cells stimulated with ponA (1 µM).

(a) Western blot analysis confirming the presence of ILK-1 from total cellular extract in both non-treated (lane 1) and ponA treated tWHCO6 cells (lane 2). The arrow indicates the ILK-1bands. (b) Densitometry of ILK-1 expression, comparing the fold difference ILK-1expression between treated and non treated tWHCO6 cells.

3.5 Discussion

With β-catenin's dual function in mind, the notion that β-catenin is an important oncogene and its abnormal expression may lead to weakened cell-cell adhesion, tumour invasion and metastasis is one of importance. Hence, recombinant DNA technology is a powerful tool for the overexpression of a gene, gene fragments and RNA such as antisense RNA and potentially allows for the isolation of unlimited quantities of a particular gene (Pikaart *et al*., 1998). Gene insertion with a view to overexpression can provide insight on the effect of a gene or gene product on various cellular events such as differentiation, proliferation, migration, invasion, and apoptosis (Pikaart *et al*., 1998).

The success of the stable transfection into WHCO6 cells allowed us to examine the possible effect of β-catenin complexing with Tcf-1 at the Tcf-1 binding sites, in the ILK-1 promoter region and the consequential outcome on ILK-1 gene expression. Co-immunoprecipitation results (figure 3.5c) confirmed that β-catenin localised with Tcf-1 in tWHCO6 cells and further established the possibility that an association between β-catenin and Tcf-1, at the proposed binding sites, activates and/or increases the gene expression of ILK-1. Hence, tWHCO6 cells were stimulated with specific concentrations of ponA to see whether an increase in exogenous β-catenin levels could be obtained.

As mentioned earlier in this chapter, transient transfections yield high gene expression in a short period, the uncertainty/variables that surround this method would require extensive analysis to determine the required parameters to prevent misinterpretations. Although transiently transfected cells results would have allowed for the opportunity to determine statistically whether there was a consistently noted increased in β-catenin expression it was not a goal or end point for this study. This study didn't deserve repeating transfections due to the time consuming nature of transfecting stable mutants rather than transient ones. For this reason repeats of the stimulation of β-catenin were not performed. According to the antibiotic resistant criteria the WHCO6 cell line was stably transfected and so the results obtained from stimulating expression

of the vector cassette were considered a true reflection of the tWHCO6 cellular events and analysed accordingly.

However, as noted in the results section of this chapter, ponA stimulation did not substantially increase cellular or nuclear β-catenin levels to the point where it can be said with certainty that an increase in β-catenin expression influenced the expression of ILK-1. It is likely that a considerable increase in β-catenin expression upon ponA stimulation was not obtained due to the tight intrinsic regulation of β-catenin. With these tight intrinsic controls in mind it allows consideration for the possibility that these regulatory mechanisms may mask any increase in β-catenin levels.

β-catenin expression with 1 μM ponA lead to an increase of 1.2 fold which was not considered to be a considerable enough increase to determine whether β-catenin does actually effect a change in ILK-1 expression. The inability to substantially increase β-catenin expression/concentration in the tWHCO6 cell line may be due to regulatory mechanisms in this particular line that compensate for the exogenous β-catenin expression, thereby masking the desired result. Regulatory mechanisms that would mask a significant increase or decrease in exogenous β-catenin would be the degradation pathways and the potential status of the different components that contribute to such pathways. A pivotal component in this degradation complex is that of APC. APC modulates β-catenin nuclear concentration by one of two ways. First, APC binds β-catenin and incorporates it into a multiple protein complex that triggers degradation (discussed in detail in chapter one) (Rosin-Arbesfeld *et al*., 2003). However, Wnt signalling inhibits β-catenin degradation to occur by preventing the phosphorylation of β-catenin by GSK3β and resulting in β-catenin translocating to the nucleaus (Neufeld *et al*., 2000). Second, APC binds nuclear β-catenin and exports it to the cytoplasm for degradation (Neufeld *et al*., 2000; Rosin-Arbesfeld *et al*., 2003). APC's involvement in β-catenin degradation is discussed in detail in chapter four, section 4.5.

Other degradation mechanisms to note would be the ubiquitin-proteosome pathways such as the β-transduction-repeat-containing protein (β-TrCP), seven in absentia homolog 1(Siah1) and RxR processes that compensate for the increase of β-catenin. The F-box protein, β-TrCP binds to phosphorylated β-catenin (which is essentially

degradation targeted β-catenin) and recruits SKp-1 and Cullin1 to form a complex (Liu *et al*., 2001). In parallel to being involved in this complex, Cullin1 binds to E2 UbcH3 and the result is ubiquitination and degradation of β-catenin (Liu *et al*., 2001). Alternatively, β-catenin is degraded by the p53 inducible Siah-1 pathway. p53 upregulates Siah1, which in turn interacts with the N-terminus of APC and recruits a ubiquitination complex to the N-terminal of β-catenin, thus targeting β-catenin for degradation (Liu *et al*., 2001; Matsuzawa and Reed, 2001) (figure 3.9). It should be noted that both the APC degradation and Siah1 pathways require β-catenin's N-terminus for successful degradation of the protein.

In addition to the above, β-catenin can also be degraded by APC independent pathways such as the RXR-mediated pathway. Retinoids are derivatives of vitamin A that regulate gene transcription through two types of nuclear receptors, namely, retinoic acid receptors (RARs) and RXRs, both of which are ligand-dependent transcription factors and each of these nuclear receptor families consists of three subtypes, (α; β; and γ) (Qiu *et al*., 1999; Xiao *et al*., 2003). Crosstalk between Wnt/β-catenin and retinoid-signalling pathways has been reported. *In vivo* RAR was found to inhibit β-catenin mediated gene transcription (Xiao *et al*., 2003). Retinoic acid has been shown to complement the Wnt signalling pathway in the upregulation of gene transcription. RAR signalling has no effect on the β-catenin protein levels within the cell, however, RXR agonists have been shown to cause degradation of RXR_{α}, RARs and thyroid hormone receptor (TR) (Qiu *et al*., 1999). This prompted Xiao *et al.* (2003) to examine the role of RXR and its ligands and their roles in β-catenin regulation (Xiao *et al*., 2003).

Xiao and colleagues (2003) showed that removal of β-catenin's N-terminus didn't prevent its degradation via RXR agonists, which is a known, necessary prerequisite for APC degradation. Furthermore, Xiao *et al*. (2003) confirmed that this pathway is independent of APC because loss of function mutations in APC and p53 did not hinder or effect β-catenin degradation by RXR agonists, neither did GSK3β inhibition by lithium chloride (LiCl) have any effect on the degradation of β-catenin by RXR agonists (Xiao *et al*., 2003). Xiao *et al*. (2003) showed that RXR agonists induced β-catenin degradation when cotransfected with RXRα. Xiao and colleagues (2003) found that degradation involves the proteosomal pathway, as proteosome inhibitors

blocked RXR agonists (Xiao *et al*., 2003). It is thought that RXRα acts as a docking protein for β-catenin and that when bound with β-catenin, the complex changes in response to RXR agonists and thus becomes a target for proteosome components and ultimately proteosome degradation (Xiao *et al*., 2003). The above highlights the many different mechanisms of β-catenin degradation all of which could have potentially affected the overexpression of β-catenin in the tWHCO6 cell line (also see figure 3.9 below).

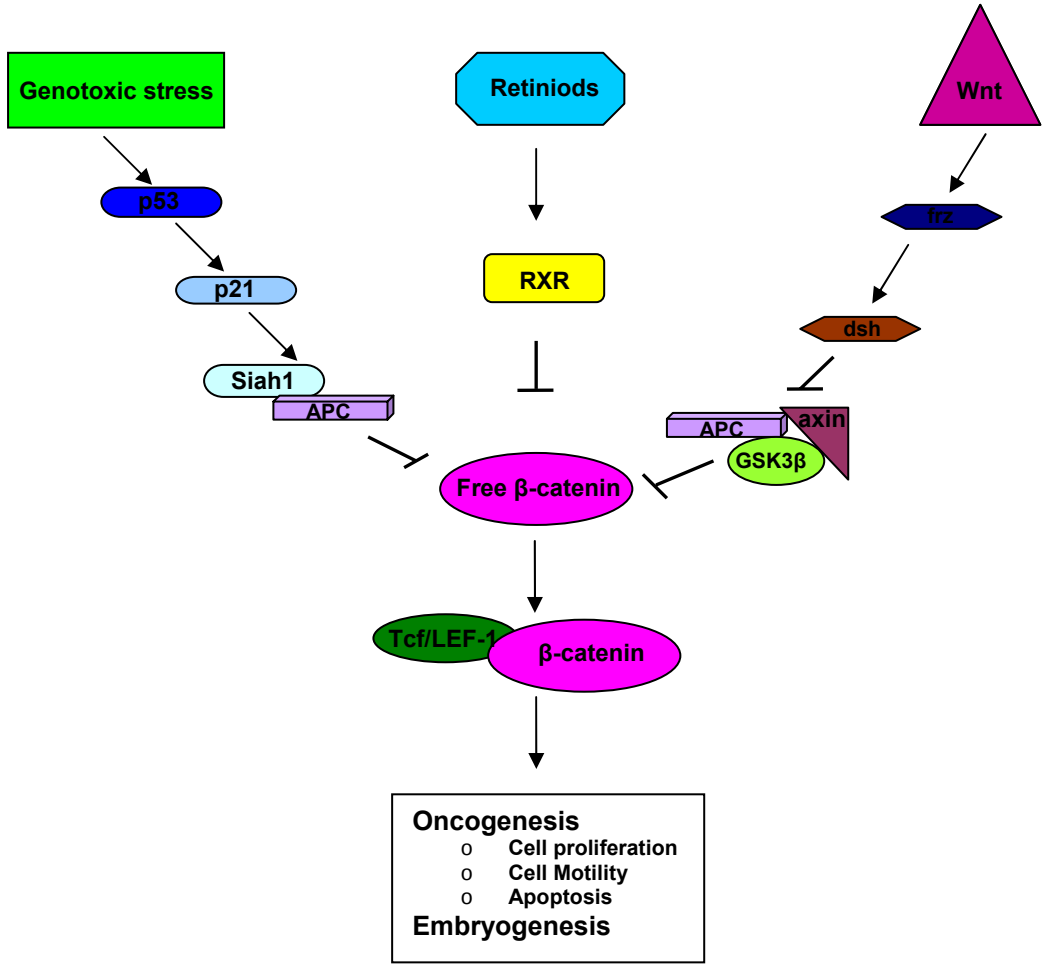


Figure 3.9: Diagram depicting 3 pathways that regulate β-catenin turnover in transformed cells.

Ubiquitin-proteosome pathways compensate for an increase of β-catenin. p53 upregulates Siah1 which interacts with the N-terminus of APC and recruits a ubiquitination complex to the N-terminal of β-catenin resulting in β-catenin degradation. RXR_{α} binds β-catenin and the complex changes in response to RXR agonists resulting in the complex becoming a target for proteosome degradation. Wnt signal inhibits the activity of GSK3β or the multiprotein complex is not formed properly (i.e. Mutation of APC or β-catenin) (Adapted from Xiao *et al*., 2003).

One is reminded that pERV3 contains a RXR receptor (figure 3.0 and figure 3.2) which is designed to form a hetrodimer with the synthetic ecdysone RXR receptor (EcBR) (Xiao *et al*., 2003). The expression of this RXR receptor as well as ponA stimulation should not have had any affect on the β-catenin expression in the tWHCO6 cells. However, it would be important to rule out any RXR and agonist that could potentially regulate β-catenin protein levels, thus masking any overexpression within the tWHCO6 cell line.

These β-catenin regulatory systems provide explanations for the lack of a substantial visible increase in β-catenin in the WHCO6 cell line and probably contributed to the slight decrease in β-catenin when the concentration of ponA was increased to 2 μM (figure 3.7).

Another possibility for the little increase of exogenous β-catenin upon ponA stimulation is that stable transfection of the two vectors (pERV3 and pEGSH-β-catenin) could have resulted in the chromosome position effect. The integration of a gene into a chromosome can result in the suppression of gene expression; known as the chromosome position effect (Xiao *et al*., 2003). DNA methylation and histone acetylation disrupts packaging of the nucleic acids and gene transcription due to integration of the 'foreign gene' (Qiu *et al*., 1999). The integration sites of both the pERV3 and the pEGSH vectors are known to affect expression.

Interestingly, Dahan (2005) transfected the WHCO1 cell line with the pERV3 pEGSH-β-catenin construct and found that stimulation of these cells with ponA lead to a 1.2 fold increase. Dahan's data in combination with the abovementioned data lends credibility to the explanation that considerable β-catenin overexpression was not accomplished due to the regulatory mechanisms in both these cell lines, which compensate for a significant increase of exogenous β-catenin. However, to further substantiate this statement statistically significant data would need to be analysed.

An alternative to consider when taking all the above comments into consideration, is that it appears that in these HOSCC cell lines, there is an increase in factors which mask or prevent a visible increase in β-catenin concentration. Competitive binding for β-catenin is known to occur between LEF-1 and APC. In colorectal carcinoma

cells Henderson *et al.* (2002) showed that LEF-1 anchors β-catenin in the nucleus by preventing export of β-catenin mediated by APC (Henderson *et al*., 2002). Shown here, Tcf-1 is expressed in HOSCC cell lines and it has been shown previously that LEF-1 and Tcf-1 have 99% homology (Henderson *et al*., 2002). Therefore a possible overexpression of Tcf-1 in WHCO6 could increase the nuclear distribution of β-catenin by anchoring β-catenin in the nucleus (just as LEF-1 has been shown to do), which in turn could affect the ILK-1 levels within this cell line.

The attempt to overexpress β-catenin in an HOSCC cell line was aimed at increasing cytoplasmic β-catenin that would result in its translocation into the nucleus and ultimately have an effect on the level of ILK-1 in the cell line. However, this approach did not lead to a substantial increase of β-catenin expression, possibly due to the factors and the cells inherent regulatory mechanisms mentioned above. An alternative approach to this study may have been to directly increase ILK-1 by creating an ILK-1 vector construct and thus determine ILK-1 effects on the HOSCC cell. Concurrently, increasing ILK-1 would have promoted β-catenin's translocation to the nucleus.

Further investigation is needed for overexpression/increased production of β-catenin in HOSCC cell lines. Accumulation of nuclear/cytoplamsic β-catenin in cancers is not necessarily due to constitutive activation of the Wnt pathway. Often mutations in key components of degradation machinery, such as axin, APC or GSK3β are responsible or contribute to the deregulation of β-catenin concentration (Bienz and Clevers, 2000, Park *et al*., 2005). The tight intracellular regulatory and degradative processes require further investigation to determine whether the increase in β-catenin levels within both the total and nuclear cell extracts in the tWHCO6 cell line are noteworthy. Although nuclear accumulation of β-catenin has been reported in HOSCC, the associated β-catenin mutations have not and so provide a potential route for obtaining intracellular increased β-catenin levels.

Chapter Four: General discussion and conclusion

β-catenin is a multifunctional protein that plays a pivotal role in both Wnt signal transduction and cell adhesion (Han *et al*., 2008). The oncogenic effects of β-catenin appear to stem from its decrease in cell adhesion capacity as well as its nuclear accumulation that consequently results in inappropriate activation of gene transcription (Daniels and Weis, 2005). It is this dysregulated gene transcription that often influences cell proliferation, morphogenesis and apoptosis. Investigation of β-catenin will provide valuable insight into the various mechanisms of cellular transformation and tumour progression for this cancer-type under study. Previous studies have focused largely on β-catenin expression and the associated implications in carcinomas derived from simple epithelia (Hoppler and Kavanagh, 2007; Segditsas and Tomlinson, 2006). However in this study β-catenin expression and its potential role in OSCC, derived from a stratified epithelium was examined. Therefore, we set out to investigate the link between β-catenin cellular/nuclear accumulation, β-catenin's ability to complex with Tcf-1 and the potential consequences this complex may have on the levels of ILK-1 protein in HOSCC, an aggressive cancer-type that is derived from a stratified epithelium.

4.1 β-catenin expression and nuclear localisation influences

One of the primary objectives of this study was to confirm the presence of intracellular β-catenin and Tcf-1, not only in the cytoplasm but specifically within the nucleus. This was achieved across all 5 HOSCC cell lines. These results however, offer fairly limited interpretation until they can be compared to the level of β-catenin in normal oesophageal tissue. Such comparisons would allow conclusions to be drawn regarding cell behaviour in the transformed state compared to that of its normal counterpart with respect to the alteration of β-catenin expression and functional activity. In normal cells it has been stated that there is very little β-catenin in the cytoplasm or nucleus due to the ubiquitination mechanism that underlies the rapid changes in protein activity and/or localisation during signal transduction (Aberle *et al*., 1997). In fact both Ninomiya *et al*. (2000) and Saeki *et al.* (2002) showed that, nuclear β-catenin is not detectable in normal oesophagus cells, nuclear β-catenin, as

seen in these HOSCC cells, is likely to be an indication of deregulated β-catenin turnover. This is in keeping with what has previously been reported for HOSCC (de Castro *et al*., 2000; Ninomiya *et al*., 2000; Saeki *et al*., 2002). A correlation between nuclear β-catenin and tumourgenesis has been well established (reviewed by Hoppler and Kavanagh, 2007; Polakis, 1999 and Willert *et al*., 2006).

4.2 Tcf-1 expression and nuclear localisation influences

Tcf-1 specific immunoblotting, conducted on resolved whole cell extracts, demonstrated the presence of numerous Tcf-1 isoforms in all five HOSCC cell lines. This is in line with the literature of van de Wetering *et al.* (1996) that discovered a multitude of Tcf-1 proteins. In terms of the isoforms that are present in the HOSCC cell lines there is a high degree of uniformity across the cell lines assessed. The homogenous nature of the Tcf-1 isoform expression in the studied lines (demonstrated by the Western blot profiles of whole cell extracts) could imply that the Tcf-1 alleles are identical across the cell lines (i.e. each genome contains the sequence for the same number of tandem repeats), that the same alternatively spliced isoforms are present.

The precise implications of the different isoforms and their DNA-binding and protein-complexing capabilities have yet to be completely identified. These Tcf-1 isoforms are not simply explained due to the alternative splice site in the C-terminus (Atcha *et al*., 2007). Perhaps the relative abundance of Tcf-1 isoforms corresponds to the relative ratio of nuclear receptors to Tcf-1 in different epithelial tissue types which could critically modify actions of nuclear receptors in these tissues (Atcha *et al*., 2007). To date the majority of studies have focused on the function of Tcf-1 within Wnt signalling, little has been done to understand the full complement of Tcf-1 isoforms and the influence/contribution of patterns during signalling. Hence our aim to look at the influence of Tcf-1 signalling on ILK-1 is one of interest. Although van de Wetering *et al.* (1996) suggested that these Tcf-1/LEF isoforms are interchangeable and redundant. Non redundancy has been noticed phenotypically with mice missing one or more Tcfs, probably due to the differences in their functional domains and in the alternatively-spliced C-terminal tails (Atcha *et al*., 2007; Merrill *et al*., 2001). Mouse knockout and transgenic experiments (removal of

the *LEF-1* or *Tcf-1* gene) resulted in partially non-redundant phenotypes in tissues where their expression overlapped, also expression of LEFs/Tcfs of dominant negative forms produced non-identical phenotypes (Merrill *et al*., 2001; Okamura *et al*., 1998).

4.3 β-catenin and Tcf-1 complexing

This study has generally taken the assumption that β-catenin is the co-activator of choice for Tcf-1 in the context of dependent regulation of gene expression. We have certainly confirmed that both β-catenin and Tcf-1 are present in the HSOCC cell lines under study. However, the Tcf -1 isoforms noted in these cell lines and their functionality could provide a different outlook on Tcf-1 function and/or association with β-catenin. For instance as mentioned in chapter two, the literature has shown that the alternate Tcf-1 promoter allows for the production of short isoforms that lack N-terminal β-catenin interaction/binding domains, but retain the ability to interact with Grg repressors, "classifying" Tcf-1 as repressor complexes (Willinger *et al*., 2006). Tcf-4 and Tcf-1 are the predominant Tcfs found in normal gut mucosa. Normal Tcf-4 expression is required for development and maintenance of stem cells in the crypt of a healthy colon (Korinek *et al*., 1998). It has been shown that knockout Tcf-4 mice (missing full length Tcf-4) have an intestine with few differentiated villi, lack a crypt stem cell compartment and die shortly after birth (Hoppler and Kavanagh, 2007; Korinek, *et al*., 1998). However, Tcf-1 (normally expressed in foetal and adult intestine) knockout mice develop intestinal and mammary adenomas (Hoppler and Kavanagh, 2007; Korinek, *et al*., 1998; Waterman *et al*., 2004), depicting Tcf-1 as a Wnt-linked tumour suppressor rather than a tumour promoter (Tcf-4). Dominant negative isoforms of Tcf-1 (truncated mRNAs missing the β-catenin binding sequence) exist and compete for binding to Wnt-response elements (WREs), thus preventing Wnt signalling (Hovanes *et al*., 2001; van de Wetering *et al*., 1996). Given that a loss of full length Tcf-4 causes a depletion of stem cells and dominant negative Tcf-1 acts more like a tumour suppressor, it has been predicted that in normal colon tissue the predominant Tcf forms are full length Tcf-4 and dominant negative Tcf-1, which act antagonistically and prevent excessive Wnt signalling (Arce *et al*., 2006; Roose *et al*., 1999). The full length Tcf-4 maintains a proliferative state

and the dominant negative Tcf-1 maintains a reduction in proliferation and promotes differentiation (Hoppler and Kavanagh, 2007). Furthermore the different Tcf-1 isoforms would also compete for WREs and so restrict Wnt signalling (Atcha *et al*., 2007; van de Wetering *et al*., 1996).

With the abovementioned noted, if we refer to the nuclear and whole cell extracts (figures 2.4 and 2.5 respectively) for Tcf-1 for this study, both full length and truncated Tcf-1 isoforms were noted among the cell lines. Tcfs functional β-catenin binding capabilities were determined by immunoprecipitation. Surprisingly, when examining the 5 HOSCC cell lines both the full length and truncated Tcf-1 isoforms were capable of complexing with β-catenin (Tcf-1E to a more noticeable degree than Tcf-1A/C/G). This was unexpected and not in line with the literature that suggests that the full length Tcf-1E is capable of binding to β-catenin, however, the truncated forms lack β-catenin binding capabilities (Atcha *et al*., 2007). Various literatures suggest that these truncated isoforms are thought to be dominant within the cells and possess Wnt signalling antagonistic properties/tumour suppressor functions (Atcha *et al*., 2007). From the co-immunoprecipitation results of this study this was not the case. Both the full length and truncated forms clearly bound to β-catenin (figure 2.6a). A variety of factors could account for this, however, one thing is for certain and that is that the Tcf-1 functionality for the cell lines under study does not fit that of normal gut mucosa. It has previously been shown that colon cancer cells predominantly express full-length LEF-1 and Tcf-1 isoforms while down regulating the expression of dominant negative isoforms. We cannot discount the possiblity that the HOSCC cell lines do not possess dominant negative isoforms of Tcf-1; there may just be another triggering factor that has inhibited the expression of the dominant negative Tcf-1s.

The shift from a suppressive activity to that of proliferative one in these cells may be largely due to the Tcf-1/Wnt signalling process. The Tcf-1 observed in the cell extracts bind to β-catenin and there does not seem to be noticeable truncated Tcf-1 that is incapable of binding β-catenin. To further validate these results it would be important to perhaps isolate the various Tcf-1 isoforms and examine the protein profiles. To look at the functionality of each of the isoforms for these cell lines cloning of the Tcf-1s and transfection may help deduce the action of the different Tcf-

1s. Another consideration is DNA sequence recognition affinity. Different DNA-protein (Tcf-1) conformations with varying degrees of stability could also affect the proliferative/suppressive effect of the Tcf-1 isoforms. For instance Atcha *et al.* (2007) showed that E-tail (E-tails of full-length Tcf-1, contain DNA-binding domains and if the WRE sequence differs from the consensus Tcf binding site, the E-tail helps stabilise the complexing of the two proteins) containing isoforms of Tcf-1 and Tcf-4 enables β-catenin to activate transcription of the LEF-1 promoter by binding to the same WRE that Tcfs are generally able to bind to. However, other isoforms of Tcf-1 do not activate the abovementioned promoter. A clear explanation for this has not been discovered, yet DNaseI footprint experiments suggest that they adopt a slightly different conformation once bound and the E-tail modifies DNA sequence affinity (Atcha *et al*., 2003; Atcha *et al*., 2007). What is not considered a possibility is that the E-tail enables a better interaction or more stable interaction with β-catenin; the reason for this is that Prieve and Waterman (1999) have shown with TOPFLASH experiments, that Tcfs without E-tails efficiently co-immunoprecipitate with β-catenin in COS-7 cells.

Thus far we have shown that there are different isoforms of Tcfs expressed in the HOSCC lines capable of binding to β-catenin, however evidence from literature show that Tcfs are diversified in form and consequently function allowing them the ability to regulate different subsets of target genes. With this in mind we decided to continue investigating whether a Tcf-1 isoform binds to the known Tcf-1 site within the ILK-1 promoter region and whether this association included β-catenin.

4.4 β-catenin/Tcf-1/DNA-complexing

A vast majority of reasons for human cancers are located within the sequence specific DNA binding domain of the protein, which suggests strongly that DNA binding is critical to tumour suppressor/activator function and is therefore extensively regulated. There is a large array of literature documenting regulation through effects on residues and modifications within protein binding regions such as phosphorylation, acetylation, deletion, antibody binding, and short basic peptides have been shown to

augment DNA-protein complexes as measured by EMSAs (Cain *et al*., 2000; Han *et al*., 2008).

The definite shift and super shift visualised in the EMSA experiments under study have indicated that the cell lines have fully functional binding capabilities with regards to Tcf-1's, DNA and β-catenin binding domain. We were able to establish that the β-catenin/Tcf-1 complex associates directly with both Tcf-1 binding sites located in the ILK-1 promoter region. From this one could draw the logical comment that it would be necessary for both β-catenin and Tcf-1 to have fully functional binding domains if inappropriate ILK-1 expression were to occur and perhaps account for abnormal cell expression.

Chapter one provides a detailed overview of the ILK-1 promoter region, describing the promoter region as a TATA-less, GC-rich promoter region with numerous *cis-acting* transcription factors-binding sites. One shouldn't forget that TATA-less promoters often rely on GC-boxes to activate transcription (Melchoir *et al*., 2002). A major transcription factor binding commonly found in TATA-less/GC rich promoters are those of the Sp1 family of proteins. Therefore it is possible that Tcf-1/β-catenin/DNA complex alone weren't enough to activate transcription of ILK-1, noticeably (Han *et al*., 2008; Melchoir *et al*., 2002; Mertin *et al*., 1999). If this is the case it would explain why a noticeable increase in ILK-1 expression was not observed when β-catenin was overexpressed in the oesophageal SCC lines examined in chapter three. To investigate this further one would need to overexpress Sp1 alone and in combination with overexpressed β-catenin to see if Sp1 is the determining factor for ILK-1 overexpression. Or the two overexpressed proteins may work synergistically and result in ILK-1 overexpression or possibly, these two proteins could provide an enhanced result. Futhermore there may be other factors that influence or even mask the actual influence of these two factors on ILK-1 expression. These considerations reiterate the complexity of signal transduction pathways.

Unfortunately the EMSA experiments detailed in chapter two do not provide evidence for the potency of the binding association complex i.e. transcriptional activation. For instance an E-tail found in certain Tcf-1 isoforms (such as Tcf-1E) binds to WREs via a bipartite recognition system and is essential for β-catenin binding. Although the

primary DNA binding of Tcf-1 is at the HMG domain, a secondary binding occurs via a cysteine clamp (C-clamp) located within the E-tail. This C-clamp provides additional binding stability, because of additional DNA contacts and results in a potent transcription activation of Wnt target genes (Atcha *et al*., 2007). This C-clamp does not replace the function of the HMG domain (Atcha *et al*., 2007). Double DNA binding transcription factors are considered unusual, however this is not unique.

Alternate splicing of the p53 gene results in a second DNA binding domain known as a C-terminal domain (CTD) (Ahn and Prives, 2001). This domain binds without any sequence specificity and stabilizes DNA binding (Ahn and Prives, 2001). Atcha *et al.* (2007) used DLD-1 colon cancer cells (contain high levels of β-catenin/Tcf-1 complexes in the nucleus) and found that over expressed dominant-negative Tcf-1E complexes (β-catenin binding site is absent) compete with β-catenin/Tcf-1 complexes and result in down regulation of Wnt target gene expression, specifically those involved in cell cycle and cell growth. However overexpression of dominant negative Tcf-1E with a mutation in the C-clamp does not (Atcha *et al*., 2007). This means that intact C-clamp DNA association of Tcf-1E is required for downregulation of some growth control genes that contain Wnt response elements (Atcha *et al*., 2007). It does not rule out that other Tcf-1 isoforms missing the C-clamp regulate Wnt target and other target genes. Atcha *et al.* (2007) showed that Tcf-1 isoforms lacking C-clamps were able to regulate Wnt targets such as *LEF-1* and *CDX1* via cooperative interactions with other factors. LEF-1 can activate *CDX1* transcription via cooperative interactions with CDX-1 protein (Beland *et al*., 2004). Atcha *et al.* (2007) showed that a β-catenin/LEF-1 fusion protein was not active until co-expressed with CDX-1. It seems that CDX-1 might be needed to stabilize LEF-1 interactions with the *CDX1* promoter (Atcha *et al*., 2007). Since over activation of growth control genes by β-catenin/Tcf-1 often lead to cancer, interference with C-clamp function might undercut these oncogenic functions. It is possible that the β-catenin/Tcf-1/DNA understudy contains a C-clamp and is a potent transcription activator however, we do not suspect this. We suspect that the Tcf-1s bound in the complexes noted in the co-immunoprecipitation and EMSA experiments actually cooperate with other transcription factors to cause activation of ILK-1. The reason for this prediction is that when examining the results of chapter three, over expression of β-catenin did not yield an increase in ILK-1 protein levels. However to confirm this it

would be of importance to over express full length Tcf-1E concurrently with β-catenin.

DNA-protein complex formation seemed to be much stronger with the Tcf-1.1 compared to the Tcf-1.2 probe. This specificity or affinity for Tcf-1.1 could have been confirmed by using mutated Tcf-1 probes which would probably not have been bound by Tcf-1; via the process of elimination, the functional/essential nucleotide bases needed for optimal DNA-protein binding could be determined. Also the disparity in DNA affinity for the two probes may be due to the fact that full-length Tcf proteins suffer a large loss in specific activity when they are purified. This property stems from the fact that purification yields unfolded, unstructured polypeptides (Daniels and Weis, 2005; Love *et al*., 2004). Another possibility for the difference in affinity for Tcf-1 between the two probes may be the flanking nucleotides to the core DNA-binding domain. Variants in DNA-binding specificity may play an important role in activation transcription of ILK-1. It has been shown that subtle preferences for nucleotides flanking the DNA binding site help achieve DNA-binding with specific transcription factors that have homology in their DNA-binding domains (Balmer and Blomhoff, 2009; Han *et al*., 2008; Mertin *et al*., 1999). Mertin *et al.* (1999) showed that different SRY-related HMG box (SOX) proteins (SOX proteins family share at least 50% homology in their HMG domains) achieve specific DNA-binding through subtle differences for nucleotides flanking their HMG domains. These preferences are likely to be dictated by signature amino acids in their HMG domains. Mertin and colleagues (1999) showed using EMSAs and DNA competition and dissociation studies that SOX9 binding was enhanced when the DNA-binding element was flanked by 5' AG and 3' GG nucleotides. This was not the case for another SOX related protein; sex-determining region Y (SRY) which preferred different 5'and 3' flanking nucleotides (Mertin *et al*., 1999). The Sox9 WT (S9WT) DNA binding element with the preferred flanking nucleotides was compared between HMG domain containing proteins Tcf-1 and SOX9. SOX9 showed a 2-fold higher affinity for the S9WT binding element (Mertin *et al*., 1999). The achievement of DNA sequence specificity via preferences for flanking nucleotide combinations, would suggest that some transcription factors may co-evolve with their DNA targets to achieve specificity (Balmer and Blomhoff, 2009). Nevertheless, to determine

whether Tcf-1 binding to the defined elements has a functional consequence, overexpression of a protein bound to these elements would be the more pertinent route for our study.

4.5 Overexpression of β-catenin in WHCO6

The attempt to overexpress β-catenin in a HOSCC cell line was aimed at increasing cytoplasmic β-catenin, in order to direct its translocation to the nucleus. The pEGSH-β-catenin vector (contains full length β-catenin cDNA) as discussed in detail in chapter three, was stably transfected together with pERV3 into the WHCO6 cell line. However, ponA stimulation of the tWHCO6 total cell population did not lead to an increase in cellular β-catenin expression (refer to chapter three). As noted in chapter three the accumulation/depletion of the "multi-tasking" protein, β-catenin, is regulated by several pathways within the cell according to cell function (Liu *et al*., 1999) (reviewed by Willert *et al*., 2006). Hence, the failure to substantially increase β-catenin within the tWHCO6 cell line may have been due to an intrinsic cellular regulatory mechanism of which there are many.

APC is considered an important "regulator" of β-catenin concentrations within the cell. Phosphorylation of APC appears to enhance its ability to promote β-catenin degradation. It has been widely suggested that APC is heterogeneously phosphorylated in cells due its ability to interact directly with the scaffold axin and its associated kinases and phosphatases (Neufeld *et al*., 2000). Ha *et al.* (2004) found that in the absence of a Wnt, signal only phosphorylated APC will have an important role in sequestering β-catenin. The enhanced affinity implies that phosphorylation of APC likely slows the release of β-catenin from the complex. To confirm whether this is the reason that an increase in β-catenin is masked, an APC construct with a mutant phosphorylation site would need to be transfected into the WHCO6 cell line.

Further to this, there have been various studies that have investigated proteins that regulate the degradation complex thus controlling the turnover subcellular localisation of β-catenin. Pin1 is a peptidyl-prolyl *cis-trans* isomerase (PPIase) that isomerizes phosphorylated Serine/Threonine-Proline peptide bonds (Ryo *et al*., 2001). Pin1

affects the activity and/or protein-protein interactions of a subset of phosphoproteins such as cdc25 and the microtubule-associated protein tau. (Ryo *et al*., 2001) A depletion of Pin1 induces apoptosis and also facilitates neural death in Alzheimer's disease (Ryo *et al*., 2001). Breast and prostate tumour cells have shown to exhibit increased levels of Pin1 along with increased transcriptional activity of cyclin D1 and c-myc among others (Wulf *et al*., 2001). Ryo *et al.* (2001) found that by manipulating Pin1 levels, Pin1 regulates the turnover of β-catenin as well as its subcellular localisation. Pin1 accomplishes this by binding directly to a phosphorylated Serine/Proline motif next to the APC-binding site in β-catenin (Ryo *et al*., 2001). This novel mechanism for regulating signalling by β-catenin, supports the possibility that an increase in β-catenin in the tWHCO6 cell line may have been masked due to the numerous levels of subcellular regulatory mechanisms.

Another aspect to consider is the turnover of the APC degradation complex. Xing *et al.* (2003) has speculated that the competitive binding of phosphorylated APC and axin to β-catenin promotes turnover of the degradation complex. This model proposes that cycles of APC phosphorylation are required for turnover of axin and release of β-catenin. Instead, Ha *et al*. (2004) noted that the greatly enhanced affinity of phosphorylated APC likely slows the release of β-catenin from the complex. Ha *et al*. (2004) suggests that the phosphorylation state of APC controls the rate of β-catenin release, thereby coupling degradation complex turnover to the cellular concentration of β-catenin. Ha and colleagues (2004) also state that it is not necessary for β-catenin to dissociate from the degradation complex in order to bind the ubiquitination machinery. It is possible that the energy-dependent unfolding activity of the proteosome-associated chaperones drives the dissociation of β-catenin from the complex, directly coupling its removal with degradation (Ha *et al*., 2004). In this way, β-catenin would be prevented from inappropriate entry into the nucleus by remaining associated with APC until it is degraded, rather than leaving the complex and possibly encountering cytoplasmic Tcf (Ha *et al*., 2004; Lee *et al*., 2001).

The β-catenin regulatory systems discussed above as well as those discussed in chapter three, provide explanations for the lack of a substantial visible increase in β-catenin in the WHCO6 cell line and probably contributed to the slight decrease in β-catenin when the concentration of ponA was increased to 2 μM (figure 3.7).

Ninomiya *et al*. (2000) examined the expression pattern of E-cadherin, α-catenin and β-catenin of 22 oesophageal squamous cell carcinoma samples with their corresponding normal oesophageal tissue. This study showed that the β-catenin level was less than half that of the corresponding normal oesophageal tissue, 40.9% (9 cases) (Ninomiya *et al*., 2000) and only 13.6% (3 cases) showed β-catenin expression in tumour tissue that was more than 2 fold that in normal tissue. For this study an increase in protein expression was defined as more than 2 fold that of corresponding normal oesophageal mucosa (Ninomiya *et al*., 2000). PCR and direct DNA sequencing confirmed that the accumulation of β-catenin was not a result from APC truncation or β-catenin gene mutation (Ninomiya *et al*., 2000). However, Ninomiya and colleagues (2000) were not able to determine the mechanism of β-catenin accumulation in these tumours. It has been demonstrated by Ochiai *et al*. (1994), Shiozaki *et al*. (2001) and Shibamoto *et al*. (1994) that phosphorylation of β-catenin is affected by other molecules such as epidermal growth factor receptor (EGFR), c-erbB-2 and hepatocyte-growth factor receptor. Therefore other mechanisms seemingly distantly involved in β-catenin degradation may be required for stabilisation of β-catenin. However, from Ninomiya's (2000) study it is clear that β-catenin accumulation is involved and does exist in oesophageal carcinogenesis.

Had there been a noticeable increase in ILK-1 expression within the overexpressed WHCO6 cell line it may have been worth examining the binding of nuclear proteins extracted from the stimulated cells with the Tcf-1 site of the ILK-1 promoter. EMSA experiments may have shown an increased binding of Tcf-1 to the Tcf-1 binding site of the ILK-1 promoter after a period of stimulation by ponA. Supershift experiments would have confirmed the presence of β-catenin in the stimulated cells. Maupas-Schwalm *et al*. (2005), showed through EMSAs an increased binding of nuclear proteins to the cyclin D1 Lef-1/Tcf site was maximal after 45 minutes of stimulation by tPA. Analysis of the stimulated WHCO6 cell line from this aspect may have shown whether in fact there is a link to an increase of binding to the Tcf-1 sites within the ILK-1 promoter and increase in ILK-1 expression alone. However, the possibility of this being masked by regulatory mechanisms should not be overlooked. Furthermore to test the functional significance of each individual Tcf-1 site reporter constructs could have been created in which one or both elements were mutated, such

that Tcf-1 could no longer bind and the stimulatory effect of β-catenin compared to the HOSCC 'wild-type' promoter could be compared.

Various studies on ILK-1 overexpression show its involvement in the β-catenin degradation pathway. ILK-1 stimulates the phosphorylation of protein kinase B (PKB) on serine 473 (Lynch *et al*., 1999; Persad *et al*., 2000). The phosphorylation of PKB plays a role in the ability of ILK-1 to down regulate GSK3β, this compromises the degradation complex and so provides a window of opportunity for an increase in cellular β-catenin that in turn translocates to the nucleus and activates gene transcription (Novak *et al*., 1998; Somasiri *et al*., 2001). This knowledge could possibly allow for a completely different angle of examination for this study and determine whether there is in fact a 'pathway loop'. Does an increase in ILK-1 that leads to an increase in cellular β-catenin, which translocates to the nucleus, possibly bind to the ILK-1 promoter region due to, the Tcf-1 binding sites and so cause a synergistic increase of ILK-1? Somasiri *et al.* (2001) transfected mouse mammary epithelial cells with wild type ILK-1 and found that overexpression caused the cells to loose E-cadherin and develop a mesenchymal morphology associated with loss of cell-cell adhesions (Somasiri *et al*., 2001). The overall loss in steady state E-cadherin levels in wild type ILK-1 overexpressing cells is an observation previously made in intestinal epithelial tissue (Somasiri *et al*., 2001; Wu *et al*., 1998). Therefore, one of the possible expectations for this study would have been a noticeable change in the morphology of the transfected cells. However, the HOSCC cell lines were not examined to determine whether these tumours naturally express increased levels of E-cadherin. Although this could be of importance as Somasiri *et al.* (2001) found that forced expression of E-cadherin caused an epithelial reversion in mesenchymal wild type ILK-1 overexpressing cells, yet no correlation could be found between the levels of wild type ILK-1 overexpression or E-cadherin expression and differentiative rescue (Somasiri *et al*., 2001).

The regulatory mechanism effects mentioned in this chapter and chapter three could possibly be reduced or minimised if a mutant β-catenin construct was transfected as opposed to the wild type β-catenin construct used in this study. Shtutman *et al.* (1999) assessed the effect of β-catenin overexpression on cyclin D1 abundance in human 293T cells. The cells were transfected with expression vectors encoding

mutant N-terminal β-catenin constructs (GFP-ΔN-β-cat and β-cat Y33) that render it stable against degradation (Shtutman *et al*., 1999). Cyclin D1 protein levels were induced 2 to 3 fold with these particular constructs, as opposed to the control construct (construct encoding GFP). Transfection of a β-catenin mutant construct into the WHCO6 cell line may have been a reasonable possibility to minimise the effects of the naturally occurring regulatory mechanisms within the cells and so determine whether ILK-1 is a direct transcriptional target for activation by the β-catenin/Tcf-1 complex.

4.6 Tcf-1 and/or β-catenin as a target for cancer therapy

Activation of oncogenes and loss of tumour-suppressor genes allow cancer cells to escape apoptosis. For this reason, the inhibition of oncogenic proteins or the reactivation of tumour suppressor genes has become the focus for the development of many anticancer drugs. The result was antibodies that block cell surface receptors such a Vectibix® (Panitumumab, a fully human anti-EGFR monoclonal antibody) and/or Erbitux® (Cetuximab, a chimeric anti-EGFR monoclonal antibody); both examples block the EGF receptor on expressing cells (Macarulla, 2008; Musch, 2008). Both the academic and industrial fields have stated that the specific inhibition of DNA-binding protein's interaction with DNA is a formidable task. However, evidence of increased activity of a limited, yet specific set of transcription factors show them to have significant roles in cancer and warrant further investigation (Darnell, 2002). It has been proposed that transcription factors are the most direct targets for treating cancer, supported by the fact that there are many more oncogenes in signalling pathways compared to oncogenic transcription factors (Darnell, 2002; Sukhdeo *et al*., 2007). So a potentially effective way of treating epithelial cancers would be by preventing the β-catenin Tcf/LEF interaction. Hovanes *et al.* (2001) has reported that Tcf-1 is most often the β-catenin partner in human colon cancers. The overactive factors of protein complexes (in particular the Tcf-1/β-catenin complex), within the nucleus offer a number of possibilities for pharmacological action and warrant further investigation (Darnell, 2002; van de Wetering *et al*., 2002).

4.7 Tcf-1/β-catenin complex may be influential in contributing to HOSCC behaviour

The prognosis of the particularly aggressive cancer type studied for this project offers one that is considerably worse than that of other digestive tract cancers (Yamamoto *et al*., 1999). Oesophageal cancer urgently needs a consistent and reliable diagnostic tool for early diagnosis as well as an effective therapeutic regime that results in non-recurrence of this cancer type. The Tcf-1/β-catenin protein complex is exceptionally dynamic and involved in numerous aspects of cell behaviour and malignancy (Segditsas and Tomlinson, 2006). This dissertation provides insight into some of these aspects, showing that β-catenin certainly binds to Tcf-1 in human oesophageal squamous cells. Further study of this interaction may show Tcf-1/β-catenin as central to events that result in HOSCC metastatic behaviour, possibly presenting this protein complex as an important therapeutic target in epithelial cancers with particular reference to the cancer type studied for this dissertation. It's probable that a coordinated contribution of several transcriptional and regulatory factors are necessary to upregulate the transcriptional efficiency of the ILK-1 gene in epithelial carcinomas of oesophageal origin. Further investigation into the effects of this dynamic protein complex and the correlation of metastatic behaviour for HOSCC deserves comprehensive investigation.

Appendix

5.0 Tissue culture

DMEM medium

1.37% DMEM

0.37% Sodium bicarbonate

2% Penicillin (500 U/ml)/streptomycin (0.5%) solution

Hams F12

1.07% Hams F12 medium

0.0118% Sodium bicarbonate

2% Penicillin (500 U/ml)/streptomycin (0.5%) solution

Mix DMEM:Hams F12 3:1

Filter, sterilize and store at $4^{o}C$

Trypsin/Ethylenediaminetetra-acetic acid (EDTA)

0.02% EDTA in PBS

0.1% Trypsin in PBS

Mix EDTA:Trypsin 1:1 to give a final concentration of 0.01% EDTA/0.05% trypsin

Phosphate buffered saline (PBS)

0.1 M Sodium chloride (NaCl)

10 mM Disodium hydrogen orthophosphate

3 mM Potassium chloride

1 mM Potassium dihydrogen orthophosphate

Adjust to pH 7.6

Make up to volume with dH_2O and autoclave

5.1 Laemmli lysis buffer

Single lysis buffer

0.05M Tris (hydroxymethyl) aminomethane (Tris)

0.15 M SDS

10% Glycerol

5% β-mercaptoethanol

Make up to volume with dH_2O and adjust pH to 6.8 and store at 4^oC

Double lysis buffer

0.05 M Tris

0.15 M SDS

20% Glycerol

10% β-mercaptoethanol

Make up to volume with dH_2O and adjust pH to 6.8 and store at 4^oC

5.2 Protein estimation

Tri-chloroactic acid (TCA)

7% TCA

Make up to volume with dH_2O

0.25% Coomassie blue

0.25% Coomassie blue

50% Methanol

Dissolve, then add 10% acetic acid and make up to volume with dH_2O

Destain

12% Ethanol

10% Acetic acid

Make up to volume with dH_2O

Elution solution

67% Methanol

32% dH_2O

1% Concentrated ammonia

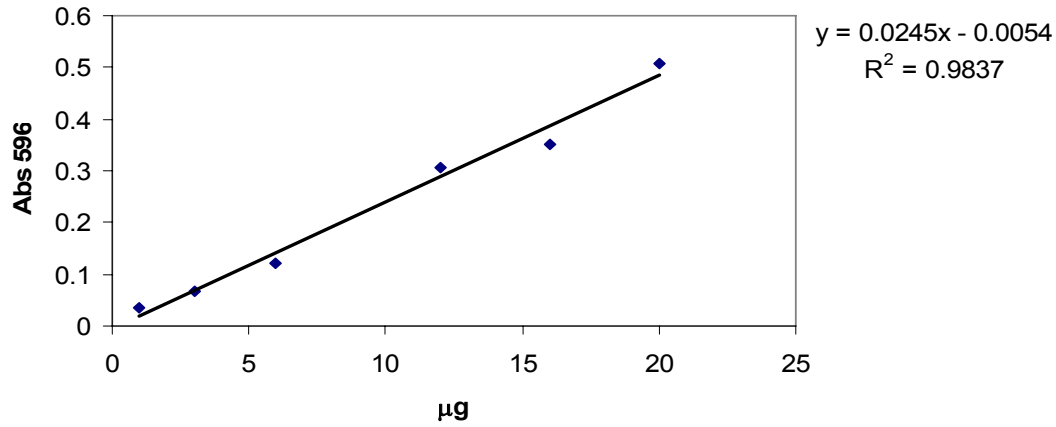


Figure 5.0: Example of a protein estimation standard curve.

The specific BSA standards, at varying concentrations in µg (x-axis) and their respective absorbance value at 596 nm (y-axis) were used to create the curve. The equation of the curve was used to calculate the unknown protein concentration in a given sample. R^2=linear regression.

5.3 SDS-PAGE

8% or 10% SDS-PAGE gel

Separating gel:

0.8/1.0 g Acrylamide (8% or 10% SDS-PAGE gel)

2.5 ml Separating buffer (1.5 M Tris pH 8.8)

400 µl SDS (50 mg/ml 0.17 M)

400 µl *N'*, *N'*-Methylenebisacrylamide (Bis) (25 mg/ml 0.16 M)

Make up to 10 ml with dH_2O, then immediately prior to pouring add:
180 µl Ammonium persulphate (APS) (1.25%)
25 µl *N*′, *N*′, *N*′, *N*′-tetranethylene-diamine (TEMED) (0.25%)

Stacking gel:
0.5 g Acrylamide
2.5 ml Stacking buffer (0.5 M Tris pH 6.8)
400 µl SDS (50 mg/ml)

Make up to 10 ml with dH_2O, then immediately prior to pouring ad:
180 µl APS
25 µl TEMED

Reservoir buffer
5.2 M Glycine
25 mM Tris pH 8.3
3 mM SDS

Make up to volume with dH_2O

0.25% Coomassie blue
0.25% Coomassie blue
50% Methanol

Dissolve, then add 10% acetic acid and make up to volume with dH_2O

Destain
10% Ethanol
10% Acetic acid

Make up to volume with dH_2O

5.4 Western blot analysis

Western blot transfer buffer

25 mM Tris pH 8.3

0.5 M Glycine

20% Methanol

Make up to volume with dH_2O

TBS Buffer

50 mM Tris-hydrochloric acid (HCl) pH 7.8

150 mM NaCl

2mM $CaCl_2.2H_2O$

Make up to volume with dH_2O

Store at 4^oC

Blocking buffer (Blotto)

50 mM Tris pH 7.8

2 mM Calcium chloride

5% Non-fat milk powder

0.01% Antifoam

0.05% Triton X-100

Make up to volume with dH_2O

Store at 4^oC

SuperSignal West Pico Chemiluminescent Working Solution

Before use mix:

50% Luminol/enhancer solution

50% Stable peroxide buffer

Store in the dark

D19B Developer

6.4 M Metol

0.6 M Sodium sulphite

80 mM Quinol

0.45 M Sodium carbonate

34 mM Potassium bromide

Fixer

0.8 M Sodium thiosulphate

0.2 M Sodium metabisulphite

5.5 Nuclear extraction buffers

Hypotonic lysis buffer

10 mM HEPES pH 7.9

1.5 mM Magnesium chloride

10 mM Potassium chloride

Nuclear preparation buffer

10 mM HEPES, pH 7.9

10 mM Potassium chloride

1.5 mM Magnesium chloride

0.5 mM DTT

0.5 mM Phenylmethylsulfonyl fluoride

0.1 mM EDTA

0.5 Nonidet P-40

Make up to a final volume with dH_2O

Store at 4^oC

Nuclear extraction buffer

20 mM HEPES, pH 7.9

1.5 mM Magnesium chloride

420 mM Sodium chloride
0.5 mM DTT
0.5 mM Phenylmethylsulfonyl fluoride
0.2 mM EDTA
25 % glycerol

Make up to a final volume with dH_2O
Store at 4^oC

5.6 Co- Immunoprecipitation

Immunoprecipitation buffer
20 mM Tris pH 8.0
0.5% Nonidet P-40
0.9% Sodium chloride

5.7 Annealed oligonucleotides

STE Buffer (1X)
0.1 M sodium chloride
10 mM Tris (hydroxymethyl)aminomethane (Tris)
1 mM EDTA

Adjust to pH 8.0
Make up to volume with dH_2O
Store at 4^oC

TE Buffer (1X)
10 mM Tris-HCl
0.1 M EDTA

Adjust to pH 8.0
Make up to volume with dH_2O

Store at 4^{o}C

5.8 Agarose gel electrophoresis

Tris-borate-EDTA (TBE) buffer (1X)

90 mM Tris

90 mM Boric acid

2 mM EDTA

Adjust to pH 8.3 with acetic acid

Make up to volume with dH_2O

Agarose gel loading buffer

50% TBE

50 % glycol

0.01 % Bromophenol blue

Ethidium bromide (EtBr)

10 ng/ml EtBr in dH_2O

Staining solution

0.5 µg/ml EtBr in 1X TBE

5.9 Native PAGE

5 % or 15% PAGE gel:

0.5/1.5 g Acrylamide (5% or 15% SDS-PAGE gel)

1 ml 5X TBE

400 µl Bis (25 mg/ml 0.16 M)

Make up to 10 ml with dH_2O, then immediately prior to pouring add:

180 µl Ammonium persulphate (APS) (1.25%)

25 µl *N*′, *N*′, *N*′, *N*′-tetranethylene-diamine (TEMED) (0.25%)

Reservoir buffer

0.5X TBE

Make up to volume with dH_2O

5.10 Ponasterone A

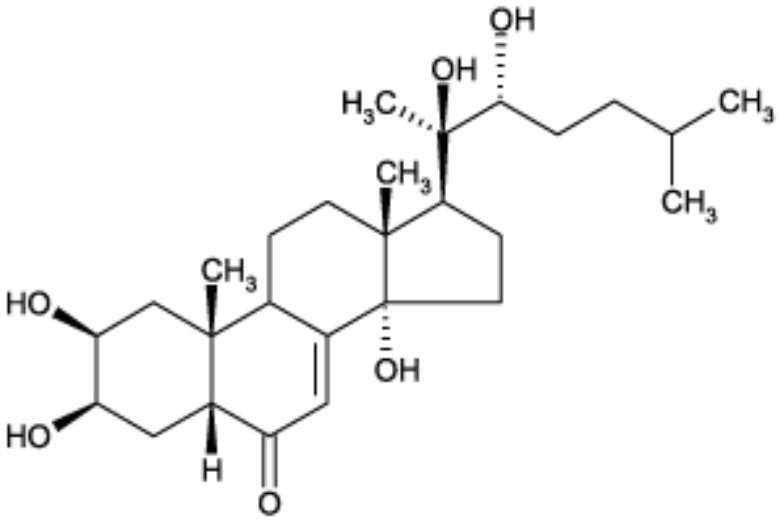


Figure 5.1: Ponasterone A (Merck)

References

Arce, L., Yokoyama, N.N., and Waterman, M.L. (2006). Diversity of LEF/TCF action in development and disease. *Oncogene*, **25**: 7492-7504.

Aberle, H., Bauer, A., Stappert, J., Kispert, A. and Kemler, R. (1997). β-catenin is a target for the ubiquitin-proteasome pathway. *The EMBO Jour*., **16**: 3793-3804.

Acton, S.L., Wong, D.H., Parham, P., Brodsky, M.F. and Jackson, A.P. (1993). Alteration of the clathrin light chain expression by transfection and gene disruption. *Mol. Biol. of the Cell*, **4**: 647-660.

Ahn, J. and Prives, C. (2001). The C-terminus of p53: the more you learn the less you know. *Nat. Struct. Biol*., **8**: 730-732.

Aricescu, A.R. and Jones, E.Y. (2007). Immunoglobulin superfamily cell adhesion molecules: zippers and signals. *Curr. Opin. Cell Biol*., **19**: 543-550.

Atcha, F.A., Munguia, J.E., Li, T.W.H., Hovanes, K. and Waterman, M.L. (2003). A new β-catenin-dependent activation domain in T cell factor. *Jour. Biol. Chem*., **278**: 16169-16175.

Atcha, F.A., Syed, A., Wu, B., Hoverter, P., Yokoyama, N.N. Ting, J.T., Munguia, J. E., Mangalam, H.J., Marsh, J.L. and Waterman, M.L. (2007). A unique DNA binding domain converts T-cell factors into strong Wnt effectors. *Mol. and Cell. Biol*., **27**: 8352-8363.

Bagnato, A. and Rosano, L. (2007). Epithelial-mesenchymal transition in ovarian cancer progression: a crucial role for the endothelin axis. *Cells Tiss. Org*., **185**: 1-3

Balmer, J.E. and Blomhoff, R. (2009). Evolution of transcription factor binding sites in mammalian gene regulatory regions: handling counterintuitive results. *Jour. of Mol. Evol*., **68**:654-664.

Beherens, J. (1999). Cadherins and catenins: role in signal transduction and tumor progression. *Can. Met. Rev.* **18**: 15-30.

Beherens, J. (2000). Cross-regulation of the Wnt signalling pathway: a role of MAP kinases. *Jour. Cell Sci.*, **113**: 911-919.

Beherens, J., von Kries, J.P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R. and Birchmeier, W. (1996). Functional interaction of β-catenin with the transcription factor LEF-1. *Nature*, **382**: 638-642.

Beland, M., Pilon, N., Houle, M., Oh, K., Sylvestre, J.R., Prinos, P. and Lohnes, D. (2004). Cdx1 autoregulation is goverened by a novel Cdx1-LEF1 transcription complex. *Mol. and Cell. Biol.*, **24**: 5028-5038.

Ben-Ze'ev, A. (1997). Cytoskeleton adhesion proteins as tumour suppressors. Curr. *Opion. in Cell Biol.*, **9**: 99-108.

Bienz, M. and Clevers H. (2000). Linking colorectal cancer to Wnt signaling. *Cell*, **103**: 311–320.

Billin, A.N., Thirlwell, H. and Ayer, D.E. (2000). Beta-catenin-histone deacetylase interactions regulate the transition of LEF1 from a transcriptional repressor to an activator. *Mol. and Cell Biol.*, **20**: 6882-6890.

Bondyk, W.H. (1995). The pCI-neo mammalian expression vector. *Prom. Notes*, **51**: 10-14.

Boyton, A.L., McKeahan, W.L. and Witfield, J.F. (1982). Ions, cell proliferation and cancer. Academic Press (New York) pg. 93-95.

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

Bramhall, S., Noack, N., Wu, M. and Loewenberg, J.R. (1969). A simple colorimetric method for determination of protein. *Anal. Biochem.*, **31**: 146-148.

Brantjies, H., Roose, J., van de Wetering, M. and Clevers, H. (2001). All Tcf HMG box transcription factors interact with Groucho-related co-repressors. *Nucl. Acids Res.*, **29**: 1410-1419.

Brodt, P. (1996). Cell adhesion and invasion in cancer metastasis. R.G. Landes Company and Chapman & Hall (Texas, USA), pg 9-10; 17-28; 35-48; 61-70; 77-86.

Cain, C., Miller, S., Ahn, J. and Prives, C. (2000). The N-terminus of p53 regulates its dissociation form DNA. *Jour. Biol. Chem.*, **275**: 39944-39953.

Cavallaro, U. and Christofori, G. (2004). Cell adhesion and signaling by cadherins and IG-CAMS in cancer. *Nature*, **4**: 118-132.

Cavallaro, U., Liebner, S. and Dejana, E. (2005). Endothelial cadherins and tumor angiogenesis. *Exp. Cell Res.*,

Clevers, H., and van de Wetering, M. (1997). TCF/LEF factors earn their wings. *TIG*, **13**: 485-488.

Crothers, D.M. (1987). Gel electrophoresis of protein-DNA complexes. *Nature*, **325**: 464-465.

Dahan, Y.L. (2005). The actin cytoskeleton and the nuclear translocation of β-catenin in human oesophageal squamous carcinoma cell lines. University of the Witwatersrand, William Cullen Research Library.

Daniels, D. and Weis, W.I. (2005). Beta-catenin directly displaces groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. *Nat. Struct. Mol. Biol.*, **12**: 364-371.

Darnell, J. E. (2002). Transcription factors as targets for cancer therapy. *Nature Rev*., **2**: 740-749.

Datar, R.V., Cartwright, T. and Rosen, C.G. (1993). Process economics of animal cell and bacterial fermentations: A case study analysis of tissue plasminogen activator. *Bio. Tech*., **11**: 349-357.

Davies, J. and Jimenez, A. (1980). A selective agent for eukaryotic cloning vectors. *Am. Jour. Trop. Med. Hyg*., **29**: 1089-1092.

de Castro, J., Gamallo, C., Palacios, G., Moreno-Bueno, G., Rodriguez, N., Feliu, J. and Gonzalez-Baron, M. (2000). β-catenin expression pattern in primary oesophageal squamous cell carcinoma. Relationship with clinicopathologic features and clinical outcome. *Virchows Arch*, **437**: 599-604.

Driver, G.A. and Veale, R.B. (2006). Modulation of integrin-linked kinase (ILK) expression in human oesophageal squamous cell carcinoma cell lines by the EGF and TCFβ1 growth factors. *Cancer Cell Int*., **6**: 1-12.

Fagotto, F., Gluck, U. and Gumbiner, B.M. (1998). Nuclear localisation signal-independent and importin/karopherin-independent nuclear import of beta-catenin. *Curr. Biol*., **8**: 181-190.

Fashena, S.J. and Thomas, S.M. (2000). Signalling by adhesion receptors. *Nat. Cell. Biol*., **2**: E225-E229.

Foulds, G.J. and Etzkorn, F.A. (1998). A capillary electrophoresis mobility shift assay for protoein-DNA binding affinities free in solution. *Nucl. Acids Res*., **26**: 4304-4305.

Franks, L.M. and Treich, N.M. (1998). Introduction to the cellular and molecular biology of cancer 3rd Ed. Oxford University Publishers (London), pg. 143-154.

Funayama, N., Fagotto, F., McCrea, P. and Gumbiner, B.M. (1995). Embryonic axis induction by the armadillo repeat domain of β-catenin: Evidence for intracellular signaling. *Jour. Cell. Biol*., **128**: 959-968.

Giancotti, F.G. and Ruoslahti, E. (1999). Integrin signaling. *Science*, **285**: 1028-1032.

Giannini, A.L., Vivanco, M.D.M. and Kypta, R.M. (2000). Analysis of β-catenin aggregation and localization using GFP fusion proteins: Nuclear import of α-catenin by the β-catenin/Tcf complex. *Exp. Cell. Res*., **255**: 207-220.

Giannis, A. (2000). β-catenin, cyclins, and more: New insights into the pathogenesis, treatment and prevention of colon cancer. *Angew. Chem. Int. Ed*., **39**: 2261-2263.

Giese, K., Cox, J. and Grosschedl, R. (1992). The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. *Cell*, **69**: 185-196.

Goeddel, D.V. (1990). Systems for heterologous gene expression. *Methods Enzymol*., **185**: 3-7.

Gore, R.M., Levine, M.s., Ghahremani, G.G and Miller, F.H. (1997). Gastric cancer. Radiologic diagnosis and staging. *Radiol. Clin. of North. Am*., **35**: 207-220.

Graham, T.A., Weaver, C., Mao, F., Kimelman, D. and Xu, W. (2000). Crystal structure of a β-catenin/Tcf complex. *Cell*, **103**: 885-896.

Ha, N., Tonozuka, T., Stamos, J., Choi, H. and Weis, W. (2004). Mechanism of phosphorylation-dependent binding of APC to β-catenin and its role in β-catenin degradation. *Mole. Cell*, 15: 511-521.

Han, C., Lim, K., Xu, L., Li, G. And Wu, T. (2008). Regulation of Wnt/β-catenin pathway by $cPLA_2\alpha$ and PPARδ. *Jour. of Cell. Biochem*., **105**: 534-545.

Hannigan, G.E., Bayani, J., Weksberg, R., Beatty, B., Pandita, A., Dehar, S. and Squire, J. (1997). Mapping of the gene encoding the integrin-linked kinase, ILK to human chromosome 11p15.5-p15.4. *Genomics*, **42**: 177-179.

He, T-C., Sparks, A.b., Rago, C., Hereking, h., Zawel, L., da Costa, L.T., Morin, P.J., Voglestein, B. and Kinzler, K.W. (1998). Identification of c-MYC as a target of the APC pathway. *Science,* **281**: 1509-1512.

Hehlgans, S., Haase, M. and Cordes, N. (2007). Signalling via integrins: Implications for cell survival and anticancer strategies. *Bioch. et Biophy. Acta*, **1775**: 163-180.

Henderson, B.R., Galea, M., Schuechner, S. and Leung, L. (2002). Lymphoid enhancer factor-1 blocks adenomatous polposis coli-mediated nuclear export and degradation of β-catenin. *The Jour. of Biol. Chem*., **277**: 24258-24264.

Hood, J.D. and Cheresh, D.A. (2002). Role of integrins in cell invasion and migration. *Nat. Rev*., **2**: 91-100.

Hoppler, S. and Kavanagh, C.L. (2007). Wnt signalling: varierty at the core. *Jour. of Cell Sci.,* 120: 385-393.

Hovanes, K., Li, T.W.H. and Waterman, M.L. (2000). The human LEF-1 gene contains a promoter preferentially active in lymphocytes and encodes multiple isoforms derived from alternative splicing. *Nucl. Acids Res*., **28**: 1994-2003.

Hovanes, K., Li, T.W.H., Munguia, J.E., Truong, T., Milovanovic, T., Marsh, J.L., Holcombe R.F. and Waterman, M.L. (2001). Beta-catenin-sensitive isoforms of lymphoid enhancer factor-1 are selectively expressed in colon cancer. *Nature Genet*., **28**: 53-57.

http://www.health24.co.za/Man/Medical/748-766,14214.asp. Health 24 Medical. Author: not stated, accessed on 09/01/2010.

Huang, Y., Li, J., Zhang, Y. and Wu, C. (2000). The roles of integrin-linked kinase in the regulation of myogenic differentiation. *Jour. Cell Biol*., **150**: 861-871.

Huber, A.H., Nelson, W.J. and Weis, W.I. (1997). Three-dimensional structure of the armadillo repeat region of β-catenin. *Cell*, **90**: 871-882.

Hülsken, J., Birchmeier, W. and Beherens, J. (1994). E-cadherin and APC compete for the interaction with β-catenin and the cytoskeleton. *Jour. Cell Biol*., **127**: 2061-2069.

Hurlstone, A. and Clevers, H. (2002). T-cell factors: turn-ons and turn-offs. *The EMBO Jour*., **21**: 2303–2311.

Hynes, R.O. (2002). Integrin: Bi-directional allosteric signalling machines. *Cell*, **110**: 673-687.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J. and Thun, M.I. (2009). Cancer Statistics, 2009. *CA Can. Jour. Clin*., **59**: 225-249.

Jones, L.J.G. and Veale, R.B. (2003). Redistribution of β-catenin in response to EGF and lithium signalling in human oesophageal squamous cearcinoma cell lines. *Can. Cell. Int*., **3**: 1-12.

Kannagi, R., Izawa, M., Koike, T., Miyazaki, K. and Kimura, N. (2004). Carbohydrate-mediated cell adhesion in cancer metastasis and angiogenesis. *Can. Sci*., **95**: 377-384.

Kawasoe, T., Furukawa, Y., Daig, Y., Nishiwaki, T., Isiguro, H., Fujita, M., Satoh, S., Miwa, N., Nagasawa, Y., Miyoshi, Y., Ogawa, M. and Nakaura, Y. (2000). Isolation and characterisation of a novel human gene, DRCTNNB1A, the expression of which is down regulated by β-catenin. *Can. Res*., **60**: 3354-3358.

Kitahara, H., Kawashiri, S., Kato, K., Ohara, T., Yoshizawa, K., Nozaki, S. and Nakagawa, K. (2008). Immunohistochemical expressions of E-cadherin and β-

catenin correlate with the invasion, metastasis and prognosis of oral squamous cell carcinoma. *Oral Surgery*, **1**: 28-34.

Klug, W.S. and Cummings, M.R. (2000). Concepts of genetics, 6th Ed. Prentice Hall, Inc. (New Jersey), pg. 636-652.

Knudson, A.G (2002). Cancer genetics. *Am. Jour. Med. Genet*., **111**: 96-102.

Korinek, V., Barker, N., Willer, K., Molenaar, M., Roose, J., Wagenaar, G., Markman, M., Lamers, W., Destree, O. and Clevers, H. (1998). Two members of the Tcf family implicated in Wnt/beta-catenin signalling during embryogenesis in the mouse. *Mol. and Cell Biol*., **18**: 1248-1256.

Labbe, E., Letamendia, A., and Attisano, L. (2000). Association of Smads with lymphoid enhancer binding factor 1/T cell-specific factor mediates cooperative signaling by the transforming growth factor-beta and Wnt pathways. *Proc. Natl. Acad. Sci. USA,* **97**: 8358–8363.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage. T4. *Nature*, **227**: 680-685.

Leber, M.F. and Efferth, T. (2009). Molecular principles of cancer invasion and metastasis (review). *Int. Jour. Oncol*., **4**: 881-895.

Lee, E., Salic, A. and Kirschner, M.W. (2001). Physiological regulation of β-catenin stability by TCF3 and CK1. *Jour. Cell Biol.*, **154**: 983–993.

Lepourcelet, M., Chen, Y.N.P., France, D.S., Wang, H., Crews, P., Peterson, F., Bruseo, C., Wood, a.W. and Shivdasani, A. (2004). Small-molecule antagonists of the oncogenic Tcf/β-catenin protein complex. *Can. Cell*, **5**: 91-102.

Levy, L., Neuveut, C., Renard, C., Charneaus, P., Branchereau, S., Gauthier, F., van Nhieu, J.T., Cherqui, D., Petit-Berton, A.F., Mathieu, D. and Buendia, M.A. (2002).

Transcriptional activation of Interleukin-8 by β-catenin-tcf4. *The Jour. of Biol. Chem.*, **277**: 42386-42393.

Lickert, H., Bauer, A., Kemler, R. And Stappert, J. (2000). Casein kinase II phosphorylation of E-cadherin increases E-cadherin/β-catenin interaction and strengthens cell-cell adhesion. *The Jour. of Biol. Chem.*, **275**: 5090-5095.

Li, F., Zhang, Y. and Wu, C. (1999). Integrin-linked kinase is localised to cell-matrix focal adhesions but not cell-cell adhesion sites and the focal adhesion localisation of integrin-linked kinase is regulated by the PINCH-binding ANK repeats. *Jour. of Cell Sci.*, **122**: 4589-4599.

Li, Q., Dashwood, W.M., Zhong, X., Al-Fageeh, M. and Dashwood, R.H. (2004). Cloning of the rat β-catenin gene (*Ctnnb1*) promoter and its functional analysis compared with the *Catnb* and *CTNNB1* promoters. *Genomics*, **83**: 231-242.

Li, T.W., Ting, J.H., Yokoyama, N. N., Bernstein, A., van de Wetering, M. and Waterman, M.L. (2006). Wnt activation and alternative promoter repression of Lef1 in colon cancer. *Mol. and Cell. Biol.*, **26**: 5284-5299.

Liu, C., Kato, Y., Zhang, Z., Do, V.M., Yanker, B.A. and He, X. (1999). β-Trcp couples β-catenin phosphorylation-degradation and regulates Xenopus axis formation. *Proc. Natl. Acad. Sci. USA*, **96**: 6273-6278.

Liu, J., Stevens, J., Rote, C.A., Yost, H.J., Hu, Y., Neufeld, K.L., White, R.L. and Matsunami, N. (2001). Siah-1 mediates a novel beta-catenin degradation pathway linking p53 to the adenomatous polyposis coli protein. *Mol. Cell.* **7**:927–936.

Love, J.J., Li, X., Case, D.A., Giese, K., Grosschedl, R. and Wright, P.E. (2002). Structural basis for DNA bending by the architectural transcription factor LEF-1. *Nature*, **376**: 791-795.

Love, J.J., Li, X., Chung, H.J., Dyson, J. and Wright, P.E. (2004). The LEF-1 high-mobility group domain undergoes a disorder-to-disorder transition upon formation of a complex with cognate DNA. *Biochem*., **43**: 8725-8734.

Lynch, D.K., Ellis, C.A., Edwards, P.A. and Hiles, I.D. (1999). Integrin-linked kinase regulates phosphorylation of serine 473 of protein kinase B by an indirect mechanism. *Oncogene*, **18**: 8024-8032.

Macarulla, T., Ramos, F.J., Elez, E., Capdevila, J., Peralta, S. and Tabernero, J. (2008). Update on novel strategies to optimize cetuximab therapy in patients with metastatic colorectal cancer. *Clin. Colorectal Can.*, **5**: 300-308.

Makrides, S.C. (1999). Components of vectors for gene transfer and expression in mammalian cells. *Prot. Expr. and Purif*., **17**: 183-202.

Mann, B., Gelos, M., Siedow, A., Hanski, M.L., Gratchev, A., Ilyas, M., Bodmer, W.F., Moyer, M.P., Riecken, E.O., Buhr, H.J. and Hanski, C. (1999). Target genes of β-catenin-T cell factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. *Proc. Natl. Acad. Sci*., **96**: 1603-1608.

Mareel, M. and Leroy., A. (2002). Clinical, cellular and molecular aspects of cancer invasion. *Phsiol. Rev*., **83**: 33-376.

Marino, M.H. (1989). Expression systems for heterologous protein production. *BioPharm*, **2**: 18-33.

Masui, O., Ueda, Y., Tsumura, A., Koyanagi, M., Hijikata, M. and Shimotohno, K. (2002). RelA suppresses the Wnt/β-catenin pathway without exerting trans-acting transcriptional ability. *Intern. Jour. of Mol. Med*., **9**: 489-493.

Matsuzawa, S.I. and Reed, J.C. (2001). Siah-1, SIP, and Ebi collaborate in a novel pathway for beta-catenin degradation linked to p53 responses. *Mol. Cell.* **7**:915–926.

Maupas-Schwalm, F., Robinet, C., Auge, N., Thiers, J.C., Garcia, V., Cambus, J.P., Salvayre, R. and Negre-Salvayre, A. (2005). Activation of the β-catenin/T-cell-specific transcription factor/lymphoid enhancer factor-1 pathway by plasminogen activators in ECV304 carcinoma cells. *Can. Res.*, **65**: 526-532.

McGlashan, N.D. (1988). Oesophageal cancer in the black peoples of South Africa 1980-82. *South Afri. Jour. of Sci.*, **84**: 92-99.

Medici, D., Hay, E.D. and Goodenough, D.A. (2006). Cooperation between Snail and LEF-1 transcription factors is essential for TGF-β1-induced epithelial-mesenchymal transition. *Mol. Biol. of the Cell*, **17**: 1871-1879.

Melchior, C., Kreis, S., Janji, B. and Kieffer, N. (2002). Promoter characterisation and genomic organization of the gene encoding intergrin-linked kinase 1. *Bio. et Biophys. Acta*, **1575**: 117 122.

Merrill, B.J., Gat, U., DasGaupta, R. and Fuchs, E., (2001). *Genes Dev.*, **15**: 1688-1705.

Mertin, S., McDowall, S.G. and Harley, V.R. (1999). The DNA-binding specificity of SOX9 and other SOX proteins. *Nucl. Acids Res.*, **27**: 1359-1364.

Mizushima, T., Nakagawa, H., Kamberov, Y.G., Wilder, E.L., Klein, P.S. and Rustgi, A.K. (2002). Wnt-1 but not epidermal growth factor induces β-catenin/T-cell factor-dependent transcription in esophageal cancer cells. *Can. Res.*, **62**: 277-282.

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O. and Clevers, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. *Cell*, **86**: 391-399.

Musch, A. (2008). Panitumumab. *Med. Monatsschr. Pharm.*, **4**: 122-126.

Nelson, W.J. and Nusse, R. (2004). Convergence of Wnt, beta-catenin, and cadherin pathways. *Science,* **303**: 1483-1487.

Neufeld, K.L., Zhang, F., Cullen, B.R. and White, R.L. (2000). APC-mediated downregulation of beta-catenin activity involves nuclear sequestration and nuclear export. *EMBO Rep*., **6**: 519-523.

Ninomiya, I., Endo, Y., Fushida, S., Fushida, S., Sasagawa, T., Miyashita, T., Fujimura, T., Nishimura, G., Tani, T., Hashimoto, T., Yagi, M., Shimizu, K., Ohta, T., Yonemura, Y., Inoue, M., Sasaki, T. and Miwa, K. (2000). Alteration of β-catenin expression in esophageal squamous-cell carcinoma. *Int. Jour. Cancer*, **85**: 757-761.

No, D., Yao, T.P., and Evans, R.M. (1996). Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc. Natl. Acad. Sci. USA*, **93**: 3346-3351.

Novak, A. and Dedhar, S. (1999). Signalling through β-catenin and Lef/tcf. *Cell. Mol. Life Sci*., **56**: 523-537.

Novak, A., Hsu, S.C., Leung-Hagesteijn, C., Radeva, G., Papkoff, J., Montesano, R., Roskelley, C., Grosschedl, R. and Dehar, S. (1998). Cell adhesion and the integrin-linked kinase regulate the LEF-1 and β-catenin signalling pathways. *Proc. Natl. Acad. Sci*., **95**: 4374-4379.

Ochiai, A., Akimoto, S., Kanai, Y., Shibata, T., Oyama, T. and Hirohashi, S. (1994). c-*erb*B-2-gene product associates with catenins in human cancer cells. *Biochem. Biophys. Res. Commun.*, **205**: 73-78.

Okamura, R., Sigvardsson, M., Galceran, J., Verbeek, S., Clevers, H. and Grosschedl, R. (1998). Redundant regulation of T cell differentiation and TCRalpha gene expression by the transcription factors LEF-1 and TCF-1. *Immunity*, **8**: 11-20.

Ozawa, M. and Keler, R. (1998). Altered cell adhesion activity by pervanadate due to the dissociation of α-catenin from the E-cadherin-catenin complex. *The Jour. of Biol. Chem.*, **273**: 6166-6170.

Ozdemir, O. (1998). The construction of a mammalian transfection vector for expression of cytosine-5 specific DNA methyltransferase gene M.Msp1 in cultured cells. *Tr. Jour. of Biol.*, **22**: 161-170.

Park, C.H., Chang, J.Y., Hahm, E.R., Park, S., Kim, H.K., Yang, C.H. (2005). Quercetin, a potent inhibitor against beta-catenin/Tcf signalling in SW480 colon cancer cells. *Biochem. Biophs. Res. Commun.*, **328**: 227-234.

Pei, D and Yi, J. (1998). A high-level mammalian expression system based on the madin-darby canine kidney cell line. *Prot. Expr. and Purif.*, **13**: 277-281.

Persad, S., Attwell, S., Gray, V., Delcommenne, M., Troussard, A., Saghera, J. and Dedhar, S. (2000). Inhibition of integrin-linked kinase (ILK) suppresses activation of protein kinase B/Akt and induces cell cycle arrest and apoptosis of PTEN-mutant prostate cancer cells. *Proc. Natl. Acad. Sci. USA*, **97**: 3207 - 3212.

Petruzzelli, L., Takai, M. and Humes, H.D. (1999). Structure and function of cell adhesion molecules. *Am. Jour. Med.*, **106**: 467-476.

Pikaart, M.J., Recillas-Targa, F. and Felsenfeld, G. (1998). Loss of transcriptional activity of a transgene is accompanied by DNA methylation and histone deacetylation and is prevented by insulators. *Genes Dev.*, **12**: 2852-2862.

Polakis, P. (1999). The oncogenic activation of β-catenin. *Curr. Opin. in Genet. & Dev.*, **9**: 15-21.

Prescott, L.M., Harley, J.P. and Klein, D.A. (1999). Microbiology. McGraw-Hill (Boston), pg. 734-738.

Prieve, M.G and Waterman, M.L. (1999). Nuclear localization and formation of β-catenin-lymphoid enhancer factor 1 complexes are not sufficient for activation of gene expression. *Mol. Cell. Biol.*, **19**: 4503-4515.

Prieve, M.G., Guttridge, K.L., Munguia, J. and Waterman, M.L. (1998). Differential importin-α recognition and nuclear transport by nuclear localization signals within the high-mobility-group DNA binding domains of lymphoid enhancer factor 1 and T-cell factor 1. *Mol. Cell Biol*., **18**: 4819-4832.

Prieve, M.G., Guttridge, K.L., Munguia, J. and Waterman, M.L. (1996). The nuclear localization signal of lymphoid enhancer factor-1 is recognised by two differentially expressed Srp1-nuclear localization sequence receptor proteins. *Jour. Biol. Chem*., **271**: 7654-7658.

Qiu, H., Zhang, W., El-Naggar, A.K., Lippman, S.M., Lin, P., Lotan, R. and Xu, X.C. (1999). Loss of retinoic acid receptor-ß is an early event during esophageal carcinogenesis. *Am J Pathol.*,**155**: 1519 -1523.

Rahmani, M., Read, J.T., Carthy, J.M., McDonald, P.C., Wong, B.W., Esfandiarei, M., Si, X., Luo, Z., Lou, H., Rennie, P.S. and McManus, B.M. (2005). Regulation of the versican promoter by the β-catenin-T-cell factor complex in vascular smooth muscle cells. *Jour. Biol. Chem*., **280**: 13019-13028.

Roel, G., van den Broek, O., Spieker, N., Peterson-Maduro, J. and Destree, O. (2003). Tcf-1 expression during Xenopus development. *Gene Expr. Patterns*, **3**: 123-126.

Roh, H., Green, D.W., Boswell, C.B., Pippin, J.A. and Drebin, J.A. (2001). Suppression of β-catenin inhibits the neoplastic growth of APC-mutant colon cancer cells. *Can. Res.,* **61**: 6563-6568.

Roose, J., Huls, G., van Beest, M., Moerer, P., van der Horn, K., Goldschmeding, R., Logtenberg, T. and Clevers, H. (1999). Synergy between tumor suppressor APC and the β-catenin-Tcf4 target Tcf1. *Science*, **285**: 1923-1926.

Rosin-Abesfeld, R., Cliffe, A., Brabletz, T. and Bienz, M. (2003). Nuclear export of the APC tumour suppressor controls β-catenin function in transcription. *The EMBO Jour*., **22**: 1101-1113.

Roura, S., Miravat, S., Piedra, J., de Herreros, A.G. and Dunach, M. (1999). Regulation of E-cadherin/catenin association by tyrosine phosphorylation. *The Jour. of Biol. Chem.*, **274**: 36734-36740.

Ryo, A., Nakamura, M., Wulf, G., Liou, Y-C. and Lu, K.P. (2001). Pin1 regulates turnover and subcellular localization of β-catenin by inhibiting its interaction with APC. *Nat. Cell Biol.*, **3**: 793-801.

Sadot, E., Sicha, I., Stutan, M., Ben-Ze'ev, A. and geiger, B. (1998). Inhibition of β-catenin-mediated transactivation by cadherin derivatives. *Pro. Natl. Acad. Sci. USA*, **95**: 15339-15344.

Saeki, H., Tanaka, S., Sugimachi, K., Kimura, Y., Miyazaki, M., Ohga, T. and Sugimachi, K. (2002). Interrelation between expression of matrix metalloproteinase 7 and β-catenin in esophageal cancer. *Digest. Dis. and Sci.*, **47**: 2738-2742.

Sambrook, J. and Russell D.W. (2001) Molecular cloning: a laboratory manual, 3rd Ed., Cold Spring Harbor Laboratory, (NewYork) pg. 45-56.

Sancho, E., Batlle, E. and Clevers, H. (2003). Live and let die in the intestinal epithelium. *Curr. Opin. in Cell Biol.*, **15**: 763-770.

Sandal, T. (2002). Molecular aspects of the mammalian cell cycle and cancer. *The Oncologist*, **7**: 73-81.

Schohl, A. and Fagotto, F. (2002). Beta-catenin, MAPK and Smad signaling during early Xenopus development. *Development*, **129**: 37-52.

Schwartz, M.a. (1997). Integrins, oncogenes and anchorage independence. *Jour. of Cell Biol.*, **55**: 505-510.

Segditsas, S. and Tomlinson, I. (2006). Colorectal cancer and genetic alterations in the Wnt pathway. *Oncogene*, **25**: 7531-7537.

Sharma, M., chuang, W.W. and Sun, Z. (2002). Phosphatidylinositol 3-kinase/Akt stimulates androgen pathway through GSK3β inhibition and nuclear β-catenin accumulation. *Jour. of Biol. Chem*., **34**: 30935-30941.

Shibamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Oku, N., Miyazawa, K., Kitamura, N., Takeichi, M. and Ito, F. (1994). Tyrosine phosphorylation of β-catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. *Cell Adhes Commun*, **1**: 295–305.

Shiozaki, H., Doki, Y., Kawanishi, K., Shamma, A., Yano, M., Inoue, M. and Monden, M. (2001). Clinical application of malignancy potential grading as a prognostic factor of human esophageal cancers. *Surgery*, **127**: 552-561.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R. and Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. *Proc. Natl. Acad. Sci. USA*, **96**: 5522-5527.

Sidorova, N.Y., Muradymov, S. and Rau, D.C. (2005). Trapping DNA-protein binding reactions with neutral osmolytes for the analysis by gel mobility shift and self-cleavage assays. *Nucl. Acids Res*., **33**: 5145-5155.

Somasiri, A., Howarth, A., Goswami, D., Dedhar, S. and Roskelley, C.D. (2001). Overexpression of the integrin-linked kinase mesenchymally transforms mammary epithelial cells. *Jour. Cell Sci*., **114**: 1125-1136.

Song, L.N., Herrell, R., Byers, S., Shah, S., Wilson, E.M. and Gelmann, E.P. (2003). β-catenin binds to the activation function 2 region of the androgen receptor and modulates the effects of the N-terminal domain and TIF2 on ligand-dependent transcription. *Mol. and Cell. Biol.*, **23**: 1674-1687.

Stead, J.A., Keen, J.F. and McDowall, K.J. (2006). The identification of nucleic acid-interacting proteins using a simple proteomics-based approach that directly incorporates the electromobility shift assay. *Molec. and Cell. Prot*., **5**: 1697-17.

Stoner, G.D. and Gupta, A. (2001). Etiology and chemoprevention of esophageal squamous cell carcinoma. *Carcinogenesis*, **22**: 1737-1746.

Sukheo, K., Mani, M., Zhang, Y., Dutta, J., Yasui, H., Rooney, M.D., Carrasco, D.E., Zheng, M., He, H., Tai, Y., Mitsiades, C., Anderson, K.C. and Carrasco, D.R. (2007). Targeting the β-catenin/TCF transcriptional complex in the treatment of multiple myeloma. *Proc. Natl. Acad. Sci. USA*, **104**: 7516-7521.

Tago, K., Nakamura, T., Nishita, M., Hydo, J., Nagi, S., Murata, Y., Adachi, s., Ohwada, S., Morishita, Y., Shibuya, H. and Akiyama, T. (2000). Inhibition of Wnt signalling by ICAT, a novel β-catenin-interacting protein. *Gen. and Dev*., **14**: 1741-1749.

Takahashi, M., Fujita, M., Furukawa, Y., Hamamoto, R., Shimokawa, T., Miwa, N., Ogawa, M. and Nakamura, Y. (2002). Isolation of a novel human gene, *APCDD1*, as a direct target of the β-catenin/T-cell factor 4 complex with probable involvement in colorectal carcinogenesis. *Can. Res*., **62**: 5651-5656.

Tan, C., Costello, P., Sanghera, J., Dominguez, D., Baulida, J., de Herreros, A.G. and Dehar, S. (2001). Inhibition of integrin-linked kinase (ILK) supresses beta-catenin-Lef/Tcf-dependent transcription and expression of the E-cadherin repressor, snail, in APC-/- human colon carcinoma cells. *Oncogene*, **20**: 133-1340.

Valenta, T., Lukas, J. and Korinek, V. (2003). HMG box transcription factor Tcf-4's interaction with CtBP1 controls the expression of the Wnt target axin2/conduction in human embryonic kidney cells. *Nucl. Acids Res*., **31**: 23692380.

van Beest, M., Dooijes, D., van de Wetering, M., Kjaerulff, S., Bonvin, A., Nielson, O. and Clevers, H. (2000). Sequence-specific high mobility group box factors recognize 10-12 base pair minor groove motifs. *Jour. Biol. Chem*., **275**: 27266-27273.

van de Wetering, M. and Clevers, H. (1992): Sequence-specific interaction of the HMG box proteins TCF-1 and SRY occurs within the minor groove of a Watson-Crick double helix. *The EMBO Jour.*, **11**: 3039-3044.

van de Wetering, M., Castrop, J., Korinek, V. and Clevers, H. (1996). Extensive alternative splicing and dual promoter usage generate Tcf-1 protein isoforms with differential transcription control properties. *Mol. and Cell. Biol.*, **16**: 745-752.

van de Wetering, M., Cavallo, R., Dooijes van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M. and Clevers, H. (1997): Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene dTCF. *Cell*, **88**: 789-799.

van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A.P., Tjon-Pon-Fong, M., Moerer, P., van den Born, M., Soete, G., Pals, S., Eilers, M., Medema, R. and Clevers, H. (2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell*, **111**: 241-250.

van Noort, M., Meeldijk, J., van der Zee, R., Destree, O. and Clevers, H. (2002). Wnt signaling controls the phosphorylation status of β-catenin. *The Jour. of Biol. Chem.*, **277**: 17901-17905.

Veale, R.B. and Thornley, A.L. (1989). Increased single class low-affinity EGF receptors expressed by human oesophageal squamous carcinoma cell lines. *S. Afr. Jour. Sci.*, **85**: 375-379.

Volgelstein, B. and Kinzler, K.W. (2004). Cancer genes and the pathways they control. *Nat. Med.*, **10**: 789-799.

Wadham, C., Gamble, J.R., Vadas, M.A. and Khew-goodall, Y. (2003). The protein tyrosine phosphatase pez is a major phophatase of adherins junctions and dephosphorylates β-catenin. *Mol. Biol. Cell*, **14**: 2520-2529.

Waterman, M.L. (2004). Lymphoid enhancer factor/T cell factor expression in colorectal cancer. *Can. and Met. Rev*., 23: 41-52.

Welch, D.R., Steeg, P.S. and Rinker-Schaeffer, C.W. (2000). Molecular biology of breast cancer metastasis: genetic regulation of human breast carcinoma metastasis. *Breast Can. Res*., **2**: 408-416.

Wen, Y., Caffrey, T.C., Wheelock, M.J., Johnson, K.R. and Hollingworth, M.A. (2003). Nuclear association of the cytoplasmic tail of MUC1 and β-catenin. *Jour. Biol. Chem*., **278**: 38029-38039.

Willert, K. and Jones, K.A. (2006). Wnt signaling: is the party in the nucleus? *Genes Development*, **20**: 1394-1404.

Willinger, T., Freeman, T., Herbert, M., Hasegawa, H., McMichael, A.J. and Callan, M.F. (2006). Human naïve CD8T cells down-regulate expression of the Wnt pathway transcription factors lymphoid enhancer binding factor 1 and transcription factor 7 (T cell factor-1) following antigen encounter in vitro and in vivo. *Jour. Immunol*., **176**: 1439-1446.

Wodarz, A. (2002). Establishing cell polarity in development. *Nat. Cell Biol*., **4**: E39-E44.

Wong, N.A.C.S. and Pignatelli, M. (2002). β-catenin- A linchpin in colorectal carcinogenesis. *Am. Jour. Pathol*., **160**: 389-401.

Wu, C. (2001). ILK interactions. *Jour. of Cell. Sci*., **114**: 2549-2550.

Wu, C. (1999). Integrin-linked kinase and PINCH:partners in regulation of cell-extracellular matrix interaction and signal transduction. *Jour. of Cell Sci*., **112**: 4485-4489.

Wulf, G.M., Ryo, A., Wulf, G.G., Lee, S.W., Niu, T., Petkova, V. and Lu, K.P. (2001). Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in

increasing the transcriptional activity of c-Jun towards cyclin D. *EMBO Jour*., **20**: 3459-3472.

Wu, S., Lim, K.-C., Huang, J., Saidi, R. F. and Sears, C. L. (1998). *Bacteroides fragilis* enterotoxin cleaves the zonula adherens protein, E-cadherin. *Proc. Natl. Acad. Sci. USA,* **95**: 14979-14984.

Wu, W.S., Wu, J.R. and Hu, C.T. (2008). Signal cross talks for sustained MAPK activation and cell migration: the potential role of reactive oxygen species. *Can. Met. Rev*., **27**: 303-314.

Xiao, J.H., Ghosn, C., Hinchman, C., Forbes, C., Wang, J, Cordrey, A., Zhao, Y. and Chandraratna, R.A.S. (2003). Adenomatous polyposis coli (APC)-independent regulation of β-catenin degradation via a retinoid X receptor-mediated pathway. *The Jour. of Biol. Chem*., **278**: 29954-29962.

Xing, Y., Clements, W.K., Kimelman, D. and Xu, W. (2003). Crystal structure of a β-catenin/axin complex suggests a mechanism for the β-catenin destruction complex. *Genes Dev*., **17**: 2753-2764.

Yamada, T., Takaoka, A.S., Naishiro, Y., Hayashi, R., Maruyama, K., Maesawa, C., Ochiai, A. and Hirohashi, S. (2000). Transactivation of the Multidrug resistance 1 gene by T-cell factor 4 / β-catenin complex in early colorectal carinogenesis. *Can. Res*., **60**: 4761-4768.

Yamamoto, H., Adachi, Y., Itoh, F., Iku, S., Matsuno, K., Arimura, Y., Endo, T., Hinoda, Y., Hosokawa, M. And Imai, K. (1999). Association of matrilysin expression with recurrence and poor prognosis in human eosophageal squamous cell carcinoma. *Cancer Res*., **59**: 3313-3316.

Zampieri, L. and Veale, R.B. (2001). P-glycoprotein expression in human oesophageal squamous cell carcinoma cell lines. *South Afri. Jour. of Sci*., **97**: 329-333.

Zhao, D.H., Hong, J.J., Guo, S.Y., Yang, R.L., Yuan, J., Wen, C.J., Zhou, K.Y and Li, C.J. (2004). Aberrant expression and function of Tcf4 in the proliferation of hepatocellular carcinoma cell line BEL-7402. *Cell Res*., **14**: 74-80.