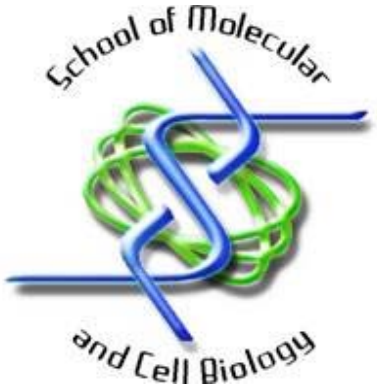
β-catenin Expression and Associated Signalling in Moderately Differentiated Human Oesophageal Squamous Carcinoma Cell Lines



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A thesis submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of PhD

Johannesburg, 2008

Declaration

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

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Lindsay Jean Gordon McCutcheon

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Mom and Dad

For always making a plan

Keir

For eternal patience, support and belief in my abilities when I had none

Abstract

β-catenin links membrane-bound cadherins to the actin cytoskeleton, regulating cellular adhesion, and consequently metastasis. Abnormal stabilization of free, cytoplasmic β-catenin enhances its transcriptional activities. Factors affecting the adhesive and signalling roles of β-catenin may be important in metastatic diseases such as oesophageal squamous cell carcinoma (SCC). This study focuses on the expression of β-catenin in moderately-differentiated oesophageal SCCs and the impact of three signalling pathways on this. The majority of β-catenin's expression within moderately-differentiated oesophageal SCCs, under standard *in vitro* conditions, was associated with the plasma membrane, rather than in the cytoplasm. This indicates that β-catenin's role is skewed towards cell-cell adhesion, rather than its signalling activities. Lithium, a Wnt pathway mimic, elevated cytoplasmic β-catenin concentrations. Membrane concentrations subsequently increased, possibly through E-cadherin sequestration of excess cytoplasmic β-catenin. TGF-β1, alone or together with lithium, did not alter β-catenin expression. EGF transiently increased cytoplasmic β-catenin, but did not alter total concentration. However, together with lithium, EGF increased stabilized cytoplasmic β-catenin concentrations, whilst decreasing membrane-associated β-catenin levels. This indicates that EGF/lithium signals synergistically provoke β-catenin redistribution. This may be induced by posttranslational modification through tyrosine phosphorylation of plasma membrane-associated β-catenin as the concentration of tyrosine, but not serine, phosphorylated β-catenin was increased by these signals. Thus, although the adhesive role of β-catenin appears intact, exogenous signals by EGF and lithium can alter, through tyrosine phosphorylation, the signalling role of β-catenin whilst reducing its adhesive role and thus, may potentiate the metastatic properties of these cells.

List of Associated Publications and Presentations

Papers

Jones*, L.J.G. and Veale, R.B. (2003). Redistribution of β-catenin in response to EGF and lithium signalling in human oesophageal squamous carcinoma cell lines. *Cancer Cell International* **3**: 1-13.

Published Abstracts

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Conference Proceedings

McCutcheon, L.J.G. and Veale, R.B. (2006). EGF and lithium, but not TGF-beta1, induces subcellular redistribution of beta-catenin in moderately differentiated oesophageal squamous cell carcinomas. *Proc. XXth South African Society of Biochemistry and Molecular Biology Conference, Pietermaritzburg, SA*; p144.

Jones, L.J.G., Schnugh, D.J. and Veale, R.B. (2005). EGF modulates the β-catenin/α-catenin Membrane Complex in Oesophageal Squamous Carcinoma Cells. *Proc. XIXth South African Society of Biochemistry and Molecular Biology Conference, Stellenbosch, SA*; p 199.

Driver, G.A., Jones, L.J. and Veale, R.B. (2005). Crosstalk Between β-catenin and ILK Signalling Cascades in Human Oesophageal Squamous Cell Carcinoma of

* Please note, a number of these publications and presentations occur under my maiden name of Jones, whilst others under my married name of McCutcheon.

the Oesophagus. *Proc. XIXth South African Society of Biochemistry and Molecular Biology Conference, Stellenbosch, SA*; p 194.

Jones, L.J.G. and Veale, R.B. (2003). EGF and Lithium synergistically induce β-catenin relocation in moderately differentiated oesophageal squamous cell carcinomas. *Proc. 18th South African Society of Biochemistry and Molecular Biology Congress, Pretoria, SA*; p 185.

Jones, L.J.G., Schnugh, D.J. and Veale, R.B. (2003). Constitutive stabilisation of β-catenin is not a property of moderately differentiated squamous cell carcinomas of the oesophagus. *Proc. 18th South African Society of Biochemistry and Molecular Biology Congress, Pretoria, SA*; p 186.

Jones, L.J.G. and Veale, R.B. (2001). The effect of EGF and Lithium on the expression of β-catenin in human oesophageal carcinoma cell lines. *First International Union of Biochemistry and Molecular Biology/South African Society of Biochemistry and Molecular Biology Special Meeting on: Biochemical and Molecular Basis of Disease, Cape Town, SA*; p 110-111.

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List of Abbreviations

AJ	Adherens junction
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
APS	Ammonium persulphate
arm	Armadillo
bp	Base pair
BSA	Bovine serum albumin (Fraction V)
βTrCP	Beta-transducin-repeat-containing protein
°C	Degrees Celsius
C	Carboxyl
Ca^{2+}	Calcium
CAM	Cell adhesion molecule
CBP	CREB-binding protein
CK	Casein kinase
cm	centimeters
CPM	Counts per minute
CtBP	C-terminal Binding Protein
dH_2O	Distilled water
DKK	Dickkopf
DMEM	Dulbecco's modified eagles medium
DNA	Deoxyribonucleic acid
Dvl	Dishevelled
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Endoplasmic Reticulum
FC(B)S	Foetal calf (bovine) serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FRAT	Frequently rearranged in advanced T-cell lymphomas
Frz	Frizzled
×g	Gravitational units
g	Gram(s)
GSK-3β	Glycogen synthase kinase-3β
GTP	Guanine thiphosphate
HBP-1	HMG-box repressor protein-1
HCl	Hydrochloric acid
HDAC	histone deacetylase
HGF	Hepatocyte growth factor
HMG	High mobility group
HRP	Horseradish peroxidase
HSD	Honesty significant difference
^{125}I	125-Iodine

ICAT	Inhibitor of β-catenin and Tcf
Ig	Immunoglobulin
IGF	Insulin-like growth factor
ILK	Integrin-linked kinase
IP	Immunoprecipitation
kD	Kilodalton
Lef	Lymphoid enhancer factor
LRP	LDRD-related protein
M	Molar
mA	Milliampere(s)
MAPK	Mitogen activated protein kinase
µCi	Microcurie
MDR1	Multidrug resistance 1
µg	Microgram(s)
µl	Microlitre(s)
ml	Millilitre(s)
mM	Millimolar
Mr	Relative molecular weight
N	Amino
NaOH	Sodium Hydroxide
ng	Nanogram(s)
NLK	NEMO-like kinase
nm	Nanometer(s)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol-3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PMSF	Phenyl-methyl-sulphonyl fluoride
PP2C/2A	Protein phosphatase 2C/2A
PPAR	Peroxisome proliferator-activated receptor
PTEN	Phosphatase and tensin homolog protein
PTP	Protein tyrosine phosphatase
R_f	Relative mobility
RGS	Regulator of G-protein Signalling
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RXR	Retinoid X Receptor
SA	South Africa
SCC	Squamous cell carcinoma
SCF	Skp1/Cullin1/F-box
SDS	Sodium dodecyl sulphate
sFRP	Secreted Frizzled-related protein
TAD	Transactivation domain
TBP	TATA-binding protein
TBS	Tris buffered saline
TCA	Trichloroacetic acid
Tcf	T-cell factor

TE	Trypsin/ETDA
TEMED	*N*,*N*,*N'*,*N'*-tetramethylethylene-diamine
TGF	Transforming growth factor
TLE	Transducin-like enhancer
TPA	12-*O*-tetradecanoylphorbol-13-acetate
Tris	Tris(hydroxymthyl)aminomethane
WHCO-	Wits human carcinoma of the oesophagus -
ZO-1	Zonula occuldens-1

List of Companies

Unless otherwise stated, the following companies are located as follows:

Company	**Country**
Bayer	South Africa
Chappel	United States of America
Dako	Denmark
Fermentas Life Sciences	United States of America
ICN	United States of America
Merck	South Africa
Molecular Probes	United States of America
MSI	United States of America
Pierce	United States of America
Serotec	United States of America
Sigma	United States of America

CHAPTER ONE

The Role of β-catenin in the Cell

1.1 The vital importance of adhesion and its pivotal role in tumour biology

Among the many aspects of tumour biology, perhaps the most lethal is the ability of the tumour cells to leave the tissue in which they arose and aggressively trespass into other tissues. This spread of cancer cells to distant sites in the body is termed metastasis (Ruoslahti, 1996). Metastasis requires the cancer cells to detach from the primary tumour mass, travel on/through the extracellular matrix (ECM), invade the surrounding tissues or vascular/lymphatic systems, extravasate and establish in a new site (Ruoslahti, 1996; Meyer and Hart, 1998; Chambers *et al*., 2002). Despite the clinical importance of metastasis in terms of patient survival and success of treatment, there remains a lack of understanding of the molecular and cellular mechanisms underlying this process (Meyer and Hart, 1998; Chambers *et al*., 2002).

Cell adhesion holds cells in their genetically determined position, maintaining the morphogenic and homeostatic integrity of a tissue (Takeichi, 1991; Gumbiner, 1996; Perez-Moreno and Fuchs, 2006). This is important in differentiation (for example, organogenesis), survival, proliferation, migration, signal transduction and immunological functions of the tissue (Shimoyama *et al.,* 1989; Hynes, 1999; Orend and Chiquet-Ehrismann, 2000; Yang *et al.,* 2001). A change in adhesion generally leads to cells that are migratory and proliferative in nature (Fujii *et al.,* 1996; Ruoslahti, 1996; Li *et al.,* 1998a; St Croix *et al*., 1998). This is advantageous for malignant cancer cells in which a loss or change of attachment can lead to the ability to invade other tissues and metastasise (Hinck *et al.,* 1994b; Fujii *et al.,* 1996; Ruoslahti, 1996; Davies *et al.,* 2000). Thus a change in cellular adhesion is a central event in the progression of a benign mass to an invasive

metastatic tumour (Ruoslathi, 1996; Meyer and Hart, 1998; Wijnhoven *et al.,* 2000).

1.2 How is cell adhesion achieved?

Cellular adhesion is promoted through the action of cell adhesion molecules (CAMs), which connect cells together and to the ECM, at specific points, such as the adherens junctions (AJs) (Ozawa *et al.,* 1990a; Takeichi, 1991; Meyer and Hart, 1998; Perez-Moreno and Fuchs, 2006). CAMs are cell surface glycoproteins that have been grouped into two classes: those that function independently of calcium (Ca^{2+}) and those that require Ca^{2+} for their binding activity (Ozawa *et al.,* 1990a). The major groups of CAMs include the cadherins, integrins, immunoglobulins, selectins and CD44 proteins. Since this study deals with cadherin interactions in particular, the remaining groups will only be introduced briefly in order to provide a broad basis to the positioning of the CAMs in adhesion.

The immunoglobulins (Igs) are members of the calcium-independent cell-surface glycoproteins, characterised by their Ig-related domains and fibronectin type III domains (Meyer and Hart, 1998; Hynes, 1999). These domains provide a mechanism for cell-ECM binding and cell-cell binding, as they are able to form both homophilic and heterophilic interactions with a variety of different ligands (Meyer and Hart, 1998; Hynes, 1999). It is important to point out that several Ig receptors can make cytoskeletal connections to the actin cytoskeleton (Hynes, 1999; Takai and Nakanishi, 2003). For example, the Ig-like intercellular adhesion molecule, nectin, can link to the cytoskeleton via the actin-binding protein afadin, and plays a role in the organisation of the adherens and tight junctions, as well as formation of synapses in neurons (Ikeda *et al*., 1999; Pokutta *et al*., 2002; Takai and Nakanishi, 2003).

The selectins, as a group of cell adhesion receptors, arbitrate cell binding via the interaction between their calcium-dependent lectin domains and cell surface

carbohydrate ligands (Mousa and Cheresh, 1997; Meyer and Hart, 1998; Hynes, 1999). This group is mainly involved in inflammatory responses and is only linked to leukocytes and endothelial cells (Mousa and Cheresh, 1997; Meyer and Hart, 1998; Hynes, 1999).

The CD44 family of glycoproteins, found on the surface of both epithelial and mesenchymal cells, acts mainly as a receptor for the ECM marker protein, hyaluronan (Ashida *et al.,* 1998). Although it does function in cell-cell and cell-ECM adhesion in some cases, it is mainly involved in the recirculation, homing, adhesion and activation of lymphocytes (Ashida *et al.,* 1998).

The integrins are a diverse family of transmembrane proteins that bind to the ECM or other CAMs (Meyer and Hart, 1998; Hynes, 1999). They are composed of two glycoprotein α- and β-subunits which non-covalently associate to form a heterodimer that binds to their ligands like "two hands grasping a pole" (Meyer and Hart, 1998). A number of α- and β-subunits exist resulting in the formation of over 20 heterodimer combinations, which determine ligand specificity (Meyer and Hart, 1998; Hynes, 1999). Their interaction with the ECM provides signals that regulate a number of cellular functions such as shape, adhesion, motility, extravasation, growth, survival, differentiation, angiogenesis and gene expression (Hannigan *et al.,* 1996; von Schlippe *et al.,* 2000). Although the role of cell-ECM adhesion is important to epithelial cell adhesion, the focus of this study is on cell-to-cell adhesion central to which is the cadherin system.

The cadherins mediate calcium-dependent cell-cell adhesion (Takeichi, 1991; Hinck *et al*., 1994b; Braga, 2000). There are a number of members of the cadherin superfamily, which are subdivided into the classical cadherins (type I and II), desmosomal cadherins, proto-cadherins and the atypical cadherin-like proteins, with the classical cadherins being the most well understood (Boggon *et al*., 2002; Gumbiner, 2005). The classical type I cadherins are important in the formation of the adherens junction, which subsequently promote the construction and maintenance of other junctional complexes, such as the desmosomal and tight

junctions (Gottardi *et al*., 2001; Takai and Nakanishi, 2003). The classical cadherins, named after the tissue in which they were discovered, include epithelial (E)-, placental (P)- and neuronal (N)-cadherin (Shimoyama *et al*., 1989; Takeichi, 1991; Gumbiner, 2005). As the focus of this study is on tissue of epithelial origin, the interactions of E-cadherin (120 kD), also known as uvomorulin in mouse, L-CAM in chicken, Arc-1 in dog or cell-CAM 120/80 in humans (Shimoyama *et al*., 1989), will be pursued. Please note, in order to make the following information more "digestible" to the reader, a summary flow diagram (Figure 1.9) has been provided at the end of this chapter.

The cadherins are glycoproteins that consist of an extracellular amino (N) terminal domain, a single-pass transmembrane domain and a highly conserved cytoplasmic carboxy (C) tail (Takeichi, 1991; Hirohashi and Kanai, 2003; Takai and Nakanishi, 2003). The monomeric cadherin forms parallel or *cis* homodimers (Braga, 2000; Hirohashi and Kanai, 2003; Gumbiner, 2005). In this formation, the extracellular domain can mediate homophilic interactions with similar receptors in the neighbouring cell as illustrated in Figure 1.1 (Nose *et al*., 1990; Takeichi, 1991; Simcha *et al*., 1998; Braga, 2000; Hirohashi and Kanai, 2003; Perez-Moreno *et al*., 2003). The extracellular domains consists of five extracellular cadherin repeats (EC 1-5) joined by calcium to form a rigid rod-like structure (Takeichi, 1991; Kemler, 1993; Pokutta *et al*., 1994; Boggon *et al*., 2002; Hirohashi and Kanai, 2003; Perez-Moreno *et al*., 2003; Takai and Nakanishi, 2003). The exact mode of the homophilic interaction is still under debate (see Gumbiner, 2005 for review), although it is well known that mutations in the calcium binding sites of the EC domains or small changes in calcium concentrations rapidly reduces adhesion (Pokutta *et al*., 1994). The homotypic binding enables cell recognition, thus facilitating cell sorting, which is needed in the assembly, segregation and rearrangement of cells during development (Shimoyama *et al*., 1989; Takeichi, 1991; Nieman *et al*., 1999). This is achieved by allowing cell migration, separation of cells into layers and maintenance of proliferation or differentiation within a cell layer (Takeichi, 1991; Gumbiner, 2005). Importantly, it is this rearranging ability of cadherins that is required for

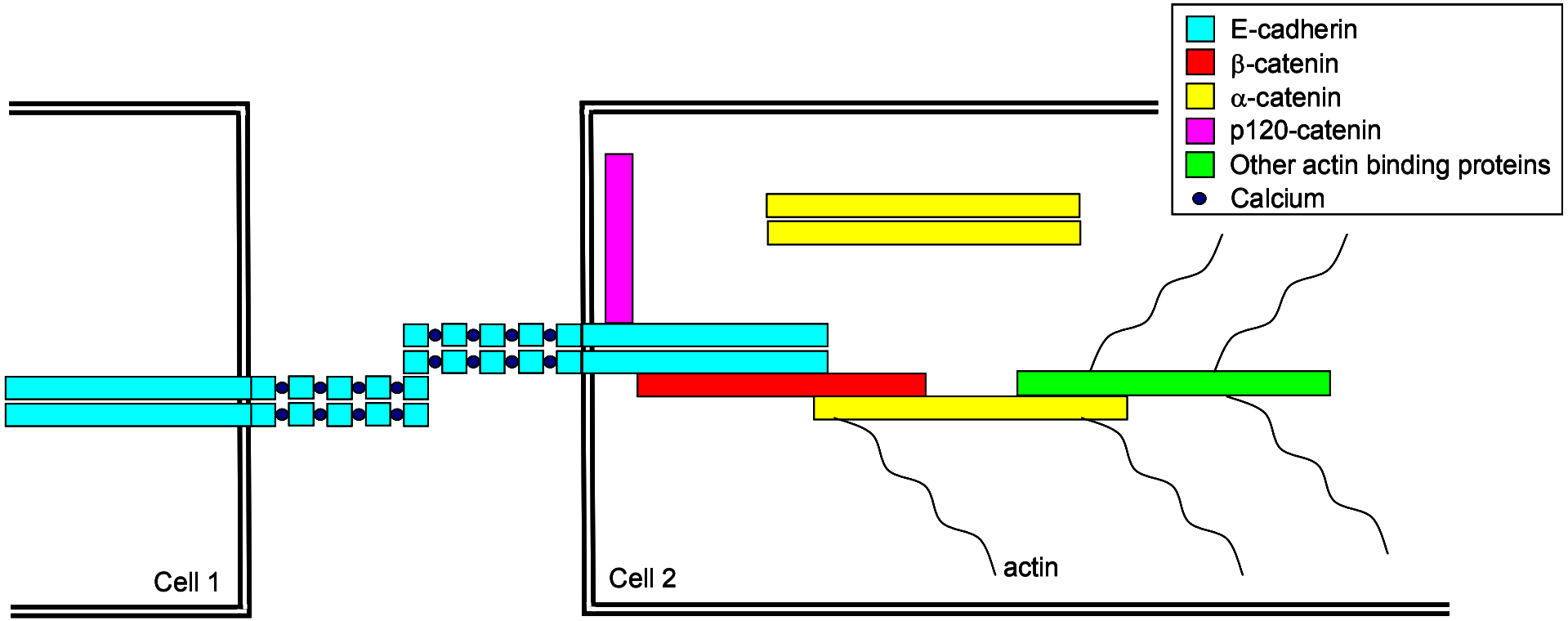


Figure 1.1: β-catenin regulates the formation of the adhesion belts in epithelial cells. Adjacent epithelial cells are linked together by homophilic interactions between the five extracellular repeat domains of the membrane-bound E-cadherin heterodimers (blue). This interaction requires the presence of calcium. E-cadherin links indirectly to the actin cytoskeleton via the catenin proteins to complete the formation of the adhesion belts. β-catenin (red) binds to the cytoplasmic domain of E-cadherin through its arm-repeat domain. Unbound α-catenin (yellow) exists as a homodimer in the cytoplasm, which dissociates and binds via its amino-terminus to β-catenin. α-catenin binds either directly, via its amino- and carboxyl-termini, or indirectly, via other actin binding proteins (such as α-actinin, vinculin or ZO-1; green) to the actin cytoskeleton. However, new evidence questions the ability of α-catenin to simultaneously associate with the E-cadherin/β-catenin complex and actin (see text for details). p120-catenin (purple) binds to a juxtamembrane domain in the E-cadherin cytoplasmic tail and appears to have a role in regulating the adhesion junction.

metaplasia and metastasis (Lilien and Balsamo, 2005).

In order to complete the formation of a mature, stable, adherens junction, the cytoplasmic domain of cadherin interacts with elements of the actin cytoskeleton, hence establishing and maintaining the phenotypic structure of the tissue (Takeichi, 1991; Yap *et al*., 1997; Braga, 2000; Adams and Nelson, 1998; Hirohashi and Kanai, 2003; Gumbiner, 2005). Although not essential for basic homophilic cadherin-mediated adhesion, anchorage to the actin cytoskeleton induces cadherin clustering, strengthening the cell contacts (Gumbiner, 1996; Yap *et al*., 1997; Adams and Nelson, 1998; Pokutta and Weis, 2000; Gumbiner, 2005). This is important in epithelial cells which are subject to mechanical stresses during compaction and polarisation into sheets and allows the sheets to function as a coordinated tissue (Gumbiner, 1996; Braga, 2000; Pokutta and Weis, 2000; Perez-Moreno *et al*., 2003). Importantly, the interaction between cadherins and the cytoskeleton is not achieved directly but rather via small, secondary or associated proteins, the catenins (Nagafuchi and Takeichi, 1989; Ozawa *et al*., 1989a; Kemler, 1993). Four types of catenins have been identified; α-, β-, γ- (or plakoglobin) and p120-catenin.

1.3 Catenins mediate the link between the cadherins and the actin cytoskeleton

β-catenin (94 kD) consists of three distinct domains; an amino terminus of approximately 149 amino acids, a carboxyl terminus (108 amino acids), and a 515 amino acid central (core) region (Huber *et al.,* 1997b; Huber and Weis, 2001). The amino- and the carboxyl-termini both contain acidic residues, with pI values of 4.4 and 4.3, respectively (Huber *et al.,* 1997b; Castaño *et al*., 2002). The core region contains 12 repeats of a 42 amino acid imperfect armadillo (arm)-repeat sequence that forms a superhelix of three α-helices that is distinctive of the Armadillo-repeat family of proteins (see Figure 1.2; Peifer *et al*., 1994a; Huber *et al.,* 1997b; Lilien and Balsamo, 2005). The superhelix bends slightly between repeats 8 and 9 producing a cleft in the helix (Huber *et al*., 1997b). The superhelix

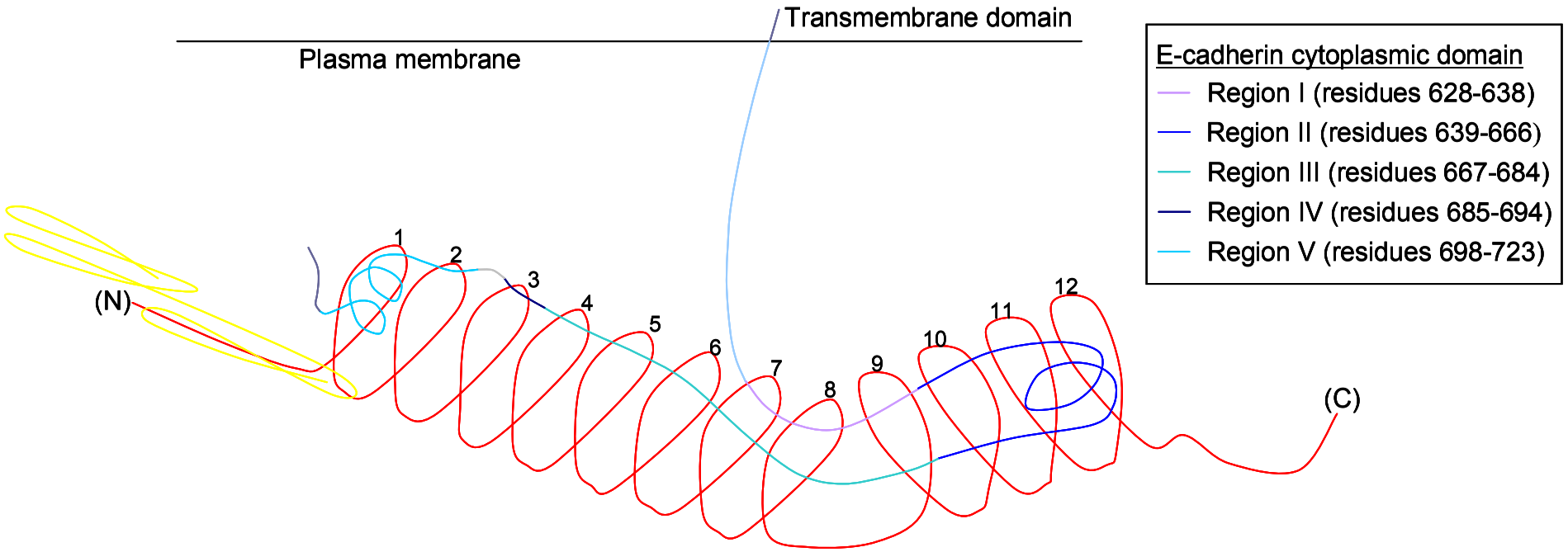


Figure 1.2: The structure of β-catenin and its association with E-cadherin and α-catenin. β-catenin (red) consists of a central superhelical domain consisting of 12 arm-repeats, flanked by an amino (N) and a carboxyl tail (C). The cytoplasmic tail of E-cadherin (shades of blue) enters β-catenin at repeat 7 and extends through to repeat 9 (Region I). It continues to run towards the carboxyl-terminus of β-catenin before forming a helix within repeats 12 and 11 (Region II). It then runs amino-terminally along the superhelical groove under itself towards the fourth repeat (Region III). This region contains a highly conserved core domain (residues 674-684) which is essential for the E-cadherin/β-catenin interaction. E-cadherin extends towards repeat 3 (Region IV) which is followed by a short, unordered sequence. It continues towards the amino-terminus of the β-catenin groove, forms two short helices that mask hydrophobic core residues at the amino-terminus of β-catenin before exiting the protein (Region V; Huber and Weis, 2001). α-catenin (yellow) binds to the amino-terminus and the first two arm-repeats of β-catenin via amphipathic helices in its own amino-terminus (Pokutta and Weis, 2000). This figure is modified from Lilien and Balsamo, (2005).

forms a groove lined with positively charged amino acids (pI 8.3), that enables association with negative (or acidic) regions of proteins (Huber *et al.,* 1997b; Lickert *et al.,* 2000). It is via this kind of ion pairing over an extended region that E-cadherin associates with β-catenin. A sequence of approximately 100 amino acids in the distal portion of E-cadherin's cytoplasmic tail interacts across the entire 12 arm-repeats of β-catenin with a high affinity (see Figure 1.2 for details; Ozawa *et al*., 1990b; Aberle *et al*., 1994; Hülsken *et al*., 1994; Jou *et al*., 1995; Huber and Weis, 2001). An acidic 30 amino acid sequence (pI 3.1-3.3) within this region appears to be the minimal binding domain for the β-catenin superhelical groove and is essential for the association between E-cadherin and β-catenin (Stappert and Kemler, 1994; Jou *et al*., 1995; Huber *et al.,* 1997b; Huber and Weis, 2001).

β-catenin is thought to play a role in controlling the levels of E-cadherin found at the plasma membrane. β-catenin associates with E-cadherin almost immediately after synthesis and is necessary for transportation of both proteins out of the endoplasmic reticulum (ER) and to the plasma membrane (Hinck *et al*., 1994c; Chen *et al.,* 1999a). It is necessary that β-catenin regulates the level of E-cadherin in this manner, as E-cadherin retained in the ER is degraded (Chen *et al.,* 1999a). Additionally, β-catenin controls the degradation of plasma membrane-associated E-cadherin. The cytoplasmic tail of E-cadherin is unstructured when unbound from β-catenin, and contains sequence motifs which are enriched in proline, glutamate, serine and threonine (PEST), that targets E-cadherin for proteasomal degradation (Huber *et al.,* 2001). β-catenin binding to this region conceals the PEST motifs, while conferring structure to the tail (Huber *et al.,* 2001). This enables selective degradation of cadherins that are not bound to β-catenin, thus strengthening adhesion (Huber *et al.,* 2001). A strong interaction between β-catenin and E-cadherin is therefore a functional necessity in the mediation of cell-cell adhesion.

β-catenin mediates the link between E-cadherin and the vinculin-related, actin binding protein, α-catenin (102 kD), and is thus thought to regulate adhesion by completing the formation of the adhesion belts (Aberle *et al*., 1994; Hinck *et al.,* 1994b; Oyama *et al.,* 1994; Jou *et al*., 1995; Gumbiner, 1996). In the cytoplasm α-catenin exists as a homodimer, which dissociates to bind to the amino-terminus and the first two armadillo repeats of β-catenin via amphipathic helices in its own amino-terminus, with a low affinity (Aberle *et al*., 1994; Hinck *et al*., 1994c; Hülsken *et al*., 1994; Oyama *et al*., 1994; Jou *et al*., 1995; Huber *et al*., 1997a; Koslov *et al*., 1997; Pokutta and Weis, 2000; Perez-Moreno and Fuchs, 2006). The binding of α-catenin to β-catenin has also proved important in increasing β-catenin's affinity for E-cadherin. Castaño *et al*. (2002) showed that the binding of α-catenin to the amino-terminal domain of β-catenin induced a conformational change to β-catenin which increased its affinity for E-cadherin.

α-catenin can also interact with the actin cytoskeleton either directly or indirectly, as is shown in Figure 1.1. Direct association occurs at various actin-binding sites in both the amino- and carboxyl-termini of the α-catenin protein (Rimm *et al*., 1995; Imamura *et al.,* 1999; Pokutta *et al*., 2002). Indirect interaction with the actin cytoskeleton is mediated by α-catenin's associations with other actin binding proteins. Thus far, such proteins include vinculin (Watabe-Uchida *et al*., 1998; Weiss *et al.,* 1998), α-actinin (Knudsen *et al*., 1995; Nieset *et al*., 1997), zonula occludens-1 (ZO-1; Itoh *et al*., 1997; Imamura *et al*., 1999), formin-1 (Kobielak *et al*., 2004), ajuba (Marie *et al*., 2003) and spectrin (Pradhan *et al*., 2001). Some of these proteins (for example, vinculin and formin-1) have been shown to play a role in recruiting actin and regulating actin polymerisation or engaging with proteins that enable this (Vasioukhin *et al*., 2000; Reinhard *et al*., 2001; Perez-Moreno *et al*., 2003; Kobielak *et al*., 2004). α-actinin is important in inducing actin bundling and cadherin clustering by mediating cross-linkages between actin filaments (Taylor and Taylor, 1994; Jamora and Fuchs, 2002). α-catenin can also link the cadherin-mediated adhesion junction with other types of adhesion junctions (for example, α-catenin binds to afadin, which forms part of the Ig-

CAM nectin adhesion complex, see Section 1.2 above), demonstrating the ability of α-catenin to organise the adherens and tight junctions (Ikeda *et al*., 1999; Pokutta *et al*., 2002; Perez-Moreno and Fuchs, 2006). Thus, α-catenin plays an essential role by attaching, organising and recruiting actin to the adhesion complex (Rimm *et al*., 1995).

The connection between the cadherins and the underlying cytoskeleton needs to be highly dynamic to enable the cell to modulate its junctions with neighbouring cells to accommodate its circumstances, such as during morphogenesis, tissue homeostasis, migration, wound healing and even metastasis (Klingelhöfer *et al*., 2003; Perez-Moreno *et al*., 2003; Drees *et al*., 2005; Perez-Moreno and Fuchs, 2006). For example, a migrating cell displays a drastically different cytoskeletal and membrane organisation to a stationary cell (Drees *et al*., 2005). The widely accepted model of the E-cadherin/β-catenin/α-catenin/actin adhesion complex was recently shown to be more dynamic in nature than originally thought. Yamada *et al*. (2005) showed that although E-cadherin/β-catenin/α-catenin can form a stable complex, α-catenin cannot bind simultaneously to β-catenin and actin. However, they did not dispute the large amount of evidence that demonstrates the importance of an interaction between the actin cytoskeleton and the cadherin/catenin complex. Instead they proposed that the cadherin/catenin complex acts to provide a pool of α-catenin that is able to locally reorganise the actin filaments and change the membrane dynamics to that of a more mature, stable junction (Drees *et al*., 2005). They showed that homodimeric α-catenin binds to and bundles actin, and suppresses actin branching while promoting the formation of linear actin cables present in mature adherens junctions (Drees *et al*., 2005). Thus, much still remains to be discovered as to the dynamics involved in the mechanics and regulation of the cadherin/catenin adhesion complex.

γ-catenin (86 kD) is structurally and functionally similar to β-catenin (Franke *et al*., 1989; McCrea *et al*., 1991; Butz *et al*., 1992; Knudsen and Wheelock, 1992; Peifer *et al*., 1992). It can weakly associate with E-cadherin (Knudsen and Wheelock, 1992; Peifer *et al*., 1992; Jou *et al*., 1995; Sacco *et al*., 1995; Wahl *et*

al., 1996) and α-catenin (Aberle *et al*., 1996), but plays a less extensive role than β-catenin in epithelial tissue and interacts mainly with the desmosomal cadherins (Cowin *et al*., 1986; Franke *et al*., 1989; Butz *et al*., 1992; Peifer *et al*.,1992; Sacco *et al*., 1995; Wahl *et al*., 1996).

p120-catenin (also called $p120^{ctn}$ or $p120^{cas}$) weakly associates with a juxtamembrane domain of the cytoplasmic tail of E-cadherin and does not link to the actin cytoskeleton in any way (Reynolds *et al*., 1994; Daniel and Reynolds, 1995; Finnemann *et al*., 1997; Lampugnani *et al*., 1997; Yap *et al*., 1998; Ohkubo and Ozawa, 1999; Thoreson *et al*., 2000). The cadherin juxtamembrane domain is known to be involved in the promotion of cadherin clustering and adhesiveness and the suppression of cell motility (Chen *et al*., 1997; Yap *et al*., 1998; Thoreson *et al*., 2000). p120-catenin is thought to play a role in regulating E-cadherin-mediated adhesion in both a positive and negative manner (Ozawa and Kemler, 1998b; Yap *et al*., 1998; Aono *et al*., 1999; Ohkubo and Ozawa, 1999; Thoreson *et al*., 2000; Ireton *et al*., 2002). In a positive sense, p120-catenin is required for the stable retention of E-cadherin at the plasma membrane by inhibiting E-cadherin endocytosis, and thus its subsequent degradation (Thoreson *et al*., 2000; Ireton *et al*., 2002; Davis *et al*., 2003; Xiao *et al*., 2003b; Xiao *et al*., 2007). It may do so by interacting or interfering with regulators of the endocytotic process (Ireton *et al*., 2002; Davis *et al*., 2003; Xiao *et al*., 2003b; Xiao *et al*., 2007), or by recycling endocytosed E-cadherin to the membrane before it is degraded (Chen *et al*., 2003; Davis *et al*., 2003; Xiao *et al*., 2003; Perez-Moreno and Fuchs, 2006; Xiao *et al*., 2007). In terms of negative regulation, posttranslational modification of p120-catenin through phosphorylation of its amino-terminus, downregulates E-cadherin levels, and thus provides a regulatory mechanism of adhesion by signalling pathways (Aono *et al*., 1999; Ireton *et al*., 2002). p120-catenin is also thought to regulate adhesion by acting as a docking protein for a number of tyrosine kinases which affect the adhesion complex both positively and negatively (see Section 1.9.4).

1.4 Aberrant expression of the adhesion molecules in the development of metastasis

Negative regulation of the E-cadherin-mediated cell adhesion is associated with cancer progression, increased invasion and metastasis (Behrens *et al*., 1989; Frixen *et al*., 1991; Vleminckx *et al*., 1991; Dorudi *et al*., 1993; Takeichi, 1993; Birchmeier and Behrens, 1994; Ihara *et al.,* 1996; Mbalaviele *et al*., 1996; Shiozaki *et al.,* 1996; Perl *et al*., 1998; Wijnhoven *et al.,* 2000; Al-Aynati *et al*., 2004). This has been demonstrated experimentally by transforming a non-invasive tumour into an invasive form by functionally blocking E-cadherin, β-catenin or α-catenin, either with antibodies or with antisense RNA (Behrens *et al*., 1989; Vleminckx *et al*., 1991; Pignatelli *et al*., 1992). Conversely, transfection of cDNA of components of the adhesion complex, into an invasive tumour reduces its invasive and metastatic ability (Chen and Öbrink, 1991; Frixen *et al*., 1991; Navarro *et al*., 1991; Vleminckx *et al*., 1991; Hirano *et al*., 1992; Breen *et al*., 1993; Watabe *et al*., 1994; Kawanishi *et al*., 1995; Mbalaviele *et al*., 1996; Li *et al*., 1998a; Perl *et al*., 1998). Thus, the adhesion complex can be thought of as a suppressor of invasion and metastasis (Vleminckx *et al*., 1991; Oyama *et al.,* 1994; Wijnhoven *et al.,* 2000).

Reduced or loss of E-cadherin expression has been noted in a wide array of tumour types (please see Table 1.1 for details). Tumours that show this tend to display infiltrative growth, with lymph node, blood and bone metastases, compared to those that have preserved E-cadherin expression (Schipper *et al*., 1991; Oka *et al*., 1992; Umbas *et al*., 1992; Doki *et al*., 1993; Dorudi *et al*., 1993; Oka *et al*., 1993; Takeichi, 1993; Kadowaki *et al*., 1994; Pignatelli *et al*., 1994; Umbas *et al*., 1994; Gagliardi *et al*., 1995; Gamallo *et al*., 1996; Shiozaki *et al*., 1996; Siitonen *et al*., 1996; Hunt *et al*., 1997; Jawhari *et al*., 1997; Bukholm *et al*., 1998; Karayiannakis *et al*., 1998; Wijnhoven *et al*., 2000; Jamora and Fuchs, 2002; Hajra and Fearon, 2002; Hirohashi and Kanai, 2003). In many instances the loss of E-cadherin expression correlates directly with tumour differentiation and clinical prognosis (Frixen *et al*., 1991; Oka *et al*., 1992; Bringuier *et al*., 1993;

Table 1.1: Cancers showing reduced expression of E-cadherin, α-catenin and β-catenin

Reduced	Type	Reference
E-cadherin	Breast adenocarcinoma	Frixen *et al*., 1991; Oka *et al*., 1993; Pierceall *et al*., 1995; Gamallo *et al*., 1996; Siitonen *et al*., 1996; Charpin *et al*., 1997; de Leeuw *et al*., 1997; Hunt *et al*., 1997; Bukholm *et al*., 1998
	Pancreatic adenocarcinoma	Pignatelli *et al*., 1994; Karayiannakis *et al*., 1998; Al-Aynati *et al*., 2004
	Gastric adenocarcinoma	Shimoyama and Hirohashi, 1991; Oka *et al*., 1992; Matsuura *et al*., 1992; Becker *et al*., 1994; Jawhari *et al*., 1997
	Prostate adenocarcinoma	Umbas *et al*., 1992; Umbas *et al*., 1994; Cheng *et al*., 1996; Richmond *et al*., 1997
	Colorectal adenocarcinoma	Dorudi *et al*., 1993; Gagliardi *et al*., 1995
	Melanoma	Danen *et al*., 1996
	Renal carcinoma	Katagiri *et al*., 1995
	Hepatocellular carcinoma	Kanai *et al*., 1997; Endo *et al*., 2000
	Ovarian carcinoma	Hashimoto *et al*., 1989; Veatch *et al*., 1994
	Thyroid carcinoma	Brabant *et al*., 1993
	Bladder carcinoma	Bringuier *et al*., 1993; Syrigos *et al*., 1995
	Lung carcinoma	Böhm *et al*., 1994
	Tongue SCC	Li *et al*., 1998a
	Head and neck SCC	Schipper *et al*., 1991
	Skin SCC	Navarro *et al*., 1991
	Oesophageal (SCC & Barretts)	Kadowaki *et al*., 1994; Nakanishi *et al*., 1997; Doki *et al*., 1993; Bailey *et al*., 1998
α-catenin	Lung carcinoma	Hirano *et al*., 1992; Shimoyama *et al*., 1992; Oda *et al*., 1993; Watabe *et al*., 1994
	Diffuse gastric carcinoma	Shimoyama *et al*. 1992; Jawhari *et al*., 1997
	Prostate adenocarcinoma	Morton *et al*., 1993; Richmond *et al*., 1997; Tomita *et al*., 2000
	Breast adenocarcinoma	de Leeuw *et al*., 1997; Bukholm *et al*., 1998
	Colorectal adenocarcinoma	Breen *et al*., 1993; Takayama *et al*., 1998; Vermeulen *et al*., 1999
	Bladder carcinoma	Shimazui *et al*., 1996
	Oesophageal SCC	Kadowaki *et al*., 1994; Nakanishi *et al*., 1997
β-catenin	Gastric adenocarcinoma	Oyama *et al.,* 1994; Kawanishi *et al*., 1995; Jawhari *et al.,* 1997
	Breast carcinomas	de Leeuw *et al*., 1997; Bukholm *et al*., 1998
	Bladder carcinoma	Shimazui *et al*., 1996
	Colorectal adenocarcinoma	Ilyas *et al*., 1997; Takayama *et al*., 1998
	Oesophageal SCC	Nakanishi *et al*., 1997

Dorudi *et al*., 1993; Pignatelli *et al*., 1994; Umbas *et al*., 1994; Syrigos *et al*., 1995; Shiozaki *et al*., 1996; Siitonen *et al*., 1996; Karayiannakis *et al*., 1998).

There are a number of mechanisms that result in reduced E-cadherin expression in tumours. Firstly, abnormal E-cadherin expression can occur by loss of one allele together with a mutation in the other allele of the E-cadherin gene (Oda *et al*., 1994; Berx *et al*., 1995). Allelic loss of E-cadherin is frequent in hepatocellular carcinomas (Slagle *et al*., 1993), breast (Berx *et al*., 1996; Hiraguri *et al*., 1998), prostate (Carter *et al*., 1990) and oesophageal adenocarcinomas (Wijnhoven *et al*., 1999). Mutation of the E-cadherin gene is limited to diffuse-type gastric (Becker *et al*., 1994; Oda *et al*., 1994; Birchmeier, 1995; Muta *et al*., 1996; Guilford *et al*., 1998; Machado *et al*., 1999; Saito *et al*., 1999b), infiltrative lobular breast (Kanai *et al*., 1994; Berx *et al*., 1995; Berx *et al*., 1996; de Leeuw *et al*., 1997; Vos *et al*., 1997) and gynaecological/endometrial cancers (Risinger *et al*., 1994; van Aken *et al*., 2001). Secondly, DNA hypermethylation of the E-cadherin gene promoter has also been shown to contribute to reduced E-cadherin expression (Graff *et al*., 1995; Hennig *et al*., 1995; Yoshiura *et al*., 1995; Hiraguri *et al*., 1998) in hepatocellular carcinomas (Yoshiura *et al*., 1995; Kanai *et al*., 1997), diffuse gastric cancers (Yoshiura *et al*., 1995; Grady *et al*., 2000; Tamura *et al*., 2000), breast (Graff *et al*., 1995; Hiraguri *et al*., 1998), prostate (Graff *et al*., 1995), bladder (Yoshiura *et al*., 1995), lung (Yoshiura *et al*., 1995) and in oesophageal adenocarcinomas (Eads *et al*., 2000). Finally, a number of cancers show increased levels of E-cadherin transcriptional repressor proteins (such as Snail, Slug, E12/E47 and SIP1) which downregulate E-cadherin expression (Batlle *et al*., 2000; Cano *et al*., 2000; Comijn *et al*., 2001; Pérez-Moreno *et al*., 2001; Bolós *et al*., 2003; Gavert and Ben-Ze'ev, 2007).

Some tumours possess normal levels of E-cadherin but still display decreased adhesion (Shiozaki *et al.,* 1996; Bukholm *et al*., 1998). This is due to alterations in the expression of the catenin proteins (Shiozaki *et al.,* 1996; Bukholm *et al*., 1998). Reduced α-catenin expression has been found in a variety of human cancers (see Table 1.1 for details). Among these, loose cell-cell adhesion and

metastasis to the lymph nodes has been noted in oesophageal squamous cell carcinomas (SCCs) that produce lower levels of α-catenin than the normal tissue (Kadowaki *et al*., 1994; Nakanishi *et al*., 1997). The mechanism underlying reduced levels of α-catenin is not yet known. A human metastatic lung carcinoma cell line, PC-9, displays reduced α-catenin proteins due to deletions in its gene resulting in aberrant mRNA production (Hirano *et al*., 1992; Shimoyama *et al*., 1992; Oda *et al*., 1993; Watabe *et al*., 1994). Mutations in the α-catenin gene have also been reported in prostate and colon tumours (Morton *et al*., 1993; Vermeulen *et al*., 1999). However, mutations of the α-catenin gene are a rare event in human cancers *in vivo* (Hirohashi and Kanai, 2003).

β-catenin is thought to play a regulatory role in cell adhesion due to its central position in the adhesion complex (Hinck *et al.,* 1994b; Gumbiner, 1996; Takayama *et al.,* 1998). Thus, aberrant expression of β-catenin, due either to a transcriptional or posttranscriptional event, has been found to correlate with invasion and metastases in a number of tumours (refer to Figure 1.9; Takayama *et al*., 1996; Takayama *et al.,* 1998). For example, two cell lines (HSC-39 and HSC-40A) both derived from a diffuse human signet ring carcinoma of the stomach, display in-frame deletions in the amino-terminus of β-catenin (amino acids 28-134) (Oyama *et al.,* 1994; Kawanishi *et al*., 1995). These deletions prevent the protein from associating with α-catenin, decreasing intercellular adhesion (Oyama *et al.,* 1994; Kawanishi *et al*., 1995). Mutations affecting the association of β-catenin with α-catenin have also been reported in colorectal cancers (Ilyas *et al*., 1997). In addition, abnormal β-catenin expression has also been found in a number of cancers, including oesophageal SCC (see Table 1.1, Nakanishi *et al*., 1997).

1.5 The structure of the β-catenin gene, *CTNNB1*

The gene coding for the β-catenin protein in humans is termed *CTNNB1* (Nollet *et al.,* 1996). It is 23.2 kilobases in size and results in a transcript that is 3362

nucleotides long (Nollet *et al.,* 1996). It consists of 16 exons, ranging from 61 to 790 base pairs (bp) in length, while the introns range from 84bp to 6700bp, with more than half being smaller than 550bp (Nollet *et al.,* 1996). *CTNNB1* contains a TATA-promoter box in its 5'-region but lacks a CCAAT-box (Nollet *et al.,* 1996). The 5'-region also contains several sites that may serve as binding regions for a number of transcription factors (Nollet *et al.,* 1996).

1.6 Regulation of unbound cytoplasmic β-catenin

Apart from its role in cell adhesion, unbound cytoplasmic β-catenin has been implicated in signal transduction pathways and the regulation of gene expression. Under normal circumstances, therefore, the level of cytoplasmic β-catenin is heavily regulated (refer to Figure 1.9 for summary). This regulation is mediated by a variety of molecules, most importantly adenomatous polyposis coli (APC), glycogen synthase kinase-3β (GSK-3β), casein kinase Iα (CKIα) and axin, which target β-catenin for its degradation as illustrated in Figure 1.3. In addition, β-catenin can be regulated in a phosphorylation-independent manner, as discussed in Section 1.6.2.

1.6.1 A phosphorylation-dependent degradation complex regulates unbound cytoplasmic β-catenin

GSK-3β plays a central role in the downregulation of β-catenin as it targets β-catenin for ubiquitination and degradation via the proteasome (Rubinfeld *et al*., 1996; Aberle *et al*., 1997). It does so by sequentially phosphorylating β-catenin on threonine 41, serine 37 and serine 33 in the amino-terminus, which identifies the protein for downstream ubiquitination and proteasomal degradation (Peifer *et al*., 1994b; Yost *et al*., 1996; Liu *et al*., 2002; Kimelman and Xu, 2006). In order for GSK-3β to mediate these phosphorylations, it requires a priming phosphorylation on serine 45 by a third party kinase, CK1α (Liu *et al*., 2002; Kimelman and Xu, 2006). This phosphorylation makes β-catenin a better substrate for GSK-3β

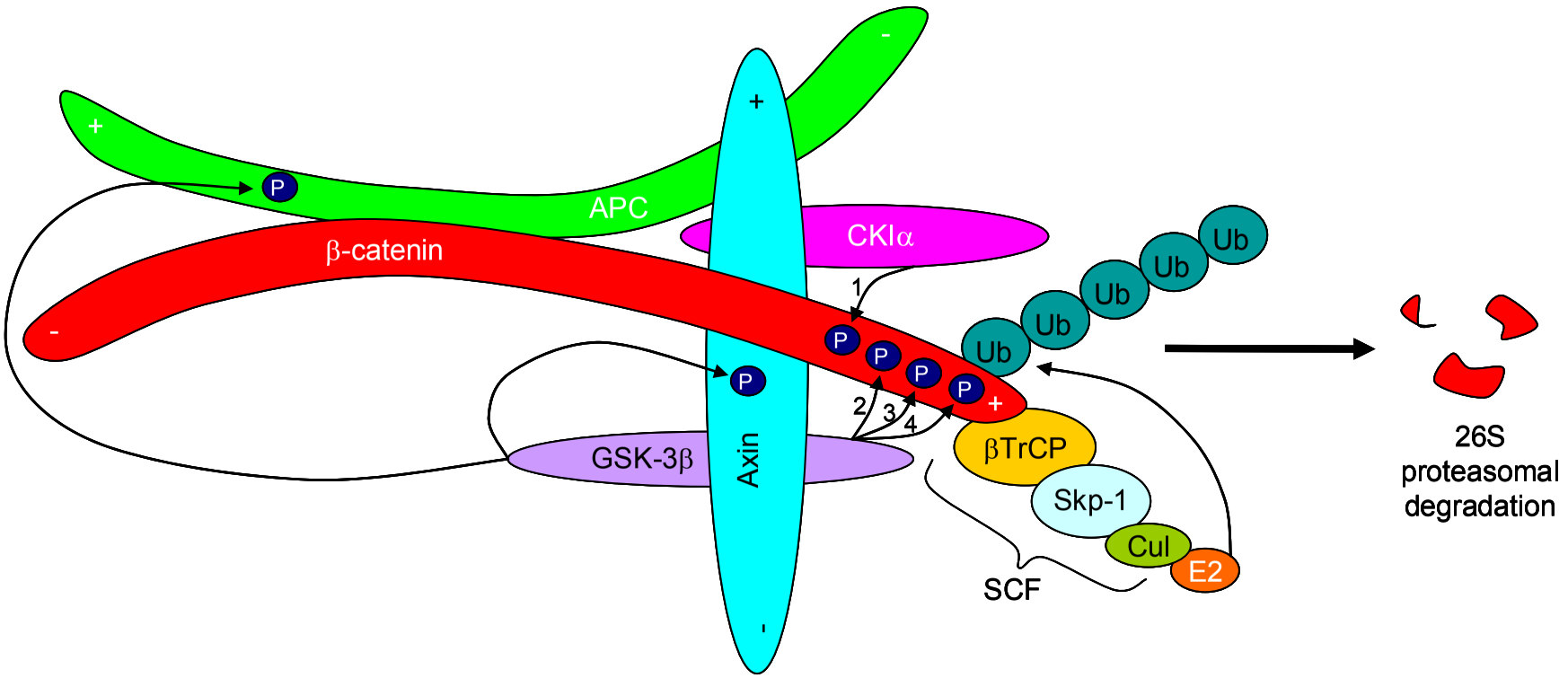


Figure 1.3: Cytoplasmic β-catenin is regulated by a degradation complex. The association of glycogen synthase kinase-3β (GSK-3β), casein kinase Iα (CKIα), adenomatous polyposis coli (APC) and axin, is essential in regulating the levels of cytoplasmic β-catenin. Axin acts as a scaffold protein binding to GSK-3β, CKIα, APC and β-catenin, bringing the former three molecules into close proximity with β-catenin. GSK-3β phosphorylates both axin and APC. This stabilizes axin and enhances the association between APC and β-catenin. CKIα mediates a priming phosphorylation of serine 45 within the amino-terminus of β-catenin (represented by arrow 1). This is required for further amino-terminal phosphorylations by GSK-3β on threonine 41, serine 37 and serine 33 (arrows 2, 3,and 4, respectively). These phosphorylations are specifically recognised by the F-box protein, β-transducin-repeat-containing protein (βTrCP), via its WD-40 domain. βTrCP binds via its F-box to Skp1, which in turn binds to Cullin1 (Cul), forming the Skp1/Cullin1/F-box (SCF) ubiquitin ligase complex. SCF in turn associates with the ubiquitin conjugating enzyme (E2), allowing for polyubiquitination (Ub) of β-catenin. In this form, β-catenin is recognised by the 26S proteasome and is degraded. Note, "+" and "-" represent amino- and carboxyl-terminus respectively. "P" represents a phosphorylation.

(Kundu *et al*., 2006). However, GSK-3β can only recognise β-catenin that is bound to APC and requires axin to phosphorylate β-catenin significantly (Hart *et al*., 1998; Hinoi *et al*., 2000).

APC is a tumour suppressor protein, whose major function is in the downregulation of excess β-catenin in the cytoplasm (Munemitsu *et al*., 1995). The central portion of APC consists of three imperfect repeats of fifteen amino acids that bind to the central region of β-catenin (Su *et al*., 1993; Rubinfeld *et al*., 1995; Kimelman and Xu, 2006). Immediately C-terminal to this, is a region containing seven related, but distinct, repeats of twenty amino acids, which can also bind to β-catenin (Rubinfeld *et al*., 1993; Munemitsu *et al*., 1995; Rubinfeld *et al*., 1995; Kimelman and Xu, 2006). This region is known to be necessary for β-catenin degradation (Munemitsu *et al*., 1995; Rubinfeld *et al*., 1997). APC encourages GSK-3β phosphorylation of β-catenin, although the exact role APC plays in the downregulation of β-catenin is still uncertain (Hinoi *et al.,* 2000; Bienz and Hamada, 2004). APC localizes to the plasma membrane in an actin-dependent manner (Rosin-Arbesfeld *et al*., 2001), and it has been speculated that its role in the degradation complex is to sequester β-catenin and target it to axin and facilitate the interaction between β-catenin and axin (Bienz and Hamada, 2004; Kimelman and Xu, 2006).

The third to seventh twenty-amino acid repeat regions of APC contains Ser-Ala-Met-Pro (SAMP) motifs which are important in its association with axin (Behrens *et al*., 1998; Hart *et al*., 1998; Kishida *et al.,* 1998; Kimelman and Xu, 2006). Axin binds to these domains via a regulator of G-protein signalling (RGS) domain in its amino terminus (Behrens *et al*., 1998; Kishida *et al.,* 1998). Although the interaction between APC and axin is not necessary for axin to mediate β-catenin downregulation, it is required for the efficient degradation of β-catenin (Fagotto *et al*., 1999; Hinoi *et al*., 2000; Salahshor and Woodgett, 2005). Axin also binds both CKIα and GSK-3β at different regions, as can be seen in Figure 1.3 (Ikeda *et al*., 1998; Sakanaka *et al*., 1998; Smalley *et al*., 1999; Liu *et al*., 2002). GSK-3β phosphorylates both axin and APC, increasing their affinity for β-catenin and

stabilizing axin (Rubinfeld *et al*., 1996; Ikeda *et al*., 1998; Fagotto *et al*., 1999; Jho *et al*., 1999; Willert *et al*., 1999; Yamamoto *et al.,* 1999). Axin interacts with β-catenin by binding directly to the arm-repeats (three through seven) of β-catenin via a domain between the CKIα and GSK-3β binding domains (Behrens *et al.,* 1998; Ikeda *et al*., 1998; Sakanaka *et al*., 1998; Liu *et al*., 2002; Kimelman and Xu, 2006). It is this binding that brings β-catenin into close proximity with both CKIα and GSK-3β, promoting the kinases' activity on β-catenin, which they would otherwise be unable to do efficiently (Hart *et al*., 1998; Ikeda *et al*., 1998; Kishida *et al.,* 1998; Sakanaka *et al.,* 1998; Hinoi *et al.,* 2000; Liu *et al*., 2002; Salahshor and Woodgett, 2005). The importance of axin in the degradation complex is highlighted by the fact that it increases the phosphorylation of β-catenin by more than twenty thousand times (Hart *et al*., 1998; Dajani *et al*., 2003). Thus, axin is a scaffold protein, coordinating the binding of APC, GSK-3β and CKIα to β-catenin and hence regulates β-catenin degradation (Hart *et al*., 1998; Ikeda *et al*., 1998; Kishida *et al*., 1998; Kikuchi, 1999; Neo *et al.,* 2000).

The GSK-3β and CKIα phosphorylation of β-catenin allows for specific ubiquitination and, hence, degradation of the protein (Figure 1.3; Aberle *et al*., 1997; Orford *et al*., 1997; Liu *et al*., 2002). Integral to this process is a Skp1/Cullin1/F-box (SCF) ubiquitin ligase complex containing the F-box motif termed human-β-transducin-repeat-containing protein (βTrCP) (Kitagawa *et al.,* 1999). βTrCP associates with the APC/GSK-3β/axin degradation complex and specifically recognises, via a WD-40 repeat domain, the destruction motif created by GSK-3β/CKIα phosphorylation of β-catenin (Hart *et al*., 1999; Kitagawa *et al.,* 1999; Liu *et al.,* 1999a; Winston *et al*., 1999; Liu *et al*., 2002; Kimelman and Xu, 2006). βTrCP is bound to the Skp1 protein, which in turn complexes with other components of the ubiquitination apparatus such as the ubiquitin-conjugating enzyme (Kitagawa *et al.,* 1999; Liu *et al.,* 1999a). This allows for a poly-ubiquitination of β-catenin that subsequently identifies the protein for 26S proteasomal degradation (Kitagawa *et al.,* 1999). Inhibition of either GSK-3β or CKIα phosphorylation of β-catenin, or of βTrCP, can result in the deregulation

and accumulation of cytoplasmic β-catenin (Hart *et al*., 1999; Liu *et al.,* 1999a; Lui *et al*., 2002). Deregulation of β-catenin in the cytoplasm is often referred to as "stabilization". It should be noted that this term reflects a negative event in the cell, with detrimental consequences, which will be discussed in Section 1.7.

1.6.2 Phosphorylation-independent degradation of β-catenin

Alternative phosphorylation-independent pathways that lead to β-catenin degradation exist. These include the p53-inducible Siah-1 protein (Liu *et al*., 2001a; Matsuzawa and Reed, 2001) and a retinoid X receptor (Xiao *et al*., 2003a).

The Siah-1 protein is normally maintained at low levels within the cell (Matsuzawa and Reed, 2001). p53 induces the expression of Siah-1, which is able to bind to ubiquitin conjugating enzymes via its amino-terminal RING domain, and target proteins for degradation (Liu *et al*., 2001a; Matsuzawa and Reed, 2001). It has been shown to reduce the levels of β-catenin in a posttranscriptional manner (Liu *et al*., 2001a). It does so by associating with a multiprotein ubiquitin ligase complex (E3), consisting of a Siah-1 interacting protein (SIP), Skp1 and the F-box protein, Ebi (Matsuzawa and Reed, 2001). SIP acts as a molecular bridge between Siah-1 and Skp, while Ebi associates with both Siah-1 and β-catenin (Matsuzawa and Reed, 2001). Unlike the βTrCP F-box protein, Ebi can interact with β-catenin independently of GSK-3β phosphorylation (Matsuzawa and Reed, 2001). However, Siah-1 is required to associate with APC in order to mediate β-catenin downregulation (Liu *et al*., 2001a).

The retinoid X receptor (RXR) has been shown to regulate β-catenin turnover in an APC-independent manner (Xiao *et al*., 2003a). Upon association with an agonist, RXRs and their dimerisation partners have been shown to be targeted for proteasomal degradation (Kopf *et al*., 2000; Osburn *et al*., 2001). Xiao *et al*. (2003a) showed that RXRα and β-catenin form a transient complex and upon agonist-association induces the degradation of both RXR and β-catenin. This group was unable to elicit the mechanism underlying this process but speculated

that RXRα could be acting as a docking and recognition protein, inducing conformational changes in its target protein, or it could be a targeting protein that carries the target protein to particular cellular compartments for modification or degradation (Xiao *et al*., 2003a).

Peroxisome proliferator-activated receptor (PPAR)-γ has also been shown to downregulate β-catenin levels, although this activity may be tissue specific (Sharma *et al*., 2004). The mechanism underlying this process is unclear, although Sharma *et al*. (2004) showed that it involves ubiquitin-mediated degradation, but was not mediated via the GSK-3β or p53/Siah-1 degradation pathways, and most likely occurs via a mechanism similar to that of the RXR-mediated degradation.

1.7 Why is the stabilization of cytoplasmic β-catenin important?

The control of cytoplasmic β-catenin levels is important for the regulation of gene transcription and apoptosis. The deregulation and increase of stable cytoplasmic β-catenin has been correlated with the entry of the protein into the nucleus, where it increases the transcription of a number of genes that will be discussed below (Behrens *et al*., 1996; Huber *et al*., 1996; Molenaar *et al*., 1996; Korinek *et al*., 1997; Rubinfeld *et al*., 1997). Overexpression of β-catenin has also been shown to inhibit apoptosis (Orford *et al.,* 1999; Chen *et al.,* 2001).

1.7.1 β-catenin acts as a transcriptional activator for a number of genes

β-catenin is able to translocate into the nucleus even though it does not possess any of the conventional nuclear import machinery (Orsulic and Peifer, 1996; van de Wetering *et al*., 1997; Fagotto *et al*., 1998; Yokoya *et al*., 1999). It was initially shown that β-catenin enters the nucleus in a passive manner by associating with a transcriptional factor complex, lymphoid enhancer factor (Lef)/T-cell factor (Tcf), which carries it into the nucleus (see Figure 1.4; Behrens *et al*., 1996; Huber *et al*.,

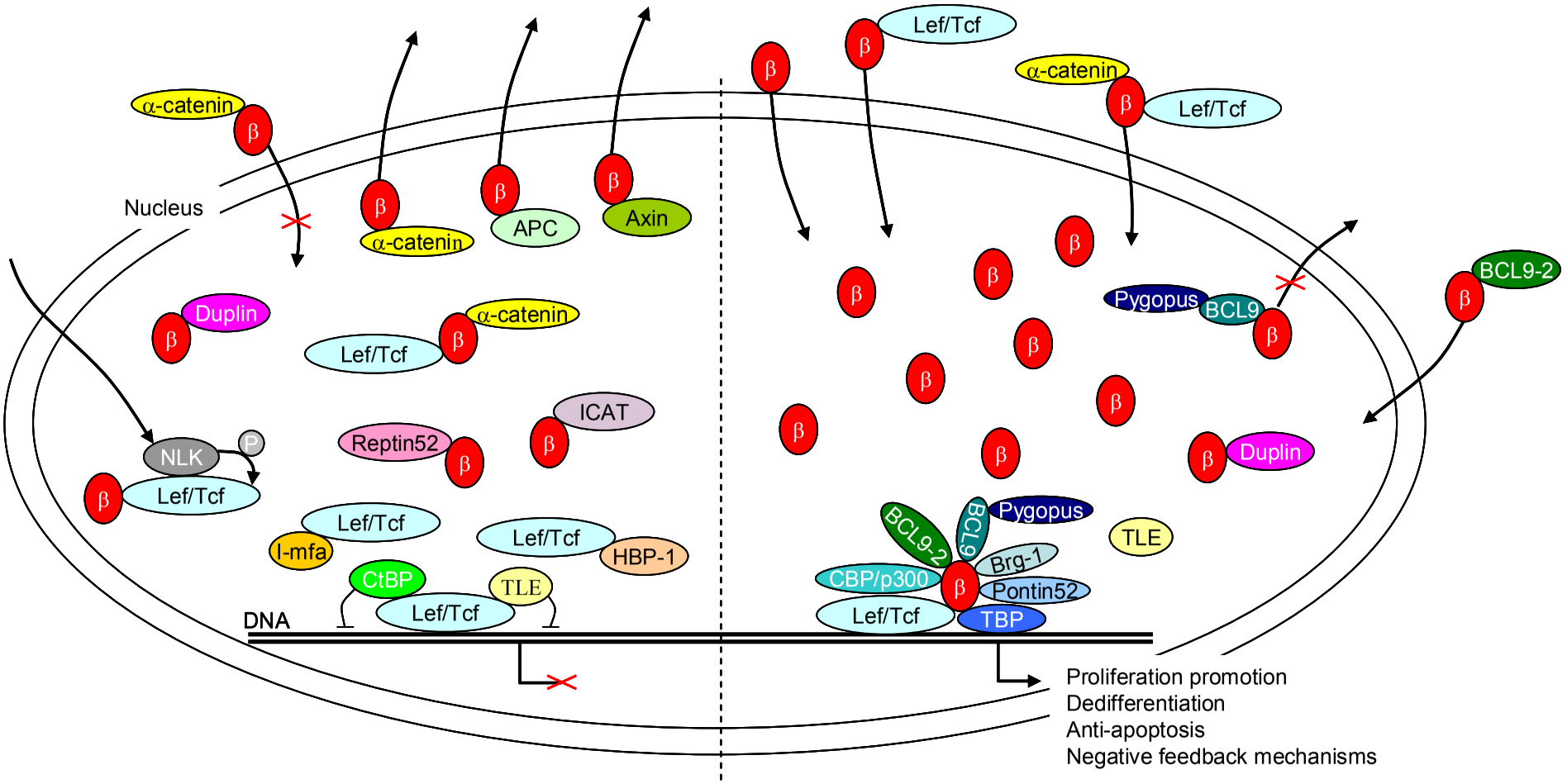


Figure 1.4: β-catenin activates transcription. β-catenin (β) enters the nucleus, either alone, or with Lef/Tcf or BCL9/pygopus or BCL9-2. α-catenin can inhibit lone β-catenin translocation. The Lef/Tcf complex is normally bound to the DNA and to the transcriptional repressors, TLE and CtBP. HBP-1 and I-mfa prevent Lef/Tcf-DNA association. β-catenin binds to Lef/Tcf, and together with TBP and Pontin 52, recruits transcriptional machinery to the promoter, activating transcription. β-catenin also removes TLE repression by interacting with CBP/p300 and Brg-1. BCL9/pygopus and BCL9-2 are essential transcriptional cofactors for β-catenin. β-catenin's transcriptional activities are repressed by α-catenin, ICAT, Duplin, Reptin52 and NLK-mediated phosphorylation (see text for details). α-catenin, APC and axin drive β-catenin from the nucleus. Note, "P" represents a phosphorylation.

1996; Molenaar *et al*., 1996). However, it has subsequently been shown to actively enter in an energy dependent manner by binding to the nuclear pores via its arm-repeats (van de Wetering *et al*., 1997; Fagotto *et al*., 1998; Yokoya *et al*., 1999). Alternatively, a nuclear factor BCL9-2 has been shown to associate with tyrosine phosphorylated β-catenin and contributes to its translocation and retention in the nucleus (Brembeck *et al*., 2004). The BCL9-2 homolog BCL9, in complex with the nuclear protein, pygopus has also been shown to mediate the nuclear retention of β-catenin (Townsley *et al*., 2004). α-catenin can inhibit nuclear entry of lone β-catenin, but it is insufficient for inhibiting β-catenin bound to Lef/Tcf (Giannini *et al.,* 2000a; Takahashi *et al.,* 2000).

In the nucleus, Lef/Tcf binds to DNA via a high mobility group (HMG)-box DNA binding domain, inducing a 130° bend in the DNA (Travis *et al*., 1991; Waterman *et al*., 1991; Giese *et al*., 1992; Behrens *et al.,* 1996). Lef/Tcf is unable to intrinsically activate transcription (Travis *et al*., 1991; Waterman *et al*., 1991; Carlsson *et al*., 1993; Giese and Grosschedl, 1993; Molenaar *et al*., 1996). Rather, it has been shown to repress transcription by associating with histone deacetylases (HDACs), either directly or indirectly via the corepressors Groucho/transducin-like enhancer protein (TLE) or C-terminal binding protein (CtBP), which modulates chromatin structure through deacetylation (Palaparti *et al*., 1997; Cavallo *et al*., 1998; Levanon *et al*., 1998; Roose *et al*., 1998; Brannon *et al*., 1999; Chen *et al*., 1999b; Billin *et al*., 2000). β-catenin contains DNA transactivation domains (TADs) in its carboxyl- and amino-termini, but has no DNA binding capacity of its own and requires an alliance with Lef/Tcf to activate transcription (van de Wetering *et al*., 1997; Hsu *et al*., 1998; Hecht *et al*., 1999). β-catenin interacts with the amino-terminus of Lef/Tcf via its arm-repeat domain (Behrens *et al*., 1996; Huber *et al*., 1996; Molenaar *et al*., 1996). This association reduces the DNA bend by 40°, inducing changes to the gene promoter structure and activates transcription (Behrens *et al.,* 1996; Tutter *et al*., 2001). β-catenin also relieves the transcriptional repression, imposed by the HDACs and Groucho/TLE or CtBP. It does so through its association with other nuclear factors such as the histone acetylatransferase CREB-binding protein (CBP)/p300

and a member of the ATP-dependent SWI/SNF complex, Brg-1, which change chromatin structure making it more accessible to the basal transcription machinery (Hecht *et al*., 2000; Miyagishi *et al*., 2000; Sun *et al*., 2000; Takemaru and Moon, 2000; Barker *et al*., 2001; Tutter *et al*., 2001). CBP/p300 can also enhance β-catenin's affinity for Tcf through acetylation (Lévy *et al*., 2004), and has been shown to act as a bridging or scaffold protein to the general transcriptional machinery (Miyagishi *et al*., 2000). β-catenin also associates with TATA-binding protein (TBP) and Pontin52, which also recruit basal transcription machinery to the promoter regions activating transcription (Bauer *et al*., 1998; Hecht *et al*., 1999; Bauer *et al.,* 2000). In addition, it has recently been shown that the nuclear association of β-catenin with BCL9-2 and the BCL9/pygopus complex plays an important role in the coactivation of β-catenin/Lef/Tcf-dependent transcription (Kramps *et al*., 2002; Brembeck *et al*., 2004; Hoffmans *et al*., 2005; Städeli and Basler, 2005).

There are a number of negative regulators of β-catenin/Lef/Tcf transcriptional activity. Inhibitor of β-catenin and Tcf (ICAT) binds to β-catenin and inhibits it from associating with both Tcf/Lef and CBP/p300, and possibly other transcriptional coactivators (Tago *et al*., 2000; Daniels and Weis, 2002; Gottardi and Gumbiner, 2004a). Reptin52 binds to β-catenin and represses its transactivational activity by an unknown mechanism, possibly by opposing Pontin52 (Behrens, 2000). Duplin, Chibby, APC, Sox 17 and 3, Kaiso and retinoic acid/vitamin D inhibit β-catenin-mediated transcription by competing with, and excluding, Lef/Tcf from binding to β-catenin (Easwaran *et al*., 1999; Zorn *et al*., 1999; Neufeld *et al*., 2000b; Sakamoto *et al.,* 2000; Takemaru *et al*., 2003; Park *et al*., 2005; Spring *et al*., 2005). The HMG-box repressor protein-1 (HBP-1) and I-mfa domain protein prevent Lef/Tcf from binding to target DNA sequences (Sampson *et al*., 2001; Snider *et al*., 2001), while activated nemo-like kinase (NLK) prevents the Lef/Tcf/β-catenin complex from associating with DNA through phosphorylation of Lef/Tcf (Ishitani *et al*., 1999; Ishitani *et al*,. 2003). α-catenin also interferes with the binding of the β-catenin/Lef/Tcf complex to DNA

and other transcription regulatory proteins (Giannini *et al.,* 2000b). α-catenin (Simcha *et al*., 1998), APC (Neufeld *et al*., 2000a; Neufeld *et al*., 2000b; Rosin-Arbesfeld *et al*., 2003) and axin (Cong and Varmus, 2004; Weichens *et al*., 2004) have all been shown to keep nuclear levels of β-catenin low by shuttling it from the nucleus.

The list of gene targets of the β-catenin/Lef/Tcf complex is ever increasing (Table 1.2). Importantly, the products of these genes have been implicated in tumourigenesis as they affect a wide range of cellular processes. These include cell cycle regulation (He *et al*., 1998; Pennica *et al*., 1998; He *et al*., 1999; Shtutman *et al*., 1999; Tetsu and McCormick, 1999; Koh *et al*., 2000), transcriptional control (Mann *et al.,* 1999), cellular adhesion (Mann *et al*., 1999; Jamora *et al*., 2003; Gavert and Ben-Ze'ev, 2007), cellular migration and invasion (Crawford *et al*., 1999; Mann *et al*., 1999; Marchenko *et al*., 2002; Gavert *et al*., 2007), growth signalling (Zhang *et al*., 2001a; Boon *et al*., 2002; Kim *et al*., 2002; Kratochwil *et al*., 2002; Shimokawa *et al*., 2003; Chamorro *et al*., 2005; Tan *et al*., 2005) and apoptosis (Yamada *et al.,* 2000; Zhang *et al*., 2001b; Longo *et al*., 2002; Kim *et al*., 2003). Although a few of these genes are downregulated, such as *E-cadherin* and *ZO-1* (Mann *et al*., 1999; Jamora *et al*., 2003; Gavert and Ben-Ze'ev, 2007), the majority of these genes are upregulated by β-catenin/Lef/Tcf (see references above). It should be noted that some of the β-catenin/Lef/Tcf target genes may serve as a regulatory mechanism for β-catenin signalling by acting as a negative feedback loop, such as *Axin2* (Jho *et al*., 2002; Leung *et al*., 2002; Lustig *et al*., 2002), *p53* (Damalas *et al*., 1999), *Tcf-1* (Roose *et al*., 1999) and *DKK1* (Chamorro *et al*., 2005).

1.7.2 Overexpression of β-catenin promotes cell survival

In order to prevent inappropriate cell growth, cells that lose their adhesive potential, such as between E-cadherin and β-catenin, usually undergo a special type of apoptosis termed anoikis (Steinhusen *et al.,* 2000; Vallorosi *et al.,* 2000; Matsubara and Ozawa, 2001). Under such circumstances β-catenin is cleaved by

Table 1.2: Gene Targets of the β-catenin/Lef/Tcf complex[2]

Function	Gene	↑/↓[3]	Reference
Cell cycle regulation	*c-myc*	↑	He *et al*., 1998
	cyclin D1	↑	Shtutman *et al*., 1999; Tetsu and McCormick, 1999
	Gastrin	↑	Koh *et al*., 2000
	peroxisome proliferator-activated receptor δ (PPARδ)	↑	He *et al*., 1999
	WISP	↑	Pennica *et al*., 1998
Transcriptional control	*c-jun*	↑	Mann *et al.,* 1999
	fra-1	↑	Mann *et al.,* 1999
Cellular adhesion	*E-cadherin*	↓	Jamora *et al*., 2003
	ZO-1	↓	Mann *et al*., 1999
	Slug	↑	Gavert and Ben-Ze'ev, 2007
Migration and invasion	*urokinase plasminogen activator receptor (uPAR)*	↑	Mann *et al*., 1999
	matrilysin/MMP-7	↑	Crawford *et al*., 1999
	MMP-26	↑	Marchenko *et al*., 2002
	ADAM10	↑	Gavert *et al*., 2007
Growth signalling	*EGFR*	↑	Tan *et al*., 2005
	Met	↑	Boon *et al*., 2002; Rasola *et al*., 2007
	VEGF	↑	Zhang *et al*., 2001a
	FGF4/18/20	↑	Kratochwil *et al*., 2002; Shimokawa *et al*., 2003; Chamorro *et al*., 2005
	BMP4	↑	Kim *et al*., 2002
Apoptotic avoidance	*human multidrug resistance 1 (MDR1)*	↑	Yamada *et al.,* 2000
	Survivin	↑	Zhang *et al*., 2001b; Kim *et al*., 2003
	COX-2	↑	Howe *et al*., 1999
	IGF-I/II	↑	Longo *et al*., 2002
Negative feedback	*Axin2*	↑	Jho *et al*., 2002; Leung *et al*., 2002; Lustig *et al*., 2002
	Tcf-1	↑	Roose *et al*., 1999
	p53	↑	Damalas *et al*., 1999
	DKK1	↑	Chamorro *et al*., 2005

[2] Note, a comprehensive list of β-catenin/Lef/Tcf gene targets may be found at www.standford.edu/~rnusse/pathways/targets.html.
[3] ↑ and ↓ denotes up- or downregulated gene expression, respectively

effector caspases-3 and -7 at both its amino- and carboxyl-termini, decreasing its transcriptional activity and its role in cell-cell adhesion (Steinhusen *et al.,* 2000; Ling *et al.,* 2001). However, overexpression of full-length cytoplasmic β-catenin has been shown to inhibit anoikis and together with Tcf-4, is able to prevent chemotherapy-induced apoptosis (Orford *et al.,* 1999; Chen *et al.,* 2001; Yang *et al*., 2006). Although there is no direct evidence yet, this may occur through transcriptional activation of the *MDR1* and *survivin* genes or possibly through its transcriptional activation of *COX-2*, which in turn increases the levels of the apoptotic inhibitor protein, Bcl-2 (Tsujii and DuBois, 1995; Howe *et al*., 1999; Yamada *et al*., 2000; Zhang *et al*., 2001b; Kim *et al*., 2003). However, the role of β-catenin in apoptosis is still controversial and appears to be dependent on cell type (van Gijn *et al.,* 2001).

1.8 Mutations can stabilize β-catenin in the cytoplasm

Regulation of β-catenin may be compromised by preventing the formation of the APC/GSK-3β/CKIα/axin degradation complex or by impeding it from binding to β-catenin (Ilyas *et al.,* 1997; see Figure 1.9 for synopsis). Mutations that result in truncated protein products of the degradation complex, or of β-catenin itself, have been implicated in the deregulation of cytoplasmic β-catenin (Ilyas *et al.,* 1997; Klymkowsky, 2005; Polakis, 2007).

A commonly reported mutation is that of the *APC* gene (on chromosome 5) that results in a truncated protein that lacks the carboxyl-terminus of the protein as well as most of the 20-amino acid repeat motifs, preventing β-catenin binding (Rubinfeld *et al*., 1993; Su *et al*., 1993; Munemitsu *et al*., 1995; Béroud and Soussi, 1996; Hinoi *et al*., 2000; Rowan *et al*., 2000; Shih *et al*., 2000). This type of mutation has been mainly associated with the early events that occur during the transformation of colorectal adenomas to carcinomas (Powell *et al*., 1992; Smith *et al*., 1993; Su *et al*., 1993; Powell *et al.,* 1994; Ilyas *et al.,* 1997; Korinek *et al*., 1997; Morin *et al*., 1997; Rowan *et al*., 2000; Shih *et al.,* 2000). This mutation has also been reported in a number of different tumours, as is summarised in Table 1.3.

Table 1.3: Mutations of APC, axin and β-catenin that result in deregulation of β-catenin in tumours

Gene	Type	Reference
APC	Colorectal	Powell *et al*., 1992; Smith *et al*., 1993; Su *et al*., 1993; Powell *et al.,* 1994; Ilyas *et al.,* 1997; Morin *et al*., 1997; Korinek *et al*., 1997; Rowan *et al*., 2000; Shih *et al.,* 2000
	Gastric	Horii *et al*., 1992a; Nakatsuru *et al*., 1992; Tamura *et al*., 1994
	Pancreatic	Horii *et al*., 1992b
	Oral SCC	Uzawa *et al*., 1994
	Hepatocellular	Hajra and Fearon, 2002
	Hepatoblastoma	Oda *et al*., 1996
	Thyroid	Laurent-Puig *et al*., 1998; Béroud and Soussi, 1996
	Ovarian	Laurent-Puig *et al*., 1998; Wu *et al*., 2001
	Breast	Abraham *et al*., 2002a; Hajra and Fearon, 2002
	Desmoid	Li *et al*., 1998b; Tejpar *et al*., 1999
Axin	Hepatocellular	Satoh *et al*., 2000
	Colorectal	Liu *et al*., 2000; Webster *et al*., 2000; Jin *et al*., 2003
	Ovarian	Wu *et al*., 2001
β-catenin	Colorectal	Ilyas *et al*., 1997; Morin *et al.,* 1997; Dashwood *et al.,* 1998; Sparks *et al*., 1998; Takahashi *et al*., 1998
	Hepatocellular	Miyoshi *et al*., 1998; de la Coste *et al*., 1998; Satoh *et al*., 2000
	Heptoblastomas	Anna *et al*., 2000; Koch *et al*., 1999
	Prostate	Voeller *et al*., 1998
	Medulloblastomas	Zurawel *et al*., 1998
	Endometrial and ovarian	Fukuchi *et al*., 1998; Palacios and Gamallo, 1998; Wu *et al*., 2001
	Thyroid	Garcia-Rostan *et al*., 1999
	Gastric	Park *et al*., 1999
	Pilomatricomas	Chan *et al*., 1999
	Melanomas	Rubinfeld *et al*., 1997
	Desmoid	Tejpar *et al*., 1999
	Pancreatic (non-ductal)	Tanaka *et al*., 2001; Abraham *et al*., 2002b; Abraham *et al*., 2002c

However, tumours, such as oesophageal SCC, do not possess mutated *APC* (and thus, loss of APC function) despite having a high frequency of loss of heterozygosity at the APC locus (Boynton *et al*., 1992; Powell *et al.,* 1994; Shibagaki *et al.,* 1994; Ogasawara *et al.,* 1996).

Mutations of the *APC* gene that result in changes to its axin binding domain have also been reported in colorectal tumours (Hinoi *et al.,* 2000; Rowan *et al*., 2000). This prevents association between the APC and axin proteins, decreasing the proficiency of the degradation complex (Hinoi *et al.,* 2000).

Loss-of-function mutations of the axin gene itself have been described in hepatocellular carcinomas and in colorectal and ovarian cancers (Liu *et al*., 2000; Satoh *et al*., 2000; Wu *et al*., 2001; Jin *et al*., 2003). These mutations prevent the formation of the degradation complex, by interfering with the binding domains for APC (RGS), GSK-3β and β-catenin and also affect association of axin with FRAT-1 and Dvl (Liu *et al*., 2000; Satoh *et al*., 2000; Webster *et al*., 2000; Wu *et al*., 2001; Jin *et al*., 2003; Polakis, 2007). In addition, loss of heterozygosity has been reported in hepatocellular carcinoma, colorectal cancer, breast cancer, neuroblastoma and other tumours (Liu *et al*., 2000; Satoh *et al*., 2000; Dong *et al*., 2001).

Somatic deletions of the third exon of the β-catenin gene, *CTNNB1*, have also been associated with deregulation of its product (Ilyas *et al.,* 1997; Miyoshi *et al.,* 1998; Palacios and Gamallo, 1998; Sparks *et al*., 1998; Zurawel *et al*., 1998). These mutations occur at the serine/threonine residues of codons 33, 37, 41 and 45, which are the GSK-3β phosphorylation target sites (Ilyas *et al.,* 1997; Morin *et al.,* 1997; Miyoshi *et al.,* 1998; Palacios and Gamallo, 1998; Sparks *et al*., 1998; Zurawel *et al*., 1998). Such mutations have been found to act as alternatives to *APC* mutations in colorectal carcinomas (Ilyas *et al*., 1997; Morin *et al.,* 1997; Dashwood *et al.,* 1998; Sparks *et al*., 1998; Takahashi *et al*., 1998). They have also been found in a large number of other cancers, as is detailed in Table 1.3. However, mutations of exon 3 of *CTNNB1* have not been found in oesophageal SCC (Sanders *et al*., 1998; Ninomiya *et al.,* 2000; De Castro *et al*., 2000; Zhang *et al.,* 2005).

1.9 Biochemical signal transduction pathways can lead to aberrant functioning of β-catenin within the cell

From the above, it can be seen that in normal adult epithelial cells the primary function of β-catenin is in cell adhesion, while the levels of unbound cytoplasmic β-catenin are kept low by the APC/GSK-3β/axin degradation complex. However, this normal state is disrupted in a number of tumours, by mutations or abnormal levels of expression, allowing for an increase in invasion and metastasis, as well as aberrant gene expression. A number of biochemical signal transduction pathways have also been associated with the regulation or deregulation of β-catenin functioning, and thus possibly play a role in tumourigenesis. These signalling pathways include Wnt, integrin-linked kinase (ILK), small GTPases, various non-receptor type tyrosine kinases and growth factor signals, which are detailed in the following sections.

1.9.1 The developmental growth-signalling pathway, Wnt, stabilizes cytoplasmic β-catenin

The most investigated pathway affecting β-catenin is that of the Wnt signalling pathway. This pathway was originally identified as a developmental signalling pathway, having a role in body axis formation in *Xenopus laevis* embryos, and is homologous to Wingless in *Drosophila melanogaster*, which plays a role in segment polarity along the antero-posterior axis of embryos (Sokol *et al*., 1991; McCrea *et al*., 1993; Funayama *et al*., 1995; Montross *et al*., 2000; also see reviews Nusse and Varmus, 1992; Miller and Moon, 1996; Cadigan and Nusse, 1997; Wodarz and Nusse, 1998). This pathway has been shown to regulate a wide range of effects, including cellular proliferation, migration, polarity, apoptosis, and differentiation (reviewed in Cadigan and Nusse, 1997; Peifer and Polakis, 2000; Longo *et al*. 2002). Wnt signalling is initiated by wnt proteins which are a large family of secreted, cysteine-rich glycoproteins (Nusse and Varmus, 1992; Smolich *et al*., 1993; Bradley and Brown, 1995; Cadigan and Nusse, 1997; Miller, 2001; Moon *et al*., 2004; Weeraratna, 2005; Mikels and Nusse, 2006). These proteins act

as short-range ligands and are spatially and temporally expressed in embryos and adults (Moon *et al*., 2004; Weeraratna, 2005; Mikels and Nusse, 2006). It has recently been discovered that the wnts mediate these effects via distinct pathways, the canonical and non-canonical pathways. The canonical pathway involves the posttranslational stabilization of β-catenin and is the most well understood (Moon *et al*., 2004; Weeraratna, 2005). The non-canonical pathways, or β-catenin-independent pathways, appears to regulate cell movements during gastrulation in a similar manner to the planar cell polarity pathway in *Drosophila* (Du *et al*., 1995; Moriguchi *et al*., 1999; Peifer and Polakis, 2000; Yamanaka *et al*., 2002; Veeman *et al*., 2003; Moon *et al*., 2004; Weeraratna, 2005). The details behind the non-canonical pathway are not pertinent to the present study and thus will not be pursued any further (reviewed in Peifer and Polakis, 2000; Veeman *et al*., 2003; Nelson and Nusse, 2004; Weeraratna, 2005).

In canonical Wnt signalling, wnt ligands bind to, and activate, members of the seven-transmembrane span, G-protein-coupled Frizzled (Frz) receptor family (Bhanot *et al*., 1996; Wang *et al*., 1996b; Slusarski *et al*., 1997; Mikels and Nusse, 2006). Wnt-1, -2, -3, -3a and -8 have been shown to mediate canonical Wnt signalling (Shimizu *et al*., 1997; Behrens and Lustig, 2004). Frz requires the presence of a single-pass transmembrane coreceptor protein, LDRD-related protein-5/6 (LRP-5/6), to mediate its signal, although the precise mechanism is unclear (Pinson *et al*., 2000; Tamai *et al*., 2000; Wehrli *et al*., 2000; Mao *et al*., 2001a; He *et al*., 2004). The Wnt signal is regulated by various proteins that associate with the wnt ligands and prevent association with Frz as can be seen in Figure 1.5 (Finch *et al*., 1997; Leyns *et al*., 1997; Wang *et al*., 1997; Miller, 2001). These include: Cerberus, Wnt-interacting factor-1 (WIF-1), and the secreted Frizzled-related proteins (sFRPs) (Finch *et al*., 1997; Glinka *et al*., 1997; Leyns *et al*., 1997; Lin *et al*., 1997; Mayr *et al*., 1997; Wang *et al*., 1997; Dennis *et al*., 1999; Hsieh *et al*., 1999; Piccolo *et al*., 1999; Miller, 2001). Members of the Dickkopf (DKK) family of regulatory proteins, in association with their receptors (Kremen1/2), bind to LRP-5/6, preventing wnt from binding to it and encourage endocytosis of the coreceptor (Glinka *et al*., 1998; Mao *et al*., 2001a; Mao *et al*., 2002; Niehrs, 2006). Wnt signalling only occurs when the wnt ligands reach a

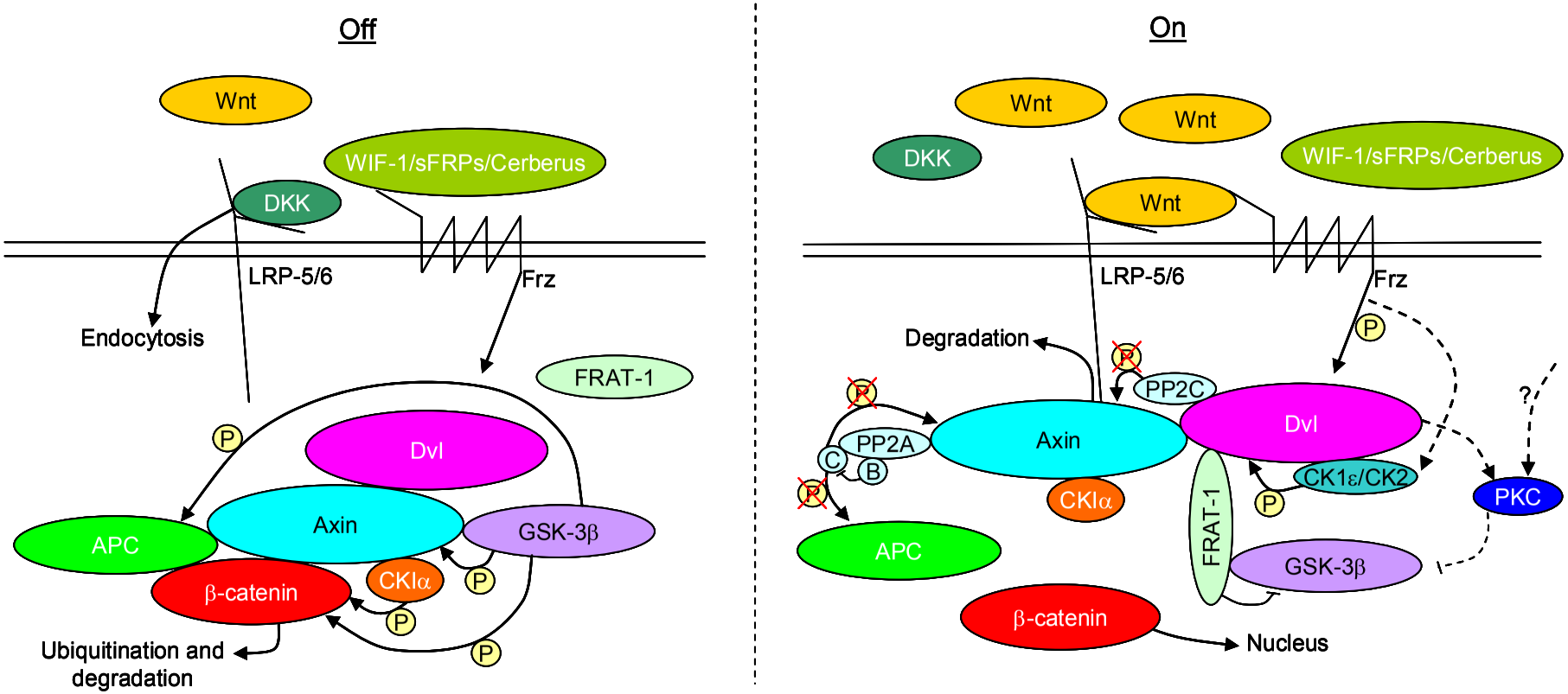


Figure 1.5: The Wnt signal transduction pathway. In the "off" state, wnts are prevented from binding to the receptor Frizzled (Frz) by various inhibitory proteins. Dickkopf (DKK) prevents wnts from binding to the coreceptor LRP-5/6 and encourages its endocytosis. The degradation complex (axin, APC, CKIα and GSK-3β) is active leading to β-catenin degradation. Dishevelled (Dvl) is also associated with this complex. The inhibitory proteins are removed from Frz and LRP-5/6 once the wnt ligands reach a particular threshold concentration. Upon binding, the wnts activate Frz, which recruits and phosphorylates Dishevelled (Dvl). Phosphorylated Dvl binds to FRAT-1, which competes with axin for GSK-3β binding and results in the dissociation of the β-catenin degradation complex. Casein kinase Iε (CKIε) and casein kinase 2 (CK2) also phosphorylate Dvl, encouraging it to bind to FRAT-1. Protein kinase C (PKC) phosphorylates and inhibits GSK-3β, but it is unknown whether it is activated by Dvl or by a parallel pathway. Protein phosphatase 2C (PP2C) and PP2A both play a role in inhibiting β-catenin degradation. This allows for stabilisation of β-catenin which can translocate into the nucleus. Note, "P" represents a phosphorylation, "P" crossed out, denotes a dephosphorylation event.

particular threshold concentration that exceeds the buffering capacity of these repressor proteins (Moon *et al*., 2004).

Upon activation, the Frz receptor requires heterotrimeric G-proteins to transduce its signal (Slusarski *et al*., 1997). Different G-proteins are used depending on the combination of which wnt binds to which Frz receptor. For example, Gαo and Gαq subunits have been shown to be important in the canonical Wnt pathway (Liu *et al*., 1999b; Liu *et al*., 2001b; Malbon *et al*., 2001). These G-proteins then activate a phosphoprotein, Dishevelled (Dvl), which is recruited to the plasma membrane (Liu *et al*., 2001b; Weeraratna, 2005).

Although parts of the pathway have been revealed, the exact sequence of events is still vague and remains to be elucidated. Axin appears to play an important role in the cascade that follows. It is known that axin is recruited to the plasma membrane where it associates with both Dvl and LRP-5/6. It was not clear whether LRP-5/6 recruits axin enabling Dvl binding, or whether Dvl recruits axin to LRP-5/6 (Mao *et al*., 2001b; Nusse, 2001; Cliffe *et al*., 2003; He *et al*., 2004; Tamai *et al*., 2004; Tolwinski and Wieschaus, 2004; Bilić *et al*., 2007; Schwarz-Romond *et al*., 2007). However, it has recently been shown that Wnt-activated Dvl may act as a platform inducing the clustering of LRP-5/6. This increase in the local concentration of LRP-5/6 triggers its phosphorylation by casein kinase 1γ (CK1γ), which leads to the recruitment of axin (Bilić *et al*., 2007). LRP-5/6 binding is thought to target axin for degradation, thus decreasing β-catenin degradation (Mao *et al*., 2001b; Tolwinski and Wieschaus, 2004). Dvl is able to bind directly to axin, and in the absence of Wnt signalling does not appear to affect the stability of the β-catenin degradation complex (Kishida *et al*., 1999; Li *et al*., 1999a; Smalley *et al*., 1999; Julius *et al*., 2000; Schwarz-Romond *et al*., 2007). However, upon hyperphosphorylation by Frz, Dvl results in a decrease of GSK-3β-mediated phosphorylation of axin (Jho *et al*., 1999; Kishida *et al*., 1999; Li *et al*., 1999a; Willert *et al*., 1999; Yamamoto *et al*., 1999). This reduces axin's stability and its affinity for β-catenin, effectively stabilizing β-catenin (Jho *et al*., 1999; Willert *et al*., 1999; Yamamoto *et al*., 1999; Schwarz-Romond *et al*., 2007). How Dvl mediates this is uncertain. It does not directly compete with GSK-3β for axin binding sites (Kishida *et al*., 1999; Smalley

et al., 1999), nor does it inhibit the catalytic activity of GSK-3β (Yamamoto *et al*., 1999). Instead it is thought that hyperphosphorylated Dvl recruits FRAT-1 (frequently rearranged in advanced T cell lymphoma-1), which induces structural changes to the degradation complex, resulting in its dissociation (Li *et al*., 1999a; Yamamoto *et al*., 1999). FRAT-1 antagonises GSK-3β activity by competing with axin for GSK-3β binding, resulting in the dissociation of GSK-3β from the degradation complex (Yost *et al*., 1998; Li *et al*., 1999a; Thomas *et al*., 1999; Farr *et al*., 2000; Dajani *et al*., 2003).

Wnt signalling activates a serine/threonine kinase, casein kinase Iε (CKIε) (Swiatek *et al*., 2004), which has been shown to be essential in Wnt-mediated stabilization of β-catenin (Peters *et al*., 1999; Sakanaka *et al*., 1999; Kishida *et al*., 2001; Gao *et al*., 2002; Cong *et al*., 2004). This kinase binds to and further phosphorylates Dvl, increasing its affinity for FRAT-1 (Peters *et al*., 1999; Sakanaka *et al*., 1999; Hino *et al*., 2003; Schwarz-Romond *et al*., 2007). Casein kinase 2 (CK2) has also been implicated in positively regulating Wnt signalling by associating with and phosphorylating Dvl and β-catenin (Willert *et al*., 1997; Song *et al*., 2000; Song *et al*., 2003).

A serine/threonine protein phosphatase, PP2C, is also involved in the Wnt signalling pathway (Strovel *et al.,* 2000). PP2C exists in a complex with Dvl, β-catenin and axin and dephosphorylates axin, leading to the stabilization of β-catenin (Strovel *et al.,* 2000). Another serine/threonine protein phosphatase PP2A, has also been implicated in Wnt signalling, although its role appears to be ambiguous. PP2A is a heterotrimer consisting of a structural A-subunit, a catalytic C-subunit and variable regulatory B-subunits (Wera and Hemmings, 1995; Salahshor and Woodgett, 2005). PP2A can play either a positive role in Wnt signalling or a negative role, depending on which B-subunit it is associated with. The B56 subunit has been shown to associate with APC and axin and downregulate β-catenin levels and its transcriptional activity, thus acting in a negative capacity (Seeling *et al*., 1999; Ratcliffe *et al*., 2000; Li *et al*., 2001a). However, other B-subunits do not show this, and in these cases the catalytic C-subunit associates with axin (Hsu *et al*., 1999; Li *et al*., 2001a). This results in dephosphorylation of both axin and APC

reducing their affinity for β-catenin, resulting in increased β-catenin stability and transcription (Hsu *et al*., 1999; Willert *et al*., 1999; Ikeda *et al*., 2000). However, there is also evidence that the positive role played by PP2A in Wnt signalling occurs downstream of β-catenin stabilization by activating Lef/Tcf (Ratcliffe *et al*., 2000; Li *et al*., 2001a).

Certain diacetylglycerol-dependent, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-sensitive isoforms of the serine/threonine protein kinase C (PKC) have been shown to phosphorylate and inhibit GSK-3β (Goode *et al*., 1992, Murray *et al*., 1999), and have been implicated in Wnt-induced stabilization of β-catenin (Cook *et al*., 1996; Chen *et al*., 2000). Although not sufficient to mediate Wnt-signalling alone, PKC does enhance canonical Wnt signalling, leading to the maximal accumulation of stabilized β-catenin (Chen *et al*., 2000). However, the exact position of PKC in the canonical Wnt pathway remains uncertain, and was initially believed to lie either downstream of Dvl or parallel to it (Cook *et al.,* 1996).

The stabilizing effect of Wnt signalling has been implicated in tumourigenesis. The wnt-1 ligand was originally identified as a protooncogene, with the ability to induce mammary hyperplasia and adenocarcinomas in mice and to transform mouse mammary epithelial cells (Brown *et al*., 1986; Tsukamoto *et al*., 1988; Jue *et al*., 1992; Nusse and Varmus, 1992; Wong *et al*., 1994; Bradley and Brown, 1995). The wnt ligands have been classified according to their ability to induce transformation, with the ligands involved in canonical Wnt signalling showing the highest transformation potential (Wong *et al*., 1994; Shimizu *et al*., 1997). Some of these highly transforming ligands have been shown to be upregulated in some cancers. For example, wnt-2 has been shown to be upregulated in oesophageal, gastric and colorectal carcinomas *in vivo* (Vider *et al*., 1996; Smith *et al*., 1999). Interestingly, this is not shown *in vitro*, suggesting that the stromal cells surrounding the tumour provide these ligands (Smith *et al*., 1999). However, it is not known whether upregulation of wnts play a fundamental role in tumourigenesis (Miller *et al*., 1999; Seidensticker and Behrens, 2000; Behrens and Lustig, 2004). It is more likely that alterations of downstream components of the Wnt pathway lead to the oncogenic stabilization of β-catenin (Seidensticker and Behrens, 2000; Polakis, 2000; Behrens

and Lustig, 2004). Alterations to the components of the β-catenin degradation complex have been shown to be most important in this respect (see above, section 1.8). Other components implicated in cancer development include a novel Frizzled receptor (FzE3), which leads to increased nuclear accumulation of β-catenin, and has been shown to be expressed in oesophageal squamous cell carcinomas (Tanaka *et al*., 1998). Overexpression or mutations of the LRP-6 coreceptor can result in an increase of stabilized β-catenin that is independent of wnt ligand stimulation (Brennan *et al*., 2004). An early event in colorectal cancer development is the inactivation of sFRP genes (Suzuki *et al*., 2002; Suzuki *et al*., 2004; Taketo, 2004; Polakis, 2007). Dvl has been shown to be overexpressed in non-small-cell lung cancers (Uematsu *et al*., 2003), while certain leukemias, gastric, pancreatic, oesophageal, cervical and breast cancers have been shown to overexpress FRAT-1and -2 proteins (Saitoh and Katoh, 2001; Saitoh *et al*., 2001; Saitoh *et al*., 2002).

1.9.2 A serine/threonine kinase, ILK, inhibits GSK-3β and affects the expression of E-cadherin

The function of β-catenin is also affected by a signal transduction pathway involving the serine/threonine kinase, integrin-linked kinase (ILK). ILK is a multidomain focal adhesion protein that associates with both the β1- and β3-subunits of the integrins (Hannigan *et al*., 1996). ILK is activated in response to cell-ECM interactions or insulin stimulation mediated by phosphatidylinositol-3-kinase (PI3K), which involves the binding of its lipid product, phosphatidylinositol-(3,4,5)-triphosphate, to ILK (Delcommenne *et al*., 1998; Somasiri *et al*., 2001). The activities of ILK are regulated by two phosphatases, ILKAP (ILK1-associated phosphatase), which is a protein phosphatase 2C (Leung-Hagesteijn *et al*., 2001), and PTEN (phosphatase and tensin homolog protein), which dephosphorylates active phosphatidylinositol-(3,4,5)-triphosphate to inactive phosphatidylinositol-(4,5)-biphosphate (Morimoto *et al*., 2000; Persad *et al.,* 2000; Persad *et al*., 2001b). ILK induces an inactivating phosphorylation of GSK-3β, either directly or indirectly by activating protein kinase B (PKB;

Delcommenne *et al*., 1998; Lynch *et al*., 1999; Persad *et al*., 2000; Persad *et al*., 2001a; Persad *et al*., 2001b; Tan *et al*., 2001). Importantly, intestinal and mammary epithelial cells that overexpress ILK display increased nuclear β-catenin levels, together with an increase of stable Lef expression, and thus elevated β-catenin/Lef-mediated transcriptional activity (Novak *et al*., 1998; Somasiri *et al*., 2001; Tan *et al*., 2001). Novak *et al*. (1998) showed that this occurred without a concomitant increase of cytoplasmic β-catenin. However, Tan *et al*. (2001) subsequently demonstrated that inhibition of ILK accelerated the degradation of β-catenin, suggesting ILK inhibition of GSK-3β does affect its ability to phosphorylate β-catenin (also see Persad *et al*., 2001b). In addition, the overexpression of ILK reduces cell-cell adhesion, and thus increases the invasive phenotype, by decreasing E-cadherin levels, possibly by elevating the levels of the transcriptional repressor protein, Snail (Hannigan *et al*., 1996; Novak *et al*., 1998; Wu *et al*., 1998; Somasiri *et al*., 2001; Tan *et al*., 2001). Following these results, overexpression of ILK has been noted in a number of cancers including Ewings sarcoma (Chung *et al*., 1998), primitive neuroectodermal tumours (Chung *et al*., 1998), medulloblastomas (Chung *et al*., 1998), prostate carcinomas (Persad *et al.,* 2000; Graff *et al*., 2001), colorectal carcinomas and other colonic cancers (Marotta *et al*., 2001; Tan *et al.,* 2001).

1.9.3 Small GTPases, Cdc42 and Rac1, regulate IQGAP1 inhibition of cadherin/catenin-mediated adhesion

The Rho family of small GTPases, which include RhoA, Rac1 and Cdc42, play a role in the regulation of actin cytoskeletal dynamics and cell motility, as well as in cell-cell adhesion (Braga *et al*., 1997; Kuroda *et al*., 1997; Takaishi *et al*., 1997; Lambert *et al*., 2002; also reviewed in Van Aelst and D'Souza-Schorey, 1997; Kaibuchi *et al*., 1999; Braga, 2000; Schmitz *et al*., 2000; Etienne-Manneville and Hall, 2002). RhoA is required in the formation of new cadherin-based adhesions. However, it appears to do so indirectly, possibly through effects on the actin cytoskeleton (Braga *et al*., 1997; Takaishi *et al*., 1997; Fukata *et al*., 1999). Rac1 and Cdc42 play a direct role in regulating the cadherin/catenin complex (Braga *et*

al., 1997; Takaishi *et al*., 1997; Kuroda *et al*., 1997; Kuroda *et al*., 1998; Fukata *et al*., 1999; Kodama *et al*., 1999; Lambert *et al*., 2002). They do so by regulating the interaction of one of their effector proteins, a RasGAP-related scaffold protein, IQGAP1 (Kuroda *et al*., 1996; McCallum *et al*., 1996; Kuroda *et al*., 1998).

IQGAP1 binds directly to both the cytoplasmic tail of E-cadherin and to β-catenin (Kuroda *et al*., 1998; Fukata *et al*., 1999). It destabilizes the adhesion complex when overexpressed by actively competing with α-catenin for β-catenin binding and results in the dissociation of α-catenin, and consequently actin, from the cadherin/catenin complex (Kuroda *et al*., 1998; Fukata *et al*., 1999; Li *et al*., 1999b). The active guanine triphosphate (GTP)-bound Rac1 and Cdc42 (but not the inactive guanine diphosphate (GDP)-form), binds to IQGAP1, preventing it from associating with β-catenin (Kuroda *et al*., 1998; Fukata *et al.,* 1999). This stabilizes the cadherin/catenin complex (Fukata *et al.,* 1999; Noritake *et al*., 2005). It may also free IQGAP1 to perform other functions such as linking the actin and microtubule networks, through its association with APC to control cell polarisation and migration (Fukata *et al*., 2002; Gundersen, 2002; Watanabe *et al*., 2004; Hanson and Miller, 2005; Noritake *et al*., 2005; Akiyama and Kawasaki, 2006).

Rac1 and Cdc42 have been shown to be activated through PI3K and EGFR signals that are stimulated upon homophilic E-cadherin contacts (Pece *et al*, 1999; Kim *et al*., 2000; Pece and Gutkind, 2000; Nakagawa *et al*., 2001; Noren *et al*., 2001; Betson *et al*., 2002; Kovacs *et al*., 2002; Lampugnani *et al*., 2002). These may activate the guanine exchange factor, Tiam1, which colocalizes with E-cadherin (Hordijk and ten Klooster, 1997; Sander *et al*., 1998; Lampugnani *et al*., 2002). p120-catenin may also play a role in regulating the activation of Cdc42 and Rac1, and thus IQGAP1, through its association with the guanine exchange factor, Vav2 (Noren *et al*., 2000; Xiao *et al*., 2007). Another molecule, calmodulin, which is a mediator of calcium-dependent signalling, has been shown to inhibit IQGAP1's activity at the plasma membrane by competing with E-cadherin for IQGAP1 binding (Li *et al.,* 1999b).

The role of the small GTPases and IQGAP1 in tumour development is only just emerging. Oncogenic Ras signals have been shown to downregulate Rac1 and Tiam1 activity in MDCK cells (Zondag *et al*., 2000b). The redistribution of IQGAP1 from the cytoplasm to the plasma membrane has been associated with negative regulation of E-cadherin functioning in gastric tumours, further advancing the progression of this type of tumour (Takemoto *et al.,* 2001). In addition, IQGAP1 is overexpressed in highly metastatic melanoma cells (Clark *et al*., 2000) and at the invasion front of advanced human colorectal carcinomas (Nabeshima *et al.,* 2002). Its overexpression has been noted to increase cell migration and invasion in several cell types, however, this effect appears to be independent of its role in cell adhesion (Mataraza *et al*., 2003).

1.9.4 The importance of posttranslational modifications by phosphorylation on β-catenin functioning

Cellular adhesion and the regulation of free β-catenin are affected by posttranslational phosphorylation events (Hoschuetzky *et al*., 1994; Kinch *et al*., 1995; Lickert *et al*., 2000). The introduction of a negative phosphate group affects the associations of β-catenin with its various binding partners (Roura *et al*., 1999; Lickert *et al*., 2000). This can work in favour of an interaction or against it. For example, serine phosphorylation of E-cadherin in the β-catenin binding domain by casein kinase II (CKII) and GSK-3β, enhances the E-cadherin/β-catenin association as it makes E-cadherin more negatively charged and thus more attractive to β-catenin (Lickert *et al*., 2000). However, the introduction of a negative charge, by phosphorylation, to the central groove of β-catenin can attenuate the association by either reducing the number of ion pairs or by partially closing the groove (Roura *et al*., 1999). Similarly, as mentioned earlier (section 1.6.1), phosphorylation of APC and axin by GSK-3β increases their affinity for β-catenin (Rubinfeld *et al*., 1996; Ikeda *et al*., 1998; Fagotto *et al*., 1999; Jho *et al*., 1999; Willert *et al*., 1999; Yamamoto *et al.,* 1999), while phosphorylation of β-catenin may reduce its affinity for APC (Shibata *et al*., 1994).

Tyrosine phosphorylation in particular has been shown to be of great importance in regulating β-catenin's interactions. This was first shown in cells transfected with the *v-Src* oncogene which resulted in an increase of tyrosine phosphorylated β-catenin (Matsuyoshi *et al*., 1992; Behrens *et al*., 1993; Hamaguchi *et al*., 1993). β-catenin dissociated from E-cadherin as a consequence of this, decreasing cell-to-cell adhesion and increasing the invasive and metastatic abilities of these cells (Tsukita *et al*., 1991; Volberg *et al*., 1991; Matsuyoshi *et al*., 1992; Behrens *et al*., 1993; Hamaguchi *et al*., 1993; Roura *et al*., 1999; Hu *et al*., 2001). It was subsequently demonstrated that phosphorylation of Tyrosine 654 (Y654), which falls into the twelfth armadillo repeat of β-catenin, was important in regulating the association between β-catenin and E-cadherin (Roura *et al*., 1999). This tyrosine phosphorylation confers a conformational change to β-catenin enabling it to more readily associate with the TATA-binding protein (TBP) in the nucleus (Piedra *et al*., 2001), thus increasing its transcriptional activities. Increased tyrosine phosphorylation of β-catenin has been noted in colorectal (Takayama *et al*., 1998), breast (Sommers *et al*., 1994; Hazan and Norton, 1998), gastric (Shibata *et al*., 1996) and lung cancers (Nishimura *et al*., 1996).

Src kinase activity has also been shown to indirectly affect the association of the cadherin/catenin complex with the actin cytoskeleton via two non-receptor tyrosine kinases, Fer and Fyn. These kinases constitutively associate with p120-catenin (Kim and Wong, 1995), which also interacts with the active Src kinase Yes (Piedra *et al*., 2003). Yes activates Fer and Fyn, which phosphorylate β-catenin at Y142, reducing its affinity for α-catenin (Ozawa and Kemler, 1998a; Rosato *et al*., 1998; Piedra *et al*., 2003). Thus, as p120-catenin interacts with E-cadherin, it may play an essential role in regulating adhesion by facilitating this interaction between these tyrosine kinases and components of the E-cadherin/catenin complex (Piedra *et al*., 2003).

Similar to Src, cells transfected with activated Ras have also been shown to affect β-catenin's interactions. Kinch *et al*. (1995) demonstrated that Ras-transformed

breast epithelia displayed reduced intercellular adhesion by destabilizing the E-cadherin/β-catenin association by elevating tyrosine phosphorylation of β-catenin. This was similarly shown in intestinal cells as well as epidermal keratinocytes (Novak *et al*., 1998; Espada *et al*., 1999). Ras activation also stabilizes free β-catenin via PI3K, by inhibiting the interaction between APC and β-catenin and thus preventing serine phosphorylation of β-catenin (Espada *et al*., 1999). *In vivo*, Ras may lie downstream of other tyrosine kinases, such as growth factors (Kinch *et al*., 1995), which will be discussed in the next section.

The studies on the effect of tyrosine phosphorylation on E-cadherin-mediated cell-cell adhesion have also shown that treatment with inhibitors to protein tyrosine phosphatases (PTPs), such as sodium orthovanadate or peroxovanadate, resulted in increased tyrosine phosphorylation of β-catenin (Tsukita *et al*., 1991; Volberg *et al*., 1991; Matsuyoshi *et al*., 1992; Ozawa and Kemler, 1998a; Soler *et al*., 1998; Müller *et al*., 1999; Hu *et al*., 2001). This demonstrated that PTPs counteract the negative regulation of cell adhesion by the tyrosine kinases, thus stabilizing the adherens junctions (Tsukita *et al*., 1991; Volberg *et al*., 1991; Matsuyoshi *et al*., 1992; Brady-Kalnay *et al*., 1995; Ozawa and Kemler, 1998a; Soler *et al*., 1998; Hu *et al*., 2001). A number of these PTPs have been identified to associate with the cadherin/catenin complex and act in distinct contexts (Brady-Kalnay *et al*., 1995; Wadham *et al*., 2003; Lilien and Balsamo, 2005). Among the first to be identified was receptor-PTPμ which associates directly with E-cadherin or p120-catenin (depending on the cell type) and modulates the phosphotyrosine content of the cadherin/catenin complex members (Brady-Kalnay *et al*., 1995; Brady-Kalnay *et al*., 1998; Zondag *et al*., 2000a). Other receptor PTPs that have been shown to associate with β-catenin and dephosphorylate it in various cell systems include: PTPκ (Fuchs *et al*., 1996), PTPλ (Wang *et al*., 1996a; Cheng *et al*., 1997; Yan *et al*., 2002), PTPβ/ζ (Meng *et al*., 2000), DEP1 (Holsinger *et al*., 2002) and PTP-LAR (Kypta *et al*., 1996; Müller *et al*., 1999). Non-receptor PTPs have also been shown to play a role in the regulation of the AJ, the most well understood being PTP1B. PTP1B binds to cadherin and dephosphorylates β-catenin at Y654 (Balsamo *et al*., 1996; Balsamo *et al*., 1998; Xu *et al*., 2002; Xu

et al., 2004). In order to achieve this, PTP1B itself is required to be tyrosine phosphorylated to be activated (Balsamo *et al*., 1996; Balsamo *et al*., 1998; Rhee *et al*., 2001; Xu *et al*., 2004). Interestingly, the Fer tyrosine kinase mediates this, suggesting that Fer may act in both a positive and a negative manner in cell adhesion (Xu *et al*., 2004). Other non-receptor PTPs involved in regulating the tyrosine phosphorylation levels of β-catenin include: PTP-PEZ (Wadham *et al*., 2003), LMW-PTP (Taddei *et al*., 2002), SHP-1 (Duchesne *et al*., 2003) and SHP-2 (Ukropec *et al*., 2000).

1.9.5 The effect of growth factors on cell-cell adhesion and the stability of cytoplasmic β-catenin

Growth factors have long been known to play an important role in cancer development and affect many aspects of the cell's functions. Among these is the regulation of cell-cell adhesion, as well as that of cytoplasmic β-catenin levels (Fukuyama and Shimizu, 1991; Hoschuetzky *et al*., 1994; Shibamoto *et al*., 1994; Eldar-Finkelman *et al*., 1995; Kinch *et al*., 1995; Shiozaki *et al*., 1995; Papkoff and Aikawa, 1998; Müller *et al*., 1999; Playford *et al*., 2000; Xu *et al*., 2003; Vogelmann *et al*., 2005). The growth factors that have been shown to affect β-catenin's functioning are insulin-like growth factor (IGF); fibroblast growth factor (FGF); hepatocyte growth factor (HGF); and epidermal growth factor (EGF) and the transforming growth factor (TGF)-β1.

Insulin-like growth factor (IGF) receptor activity induces tyrosine phosphorylation of β-catenin (see Figure 1.6.a), leading to its dissociation from the E-cadherin complex, thus increasing cell migration, and its relocation to the cytoplasm in colorectal cancer cell lines (Playford *et al.,* 2000). IGF-I and IGF-II have also been shown to enhance the stability of β-catenin by activating PKB via the ras gene product (Playford *et al.,* 2000; Morali *et al*., 2001). This was sufficient to induce β-catenin's transcriptional activity in hepatoma cells (Desbois-Mouthon *et al*., 2001) but not in human embryonic kidney cells (Playford *et al.,* 2000), and thus the effect of IGF appears to be cell-type

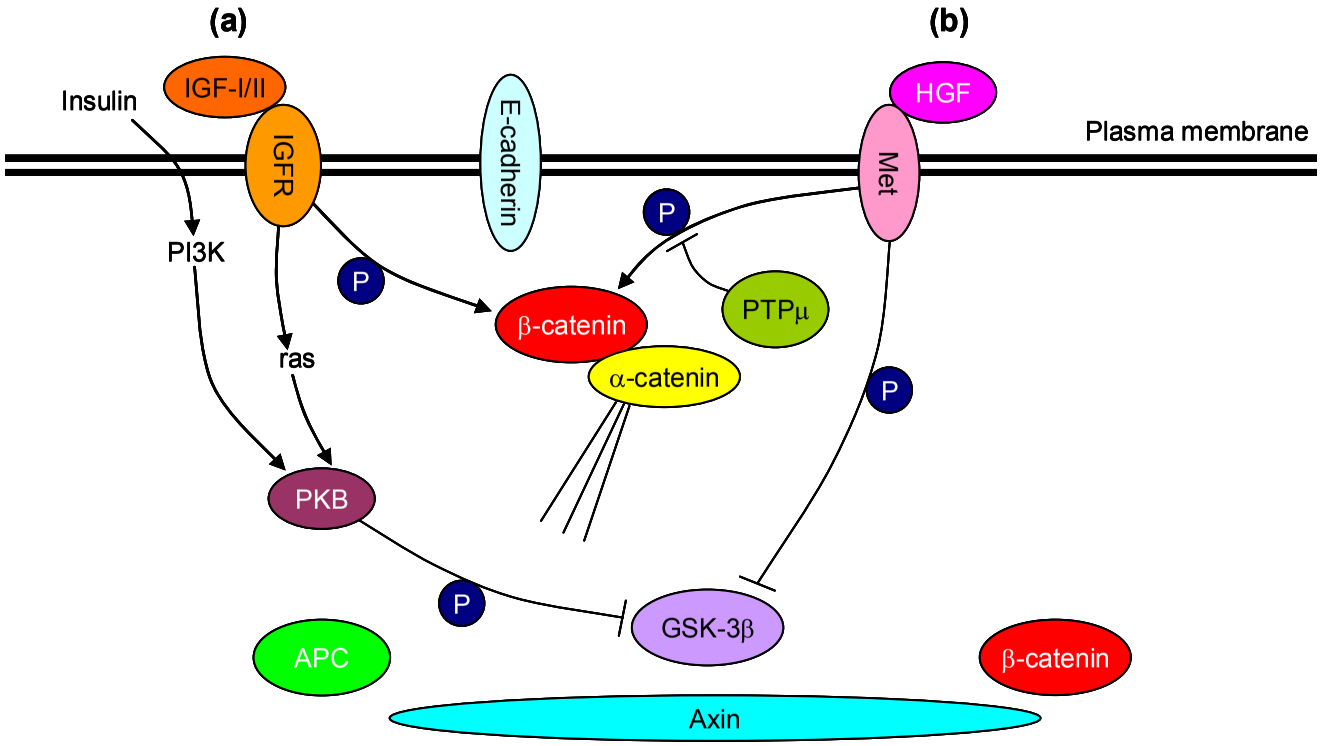


Figure 1.6: The effects of growth factors on β-catenin functioning. The growth factors **(a)** insulin-like growth factor (IGF) and **(b)** hepatocyte growth factor (HGF) have an effect on β-catenin's function in cell adhesion as well as its degradation in the cytoplasm. **(a)** The activated IGF receptor (IGFR) phosphorylates β-catenin, breaking its association with E-cadherin, thus reducing adhesion. It induces the translocation of β-catenin to the cytoplasm, where it briefly stabilizes β-catenin. Similarly, insulin activates PKB via PI3K, which in turn phosphorylates and inactivates GSK-3β inducing a low level of β-catenin stabilisation. **(b)** The HGF receptor, Met also interrupts the E-cadherin/β-catenin association by phosphorylating the latter. PTPμ may inhibit this event. Met also increases the levels of free β-catenin possibly through phosphorylating and inhibiting GSK-3β. Note, "P" denotes a phosphorylation event.

dependent. This is similar to insulin-stimulated PI3K activation of PKB, which phosphorylates and inhibits GSK-3β activity (Cross *et al*., 1995; Yuan *et al.,* 1999; Ding *et al.,* 2000). This PKB inhibition of GSK-3β alone is also not sufficient to result in the accumulation and transcriptional activity of β-catenin (Yuan *et al.,* 1999; Ding *et al.,* 2000). However, it is able to augment β-catenin's transcriptional activity induced by Wnt or FRAT-1 stimulation, possibly by decreasing GSK-3β's affinity for β-catenin (Yuan *et al.,* 1999; Ding *et al.,* 2000). Apart from colorectal cancers (Freier *et al*., 1999; Hakam *et al*., 1999), IGF receptors are overexpressed in ovarian carcinomas (Resnicoff *et al*., 1993) and rhabdomyosarcomas (Shapiro *et al*., 1994), possibly implicating the activity of this receptor in the progression of these diseases.

FGF-2 has been shown to free β-catenin from the adhesion complex (Halama *et al*., 2001; Katoh and Katoh, 2006) and increases its transcriptional activity through decreasing the activity of GSK-3β in endothelial cells (Holnthoner *et al*., 2002; Katoh and Katoh, 2006). FGF-1 also decreases GSK-3β activity, and can increase the translocation of β-catenin to the nucleus in neuronal cells (Hashimoto *et al*., 2002). However, this does not occur in epithelial cells (El Hariry *et al*., 2001a; El Hariry *et al*., 2001b).

The cytokine hepatocyte growth factor (HGF, also referred to as scatter factor or SF) results in the loss of adhesion and increase of cell motility by activating its tyrosine kinase receptor Met, as can be seen in Figure 1.6.b (Stoker and Perryman, 1985; Stoker *et al*., 1987; Weidner *et al*., 1990; Naldini *et al*., 1991; Shibamoto *et al*., 1994; Hiscox and Jiang, 1999a). Met associates with E-cadherin and β-catenin (Hiscox and Jiang, 1999b; Monga *et al*., 2002; Rasola *et al*., 2007) and phosphorylates β-catenin directly, disrupting E-cadherin-mediated adhesion and can stimulate motility of epithelial and cancer cells (Shibamoto *et al*., 1994; Hiscox and Jiang, 1999a; Müller *et al*., 2002; Brembeck *et al*., 2004; Rasola *et al*., 2007). This activity of HGF may be opposed by the protein tyrosine phosphatase, PTPμ which associates with Met (Hiscox and Jiang, 1999b). Activated Met has

also been shown to increase the levels of free β-catenin, as well as its transcriptional activities, possibly by increasing its affinity for BCL9-2 (Papkoff and Aikawa, 1998; Danilkovitch-Miagkova *et al*., 2001; Liou *et al*., 2002; Monga *et al*., 2002; Müller *et al*., 2002; Brembeck *et al*., 2004; Gavert and Ben-Ze'ev, 2007; Rasola *et al*., 2007). This may occur through inhibiting GSK-3β activity (Papkoff or Aikawa, 1998), or by a mechanism independent of this which induces direct translocation of β-catenin from the plasma membrane to the nucleus, possibly by cooperating with other signal transduction pathways (Liou *et al*., 2002; Monga *et al*., 2002; Müller *et al*., 2002). HGF and Met are inappropriately expressed and activated in a number of metastatic tumours, including colorectal (Shibamoto *et al*., 1994; Di Renzo *et al*., 1995), mammary (Jin *et al*., 1997; Ghoussoub *et al*., 1998; Camp *et al*., 1999; Hiscox and Jiang, 1999b), gastric (Shibamoto *et al*., 1994; Nakajima *et al*., 1999) and hepatocellular (Hu *et al*., 1999) cancers.

The 170 kD EGF transmembrane glycoprotein receptor (EGFR; also referred to as human EGF receptor-1 or HER1; or c-erbB1) localizes in close proximity to the E-cadherin/catenin complex and thus is of particular interest to cell adhesion (Fukuyama and Shimizu, 1991; Takahashi and Suzuki, 1996; Wells, 1999). Association of the extracellular domain of EGFR with the 53-amino acid EGF ligand, or with TGF-α (Ochiai *et al*., 1994), causes receptor dimerisation, inducing a self-phosphorylation, which activates intrinsic tyrosine kinase activity in its cytoplasmic tail (Yarden and Schlessinger, 1987; Wells, 1999). As can be seen in Figure 1.7, the activated receptor binds directly to the armadillo repeat region of β-catenin and phosphorylates it on tyrosine residues (Hoschuetzky *et al*., 1994; Ochiai *et al*., 1994; Shibamoto *et al*., 1994; Shiozaki *et al*., 1995; Takahashi *et al*., 1997; Hazan and Norton, 1998; Schroeder *et al*., 2002). Hoschuetzky *et al*. (1994) showed that both the carboxyl- and amino-termini can be phosphorylated by EGFR. Piedra *et al*. (2003) stated that Y654 is phosphorylated by EGFR and, as mentioned above, this is known to affect the association of β-catenin with E-cadherin (Roura *et al*., 1999). However, a number of other studies have shown that EGFR stimulation does not affect the E-cadherin/β-catenin bond, but causes

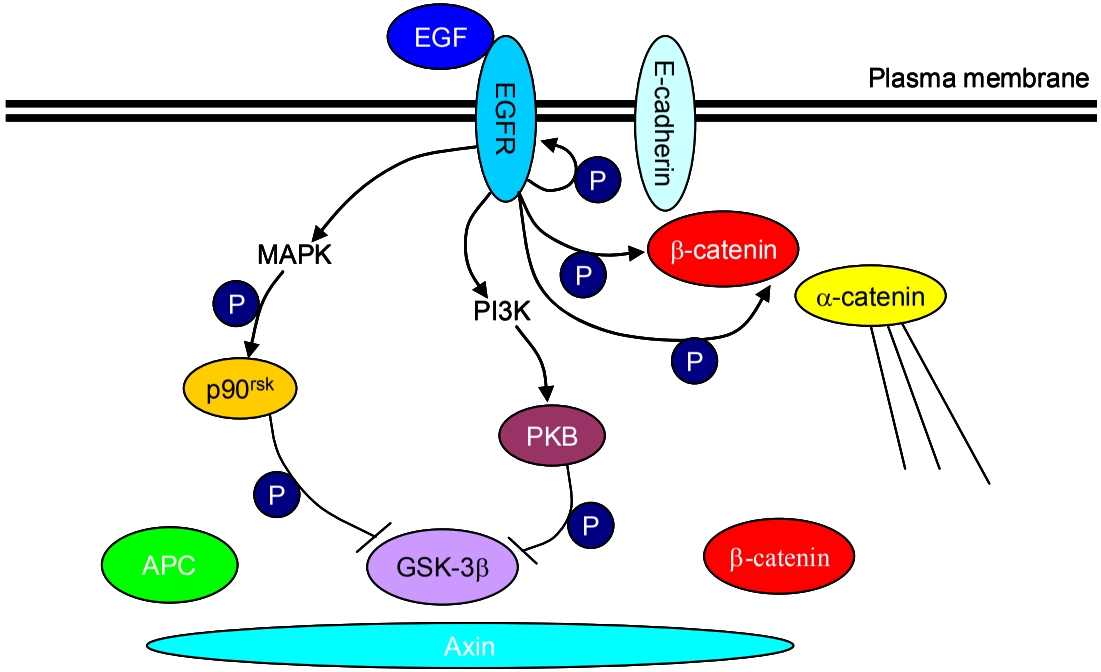


Figure 1.7: The effect of activated EGFR on β-catenin functions. EGF associates with its receptor (EGFR) inducing an activating self-phosphorylation of the receptor. Activated EGFR phosphorylates β-catenin, disrupting the association of the E-cadherin/catenin complex with the actin cytoskeleton, decreasing adhesion. EGFR activates the MAPK pathway, which activates $p90^{rsk}$, which in turn phosphorylates and inhibits GSK-3β. EGFR can also activate the PI3K/PKB pathways, which can also inhibit GSK-3β through phosphorylation. Note, "P" denotes a phosphorylation event.

dissociation of this complex with the actin cytoskeleton (Takahashi *et al*., 1997; Hazan and Norton, 1998; Kuwada *et al*., 1998; Schroeder *et al*., 2002), suggesting that the association between β-catenin and α-catenin is affected by EGFR stimulation. Either way, EGFR stimulation leads to the dissociation of the adherens junction from the actin cytoskeleton, thus opposing E-cadherin-mediated adhesion and stimulating cell scattering and migration, which is imperative for tumour metastasis (Hoschuetzky *et al*., 1994; Ochiai *et al*., 1994; Shibamoto *et al*., 1994; Shiozaki *et al*., 1995; Fujii *et al*., 1996; Takahashi *et al*., 1997; Hazan and Norton, 1998). This is further supported by various studies that have found that the expression of EGFRs are upregulated in metastasising cancers including breast adenocarcinomas (Sainsbury *et al*., 1985; Hazan and Norton, 1998; Kobayashi *et al*., 2002), bladder transitional cell carcinomas (Berger *et al*., 1987; Neal *et al*., 1990), gastric adenocarcinomas (Tahara *et al*., 1986), thyroid tumours (Duh *et al*., 1985), and a number of squamous cell carcinomas, such as human cutaneous (Ozanne *et al*., 1986; Fujii *et al*., 1996), oral (Odajima *et al*., 2005), head and neck (Ozanne *et al*., 1986), cervix (Ozanne *et al*., 1986) and oesophageal (Ozawa *et al*., 1989b; Veale and Thornley, 1989; Mukaida *et al*., 1991; Yano *et al*., 1991; Iihara *et al*., 1993) squamous cell carcinomas.

EGFR has also been shown to stimulate the mitogen activated protein kinase (MAPK) pathway, which activates the serine/threonine kinase $p90^{rsk}$, which in turn phosphorylates and inhibits GSK-3β activities (Stambolic and Woodgett, 1994; Eldar-Finkelman *et al.,* 1995). In addition, PKB, which is also known to inhibit GSK-3β activity (Cross *et al*., 1995), can be activated through EGFR stimulation of PI3K (Okano *et al*., 2000; Andl *et al*., 2003). It has been reported that in cells migrating after EGF stimulation, an increased pool of cytoplasmic and nuclear tyrosine phosphorylated β-catenin was detected (Kuwada *et al*., 1998; Müller *et al.,* 1999). However, Mizushima *et al*. (2002) have reported that the activation of the EGFR does not increase β-catenin/Tcf transcriptional activity in oesophageal SCC.

The cytokine transforming growth factor (TGF)-β1 plays an opposing role in tumour development. In the normal tissue and early stages of tumourigenesis, TGF-β1 acts as a tumour suppressor by inhibiting cellular proliferation and growth and induces apoptosis (Cui *et al*., 1996; Massagué *et al*., 2000; Xu *et al*., 2003; Satterwhite and Neufeld, 2004). However, in late stage tumours, it loses this suppressor function and promotes invasion and metastasis (Cui *et al*., 1996; Janji *et al*., 1999; Massagué *et al*., 2000; Weeks *et al*., 2001). In these late stage tumour cells, TGF-β1 induces dissociation of the adherens junction complex, detachment from the cytoskeleton and the reorganisation of the cytoskeleton (Portella *et al*., 1998; Janji *et al*., 1999; Bakin *et al*., 2000; Weeks *et al*., 2001; Tian and Phillips, 2002; Tian *et al*., 2003; Xu *et al*., 2003). This ultimately results in changes to cellular morphology which is associated with epithelial cell dedifferentiation to a more mesenchymal phenotype (Cui *et al*., 1996; Portella *et al*., 1998; Akhurst and Balmain, 1999; Janji *et al*., 1999; Ellenrieder *et al*., 2001; Tian and Phillips, 2002; Tian *et al*., 2003; Xu *et al*., 2003). These changes appear to be mediated by differential TGF-β1 signals. For example, the reorganisation of the cytoskeleton appears to be modulated by TGF-β1 stimulation of the Rho-GTPase-ROCK pathway (Tian *et al*., 2003), while the disruption of the adherens junction is mediated by TGF-β1 activation of the PI3K-PKB/Akt pathway and Ras activity (Bakin *et al*., 2000; Tian *et al*., 2003; Vogelmann *et al*., 2005).

Vogelmann *et al*. (2005) demonstrated in pancreatic carcinoma cells that both PI3K and the PTEN phosphatase associate with β-catenin at the adherens junction, where PTEN inhibits PI3K activity (see Figure 1.8). However, upon TGF-β1 stimulation, the level of PTEN associated with β-catenin decreases and PI3K is able to increase tyrosine phosphorylation of β-catenin and α-catenin, resulting in the dissociation of the E-cadherin/catenin complex from the cytoskeleton. Tian and Phillips (2002) similarly showed an increase of tyrosine phosphorylation of β-catenin and its dissociation from the E-cadherin and α-catenin in response to TGF-β1 in renal proximal tubule cells.

TGF-β1 stimulation has also been coupled with an increase in the cytoplasmic and

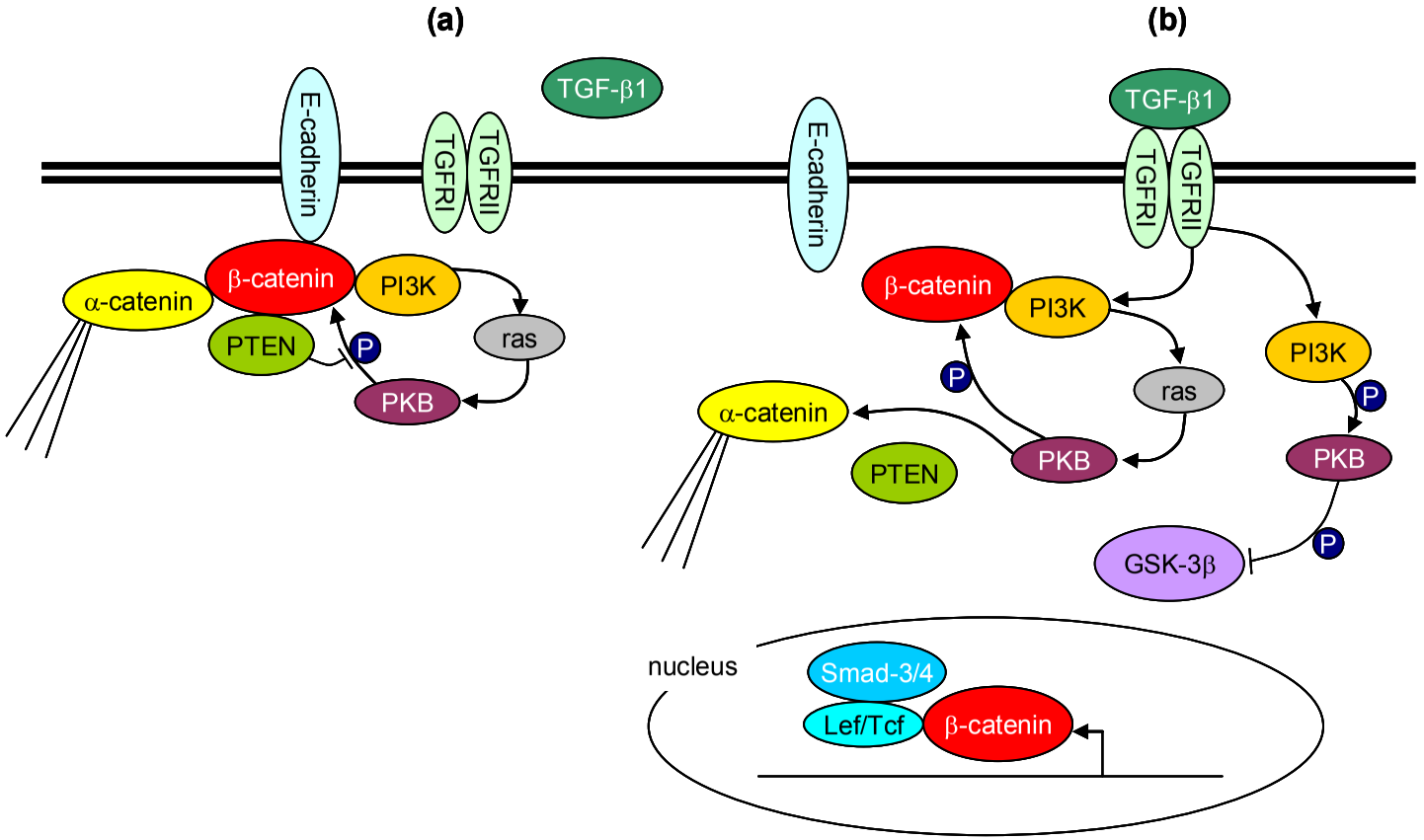


Figure 1.8: The role of TGF-β1 in regulating β-catenin functions in adhesion and signal transduction and gene expression. Both PTEN and PI3K can associate with β-catenin, where PTEN inhibits PI3K activity **(a)**. Upon TGF-β1 binding, the levels of PTEN associated with β-catenin decline and PI3K is able to effectively phosphorylate β-catenin and α-catenin, leading to a decrease in the association of the cadherin/catenin complex with the actin cytoskeleton **(b)**. TGF-β1 is also able to increase the cytoplasmic and nuclear pool of β-catenin, possibly through activating the PI3K/PKB pathway, which inhibits GSK-3β. TGF-β1 also increases the transcriptional activity of the β-catenin/Lef/Tcf complex, through its effector proteins, Smad-3 and -4.

nuclear pool of β-catenin (Janji *et al*., 1999; Tian and Phillips, 2002; Xu *et al*., 2003; Masszi *et al*., 2004; Satterwhite and Neufeld, 2004; Edlund *et al*., 2005). This increase could be a direct result of β-catenin redistributing from the plasma membrane into the cytoplasm as a result of the dissociation of the cadherin/catenin complex as mentioned above (Janji *et al*., 1999; Weeks *et al*., 2001). In addition, TGF-β1 signalling can mediate an inhibitory phosphorylation of GSK-3β's serine 9 via activation of PKB, further stabilizing the free pool of β-catenin (Cheon *et al*., 2004; Edlund *et al*., 2005). TGF-β1 signalling has also been shown to decrease the expression of APC and increase *de novo* expression of β-catenin (Satterwhite and Neufeld, 2004).

In the nucleus, TGF-β1 signalling has been shown in a number of cells to increase the transcriptional activity of the β-catenin/Lef/Tcf complex (Labbé *et al*., 2000; Xu *et al*., 2003; Cheon *et al*., 2004; Masszi *et al*., 2004; Warner *et al*., 2005). However, this may be dependent on cell-type and context, such as cell density (Tian *et al*., 2003; Masszi *et al*., 2004). The TGF-β1 effector proteins, Smad-3 and -4 have been shown to associate directly with Lef, and indirectly with β-catenin, and may modulate their transcriptional activity (Labbé *et al*., 2000; Tian and Phillips, 2002). In addition, TGF-β1 may also negatively modulate the activity of β-catenin in the nucleus by activating the TAK1/NLK pathway (Ishitani *et al*., 1999; Ishitani *et al*., 2003; see Section 1.7.1 above).

The level of TGF-β1 is elevated in a number of cancers and is associated with tumour progression, invasion and metastasis. These include hepatocellular carcinomas (Shirai *et al*., 1994; Xu *et al*., 2003), renal cell carcinomas (Junker *et al*., 1996), colorectal carcinomas (Tsushima *et al*., 1996; Narai *et al*., 2002), gastric carcinomas (Saito *et al*., 1999a; Xiangming *et al*., 2001), bladder carcinomas (Kim *et al*., 2001; Shariat *et al*., 2001), thyroid neoplasias (Jasani *et al*., 1990), breast cancers (Gorsch *et al*., 1992; Chakravarthy *et al*., 1999), pancreatic cancers (Friess *et al*., 1993) and oesophageal SCCs (Natsugoe *et al*., 2002; Fukai *et al*., 2003; Fukuchi *et al*., 2004).

1.10 Why is it important to examine β-catenin function in oesophageal squamous cell carcinoma?

The importance of β-catenin has already been highlighted in a number of cancers, especially in colorectal adenocarcinomas and melanomas. However, its role in squamous cell carcinoma, especially that of oesophageal SCC, is yet to be fully elucidated.

1.10.1 Epidemiology of oesophageal carcinomas

Oesophageal cancer has a poor prognosis and is among the leading causes of cancer deaths worldwide (Boynton *et al.,* 1992; Huang *et al.,* 1992; Shibagaki *et al.,* 1994; Gore, 1997; Lu, 2000; Stoner and Gupta, 2001). There are two main forms of oesophageal cancer: the more rare adenocarcinoma, which is associated with Western countries (such as the United States of America and parts of Europe), and the common (90% of cases), squamous cell carcinoma (Huang *et al.,* 1992; Powell *et al.,* 1994; Lu, 2000; Stoner and Gupta, 2001). The incidence of SCC is especially high in regions of South Africa, China, Iran, Uruguay, France and Italy (Lu, 2000; Stoner and Gupta, 2001). For example, up to 50% of all registered cancers in the Transkei region of South Africa are oesophageal (Gore, 1997).

Oesophageal SCC is predominantly prevalent in males and is of particular importance in South Africa where a high proportion of the working population is involved in activities such as mining where there is an increased risk of exposure to carcinogens. Other important contributory factors include the duration of tobacco intake, "binges" of alcohol in short but high doses, *N*-nitrosamines from food sources or digestive (endogenous) products, as well as nutritional deficiencies, especially in vitamins and minerals (Launoy *et al.,* 1997; Lu, 2000; Saeki *et al.,* 2000; Stoner and Gupta, 2001; Isaacson, 2005).

1.10.2 Oesophageal SCCs are classified into three broad categories

Diagnosis and prognosis of oesophageal SCCs at present falls into three categories, well-, moderately- and poorly-differentiated tumours. These categories are broadly based on the morphology of the tumour with information regarding these categories being both patchy and scattered, adding to the dubious value of this classification system. The following represent the available data. Well-differentiated tumours are defined as those that display keratinisation, moderate nuclear atypia and minimal necrosis, while the more poorly-differentiated tumours exhibit a high incidence of mitosis and large areas of necrosis (Stoner and Gupta, 2001). These established diagnostic categories are extremely broad, especially the moderately-differentiated category, into which borderline cases are often placed. In addition, it is becoming increasingly evident that this visual classification provides a gross level of data, while the information collected at a molecular level (such as that collected from E-cadherin, integrin and other studies) is more detailed and consistent (Sarbia *et al*., 1995). Hence, the visual classification of oesophageal SCCs, especially that of moderately-differentiated SCCs, will need to be revised if it is to be useful to diagnosis/prognosis. The development of molecular markers is likely to lead to a more beneficial form of diagnosis and/or prognosis.

1.10.3 How can β-catenin be useful in staging and further understanding oesophageal SCCs?

A change in cell-cell adhesion has been correlated with increased metastasis, and thus is an important consideration when staging a cancer. Metastasis is of particular importance in oesophageal SCCs as the majority of patients present with advanced metastases (Stoner and Gupta, 2001). In addition, recurrence of the disease, after tissue resection, occurs in nearly half of all patients due to the presence of micro-metastases (Thorban *et al.,* 2000). The occurrence of micro-metastases reduces the prognostic 5-year survival rates to below 10% (Stoner and Gupta, 2001). The critical regulatory position β-catenin holds in the adhesion

complex, suggests that it may serve as an ideal marker for differences in intercellular adhesion, and thus metastasis, in oesophageal SCCs.

β-catenin has also been implicated in the upregulation of a number of oncogenes, such as *c-myc* and *cyclin D1*, when stabilized in the cytoplasm. This may be important in the progression of oesophageal SCCs as it is a slow growing solid tumour that most probably arises through accumulation of genetic alterations and the amplification of the protooncogenes such as *c-myc*, *int-2*, *hst-1*, *EGF*, *EGFR* and *cyclin D1* (Boynton *et al.,* 1992; Huang *et al.,* 1992; Powell *et al.,* 1994; Shibagaki *et al.,* 1994, Stoner and Gupta, 2001). Thus, there is a real possibility that β-catenin may play a role in the development and progression of oesophageal SCCs, thereby increasing its potential as a molecular marker for this disease.

In addition, the role of cytoplasmic β-catenin stabilization on its adhesive function is not known. This requires deeper investigation as it may provide further clues useful in understanding the metastatic behaviour of oesophageal SCCs. It should be remembered that a number of signal transduction pathways affect β-catenin functioning and thus may provide insight as to how the balance between β-catenin's roles in adhesion and in the cytoplasm is mediated. Of particular interest to oesophageal SCC is EGF signalling as the EGFRs are known to be overexpressed in moderately-differentiated oesophageal SCCs (Veale and Thornley, 1989).

1.11 Aims

This study aims to:

- identify and determine the localization of β-catenin, as well as the level of β-catenin expression, in cell lines derived from human oesophageal SCCs that have been traditionally classified as being moderately-differentiated. Comparison of cell lines of a single pathological grade may highlight any similarities or differences that occur *within* this category, and thus aid in further defining this broad category. Investigating the expression and

distribution of β-catenin will also assist in determining the functional role (adhesion or signalling) that this protein may play in the development of moderately-differentiated oesophageal SCCs, and may identify the protein as a molecular marker for this stage of the disease.

- examine how the balance between plasma membrane-associated β-catenin and cytoplasmic β-catenin is affected by the signalling induced through growth factor stimulation (by EGF and TGF-β1) and a Wnt-like stabilization of cytoplasmic β-catenin, and to determine whether crosstalk between these pathways occurs.

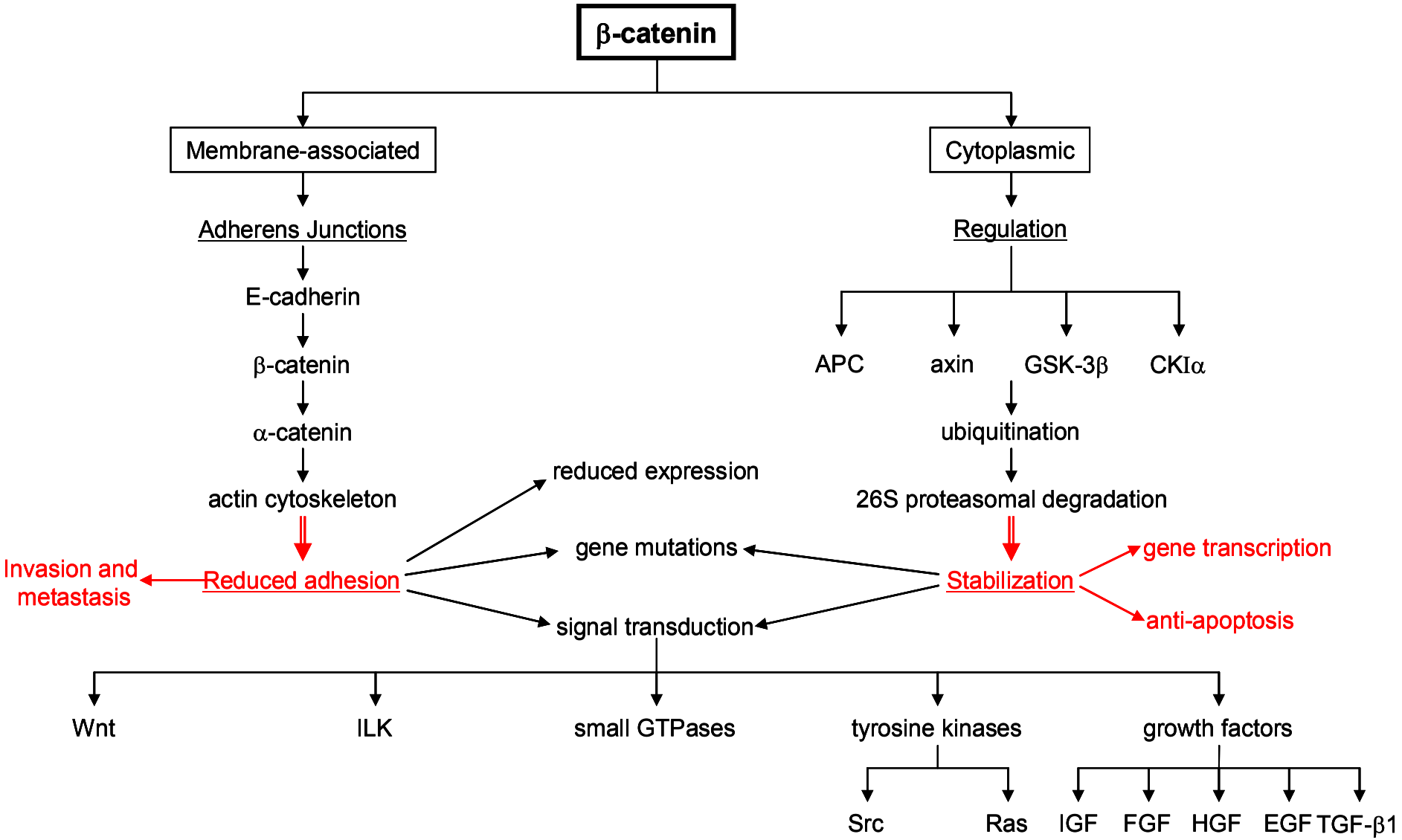


Figure 1.9: A flow diagram of the functions of β-catenin in the cell. The diagram acts as a synopsis of the important roles of β-catenin in the cell, namely cell adhesion when membrane-associated, and its regulation in the cytoplasm. The diagram also shows how aberrations in these functions (reducing adhesion and its stabilization), by decreased expression, gene mutations and various signal transduction pathways, can potentiate tumour progression.

CHAPTER TWO

The Expression of β-catenin in Moderately-Differentiated Human Oesophageal Squamous Cell Lines

Cell adhesion is fundamental in the maintenance of epithelial tissue structure and differentiation (Hynes, 1999; Orend and Chiquet-Ehrismann, 2000; Yang *et al*., 2001; Perez-Moreno *et al*., 2003). Changes to cellular adhesion through alterations in the expression or structure of the adherens junction components, have been linked to the development, progression, invasion and metastasis of a tumour (Ruoslahti, 1996; Davies *et al*., 2000; Al-Aynati *et al*., 2004). As mentioned in Chapter 1, the strength and stability of cell-cell adhesion lies in the associations of the plasma membrane-binding cadherin proteins and the actin cytoskeleton (Pokutta and Weis, 2000; Perez-Moreno *et al*., 2003). Whether the interaction with the actin cytoskeleton and the cadherin complex is direct or not (see Section 1.3; Drees *et al*., 2005; Yamada *et al*., 2005), β-catenin remains vital to the arbitration of this interaction as it brings the α-catenin/actin complex to the cadherin protein. Thus, due to this central position held by β-catenin in the adhesion sequence, β-catenin is thought to be a key regulator of cell adhesion (Hinck *et al*., 1994b; Gumbiner, 1996; Takayama *et al*., 1998).

Aberrant expression of β-catenin at the plasma membrane leads to weakened adhesion and increases the invasive and metastatic potential of a tumour (Takayama *et al*., 1998; Odajima *et al*., 2005). This has been demonstrated in a number of cancers, in particular colorectal cancer tissue (Oyama *et al*., 1994; Kawanishi *et al*., 1995; Jawhari *et al*., 1997; Ilyas *et al*., 1997; Takayama *et al*., 1998; Klymkowsky, 2005). Thus, the expression of β-catenin has important implications with respect to the development and progression of the disease, and may therefore have the potential to serve as a molecular marker for determining the progressive stage of a given tumour.

β-catenin can also be found "free" in the cytoplasm. Accumulation of β-catenin in this unbound state enables it to enter the nucleus and act as a transcriptional activator for certain genes including a number of oncogenes (Behrens *et al*., 1996; Huber *et al*., 1996; Molenaar *et al*., 1996; Korinek *et al*., 1997; Rubinfeld *et al*., 1997). Free β-catenin is usually tightly regulated by a degradation complex, consisting of GSK-3β, CKIα, APC and axin, which targets the protein for ubiquitination and proteasomal degradation by phosphorylation (Munemitsu *et al*., 1995; Aberle *et al*., 1997; Kishida *et al*., 1998; Liu *et al*., 2002). Reduced E-cadherin-mediated adhesion and/or inhibition of this degradation complex, such as by specific mutations or by Wnt signalling (see Chapter 1.8 and 1.9.1), leads to the accumulation of stabilized cytoplasmic β-catenin (Hinck *et al*., 1994a; Munemitsu *et al*., 1995; Morin *et al*., 1997; Rubinfeld *et al*., 1997; Simcha *et al*., 1998; Orsulic *et al*., 1999; Gottardi *et al*., 2001; Stockinger *et al*., 2001; Moon *et al*., 2004). This has been shown to be important in the development of a number of tumours, especially colorectal and hepatocellular carcinomas (Ilyas *et al*., 1997; Morin *et al*., 1997; Miyoshi *et al*., 1998; Satoh *et al*., 2000; Jin *et al*., 2003).

Currently, human oesophageal squamous cell carcinomas (SCCs) are histopathologically classified into poorly-, moderately- and well-differentiated tumours, with metastasis being a common phenomenon regardless of the state of differentiation. These classifications broadly reflect the metastatic potential of the tumour at the time of surgical resection. However, there is a considerable lack of understanding at a molecular level as to the mechanisms underlying the progression of this disease, which may prove useful in refining these broad categories. β-catenin makes for an ideal molecular candidate based on its documented association with metastasis in other tumours, as well as in abnormal signal transduction (refer to Chapter 1.4; 1.7; 1.8 and 1.9 for details). Thus, the following investigation focuses on the role played by β-catenin in human oesophageal SCCs. The study examines and compares the expression of β-catenin in five cell lines derived from moderately-differentiated human squamous cell carcinomas of the oesophagus, to analyse expression characteristics to determine whether a pattern of expression exists. The investigation also attempts to assess

whether cytoplasmic β-catenin is stabilized intracellularly, which may implicate it in aberrant signal transduction pathways and possible gene activation.

2.2 Methods and Materials

2.2.1 Cell lines

In vitro cultures of five South African cell lines of human oesophageal squamous cell carcinomas (SCCs) termed WITS Human Carcinoma of the Oesophagus 1 (WHCO1), WHCO3, WHCO5, WHCO6 and SNO (passage numbers 32, 11, 17, 19 and indeterminate, respectively) were obtained from the Cell Biology Laboratory, School of Molecular and Cell Biology, University of the Witwatersrand (Veale and Thornley, 1989; Bey *et al*., 1976). These cell lines were derived from moderately-differentiated tumour resections from patients presenting with metastatic disease. The cell lines were maintained in DMEM:Hams F12 (3:1) supplemented with 10% Foetal Calf (or Bovine) Serum (FCS or FBS) on Corning® tissue culture dishes at 37°C in a 5% carbon dioxide (CO_2) atmosphere. All cell lines were mycoplasma free according to the MycoFluor Mycoplasma Detection Kit (Molecular Probes).

The human colon adenocarcinoma CACO-2 cell line was a gift from the School of Anatomical Sciences, University of Witwatersrand Medical School. This cell line has been reported to have somatic *APC* mutations, as well as mutations in the β-catenin gene, *CTNNB1*, which promotes escape from APC-dependent regulation of β-catenin (Ilyas *et al*., 1997). Sampson *et al*. (2001) similarly reported that the CACO-2 line displays a constitutively active Wnt signal transduction pathway. Hence this cell line was used as a positive control for cells displaying stabilized cytoplasmic and nuclear β-catenin. The cells were maintained as for the oesophageal cell lines. Please note; although healthy oesophageal epithelium is an ideal negative control for these experiments, it was not used due to the difficulties in obtaining and culturing normal oesophageal squamous cells.

2.2.2 Antibodies

β-catenin was specifically detected with a polyclonal antibody obtained from Sigma. Monoclonal anti-E-cadherin was obtained from Sigma. Polyclonal horseradish peroxidase (HRP)-bound goat anti-rabbit secondary antibody (Sigma) and monoclonal HRP-bound rabbit anti-mouse secondary antibody (Serotec) were used in immunoblot experiments. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody from Chappel was used in the immunofluorescence experiments. ^{125}I Protein A used in the radioimmunoassays was obtained from ICN.

2.2.3 Identification of β-catenin: cell fractionation, SDS-PAGE and immunoblot

2.2.3.1 Whole cell extraction

Oesophageal SCC cell cultures were seeded into 10 centimeter plates and allowed to proliferate until becoming approximately 70% confluent plated. Two plates per cell line were rinsed three times with phosphate buffered saline (PBS) (see Appendix 1.1.1 for details). The cells were scraped into the PBS with a rubber policeman and transferred to a 1.5 ml eppendorf tube. The cells were pelleted in a TOMY-desktop centrifuge (1145 ×g) for 5 minutes and the supernatant discarded. The pellet was resuspended in Laemmli sample buffer (Laemmli, 1970; see Appendix 1.2.1), by gentle pulses in a vortex. The sample was boiled for 5 minutes, briefly vortexed and centrifuged in the TOMY-desktop centrifuge for 5 minutes.

2.2.3.2 Cell fractionation into plasma membrane and "cytoplasmic" components

The plasma membrane of the cells was separated from the rest of the cellular components following the protocol described by Chen *et al*. (2000). This latter fraction, which contains essentially the cytoplasmic and nuclear material, and thus the "free" component of β-catenin (see Jones and Veale, 2003), will hereafter be referred to as the cytoplasmic fraction. Cell cultures of the five human oesophageal SCC cell lines and the CACO-2 cell line were rinsed twice with PBS and once with a phenyl-methyl-sulphonyl fluoride (PMSF, Merck)/Aprotinin (Trasylol®, Bayer) protease inhibitor stock solution (see Appendix 1.2.3). The cells were scraped into this solution with a rubber policeman and transferred to an eppendorf tube. The cells were pelleted in a TOMY-desktop centrifuge and the supernatant discarded. 500 µl of a hypotonic buffer (see Appendix 1.2.4) was added to the pellet, which was immediately frozen at -70°C.

The cells were thawed slowly on ice and homogenized by 30 strokes with an eppendorf-dounce homogenizer. To ensure that no protein was left on the homogenizer, the instrument was rinsed with 50 µl of the hypotonic buffer. The homogenate was transferred to polycarbonate ultracentrifuge tubes, and the eppendorf tube was rinsed with 50 µl hypotonic buffer to ensure that all the homogenate was transferred. The samples were centrifuged at 100 000 ×g, in a Beckman L7-65 ultracentrifuge for 30 minutes at 4°C.

The resultant supernatant containing the cytoplasmic material, was transferred to a clean eppendorf tube and frozen at -70°C. 500 µl of 0.1% sodium dodecyl sulphate (SDS) in 20 mM Tris.HCl (pH 7.4) was added to the insoluble plasma membrane fraction (pellet). The pellet was slowly allowed to resuspend into solution and was transferred to a clean eppendorf tube. The centrifuge tube was rinsed with 100 µl of 0.1% SDS in 20 mM Tris.HCl (pH 7.4) and was transferred to the eppendorf tube to ensure all the protein was captured. The samples were

frozen at -70°C. Note, extractions of all the cell lines were performed simultaneously.

2.2.3.3 Protein estimation

An estimation of the total protein content was conducted on the plasma membrane and cytoplasmic cellular fractions of cell lines, as well as on the whole cell extractions. The protein estimation was accomplished by performing a Bradford assay following the protocol from Bramhall *et al*. (1969). This method was preferred over the commercial BioRad assay as the detergent levels in the extraction interfere with the BioRad assay and dilution of the extraction buffer ran the risk of reducing the solubility of the protein. Samples were concentrated by lyophylisation as the initial protein estimation indicated very low levels of protein in the samples.

Whatmann® GF/C discs were rinsed with dH_2O for 20 minutes to rid the discs of any loose fibres. The discs were rinsed briefly with 95% ethanol, 100% ethanol and finally with acetone to dehydrate the discs and were allowed to air-dry. An appropriate standard curve for each extraction was constructed using bovine serum albumin (BSA; see Appendix 1.3.1). 5 µl of hypotonic buffer, containing the protease inhibitors used in the extraction, was added to a separate disc to control for the possibility that these inhibitors reacted positively, thus skewing the total concentration of protein in the extract. 5 µl of the cellular fractions were dotted on the remaining discs. All discs were duplicated to enhance accuracy of results.

The sample discs were allowed to air-dry before being placed in 7.5% Trichloroacetic Acid (TCA; see Appendix 1.3.2) for 30 minutes with gentle swirling. The TCA was drained off and the sample discs were covered with Coomasie blue stain (see Appendix 1.3.3) for 5 minutes to ensure that the residual TCA was removed and then was replaced with fresh Coomasie blue for 1 hour with gentle swirling. The discs were then destained in acetic acid:methanol:water

(10:12:100) until the background of the discs had cleared. This step was conducted in the dark to avoid any possible light degradation of the dye. The samples were allowed to air-dry in the dark and the stained area was cut out and placed in glass tubes. Duplicate samples of background staining were also added to a tube to control for any remaining background staining of the disc. 5 ml of an elution solution (see Appendix 1.3.5) was added, and the samples were eluted in the dark for 1 hour to ensure that the dye was fully released from the discs. The absorbance of each solution was measured by spectrophotometry (λ = 596 nm). The concentrations of the total protein of the samples were calculated from the BSA standard curve ($y = 0.0201x + 0.02$, $r^2 = 0.996$ for whole cell extracts; $y = 0.0259x + 0.0021$, $r^2 = 0.995$ for the membrane extracts and $y = 0.0199x + 0.0058$, $r^2 = 0.993$ for the cytoplasmic extracts; see Appendix 2, Figure A).

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

10% polyacrylamide gels were prepared as detailed in Appendix 1.4.1. Plasma membrane and cytoplasmic samples were boiled for 5 minutes in Laemmli sample buffer. 5 μg of protein from the whole cell extracts and the plasma membrane and cytoplasmic fractions were loaded and electrophoresed at 20 mA for approximately 1 hour until the protein ladder (PageRuler™, Fermentas Life Sciences) was 1 cm from the bottom of the gel. The gels were stained with Coomasie Blue for 1 hour and destained in acetic acid:methanol:water (1:1:10). The position of the 94 kD β-catenin band was calculated using the equation obtained from plotting the relative mobility (R_f) values of the molecular weight marker against the $\log_{10}$ values of the molecular weights ($y = -1.16x + 2.31$, $r^2 = 0.987$; $y = -1.17x + 2.30$, $r^2 = 0.997$ and $y = -1.19x + 2.25$, $r^2 = 0.995$ for the whole cell, membrane and cytoplasmic extracts, respectively; see Appendix 3, Figure B).

2.2.3.5 Immunoblotting

An immunoblot (western blot) was conducted to determine the presence of β-catenin in the plasma membrane and cytoplasmic extracts. To ensure that the electrophoretic separation was successful, two extra lanes were included and used to stain and destain as for the standard SDS-PAGE.

Protein on the rest of the gel was transferred to NitroBind nitrocellulose transfer membrane (MSI) using a BioRad Criterion™ Blotter at 400 mA for 2 hours (4°C) in Western Blot Transfer Buffer (see Appendix 1.5.1). After transferal, the nitrocellulose membrane was rinsed twice with Tris buffered saline (TBS; see Appendix 1.5.2) and stored overnight at 4°C.

Each membrane was blocked with a casein-based blocking buffer, BLOTTO (see Appendix 1.5.3) for 1 hour and then rinsed six times with TBS. The blot was incubated in polyclonal anti-β-catenin antibody (1:5 000) for 1 hour and then rinsed six times for 5 minutes each with TBS. An incubation with the polyclonal HRP-bound goat anti-rabbit secondary antibody (1:10 000) followed for 1 hour in the dark. The blot was rinsed six times with TBS as above before being exposed to the SuperSignal® West Pico Working Solution (see Appendix 1.5.4), from the SuperSignal® West Pico Chemiluminescent Substrate kit (Pierce), for 5 minutes. The blot was sealed in polyethylene "cling wrap" and exposed to CL-XPosure™ Film (Pierce) for 1.5 minutes. The film was developed in D19B developer (see Appendix 1.5.5) for 5 minutes, rinsed briefly in water before fixing (see Appendix 1.5.6) for 5 minutes, followed by a brief rinse in water and then air-dried.

The success of protein transfer to the nitrocellulose was evaluated by Fast green staining. Although radiolabelling is often the method of choice for assessing protein transfer, Fast green staining is efficient, sensitive, safer and more cost-effective. Therefore, replicate samples were transferred to nitrocellulose, stained

with Fast green (see Appendix 1.5.7) for 20 minutes, and destained as for SDS-PAGE.

2.2.3.6 Efficacy of plasma membrane extraction

In order to determine the efficacy of separating the plasma membrane fraction from the rest of the cellular components, an immunoblot of a plasma membrane protein, E-cadherin, was conducted. E-cadherin is a 120 kD transmembrane glycoprotein (Shimoyama *et al*., 1989; Takeichi, 1991). It is rapidly transported out of the ER to the plasma membrane after synthesis, as E-cadherin that is retained in the ER is degraded (Hinck *et al*., 1994c; Chen *et al*., 1999a). Plasma membrane and cytoplasmic fractions (10 µg) from the SNO cell line were electrophoresed on a 10% SDS-PAGE gel. A whole cell extraction was also loaded as a reaction control. It should be noted that more protein (20 µg) was loaded from this extract than the plasma membrane and cytoplasmic extracts. The protein was transferred to nitrocellulose as in Section 2.2.3.5. The immunoblot was conducted as described above (see Section 2.2.3.5), using an anti-E-cadherin antibody (1:500) as the primary antibody (incubating for 3 hours) and a monoclonal rabbit anti-mouse HRP-conjugated antibody (1:4 500) as the secondary antibody.

2.2.4 Localization of β-catenin in human oesophageal SCC cells using indirect immunofluorescence

The localization of β-catenin was determined in the five oesophageal SCC cell lines by indirect immunofluorescent microscopy. The CACO-2 cell line was used as a control for both cytoplasmic and nuclear localization of the protein. Cells were seeded onto sterile cover slips and allowed to proliferate until becoming approximately 70% confluent. The cover slips were rinsed four times with PBS and fixed for 30 minutes with 4% paraformaldehyde (see Appendix 1.6.1). Paraformaldehyde was used as the fixative of choice as it has been shown to be the most appropriate fixative for the current application (Simcha *et al*, 1998). The

cells were rinsed five times with PBS and the membrane was further permeabilised by incubating the cells in 0.25% Triton X-100 for 10 minutes. After rinsing twice with PBS, the cover slips were dipped in dH_2O and allowed to dry partially. This permitted maximum adherence of the DakoCytomation© (Dako) pen ink to the cover slip, which was used to create two fluid barriers or "wells" (approximately 0.5 cm in diameter) per cover slip. The cells within these wells were rehydrated with PBS for 30 minutes. Anti-β-catenin antibody (1:300) was added to the experimental well and PBS to the second well as a control. After a 1.5 hour incubation the antibody was removed and both wells were rinsed five times with PBS. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (1:500) was added to both wells and incubated in the dark for 1.5 hours. The antibody was removed and the wells were rinsed six times with PBS. The cover slips were mounted using Elvanol mounting agent (see Appendix 1.6.2) to sterilised glass slides and were viewed under a Zeiss LSM 410 confocal microscope (FITC excitation 490, emission 525). Photomultiplier tube settings remained at the manufacturer's setting at all times. Composite images represent the entire thickness of the cell. Description of the fluorescent antibody reaction will be colloquially referred to as "staining", although this reaction is not true "staining" in the histochemical sense.

2.2.5 Radioimmunoassay (RIA) quantification of the cellular component β-catenin under standard culture conditions

β-catenin was quantified in each of the five oesophageal SCC lines by using a radioimmunoassay (RIA) modified specifically for this purpose (see Zampieri and Veale, 2001). An equal number of cells were seeded into Nunc™ 24 multiwell tissue culture dishes in triplicate, plus extra wells for cell counts 24 hours later. The cells were rinsed three times with cold PBS to remove any culture medium and were fixed with 4% paraformaldehyde in PBS (see Appendix 1.7.1), twice for 5 minutes. Following a PBS rinse, the cells were further permeabilised with 0.25% Triton X-100 (10 minutes). The cells were rinsed, blocked with BLOTTO for 20 minutes and rinsed again. The experimental wells were incubated with 250

µl anti-β-catenin antibody (1:300) for 1.5 hours, while PBS was added to the control wells for this period. After a further rinse, 250 µl ^{125}I-Protein A (1.1 µCi/ml) was added to all wells for 1 hour. After a final rinse, the cells were incubated in 2 M NaOH (500 µl per well) for 1 hour to solubilise the cells. A further 1 ml of NaOH was added to ensure complete solubilisation. A Packard Cobra Auto-Gamma® counter was used to determine the specific antibody binding to β-catenin. As ^{125}I is a strong gamma emitter the distinction between disintegrations per minute (DPM) and counts per minute (CPM) is negligible, therefore, CPM was used to express the specific binding. The data subjected to statistical analysis was obtained from triplicate samples with the experiment repeated three times.

In order to ascertain whether a significant difference existed between the cell lines with regard to the expression of β-catenin, the CPM per 100 000 cells were subjected to a two-way analysis of variance (ANOVA) and Tukey's Honesty Significant Difference (HSD) test using Statistica 5.1.

2.2.6 Densitometry

As the RIA provided a measure of the total β-catenin concentration in the cell, it has limited application in differentiating between cellular fractions. Therefore, densitometric analysis (using Labworks ^{TM} Image Acquisition and Analysis software, version 4.5), of images of the immunoblots (captured on a High Resolution Scanner HP ScanJet 4400c), was used to semiquantitatively compare the levels of β-catenin at the plasma membrane and in the cytoplasm. The area under the peak for each sample was used as a basis for comparison between samples. Expression levels were represented as a percentage of the maximum per 5 µg of protein (see also Chen *et al*., 2000).

2.3 Results

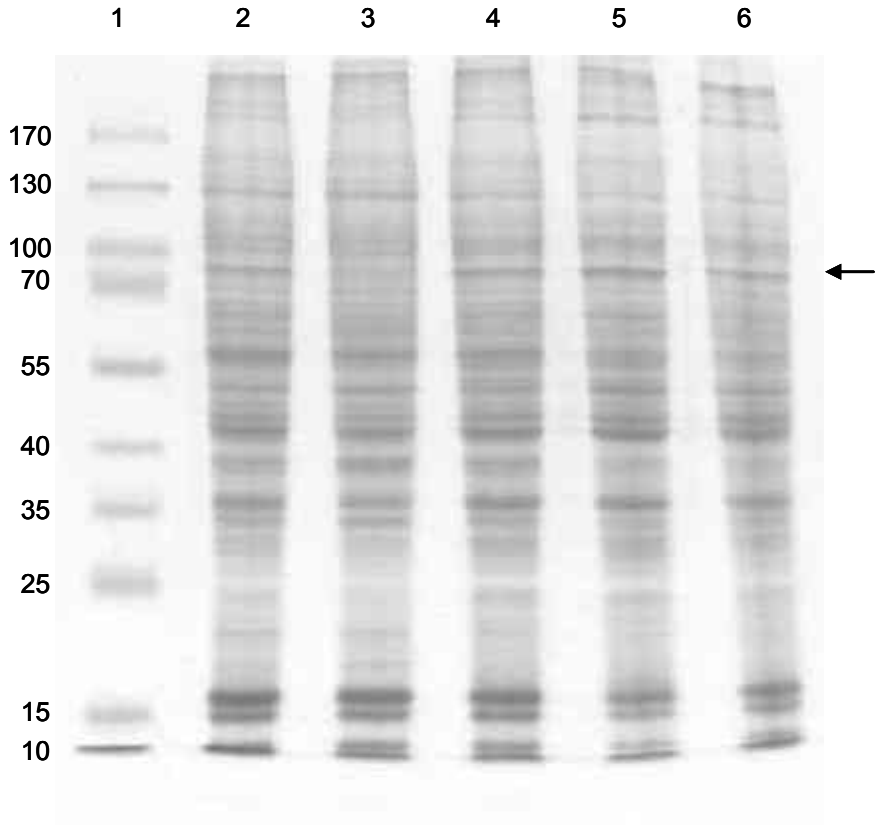
2.3.1 SDS-PAGE separation of cellular proteins and identification of β-catenin by immunoblot

Whole cell extractions of the oesophageal SCC cell lines contained a large compliment of proteins across the entire electrophoretic mobility range of the 10% SDS-PAGE gel, although a higher concentration of polypeptides occurred in the mid-range (100-40 kD, refer to Figure 2.1). A polypeptide of 94 kD was identified, suggesting the presence of β-catenin in these cell lines. The equal staining of the lanes provided a visual indication as to the efficacy of the protein estimation.

The plasma membrane fraction of the oesophageal SCC cells (Figure 2.2.a) displayed an entire range of polypeptides across the separating SDS-PAGE, although there appeared to be an enrichment of polypeptides with the molecular weights between 66 and 45 kD. The cytoplasmic[§] fractions (Figure 2.2.b) displayed a scattering of polypeptides between 100 and 30 kD, with lower molecular weight proteins being under represented. The presence of a band at 94 kD in both fractions (as indicated by the arrows) positively identifies a polypeptide with a molecular weight corresponding to that of β-catenin. The cellular fractions of the CACO-2 control cells displayed a similar expression pattern to the oesophageal SCC cell lines (see Figure 2.2.c).

The efficacy of separation of the plasma membrane fraction from the rest of the cellular (cytoplasmic) components was established by immunoblotting for the plasma membrane protein, E-cadherin (see Figure 2.3). Please note, the successful transfer of protein from the SDS-PAGE to the nitrocellulose membrane was established by Fast green staining of the nitrocellulose membrane (see Appendix 3, Figure C). The plasma membrane fraction showed a specific reaction at the correct molecular weight for E-cadherin (120 kD) as determined by comparison to

[§] Refer to Section 2.2.3.2



<u>Figure 2.1</u>: The protein profile of whole cell extractions of human oesophageal SCC cell lines. Separation of whole cell extracts (5 μg) from oesophageal SCC cell lines by SDS-PAGE showed a large compliment of polypeptides across the entire electrophoretic mobility range of the gel, although the majority of polypeptides occurred within the mid-range of molecular weights (100-40 kD). The arrow indicates 94 kD. The molecular weight marker (170, 130, 100, 70, 55, 40, 35, 25, 15 and 10 kD) is shown in lane 1. WHCO1, WHCO3, WHCO5, WHCO6 and SNO are represented in lanes 2-6, respectively. (Gel = 10%, stained with Coomasie blue).

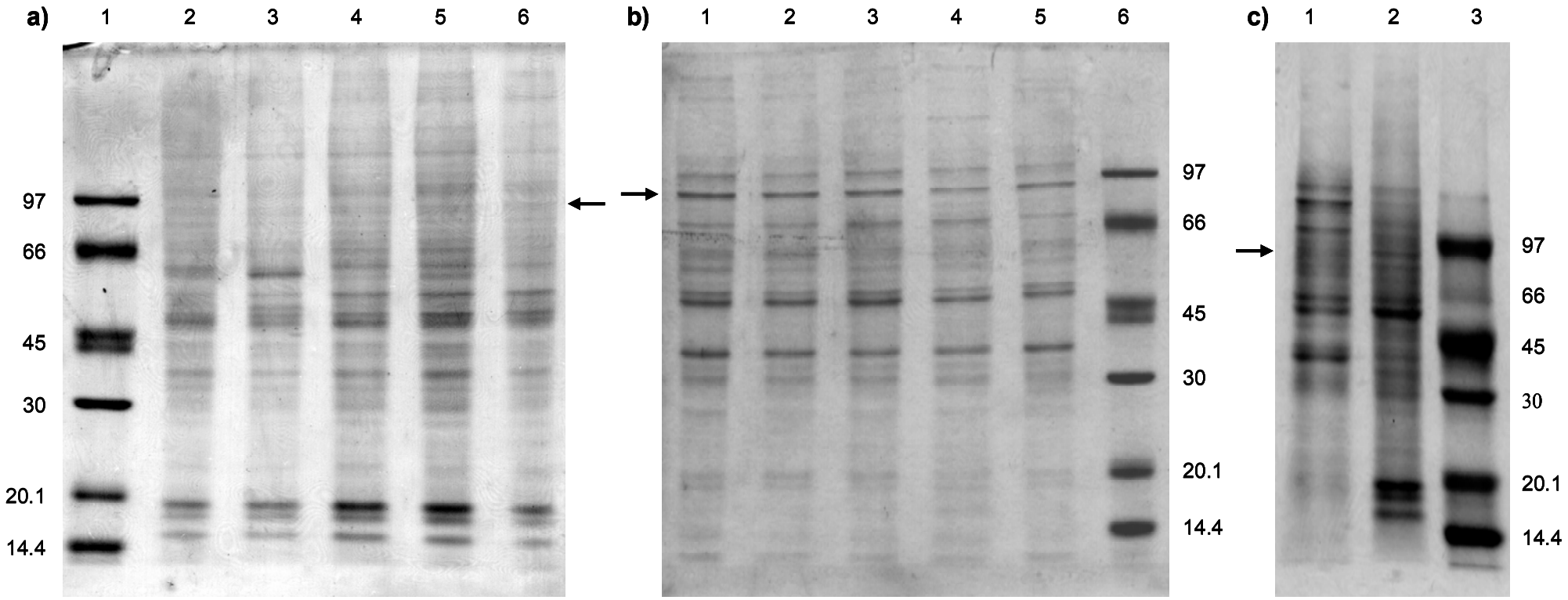


Figure 2.2: Separation of plasma membrane and cytoplasmic extracts of the oesophageal SCC cell lines by SDS-PAGE. **a)** The plasma membrane extraction (5 μg) of the oesophageal SCC cells enriches polypeptides ranging between 66 and 45 kD, although proteins occurred across the electrophoretic range of the gel. WHCO1, WHCO3, WHCO5, WHCO6 and SNO are represented in lanes 2-6. **b)** The cytoplasmic fractions (5 μg) displayed a scattering of polypeptides between 100 and 30 kD, with the lower molecular weight proteins being under represented (WHCO1, WHCO3, WHCO5, WHCO6 and SNO are represented in lanes 1-5). The arrows indicate 94 kD. **c)** The protein profile of the cytoplasmic (lane 1) and membrane (lane 2) fraction of the CACO-2 control cell line was similar to that of the oesophageal SCC cell lines. The molecular weight marker (97, 66, 45, 30, 20.1, and 14.4 kD) of **a**, **b** and **c** are represented in lanes 1, 6 and 3 respectively. (Gel = 10%, stained with Coomasie blue).

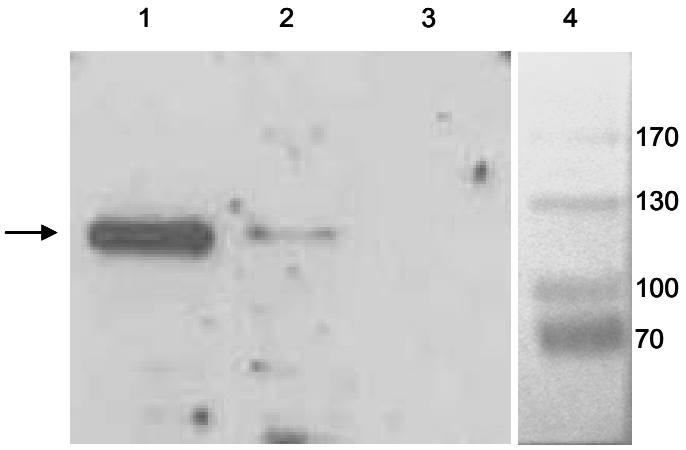


Figure 2.3: No contamination of the plasma membrane and cytoplasmic fractions occur. E-cadherin (120 kD), a known plasma membrane protein, was detected in the plasma membrane extracts (lane 2, indicated by the arrow), but was not in the cytoplasmic fractions (lane 3) by immunoblotting (NOTE, 10 μg). E-cadherin was also present in the whole cell control extractions, as expected (lane 1; NOTE, 20 μg). This indicates that no contamination of the extracts occurs. Extracts (SNO cell line shown) were electrophoresed through a 10% SDS-PAGE and immunoblotted with anti-E-cadherin (Sigma). Molecular weight was determined from the protein ladder transferred from the PAGE gel to the nitrocellulose membrane during immunoblotting (lane 4; 170, 130, 100 and 70 kD).

the visible PageRuler™ protein ladder (Fermentas Life Sciences), which was simultaneously transferred to the nitrocellulose with the protein. As expected, a similar result was found in the whole cell extracts, which was loaded as a reaction control. However, E-cadherin was not detected in the cytoplasmic fraction. This indicates that no contamination of the extracts occurred.

A strong presence of β-catenin (94 kD) was found in the plasma membrane fraction of all the human oesophageal SCC cell lines as detected by immunoblotting (see Figure 2.4.a, indicated by the black arrow). β-catenin was also identified in the cytoplasmic fractions of all the oesophageal SCC cell lines (see black arrow in Figure 2.4.b), although required twice the amount of total protein (5 μg) than was needed for the detection in the membrane fraction (2.5 μg). Interestingly, an 80 kD band occurred consistently in the immunoblots of the plasma membrane fraction, as well as a number of low molecular weight polypeptides (indicated by the red arrows). These most likely represent degradation products (see Discussion for details). The CACO-2 cell line was used as a positive control for cytoplasmic β-catenin staining as it possesses a constitutive Wnt-like effect due to stabilizing mutations of the *APC* and *CTNNB1* genes (Ilyas *et al*., 1997; Sampson *et al*., 2001). This cell line showed β-catenin was strongly expressed in both the membrane (5 μg, Figure 2.4.a, lane 7) and the cytoplasmic (5 μg, Figure 2.4.b, lane 7) fractions. The presence of β-catenin in the cytoplasmic fraction of the CACO-2 control indicates that the cytoplasmic extraction was efficient and successful. From these results, it appears that in the human oesophageal SCCs examined the majority of β-catenin is associated with the plasma membrane with lower amounts elsewhere in the cell, suggesting that the cytoplasmic fraction is indeed regulated most likely by the degradation pathway (see Chapter 1.6).

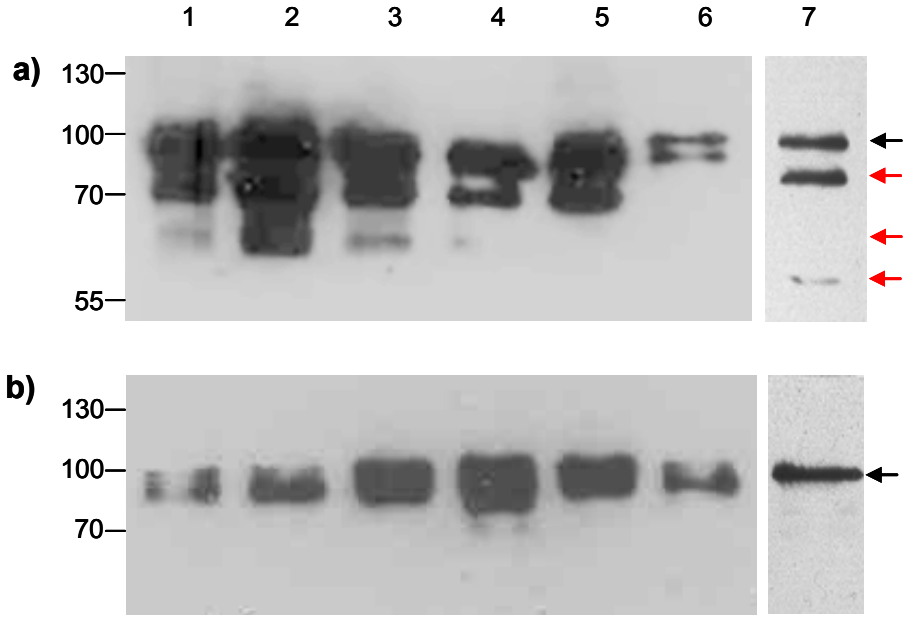


Figure 2.4: β-catenin expression occurs in both the plasma membrane and the cytoplasmic fractions of oesophageal SCC cell lines. Protein from **a)** the plasma membrane (2.5 μg) and **b)** the cytoplasmic (5 μg) cellular fractions were separated and blotted specifically for β-catenin. **a)** The presence of β-catenin was identified in the membrane fractions of all cell lines as strong bands were detected at 94 kD (black arrow). An 80 kD band and a number of smaller polypeptides were also exposed in this fraction (red arrows) and are likely to represent partial degradation products (Ninomiya *et al.,* 2000; Takahashi *et al.,* 1998). **b)** CACO-2 cell line, which reportedly has stabilized cytoplasmic β-catenin (Sampson *et al.,* 2001), exposed a strong β-catenin band in this fraction (black arrow). β-catenin was also detected in the cytoplasm extracts of the oesophageal SCC cell lines, although at lower levels than the plasma membrane, suggesting that cytoplasmic and nuclear β-catenin is regulated in the oesophageal SCCs. Lanes 1-5 and 7 represent WHCO1, WHCO3, WHCO5, WHCO6, SNO and CACO-2 cell lines, respectively. Protein from a standardized control extraction (5 μg), which enabled comparisons between immunoblots, is represented in lane 6. Molecular weight markers (130, 100, 70 and 55 kD) are indicated on the left.

2.3.2 β-catenin is located mainly at the plasma membrane in human oesophageal SCCs

In human oesophageal SCC E-cadherin has been previously localized to points of cell-cell contact in these cell lines, giving the typical "cobblestone" appearance (Jones and Veale, 2003). Since the greatest concentration of β-catenin was identified in the membrane fraction, it is assumed to be interacting with E-cadherin, and thus would be expected to be localized just below the plasma membrane. Figure 2.5 (a-e) shows that in the human oesophageal SCC cells β-catenin locates at the plasma membrane at points of cell-cell contact (as indicated by the yellow arrows), but is weak or absent at contact-free edges (blue arrow in 2.5.b). This pattern is similar to that of E-cadherin staining in these cell lines (Jones and Veale, 2003). Thus, as described for other cell types, β-catenin colocalizes to facilitate its association with E-cadherin in oesophageal cells. However, dual labelling of both β-catenin and E-cadherin with different fluorophores would be required to positively ascertain this.

The CACO-2 control (Figure 2.5.f) displays greatly enhanced nuclear staining (as indicated by the white arrow), with concomitantly reduced plasma membrane staining. The WHCO3 cell line showed weak staining in the cytoplasm (indicated by the pink arrow) and the faint but apparent central staining seen in WHCO1 and WHCO5 (indicated by white arrows) indicates a minimal presence of β-catenin in the nucleus in these cells.

2.3.3 Comparison of the total level of β-catenin expression between moderately-differentiated human oesophageal SCC cell lines

Having shown the presence and localization of β-catenin in the five moderately-differentiated human oesophageal SCC cell lines, the level of β-catenin expression in these cell lines was assessed by RIA. Comparison of the counts per minute (CPM)/10^5 cells using a two-way ANOVA followed by Tukey's Honesty

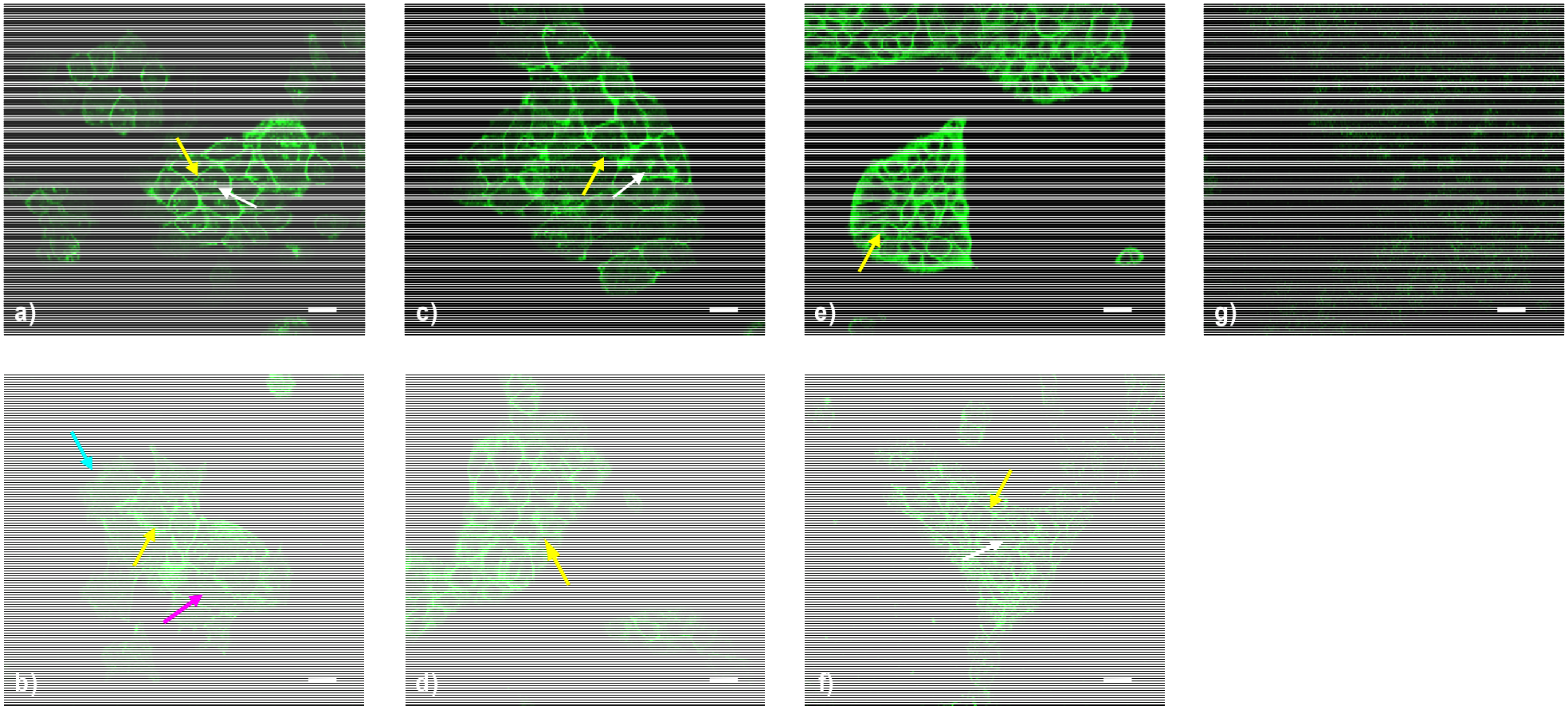


Figure 2.5: Composite confocal images of β-catenin staining by indirect immunofluorescence. The fluorescent green image represents the localization of β-catenin in **a)** WHCO1, **b)** WHCO3, **c)** WHCO5, **d)** WHCO6, **e)** SNO and **f)** CACO-2 cells. **a-e)** The majority of β-catenin locates at the plasma membrane at points of cell-cell adhesion (indicated by the yellow arrows), but is absent at contact-free edges (shown by the blue arrow in **b**). This is similar to the reported localization of E-cadherin in these cells (Jones and Veale, 2003), suggesting a possible association between these proteins. Weak cytoplasmic staining was noted in WHCO3 (shown by the pink arrow) and the faint central staining in WHCO1 and WHCO5 (white arrows) suggests β-catenin is present in the nucleus. **f)** CACO-2 (control) displays nuclear localization of β-catenin (white arrow), with low levels present at the plasma membrane (yellow arrow). **g)** The negative control (for example WHCO6 cells) shows that non-specific binding by the secondary antibody is negligible. Scale bar = 25 mm (FITC excitation 490, emission 525).

Significant Difference (HSD) test indicated that no significant difference ($F_{(4, 42)} = 1.09$; $p = 0.37$) occurred in the level of β-catenin expressed between the oesophageal SCC cell lines (see Figure 2.6). However, although the differences are not significant it is worth noting that the WHCO3 and SNO cell lines display a lower level of β-catenin than the other three lines.

The ANOVA also indicated that there was no skewing of the results due to background effects as the difference between the experiment and the control is highly significant ($F_{(1, 42)} = 60.69$; $p < 0.001$). In addition, no interaction occurred between the experiment and the control ($F_{(4, 42)} = 0.79$; $p = 0.54$). Detailed statistics tables can be found in Appendix 4.1 (Tables A and B).

2.3.4 Comparative distribution of β-catenin expression

Densitometry of immunoblotted extracts from the plasma membrane and cytoplasm enabled a semiquantitative comparison of the levels of β-catenin occurring in these two regions. The level of membrane-associated β-catenin (see Figure 2.7, blue bars) observed in WHCO1 (48%) and SNO (68%) cells are substantially lower than those found in WHCO3 (100%) and WHCO6 (98%) cells, while WHCO5 cells appear to express a medium level of membrane β-catenin (85%). Thus, considerable variation in the levels of β-catenin associated with the membrane components occurs between the five oesophageal SCC cell lines. The expression levels were represented as a percentage of the maximum per 5 μg of protein, which occurred in the membrane fraction of WHCO3 cells. Note, in order to obtain a clear image, only 2.5 μg total protein from the plasma membrane fraction was used to detect β-catenin in the immunoblot (refer back to Figure 2.4). Thus, densitometry of this immunoblot was adjusted to represent the level of β-catenin per 5 μg of total protein to facilitate direct comparison between sample extracts.

The levels of cytoplasmic β-catenin (yellow bars) were compared to that found in the membrane. As mentioned above (2.2.1), the CACO-2 cell line has been

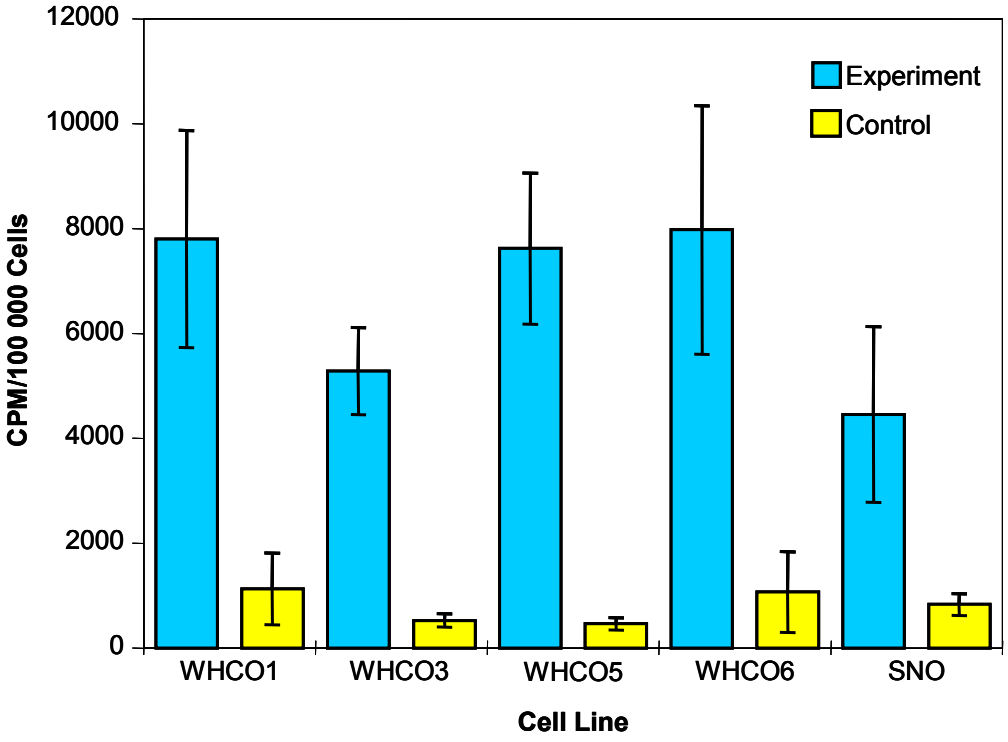


Figure 2.6: Total β-catenin expression in human oesophageal SCCs. The level of β-catenin was assessed by a modified RIA and expressed as CPM/10^5 cells. β-catenin expression (blue) does not differ significantly between the five moderately-differentiated oesophageal SCC cell lines ($F_{(4, 42)} = 1.09$; $p = 0.37$). However, it should be noted that WHCO3 and SNO display slightly lower levels of the protein than the other three cell lines. The control (yellow) indicates non-specific binding of ^{125}I-Protein A is statistically negligible ($F_{(1, 42)} = 60.69$; $p < 0.001$) and no interaction occurred between the experimental and control groups ($F_{(4, 42)} = 0.79$; $p = 0.54$).

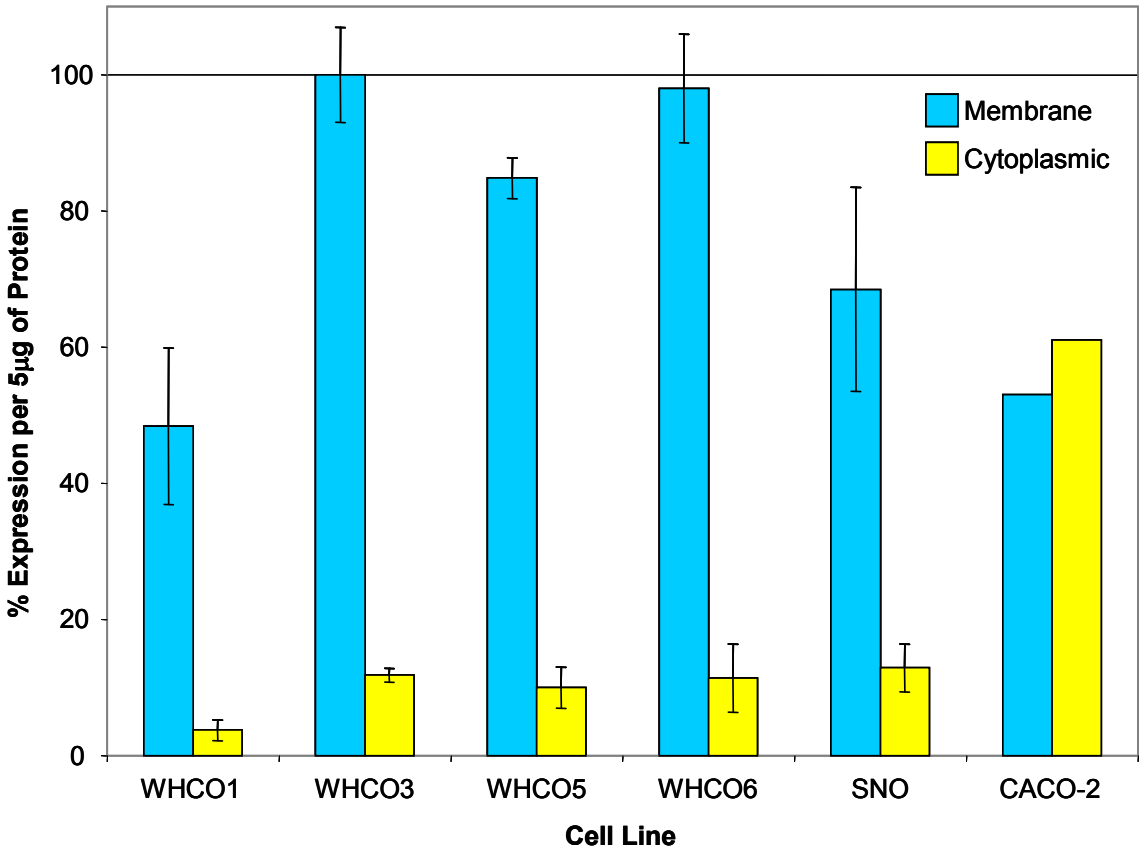


Figure 2.7: Comparative analysis of β-catenin expression in the plasma membrane and cytoplasmic fractions. The expression of β-catenin present in the plasma membrane (blue) and in the cytoplasm (yellow) fractions were analysed semi-quantitatively by densitometry of the immunoblot. The plasma membrane levels of β-catenin varied among the five oesophageal SCC cell lines with WHCO1 and SNO expressing the lowest levels, while WHCO3 and WHCO6 expressing the highest levels. The level of cytoplasmic β-catenin in the oesophageal cell lines is low (4-13%) compared to the membrane-associated β-catenin (48-100%). The CACO-2 control shows that the cytoplasmic component of β-catenin is substantially increased by its constitutive Wnt stimulation (61%), and is higher than the membrane component (53%). Expression levels are represented as a percentage of the maximum per 5 µg of protein from each fraction. Note, no standard error bars were included for the CACO-2 cells (refer to text 2.3.4).

characterised as having constitutively active Wnt signalling, as well as mutations of the *APC* and *CTNNB1* gene, which lead to stabilization of cytoplasmic β-catenin (Ilyas *et al*., 1997; Sampson *et al*., 2001). Ilyas *et al*. (1997) also reported that the mutated β-catenin in this cell line still associates with E-cadherin and α-catenin, retaining some of its membrane association. Thus, as expected, the level of β-catenin detected in the cytoplasmic fraction of these cells was high (61%) and in fact, was in slight excess of its membrane fraction (53%). As this was in accordance with the previously published information, this was not repeated and thus did not lend itself to the inclusion of standard error bars. In contrast, the level of cytoplasmic β-catenin in the human oesophageal SCC cell lines is extremely low (4-13%) in comparison to the level of membrane-associated β-catenin (48-100%). This modest level of expression of β-catenin in the cytoplasm indicates that in these cell lines, free, cytoplasmic β-catenin is actively degraded and is not constitutively stabilized.

2.4 Discussion

β-catenin has been implicated in the regulation of the strength of adhesion as it plays a central role in linking the plasma membrane-bound cadherin molecule to the cell's cytoskeleton (Hinck *et al*., 1994b; Gumbiner, 1996; Takayama *et al*., 1998). Reduction of β-catenin at cell-cell contacts is associated with the progression and invasion of many tumours (Oyama *et al*., 1994; Kawanishi *et al*., 1995; Ilyas *et al*., 1997; Jawhari *et al*., 1997; Takayama *et al*., 1998; Odajima *et al*., 2005). Also, deregulation of the cytoplasmic levels of this protein has been linked to the progression of many tumours, such as colorectal carcinomas, due to its ability to activate transcription (Munemitsu *et al*., 1995; Behrens *et al*., 1996; Korinek *et al*., 1997; Morin *et al*., 1997; Rubinfeld *et al*., 1997). The present set of experiments focus on whether human oesophageal SCC cell lines derived from moderately-differentiated tumours express similar levels of β-catenin. Further, determining the distribution of the β-catenin protein in these lines would be indicative of its status in signal transduction and gene expression.

There have been a number of attempts to evaluate the levels of β-catenin in oesophageal SCCs. These studies employed immunohistochemical techniques and a scoring method to determine the level of β-catenin expression. These studies were conducted on tissue sections taken from tumours across the range of pathological grades of oesophageal SCCs and compared to normal oesophageal mucosal expression. Although one group found that β-catenin levels were elevated in oesophageal SCC (Shiozaki *et al*., 2000), most found that oesophageal SCCs expressed lower levels of β-catenin than normal oesophageal mucosa (Nakanishi *et al*., 1997; Sanders *et al*., 1998; De Castro *et al*., 2000; Zhao *et al*., 2003; Lin *et al*., 2004; Zhang *et al*., 2005). However, these researchers admitted to inconsistencies between these results with reports of tumours displaying reduced β-catenin expression ranging from 73% to only 35%. The subjectivity of the immunohistochemical technique is cited as the likely cause of the observed variation (Shiozaki *et al*., 2000; Zhao *et al*., 2003; Lin *et al*., 2004). In addition,

there were contradictions with regard to the statistical significance between β-catenin expression and clinopathological features, such as survival rates and tumour grade. Thus, in order to reduce complications arising from using differing pathological grades, the present study determined the expression of β-catenin within a single pathological grade of oesophageal SCC, the moderately-differentiated grade. In addition, instead of using immunohistochemical means to evaluate β-catenin levels, a modified RIA was employed in an attempt to provide a less subjective and more quantifiable method of assessing β-catenin expression.

The potential of β-catenin as a molecular marker for this particular pathological grade of oesophageal SCC appears promising as RIA analysis of the level of β-catenin expressed showed that no significant variation occurred between the five cell lines. Ultimately, however, similar data from the poorly- and well-differentiated grades would need to be obtained for comparative purposes. However, as far as it is known, there are no *bona fide* cell lines representing these grades in South Africa due to the difficulties in establishing these lines. RIA is useful in quantifying the total level of protein expression, however it does not provide information as to the distribution of that protein within the cell. As many tumours have displayed an altered distribution of β-catenin (Klymkowsky, 2005), it is important to identify its localization which would imply the functional role it may play in the development of this type of tumour.

In normal epithelia, β-catenin is found just below the plasma membrane in association with the cadherins that are involved in cell-cell adhesion (Sanders *et al*., 1998; Takayama *et al*., 1998; Shiozaki *et al*., 2000). The oesophageal SCC cells examined here display the "cobblestone" pattern of β-catenin staining at the plasma membrane at points of cell-cell contact (see Figure 2.5). This pattern parallels the E-cadherin staining noted by Jones and Veale (2003) in the same cell lines, suggesting that the association between E-cadherin and β-catenin is not compromised in this type of tumour. This data is corroborated by two immunohistochemical studies which showed that although β-catenin levels were

reduced in oesophageal SCCs, the majority of β-catenin was preserved at the plasma membrane (Sanders *et al*., 1998; Lin *et al*., 2004).

A previous study (using densitometry of immunoblots) indicated that β-catenin concentrations at the plasma membrane were reduced in oesophageal SCC tumour sections in comparison to normal oesophageal mucosa (Kimura *et al*., 1999). These comparisons made use of a range of histological grades but did not determine if differences of β-catenin occurred within a particular grade. The current investigation showed that within the moderately-differentiated oesophageal SCC cell lines, the level of β-catenin at the plasma membrane varied from 48% to 100%, with the lowest level seen in WHCO1 and SNO cells (see Figure 2.7). One could speculate that these cells have a higher metastatic potential than the other three lines through the reduction in cell-cell adhesion.

As mentioned above, stabilization of β-catenin in the cytoplasm has been linked to the progression of multiple types of tumours. Most studies have focused on mutations that lead to the deregulation of cytoplasmic β-catenin. Mutations in the β-catenin gene, *CTNNB1*, that result in the deletion of the serine GSK-3β phosphorylation sites, have been noted in colorectal, prostate, hepatocellular, thyroid, endometrial, ovarian carcinomas, as well as in melanoma cell lines and in medulloblastomas (Morin *et al*., 1997; Rubinfeld *et al*., 1997; Fukuchi *et al*., 1998; Miyoshi *et al*., 1998; Palacios and Gamallo, 1998; Takahashi *et al*., 1998; Voeller *et al*., 1998; Zurawel *et al*., 1998; Garcia-Rostan *et al*., 1999). However, similar assays performed on oesophageal SCC did not detect mutations of *CTNNB1* (Sanders *et al*., 1998; Ninomiya *et al*., 2000; De Castro *et al*., 2000; Zhang *et al*., 2005), nor was this detected in the cell lines used in the present study (Schnugh, 2001). Specific mutations of *APC* that lead to the deregulation of cytoplasmic β-catenin in colorectal carcinomas, were also reported by Powell *et al*. (1994) to be rare in oesophageal SCCs. However, it has been noted in a number of tumours that mutations of *CTNNB1* or *APC* are not required for β-catenin stabilization and nuclear accumulation (Omholt *et al*., 2001; Al-Aynati *et al*., 2004; Odajima *et al*., 2005). Two immunohistochemical studies described that

a small percentage of cells from oesophageal SCC tumour sections showed cytoplasmic and nuclear β-catenin staining and that this occurs more frequently in the more poorly-differentiated tumours (Sanders *et al*., 1998; De Castro *et al*., 2000). The current investigation showed through immunoblotting, that β-catenin was present in the cytoplasm of the moderately-differentiated oesophageal SCC cell lines (see Figure 2.4). However, the level at which it was expressed was considerably lower than that found at the plasma membrane (Figure 2.7), suggesting that the primary role for β-catenin remains in mediating cell-cell adhesion in these cells. Kimura *et al*. (1999) also used densitometry as a means to measure the levels of β-catenin found in the cytoplasm of oesophageal SCC tissue sections taken over a range of pathological grades. They found that two distinct groups occur with regard to cytoplasmic β-catenin expression. The first group expressed cytoplasmic β-catenin levels equivalent to that found in normal oesophageal mucosa, while the second group show levels similar to that found in colorectal carcinomas with known β-catenin stabilization. The present study showed that in comparison to the CACO-2 cell line, which has constitutively active Wnt signalling (Ilyas *et al*., 1997; Sampson *et al*., 2001), and thus display a high level of cytoplasmic β-catenin, the oesophageal SCC cell lines express low, possibly normal, levels of β-catenin in the cytoplasm. This was further demonstrated by immunofluorescence that showed little, if any, cytoplasmic and nuclear β-catenin staining in the oesophageal SCC cell lines. In contrast, a strong presence of β-catenin was noted in the nucleus of the CACO-2 cells. Thus, from these data the logical suggestion is that moderately-differentiated oesophageal SCCs fall within the first group noted by Kimura *et al*. (1999) and that elevation of cytoplasmic β-catenin levels is not a feature of this cohort of oesophageal SCCs. However, Kimura *et al*. (1999) was unable to find a correlation between the levels of cytoplasmic β-catenin and histopathological grade. Thus, the current study suggests that regardless of tumour grade, β-catenin is not constitutively stabilized in oesophageal SCC, and that the elevation of β-catenin in the cytoplasm noted by Kimura *et al*. (1999) may be the result of a transient stabilization by some inducible factor, such as a growth factor.

The 80 kD band and occasional smaller molecular weight polypeptides noted in all the membrane immunoblots (see red arrows of Figure 2.4.a), but rarely in the cytoplasmic blots, is perplexing. Although this band is commonly reported in most other studies, very few researchers comment on its presence. A report by Schroeder *et al*. (2002) found that two "species" of β-catenin, of approximately the same size difference (3-4 kD), exist in transgenic mice models. However, this has not been reported in humans. Giesberts *et al*. (1999) pronounced that hypophosphorylation of β-catenin increased its electrophoretic mobility and hence the presence of the smaller bands represented phosphorylated isoforms of the protein in human embryonal carcinoma cells used in the study. However, this cannot be the case, as the addition of phosphates by phosphorylation would not change the size of the protein sufficiently to be easily detected on a one-dimensional SDS-PAGE, and would require a two-dimensional SDS-PAGE to determine changes in the charge. The most common, and plausible, explanation for the second band in humans has been provided by Jou *et al*. (1995), Takahashi *et al*. (1998) and Ninomiya *et al*. (2000), who simply stated that the band represented a degradation product, but gave no further explanation. The addition of protease inhibitor mix to the extraction buffer suggests that this degradation must have occurred either despite their presence, or extremely rapidly before the proteins were extracted, or was due to mechanical degradation. It is more probable that these products are a consequence of a mechanical degradation event rather than via proteasomal action, as it is unlikely that proteins associated with the proteasome would found in the membrane fraction. These cleaved protein fractions may be sequestered by E-cadherin, which protect them from further degradation. This explanation is certainly plausible as Steinhusen *et al*. (2000) showed that β-catenin, truncated by caspases during apoptosis, could still associate with E-cadherin, provided that the arm-repeat region of β-catenin was not damaged. E-cadherin is known to protect β-catenin from degradation (Sadot *et al*., 1998), and thus any further cleavage and degradation of this protein is halted. The 80 kD cleaved protein fragment must still retain the epitope for the antibody, explaining its presence in the immunoblot. In the cytoplasmic fraction however,

the cleaved protein is not protected by an association with E-cadherin and therefore will continue to be cleaved further and degraded, and hence would not be detected in the immunoblot.

The data presented in this chapter indicates that the primary role of β-catenin remains in cell-cell adhesion in the oesophageal SCC cell lines, although at inconsistent levels. Also, constitutive stabilization of β-catenin in the cytoplasm does not appear to be a leading factor in the development of human oesophageal SCC. From these results it would be tempting to suggest that β-catenin may not be a major feature of the abnormal behaviour observed in these cancer cells. However, β-catenin is involved in a number of signalling pathways that affect its function/s (see Chapter 1.9). Thus, the following chapter describes an examination of how signalling pathways stimulated by epidermal growth factor (EGF) and transforming growth factor-β1 (TGF-β1), as well as the stabilization of cytoplasmic β-catenin, affect the balance between plasma membrane-associated β-catenin and that found elsewhere in the cell.

CHAPTER THREE

The Effect of Wnt and Growth Factor Stimulation on the Distribution of β-catenin in Moderately-Differentiated Oesophageal SCC Cells – Is there Crosstalk between these Pathways?

β-catenin has been shown to participate in i) cell adhesion, when associated with the plasma membrane-bound cadherin proteins, and ii) signal transduction pathways, when stabilized in the cytoplasm (refer to Chapter 1.3 and 1.9 for details). Anomalies to either of these functions can be significant in the development, invasion and metastasis of a tumour (see Chapter 1.4, 1.7 and 1.9). It is therefore, important to understand the balance that occurs between cytoplasmic and plasma membrane-associated β-catenin and the factors that affect this. A number of signalling pathways, in particular those induced by growth factors and Wnt, impinge on β-catenin's structural and signalling role. Thus, the following work aims to track changes, induced by exogenous signals (lithium and growth factors), to the expression of plasma membrane-associated β-catenin and that found elsewhere in the cell.

Canonical Wnt signalling induces stabilization of cytoplasmic β-catenin by inhibiting its degradation complex (reviewed in Behrens and Lustig, 2004; Moon *et al*., 2004; Weeraratna, 2005; also see Chapter 1.9.1). This enables β-catenin to translocate to the nucleus where it, together with Lef/Tcf, activates transcription of a number of genes, including oncogenes (Behrens *et al*., 1996; Huber *et al*., 1996; Molenaar *et al*., 1996; Korinek *et al*., 1997; Rubinfeld *et al*., 1997; also see Chapter 1.7.1 for details). Although there has been a concerted effort to uncover the mechanisms surrounding this aspect of β-catenin signalling, little is known about the effect the increase of cytoplasmic β-catenin has on its adhesive function. From the previous chapter it was established that there is no significant innate constitutive stabilization of β-catenin in human oesophageal SCC cells. However,

Tanaka *et al*. (1998) showed that oesophageal SCCs express a novel Frizzled receptor (FzE3), which leads to the increase of nuclear β-catenin. This suggests that oesophageal SCCs are receptive to wnt-induced signalling. Thus, in order to establish whether wnt-induced stabilization of β-catenin has an effect on its structural role in oesophageal SCCs, an exogenous mimic of the Wnt signal will be used. Lithium induces similar effects to wnt stimulation during *Drosophila* and *Xenopus* development and is widely considered to be an effective mimic of the Wnt signal (Pierce and Kimelman, 1995; Klein and Melton, 1996; Schneider *et al*., 1996; Stambolic *et al*., 1996; Hedgepeth *et al*., 1997). It is able to inhibit GSK-3β activity by inhibiting autoregulatory phosphatases that maintains GSK-3β in an activated, dephosphorylated state, and is able to increase the levels of stable cytoplasmic β-catenin (Harwood *et al*., 1995; Hedgepeth *et al*., 1997; Orford *et al*., 1997; Willert *et al*., 1999; Chen *et al*., 2000; Zhang *et al*., 2003).

EGFR activity induces cell scattering and has been linked to changes in cell morphology, such as plasma membrane ruffling and cell rounding, both of which are needed for cell migration and division (Hoschuetzky *et al*., 1994; Shibamoto *et al*., 1994; Shiozaki *et al*., 1995; Fujii *et al*., 1996). The receptors concentrate at the plasma membrane with the cadherins (Fukuyama and Shimizu, 1991; Takahashi and Suzuki, 1996), and disrupt association with the cytoskeleton through tyrosine phosphorylation of β-catenin, thus altering adhesion and increasing the propensity to metastasise (Hoschuetzky *et al*., 1994; Ochiai *et al*., 1994; Shibamoto *et al*., 1994; Shiozaki *et al*., 1995; Fujii *et al*., 1996; Shibata *et al*., 1996; Takahashi *et al*., 1997; Hazan and Norton, 1998).

The EGFR is also thought to indirectly inactivate GSK-3β via its activation of the MAPK pathway, which in turn activates the GSK-3β inhibitor $p90^{rsk}$ (Stambolic and Woodgett, 1994; Eldar-Finkelman *et al*., 1995). In addition, EGFR may also stimulate the activation of another GSK-3β inhibitor, PKB through PI3K (Cross *et al*., 1995; Okano *et al*., 2000; Andl *et al*., 2003). Thus, a potentially important outcome of this is that EGF signalling may lead to the accumulation of stabilized β-catenin.

Veale and Thornley (1989) demonstrated that South African WHCO- and SNO cell lines have significantly increased expression of single cell low-affinity EGFRs (especially in the WHCO3 cell line) to that of normal human oesophageal keratinocytes. Hence, overactivity of this receptor is suspected to play a role in the development of this type of tumour, and therefore the effects of EGFR on β-catenin may provide an indication as to its influence in this process.

TGF-β1 has been shown to play a paradoxical role in tumour development, initially as a suppressor of proliferation and growth, and subsequently as a promoter of invasion and metastasis (Janji *et al*., 1999; Massagué *et al*., 2000; Weeks *et al*., 2001; Satterwhite and Neufeld, 2004; Xu *et al*., 2003). In these late stage tumours, TGF-β1 stimulates dissociation of the adherens junctions (Bakin *et al*., 2000), loss of contact with the cytoskeleton and subsequently alterations to the actin cytoskeleton that induce changes to cellular morphology (Cui *et al*., 1996; Portella *et al*., 1998; Akhurst and Balmain, 1999; Janji *et al*., 1999; Ellenrieder *et al*., 2001; Weeks *et al*., 2001; Tian and Phillips, 2002; Tian *et al*., 2003; Xu *et al*., 2003). TGF-β1 has been shown to alter the expression of adherens junction proteins in various epithelial cell lines. For example, TGF-β1 reduced the levels of E-cadherin in a metastasising melanoma cell line (HT-144; Janji *et al*., 1999), a hepatocellular carcinoma cell line (SMMC-7721; Xu *et al*., 2003), and in renal proximal tubular epithelial cells (HK-2; Tian and Phillips, 2002). TGFβ-1 has also been shown to increase the levels of tyrosine phosphorylated β-catenin resulting in its dissociation from E-cadherin and α-catenin (Tian and Phillips, 2002; Vogelmann *et al*., 2005).

TGF-β1 signalling has also been noted to increase the levels of stable cytoplasmic and nuclear β-catenin (Janji *et al*., 1999; Tian and Phillips, 2002; Xu *et al*., 2003; Masszi *et al*., 2004; Satterwhite and Neufeld, 2004; Edlund *et al*., 2005). It may do so by inducing a redistribution of β-catenin from the plasma membrane (Janji *et al*., 1999; Weeks *et al*., 2001). Alternatively, it may interfere with β-catenin's

degradation complex by either activating PKB, which inhibits GSK-3β activity through phosphorylation (Cheon *et al*., 2004; Edlund *et al*., 2005), or by decreasing the expression of APC (Satterwhite and Neufeld, 2004). TGF-β1 signalling is also able to modulate the transcriptional activity of the β-catenin/Lef/Tcf complex through the Smad proteins (Ishitani *et al*., 1999; Labbé *et al*., 2000; Ishitani *et al*., 2003; Xu *et al*., 2003; Cheon *et al*., 2004; Masszi *et al*., 2004; Warner *et al*., 2005).

Of importance to the study of oesophageal SCCs in particular, Ozanne *et al*. (1986) showed that TGF-β did not induce growth inhibition in oesophageal SCCs as it does in normal keratinocytes, suggesting that the oesophageal SCCs respond to TGF-β as an activator rather than a suppressor of tumour progression. This has been supported by studies that have shown that the expression of the TGF-β1 receptor-I (TβRI), which is associated with its role as a tumour suppressor, is lower in oesophageal SCCs than normal oesophageal mucosa and is associated with a poorer prognosis (Koliopanos *et al*., 2002; Fukai *et al*., 2003). The levels of TGF-β1 have also been reported to be elevated in approximately 50% of oesophageal SCCs (Koliopanos *et al*., 2002; Natsugoe *et al*., 2002; Fukai *et al*., 2003; Fukuchi *et al*., 2004). In addition, the expression of the TGF-β1 effector molecule, Smad-4, has been shown to correlate with the stage of tumour progression (such as invasion depth and lymph node metastasis) and the rate of survival in oesophageal SCCs (Natsugoe *et al*., 2002). Thus, TGF-β1 is likely to play an important role in oesophageal tumour development and its effect on β-catenin needs to be explored.

The regulation of various cellular functions is often mediated, not by a single signal transduction pathway, but through a coordinated cooperation between pathways. Crosstalk between Wnt signal transduction and growth factors has been suggested by a number of researchers. For example, crosstalk between TGF-β1 and Wnt has been shown to be important in chondrocyte differentiation (Tuli *et al*., 2003; Zhou *et al*., 2004). TGF-β1 has also been shown to augment Wnt-

induced stabilization of β-catenin and increase Lef/Tcf-mediated transcription of target genes in a number of cell lines, such as the murine embryonic maxillary mesenchymal (MEMM) cell line and in a gastric adenocarcinoma cell line (AGS) (Lei *et al*., 2004; Warner *et al*., 2005). In addition, Chen *et al*. (2000) showed that other growth factors, in particular EGF, enhanced lithium-induced accumulation of cytoplasmic β-catenin in the mouse mammary epithelial cells (C57MG) and the human transformed embryonal epithelial kidney cells (HEK-293). Similarly, the transcriptional activity of β-catenin was found to be enhanced by EGF (or HGF) in a rat bladder carcinoma cell line (NBT-II), which expresses a stabilized mutant form of β-catenin (Müller *et al*., 2002). Thus, the present study investigates the singular effect of lithium and the growth factors EGF and TGF-β1, as well as the combined effect of these exogenous signals on the distribution of β-catenin in moderately-differentiated human oesophageal SCC cell lines. The data presented here shows that EGF, but not TGF-β1, is able to enhance lithium-induced stabilization of β-catenin possibly through redistribution of β-catenin from the plasma membrane into the cytoplasm.

3.2 Methods and Materials

3.2.1 Treatment of cell cultures with lithium, EGF and TGF-β1

The oesophageal SCC cultures were treated with EGF (10 ng/ml, Sigma) for 20 minutes and 3 hours, prior to extraction. 10 ng/ml EGF has been shown to be sufficient for inducing changes in cellular morphology, which promotes dissociation and increases the invasive ability of poorly-differentiated human oesophageal SCCs (Shiozaki *et al*., 1995). The cell cultures were also treated with lithium (10 mM; Merck) alone, or together with EGF, for 3, 12 and 24 hours. The concentration of lithium (10 mM) has been shown to be efficient for inhibiting GSK-3β function in various other cell lines (Stambolic *et al*., 1996; Chen *et al*., 2000). In addition, the cells were exposed to TGF-β1 (1 ng/ml, Sigma) alone, or together with lithium, for 0.5, 1, 3, 6, 12 and 24 hours. 1 ng/ml TGF-β1 has previously been shown to provide optimum effect in these cell lines. Note, all cultures were treated while the cells were in an exponential growth phase (~70% confluent).

3.2.2 Densitometric quantification of plasma membrane and cytoplasmic β-catenin levels when under EGF or TGF-β1 signals alone, or together with lithium

The treated cell cultures were fractionated into plasma membrane and cytoplasmic components following the protocol described in Chapter 2.2.3.2, and the protein concentration was estimated as in Chapter 2.2.3.3. Protein from each extract was separated by electrophoresis (as performed in Chapter 2.2.3.4), and followed by immunoblotting for β-catenin (as described in Chapter 2.2.3.5).

A normalized loading control for the immunoblots was conducted in order to enable for subsequent comparisons between the immunoblots for densitometry. Following the same procedure as described for β-catenin (Chapter 2.2.3.5), extracts of the cell lines grown under standard tissue culture conditions, as well as

those treated as described above, were immunoblotted with polyclonal anti-α-actin antibody (1: 2 000; Sigma) for 1 hour, followed by an incubation with the polyclonal HRP-bound goat anti-rabbit secondary antibody (1:10 000) for 1 hour.

Densitometry was used to quantify the intensity of the β-catenin bands exposed in the immunoblots. The plasma membrane and cytoplasmic levels of β-catenin were expressed as a fold increase (or decrease) with respect to the standard expression of β-catenin per 5 μg total protein in these fractions in the human oesophageal SCC cell lines (= 1).

3.2.3 Radioimmunoassay evaluation of the total level of β-catenin expression when exposed to EGF

Cells were seeded into Nunc™ 24 multiwell tissue culture dishes 24 hours prior to treatment with 10 ng/ml EGF (Sigma) for 0, 0.25, 1, 3, 6, 12 and 24 hours. A RIA was performed as in Chapter 2.2.5. An analysis of covariance (ANCOVA) and Tukey's HSD (Statistica 5.1) of CPM/10^5 cells were used to assess whether the expression of β-catenin differs significantly between cell lines and at various lengths of treatment. The data subjected to statistical analysis was obtained from triplicate samples with the experiment repeated three times.

3.2.4 Indirect immunofluorescence was used to determine β-catenin localization subsequent to EGF and/or lithium treatment

Cells were seeded onto sterile cover slips and allowed to proliferate until approximately 70% confluent. The cultures were treated with lithium and/or EGF as above (see 3.2.1). Indirect immunofluorescence was conducted as described in Chapter 2.2.4, to visually track whether these signals induce a change in the localization of β-catenin.

3.3 Results

3.3.1 Lithium briefly elevates cytoplasmic β-catenin concentrations, while a delayed increase occurs in the membrane fraction

Wnt stimulation is known to induce the stabilization of cytoplasmic β-catenin, however, its effect on the plasma membrane-associated β-catenin remains vague. Thus, the oesophageal SCC cell lines were exposed to an established exogenous mimic of the Wnt signal, lithium, to determine the effect on the cellular distribution of β-catenin. The oesophageal SCC cultures were exposed to lithium for 3, 12 and 24 hours prior to extraction into plasma membrane and cytoplasmic fractions. These times were decided upon as Chen *et al*. (2000) described changes of cytoplasmic β-catenin levels in C57MG mouse mammary epithelial cells and HEK-293 cells after 3 hours of exposure to lithium, and an increase in its transcriptional activity in the nucleus after 16 hours. A 94 kD polypeptide was identified in both the plasma membrane and the cytoplasmic fractions of the oesophageal SCC cell lines treated with lithium, separated by gel electrophoresis (results not shown due to the similarity to those shown in Chapter 2.3.1, Figure 2.2). This band was identified as β-catenin through immunoblotting, and was shown to be present in the plasma membrane fraction of all the oesophageal SCC cell lines (see Figure 3.1.a, black arrows), as well as in all the cytoplasmic fractions (Figure 3.1.b), with the exception of WHCO6 treated for 3 hours (see Figure 3.1.b.ii). The 80 kD degradation product (red arrows), noted in the standard extracts (Figure 3.1.a.i), was also detected in the membrane fractions under the influence of lithium (Figure 3.1.a.ii-iv).

Lithium (3 hours) did not alter plasma membrane-associated β-catenin levels from standard concentrations, as assessed by densitometry of immunoblots (see Figure 3.2.a). However, the levels of cytoplasmic β-catenin were elevated in WHCO1, WHCO3 and WHCO5 cells at this time (Figure 3.2.b). This augmentation was most notable in the WHCO3 cells, which showed a 9.2-fold increase. The concentration of β-catenin in the SNO cells was minimally reduced at this time,

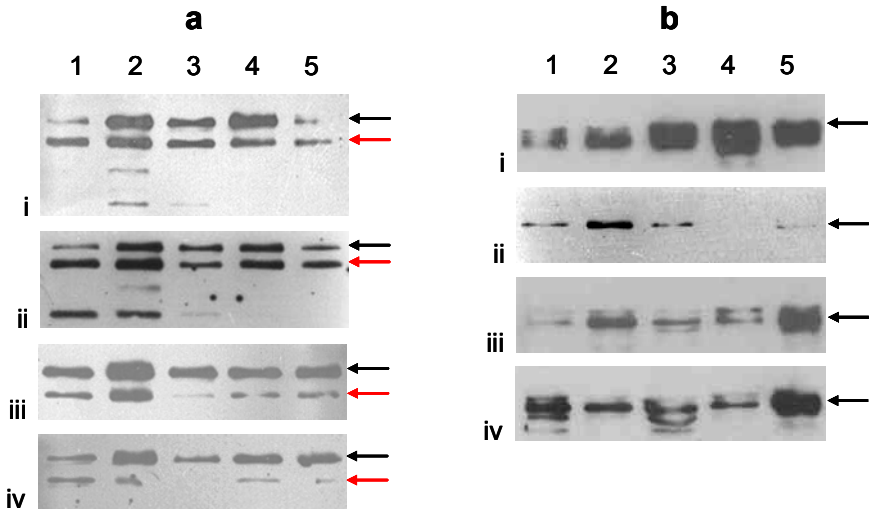


Figure 3.1: β-catenin is present in both the membrane and cytoplasmic fractions in oesophageal SCC cell lines exposed to lithium. β-catenin (94 kD, indicated by the black arrows) was identified in all the plasma membrane **(a)** and cytoplasmic **(b)** fractions of cells exposed to lithium (10 mM) for 3, 12 and 24 hours (**ii**, **iii** and **iv**, respectively) through immunoblotting. The one exception to this occurred in the WHCO6 cell line which did not show a reaction for β-catenin in the cytoplasmic fraction at 3 hours of exposure (**b**, **ii**). The immunoblot of β-catenin in these cell lines under standard tissue culture conditions is shown in (**i**). The 80 kD bands (red arrows) noted in the plasma membrane fractions represent partial degradation products (see Discussion in Chapter 2). Lanes 1-5 represent WHCO1, WHCO3, WHCO5, WHCO6 and SNO, respectively. All experiments were performed in duplicate. Please note, due to loading restrictions on the gel, various concentrations of total protein were loaded. These concentrations were noted and adjusted in subsequent analysis.

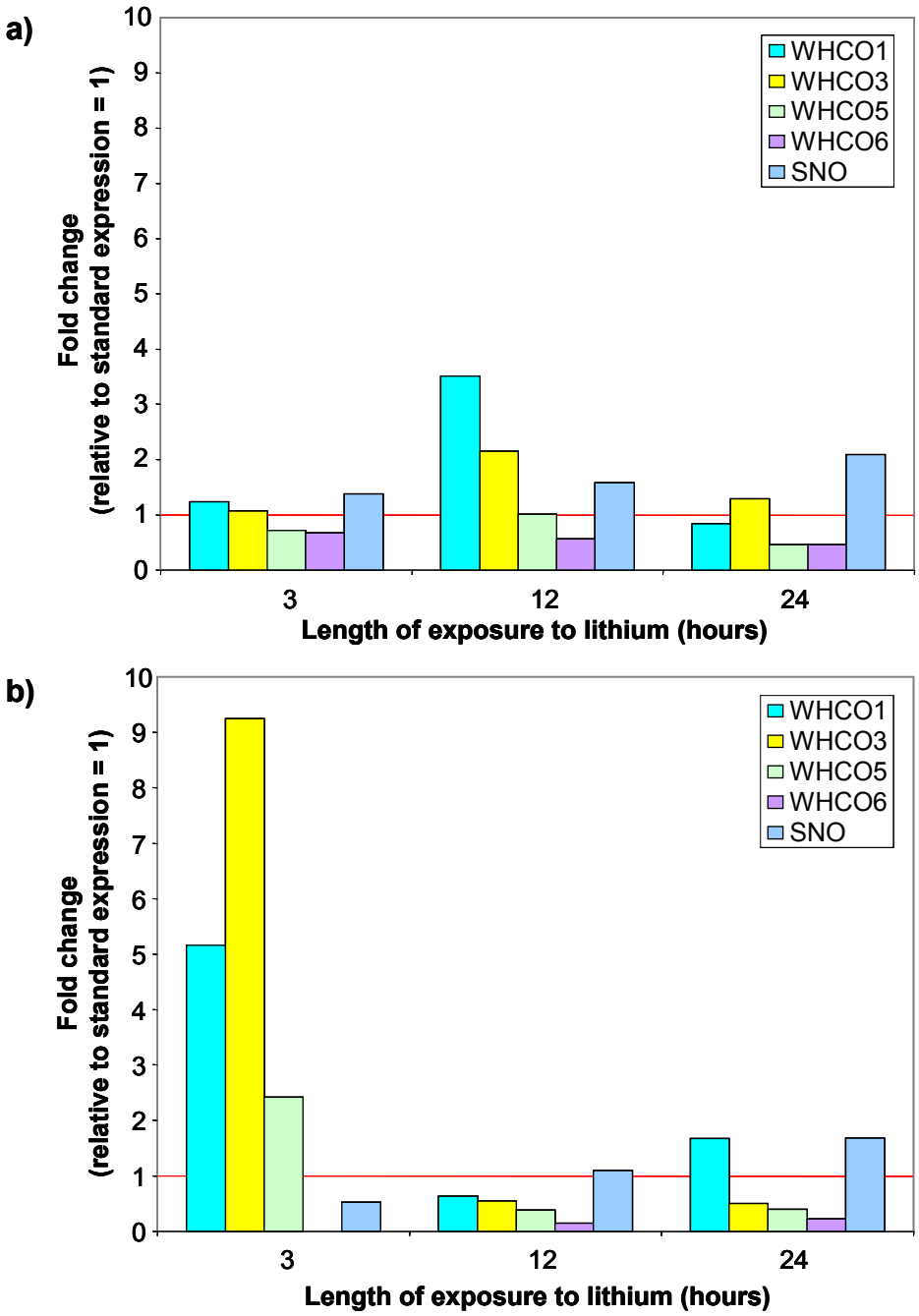


Figure 3.2: Lithium briefly induces elevated β-catenin in the cytoplasm, while a delayed increase occurs in the membrane fraction. (a) The concentration of plasma membrane-associated β-catenin (assessed by densitometry of immunoblots) was altered only after 12 hours of lithium stimulation, where an increase from standard expression (= 1, indicated by the red line*) was noted in WHCO1, WHCO3 and SNO. These levels returned to standard expression in WHCO1 and WHCO3, but rose slightly in SNO after 24 hours of exposure. **(b)** Lithium induced an appreciable increase of cytoplasmic β-catenin concentrations in WHCO1, WHCO3 and WHCO5 after 3 hours of exposure. This increase however, was short-lived as β-catenin levels returned to standard expression after 12 and 24 hours.
*The concentration of β-catenin is expressed as a fold increase (or decrease) relative to membrane (**a**) and cytoplasmic (**b**) standard expression per 5 μg of total protein.

while β-catenin was not detected in the WHCO6 cells at this duration of exposure.

After 12 hours of lithium exposure, β-catenin levels in the plasma membrane fraction increased from standard expression in WHCO1, WHCO3 and SNO by ~3.5-, 2.2- and 1.6-fold, respectively. No marked increase was noted in WHCO5 or WHCO6 at this time. The high level of membrane-associated β-catenin continued in SNO (2.1-fold increase) at 24 hours of lithium treatment, while the other cells lines returned to standard concentrations. The concentration of β-catenin in the cytoplasmic fractions however returned to levels slightly below the standard after 12 hours of exposure. This was similarly noted after 24 hours of exposure, although WHCO1 and SNO did show a small increase of 1.7-fold each. This suggests that the effect of the lithium signal on β-catenin expression in oesophageal SCC cells is short-lived and the regulatory mechanisms for β-catenin are reestablished after 12 hours of exposure.

3.3.2 EGF does not affect the total concentration of β-catenin but alters the cytoplasmic pool in moderately-differentiated oesophageal SCCs

EGF does not change the total concentration of β-catenin expression over time in the five oesophageal SCC cell lines. The total concentration of β-catenin (assessed by a modified RIA and expressed as CPM/10^5 cells) does not vary significantly at different lengths of EGF exposure within each cell line as was established by an ANCOVA of the data ($F_{(7, 205)} = 1.72$; $p = 0.11$). However, it should be noted that within the first hour of EGF treatment, a trend was observed that β-catenin levels appeared to decrease slightly in all cell lines, particularly the WHCO5 cell line (see Figure 3.3). Interestingly, the ANCOVA also highlighted that the level of β-catenin expression differs significantly between the cell lines ($F_{(4, 208)} = 8.13$; $p < 0.001$) when exposed to EGF. A Tukey's HSD test further revealed that it is the WHCO3 and WHCO5 cells that differ from both WHCO6 ($p = 0.016$ and $p = 0.015$, respectively) and SNO ($p < 0.001$ in both cases) cells. For detailed tables of statistical analyses see Appendix 4.2 (Tables C, D and E).

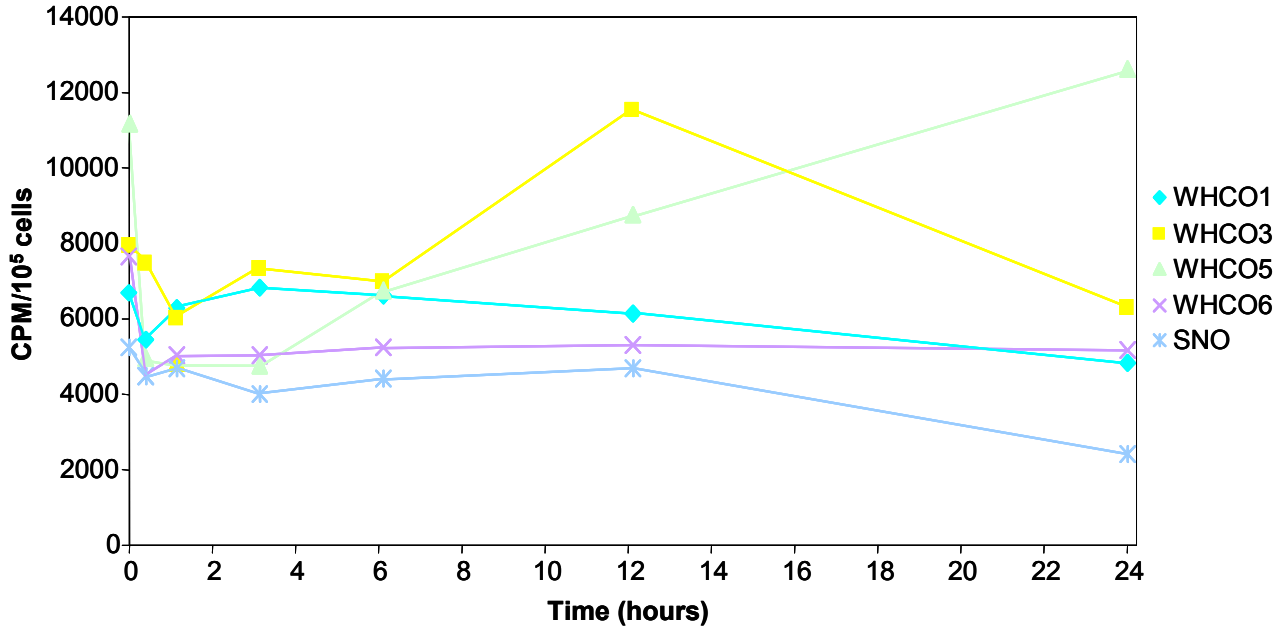


Figure 3.3: EGF does not change the total concentration of β-catenin over time. Although β-catenin concentrations (CPM/10^5 cells) tended to decrease initially (within the first hour) when exposed to EGF (10 ng/ml), no significant difference occurred between β-catenin levels *within* each cell line over the 24 hour time course of EGF treatment ($F_{(7, 205)}$ = 1.72; p = 0.11), as assessed by ANCOVA analysis of results obtained through a modified RIA. A significant difference however, was identified *between* the cell lines ($F_{(4, 208)}$ = 8.13; p < 0.001). A Tukey's HSD test identified that WHCO3 differed from WHCO6 (p = 0.016) and SNO (p < 0.001), while WHCO5 differed from WHCO6 (p = 0.015) and SNO (p < 0.001).

The addition of EGF to cells in a logarithmic growth phase is known to induce the activation and autophosphorylation of the EGFR within 5 minutes and the receptor remains in this active state for approximately 30 minutes (Takahashi and Suzuki, 1996). Due to this rapid response, the oesophageal SCC cultures that were still in an exponential growth phase, were exposed to EGF briefly (20 minutes), as well as for a longer period (3 hours) to determine the effects of EGF on the concentration of the different pools of β-catenin (i.e. membrane and cytoplasmic) in the short- and longer-term. β-catenin was identified (through electrophoresis and immunoblotting) in all the membrane extracts of these cells, with the exception of the SNO cell line after 20 minutes of EGF exposure (see Figure 3.4.a.ii). Strong bands of β-catenin immunoreacted from the electrophoresed cytoplasmic fractions of cells treated with EGF for 20 minutes, especially in WHCO1 and WHCO3 (see Figure 3.4.b.ii). This reaction was notably lower after 3 hours of EGF exposure as only faint bands of β-catenin were detected (Figure 3.4.b.iii). Quantification of these reactions, through densitometry, showed that EGF stimulation for either 20 minutes or 3 hours did not alter the concentration of plasma membrane-associated β-catenin from standard concentrations (see Figure 3.5.a). However, the concentration of β-catenin in the cytoplasmic fraction was dramatically increased in the WHCO1 and WHCO3 cell lines (~19- and 8-fold, respectively) after a short exposure to EGF (20 minutes, Figure 3.5.b). The SNO cell line showed a smaller increase at this time (~2-fold), while the WHCO5 and WHCO6 cell lines did not change. However, this increase was short-lived as the concentration of β-catenin in this fraction decreased to below standard levels after 3 hours of exposure.

3.3.3 EGF augments lithium-induced stabilization of cytoplasmic β-catenin, while decreasing membrane-associated β-catenin

There is increasing evidence that signal transduction pathways cooperate with one another to regulate cellular functions. Simultaneous exposure of the oesophageal SCC cell lines to lithium and EGF (3, 12 and 24 hours) induced interesting

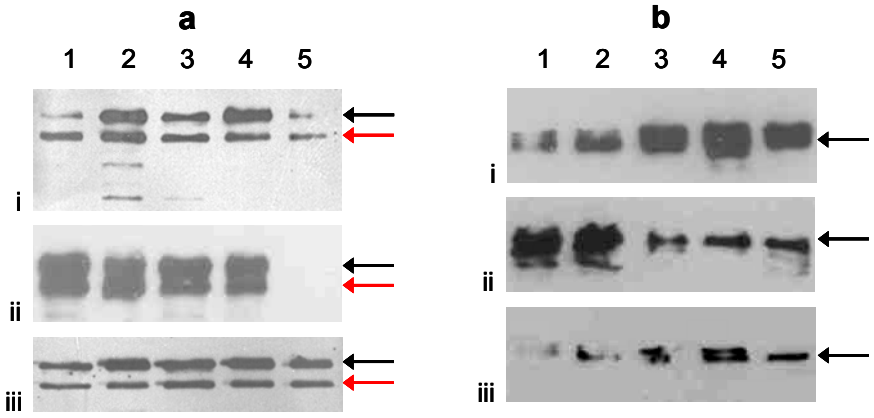


Figure 3.4: β-catenin detection in oesophageal SCC cellular fractions treated with EGF. β-catenin (94 kD, indicated by the black arrows) was detected (by immunoblotting) in the membrane fractions **(a)** of the oesophageal cell lines exposed to EGF (10 ng/ml) for 20 minutes and 3 hours (**ii** and **iii**, respectively). The SNO cell line (20 minutes) was the exception as it did not show a reaction in this fraction (**a**, **ii**). The standard expression of β-catenin at the membrane in these cell lines is shown in (**i**). The 80 kD degradation product is indicated by the red arrow (see Discussion in Chapter 2). Strong bands of cytoplasmic β-catenin **(b)** reacted after 20 minutes of EGF stimulation (**ii**), especially in WHCO1 and WHCO3. The bands detected at 3 hours of EGF exposure (**iii**), were slightly fainter than those exposed for under standard culture conditions (**i**). Lanes 1-5 represent WHCO1, WHCO3, WHCO5, WHCO6 and SNO, respectively. An α-actin loading control was conducted to enable comparisons between the blots (see Appendix 5). All experiments were performed in duplicate. Note, due to loading restrictions on the gel, various concentrations of total protein were loaded.

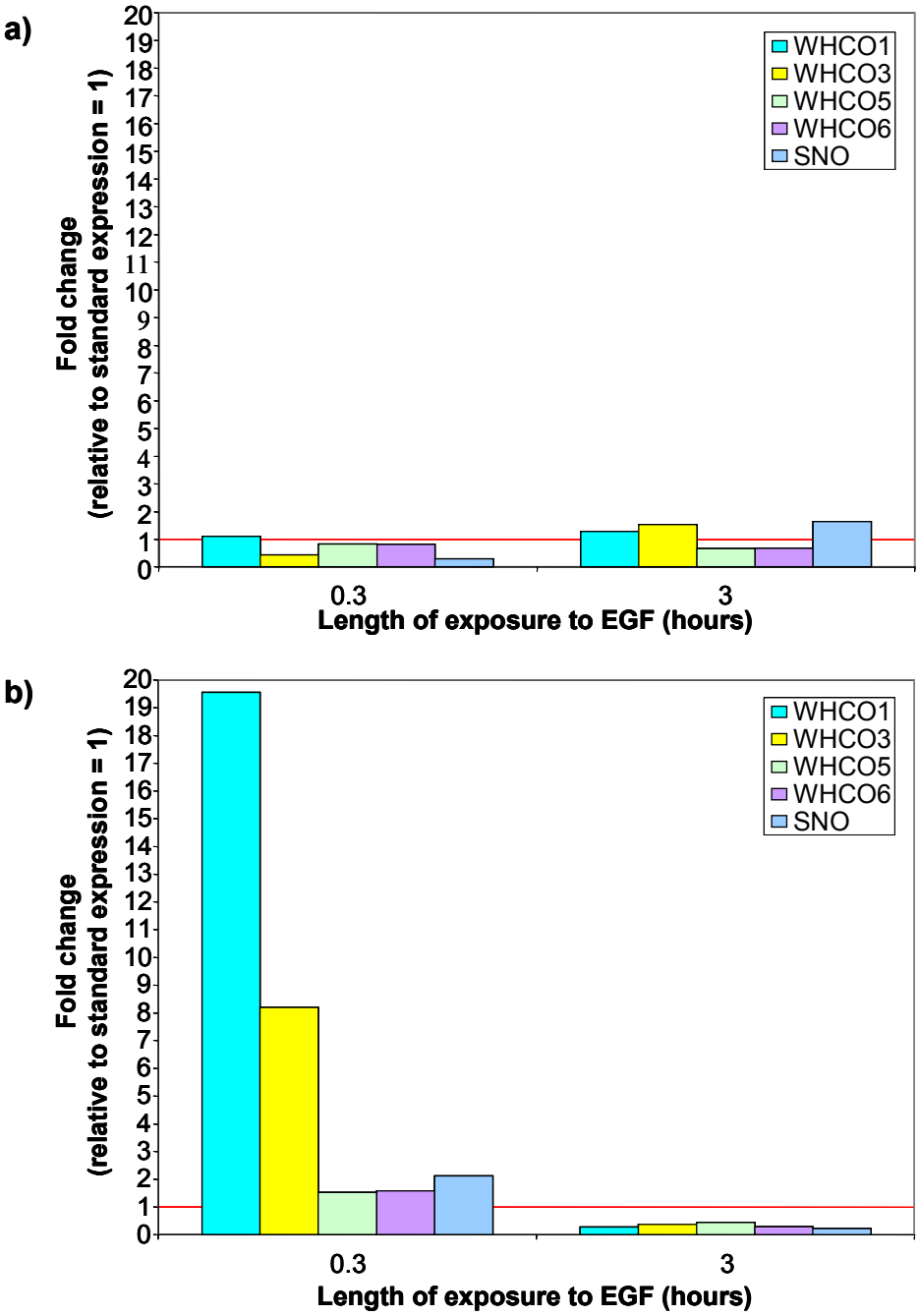


Figure 3.5: EGF briefly elevates the cytoplasmic concentration of β-catenin. EGF exposure (20 minutes and 3 hours) did not alter the concentration of β-catenin associated with the plasma membrane **(a)** from standard expression (= 1, indicated by the red line*) as determined by densitometry of the immunoblots. However, a brief exposure to EGF (20 minutes) induced a large increase of β-catenin in the cytoplasmic fractions **(b)** of the WHCO1 and WHCO3 cell lines and to a lesser extent in the SNO cell line. This increase was short-lived as the concentration of β-catenin in this fraction decreased to slightly below standard levels after 3 hours of exposure.

*The concentration of β-catenin under EGF stimulation is expressed as a fold increase (or decrease) relative to the membrane (**a**) and the cytoplasmic (**b**) standard expression per 5 μg of total protein.

changes to the concentration of β-catenin in both the membrane and cytoplasmic pools of β-catenin. The presence of β-catenin in the membrane and cytoplasmic fractions was established through immunoblotting of the electrophoresed extracts, in all the oesophageal cell lines under these conditions, with the exception of the membrane fraction of WHCO1 after 3 hours of exposure (see Figure 3.6). The concentration of β-catenin (established through densitometry of the immunoblots) in the membrane fractions was shown to be lower than standard expression after 3 hours of exposure in all five of the cells lines, as is shown in Figure 3.7.a. The cells recovered after 12 hours, although WHCO5 and WHCO6 β-catenin levels remained slightly lower than the standard concentration. After 24 hours, the cells remained at, or just below, standard concentrations of β-catenin, with the exception of WHCO1 which increased its expression in this fraction by ~2.5-fold the standard concentration.

Interestingly, the cytoplasmic component of the oesophageal SCCs under these conditions increased substantially (over 8-fold in WHCO3) after 3 hours of treatment, except for WHCO1, which only showed an increase after 12 hours of exposure (see Figure 3.7.b). The increase of β-catenin in this fraction is similar to that found with lithium alone for WHCO3 and WHCO5. However, in the WHCO6 and SNO cell lines, EGF signalling appeared to augment the concentration of β-catenin in this fraction. The enhancing effect of EGF stimulation on lithium-induced accumulation of cytoplasmic β-catenin parallels the results found by Chen *et al*. (2000) in C57MG and HEK-293 cells. In addition, concurrent treatment with lithium and EGF induced a prolonged elevation of β-catenin levels in the cytoplasmic fraction (as seen after 12 hours of exposure) of WHCO1, WHCO3 and WHCO6, while these cells treated with lithium alone returned to standard concentrations after this time. Again, these results are analogous to the enhanced nuclear activity of β-catenin noted in C57MG and HEK-293 cells, after 16 hours of stimulation (Chen *et al*., 2000). The concentration of β-catenin in the oesophageal cell lines dropped to slightly below standard concentrations after 24 hours of stimulation.

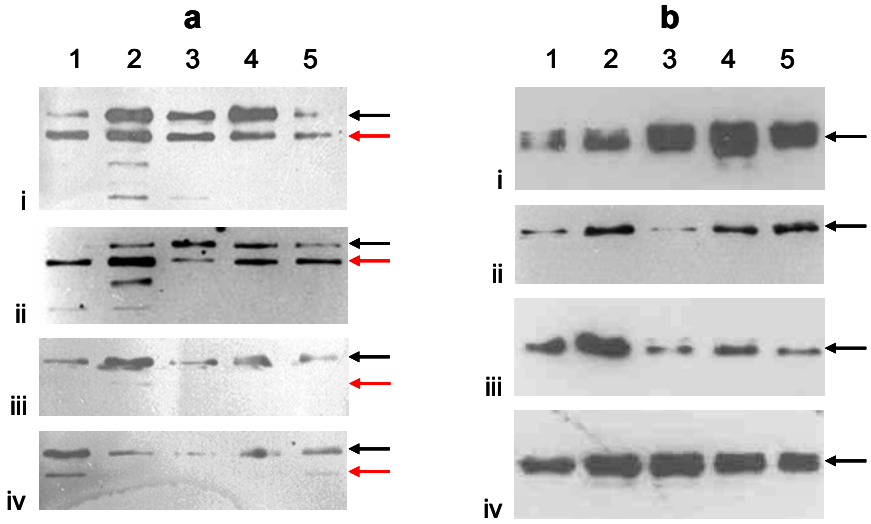


Figure 3.6: β-catenin occurs in membrane and cytoplasmic fractions in oesophageal SCC cell lines simultaneously exposed to lithium and EGF. β-catenin (94 kD, indicated by the black arrows) was identified through immunoblot in all the plasma membrane fractions **(a)** of the oesophageal SCC cell cultures exposed simultaneously to lithium (10 mM) and EGF (10 ng/ml) for 3, 12 and 24 hours (**ii**, **iii** and **iv**, respectively), with the exception of WHCO1 after 3 hours of treatment. The immunoblot of β-catenin from the plasma membrane under standard culture conditions is represented in (**i**). The 80 kD degradation product (red arrows) are present (see Discussion in Chapter 2). All the cell lines showed a strong presence of β-catenin in the cytoplasmic fractions **(b)** after 3, 12 and 24 hours of treatment (**ii**, **iii** and **iv** respectively), similar to that found under standard conditions (**i**). Lanes 1-5 represent WHCO1, WHCO3, WHCO5, WHCO6 and SNO, respectively. An α-actin loading control was conducted to enable comparisons between the blots (see Appendix 5). All experiment were performed in duplicate. Note, various concentrations of total protein were loaded due to loading restrictions on the gel.

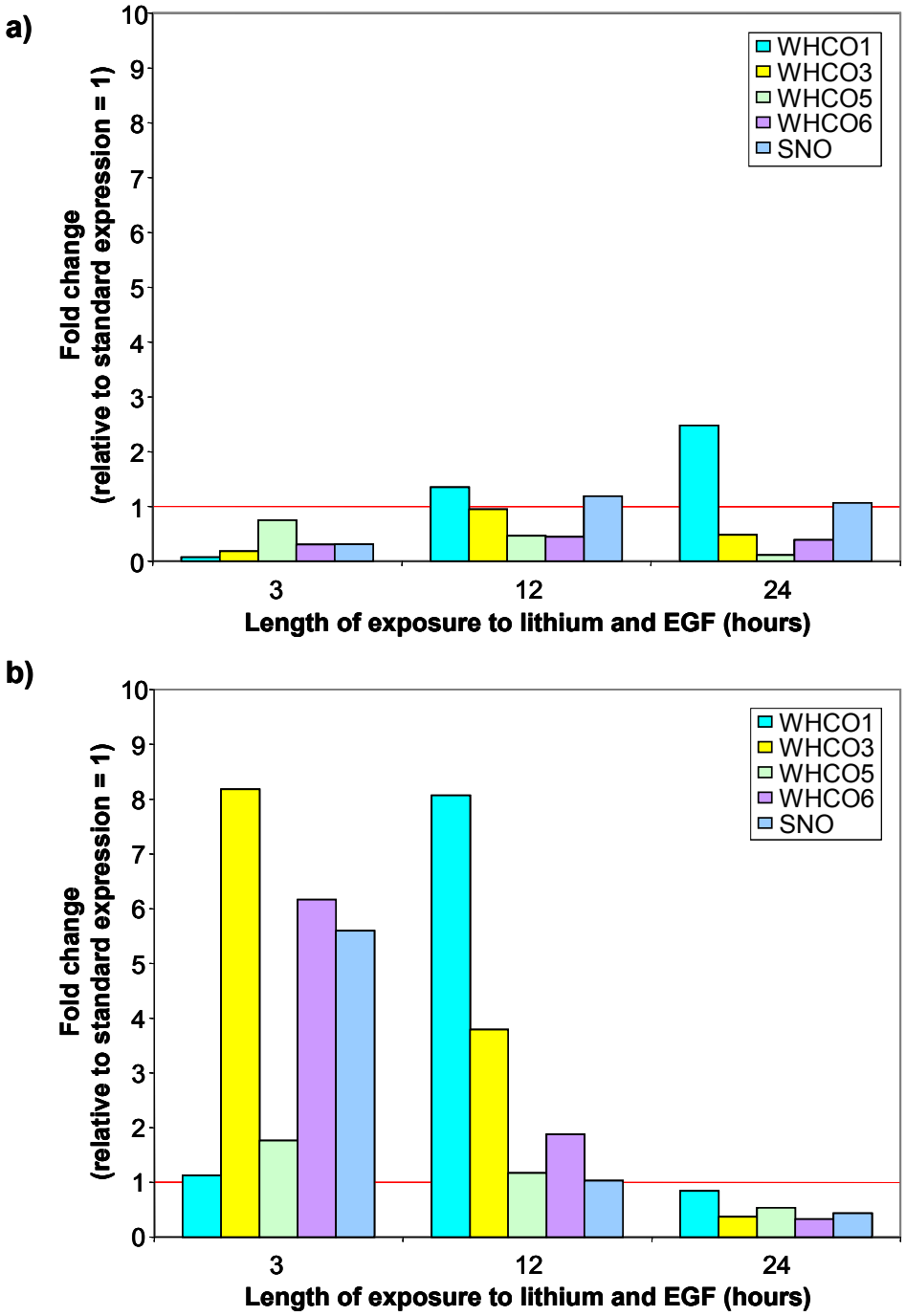


Figure 3.7: Simultaneous exposure of oesophageal SCC cells to lithium and EGF results in a prolonged increase of cytoplasmic β-catenin, while reducing the membrane-associated concentration of the protein. (a) Combined lithium and EGF signalling (3 hours) resulted in a decline of membrane-associated β-catenin from standard concentrations (= 1, indicated by the red line*) as analysed by densitometry of immunoblots. WHCO1, WHCO3 and SNO recovered after 12 and 24 hours of exposure, with WHCO1 increasing expression after 24 hours. **(b)** Cytoplasmic β-catenin increased substantially after 3 hours of exposure, except WHCO1, which only showed an increase after 12 hours. The concentration of cytoplasmic β-catenin remained elevated in WHCO1, WHCO3 and WHCO6 after 12 hours of treatment. However, all cells returned to standard concentrations after 24 hours of treatment.
*The concentration of β-catenin is expressed as a fold increase (or decrease) relative to the standard expression per 5 μg of total protein of the membrane (**a**) and the cytoplasmic (**b**) fractions.

3.3.4 The subcellular localization of β-catenin when treated with lithium and/or EGF

β-catenin remained strongly localized at points of cell-cell contact at the plasma membrane in all the oesophageal SCC cell lines treated with EGF and/or lithium as tracked by indirect immunofluorescence (indicated by the white arrows in Figure 3.8). EGF exposure (3 hours) did not appear to affect the standard distribution of β-catenin in any of the cell lines, as is shown in Figure 3.8.b for the WHCO1 cell line. Cytoplasmic staining (blue arrow) was noted in WHCO5 cells after 3 hours of lithium stimulation (see Figure 3.8.d), but returned to standard localization by 12 and 24 hours. No change in the distribution of β-catenin was detected in the other cell lines. Cytoplasmic and nuclear β-catenin staining (as indicated by the blue and yellow arrows, respectively) was noted in WHCO5 in response to 3 hours of concurrent signals from lithium and EGF (Figure 3.8.e), while low intensity staining in the cytoplasm was noted in SNO at this time (Figure 3.8.h). At 12 and 24 hours of exposure, however, β-catenin appeared to return to standard distributions in these cell lines (Figure 3.8.f represents β-catenin staining in WHCO5 after 24 hours of exposure). A change in the localization of β-catenin in the other cell lines was not detected under these conditions as is shown by WHCO6 cell line (Figure 3.8.j) after 3 hours of exposure.

3.3.5 TGF-β1, alone or together with lithium, does not noticeably influence the membrane or cytoplasmic concentration of β-catenin

It was of interest to investigate the effect of TGF-β1 on the distribution of β-catenin in the oesophageal SCC cell lines, as this cytokine has previously been reported to influence β-catenin in other cellular systems (Tian and Phillips, 2002; Satterwhite and Neufeld, 2004; Xu *et al*., 2003). In addition, since crosstalk between TGF-β1 and Wnt signalling has also been noted in other cells (Lei *et al*.,

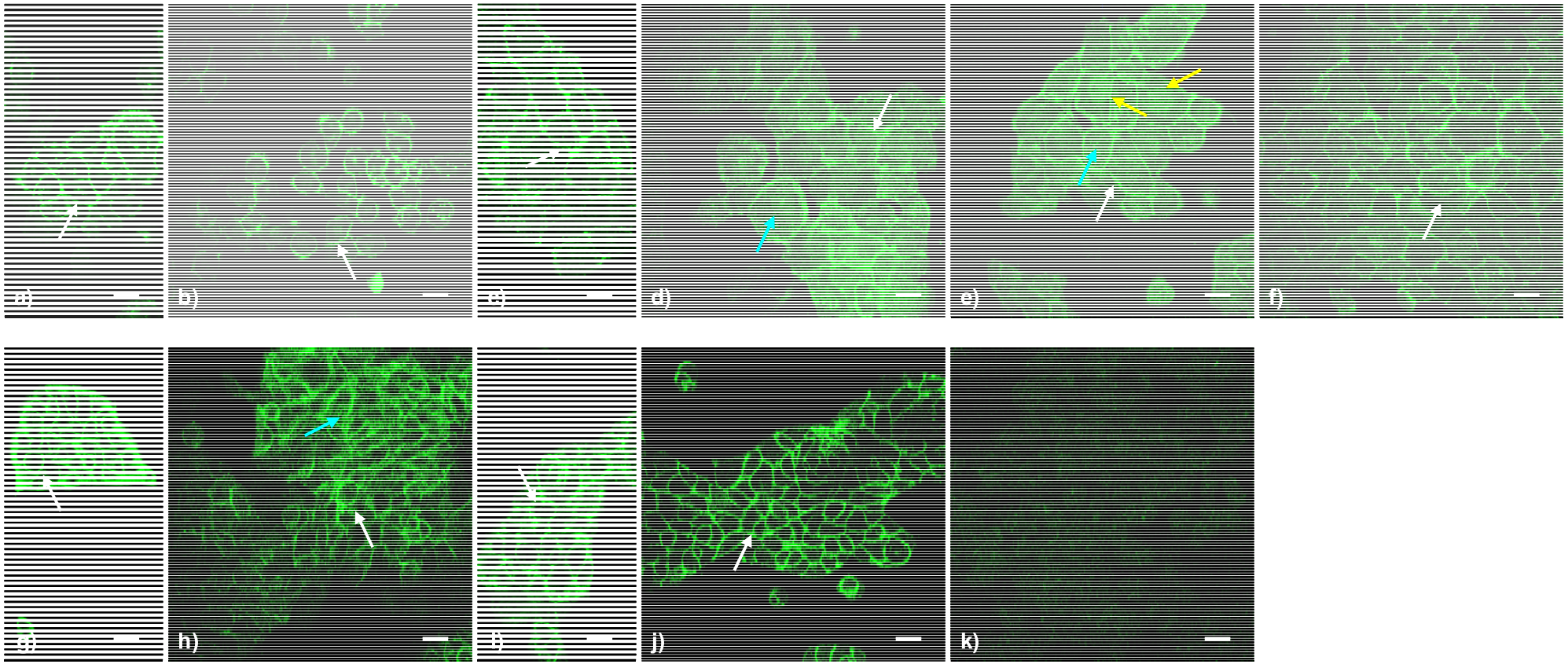


Figure 3.8: The subcellular localization of β-catenin subsequent to treatment with EGF and/or lithium. Under all EGF and/or lithium treatments, the membrane staining of β-catenin (see white arrows) remained intense. EGF signalling (3 hours) did not appear to alter the standard distribution of β-catenin in any of the five cell lines, as is indicated by WHCO1 **(b)**. Three hours of lithium stimulation resulted in β-catenin localizing in the cytoplasm in WHCO5 (**d**, as indicated by the blue arrow). Similarly, cytoplasmic β-catenin staining (blue arrows) was noted in both WHCO5 and SNO after 3 hours of concurrent exposure to EGF and lithium (**e** and **h**, respectively). Under these conditions, nuclear staining was also noted in WHCO5 (**e**, see yellow arrows). However, β-catenin returned to standard locations after 12 and 24 hours of lithium, or combined EGF and lithium, stimulation, as is shown by WHCO5 after exposure to EGF and lithium for 24 hours **(f)**. None of the other cells lines appeared to change from the standard under these conditions, as is indicated by WHCO6 after 3 hours of EGF and lithium exposure **(j)**. A negative control **(k)** represented by WHCO3 cells after 24 hours of EGF and lithium stimulation, shows that non-specific binding is negligible. Note, for comparative reasons, the localization of β-catenin under standard *in vitro* conditions for WHCO1, WHCO5, SNO and WHCO6 (**a**, **c**, **g** and **i**, respectively) have been included. All photographs are composite confocal images. Scale bar = 25 mm. (FITC excitation 490, emission 525).

2004; Warner *et al*., 2005), it was important to investigate whether TGF-β1 and lithium together alter the subcellular pools of β-catenin in the oesophageal SCC cell lines. Thus, the oesophageal SCC cell lines were exposed to TGF-β1 alone, or together with lithium, for 0.5; 1; 3; 6; 12 and 24 hours prior to extraction into the membrane and cytoplasmic fractions. The intervals of exposure were decided upon as the reported temporal responses to TGF-β1 in other cellular systems varied from a few hours to days (Tian and Phillips, 2002; Satterwhite and Neufeld, 2004; Xu *et al*., 2003). β-catenin was identified in all the membrane and cytoplasmic fractions of the oesophageal SCC cell lines exposed to TGF-β1 and/or lithium (see Figure 3.9). Note the lower molecular weight products (which lend an inconsistent "pattern" of bands in these immunoblots) likely repesent mechanical degradation products (see Discussion, Chapter 2). Only the 94 kD bands were scanned in subsequent denisometry of these immunoblots.

The oesophageal cells (WHCO3, WHCO6 and SNO) treated with TGF-β1 alone, tended to exhibit slightly lower β-catenin concentrations at the plasma membrane than standard concentrations over the 24 hours period (ascertained by densitometry of immunoblots), although there were small differences in the levels of expression at various time points (refer to Figure 3.10.a). WHCO5 showed a general increase of β-catenin concentration (~2-fold) in response to TGF-β1 exposure, except at 1 and 3 hours where levels were lower than standard. β-catenin concentration did not change from standard expression in WHCO1, except at 6 and 24 hours which displayed an increased β-catenin concentrations of 1.6- and 2.1-fold, respectively.

TGF-β1 stimulation did not considerably change the level of β-catenin in the cytoplasmic fractions from standard levels in WHCO1, WHCO3, WHCO6 and SNO; although there were variations in expression over the 24 hour period (Figure 3.10.b). For example, WHCO1 displayed increased β-catenin levels at 0.5 hours but was decreased after 12 hours; WHCO3 dropped slightly below standard concentration at 3 and 6 hours; and WHCO6 was decreased at 3 hours. β-catenin

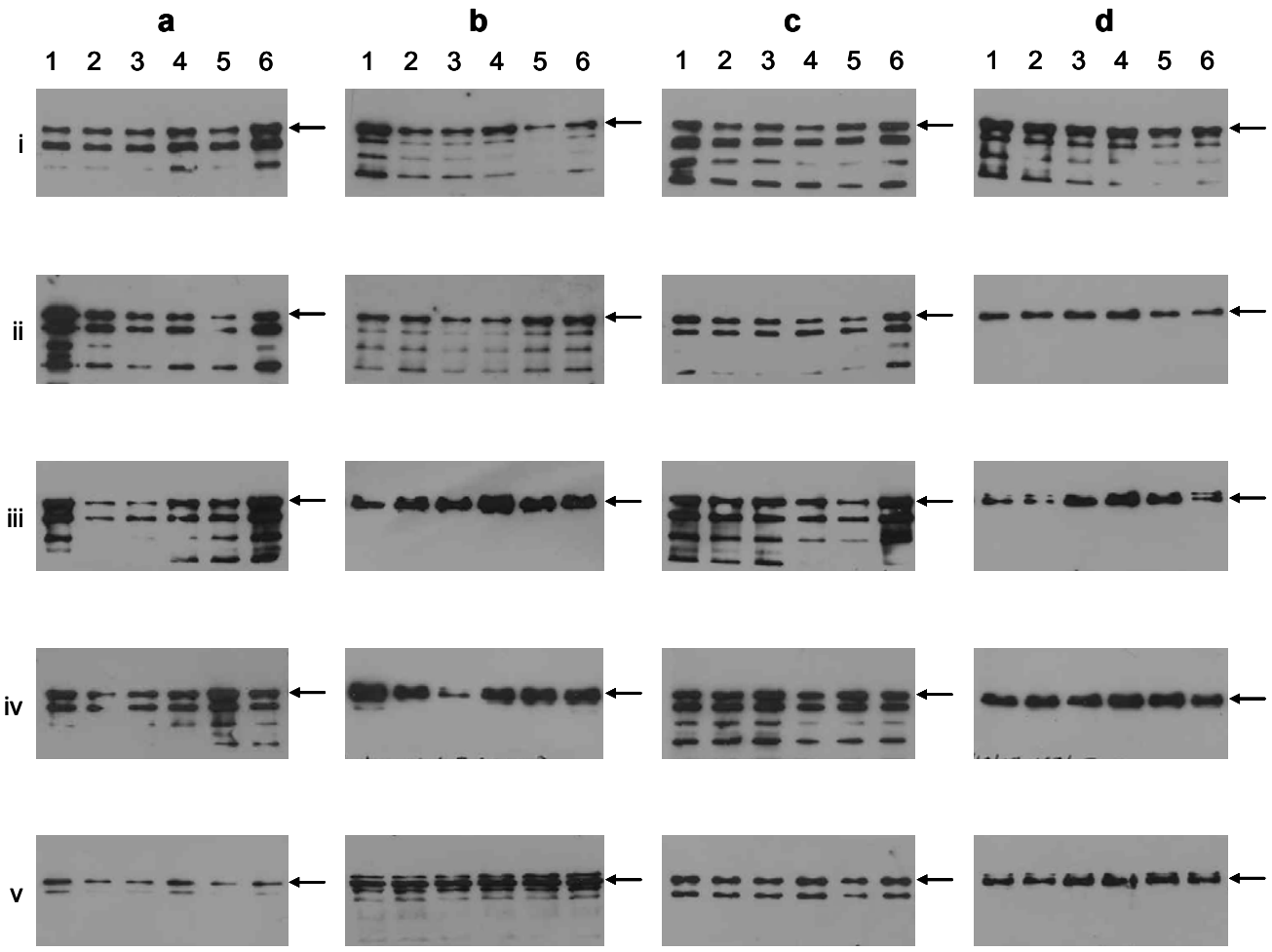


Figure 3.9: β-catenin occurs in all oesophageal SCC cellular fractions when treated with TGF-β1 alone, or together with lithium. β-catenin (94 kD, indicated by black arrows) was found to be expressed (by immunoblotting) in all the plasma membrane **(a)** and cytoplasmic **(b)** fractions of the oesophageal SCC cells treated with TGF-β1 (1 ng/ml) alone. The lower molecular weight bands represent degradation products (see Discussion, Chapter 2). Similarly, β-catenin was also found in the plasma membrane **(c)** and cytoplasmic **(d)** fractions of cells treated with TGF-β1 together with lithium (10 mM). Lanes 1-6 represent 0.5, 1, 3, 6, 12 and 24 hours of treatment, respectively. **i-v** represent WHCO1, WHCO3, WHCO5, WHCO6 and SNO, respectively. All experiments were performed in duplicate.

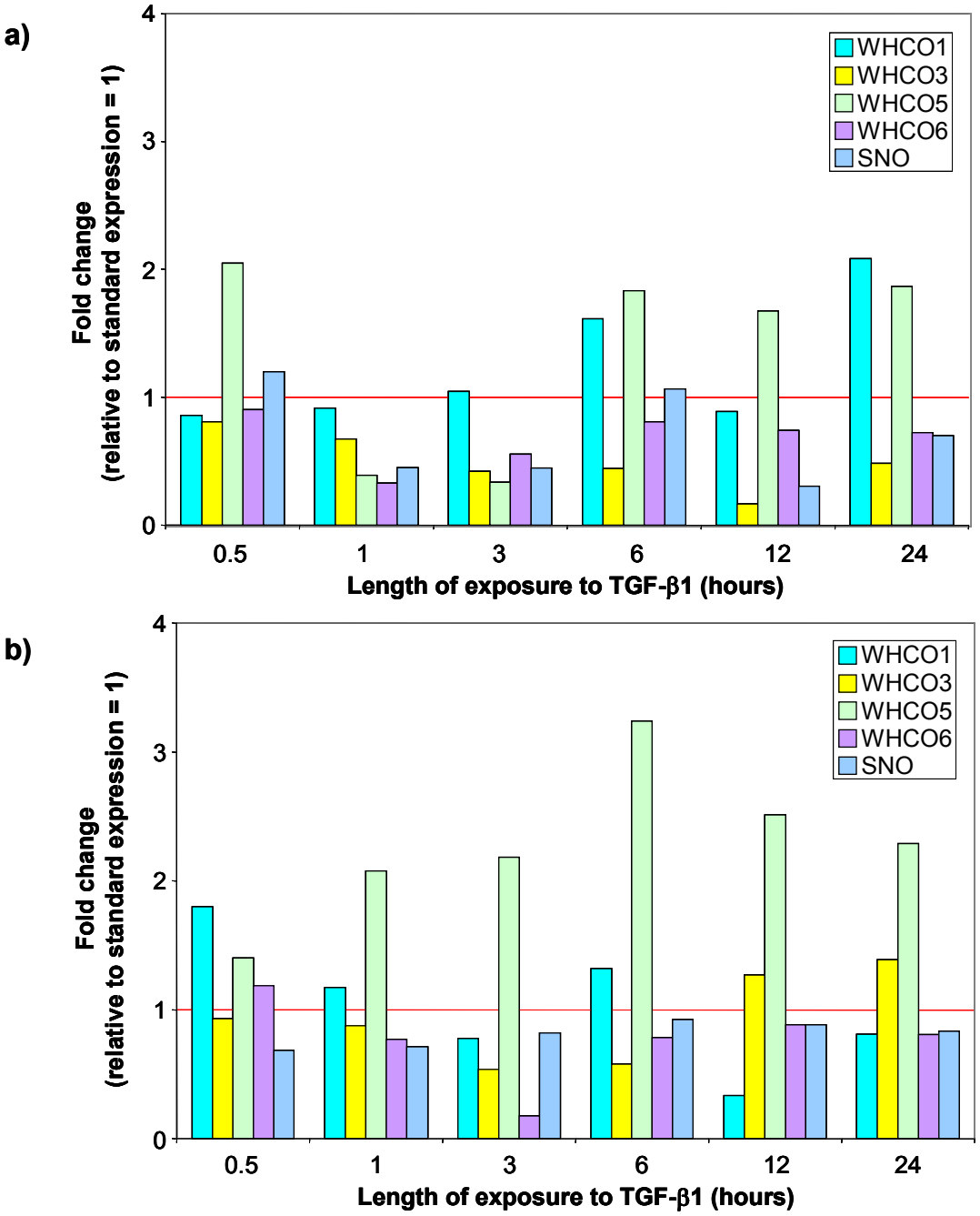


Figure 3.10: TGF-β1 does not extensively affect the concentration of the membrane and cytoplasmic pools of β-catenin. (a) The concentration of plasma membrane-associated β-catenin (ascertained by densitometry of immunoblots) in the WHCO5 cell line showed a small increase of β-catenin concentrations (~2-fold) in response to TGF-β1 stimulation, except at 1 and 3 hours where levels were lower than standard (= 1, indicated by the red line*). WHCO1 also displayed slightly higher concentration of β-catenin to standard levels but only at 6 and 12 hours of exposure. WHCO3, WHCO6 and SNO all tended towards slightly lower than standard in response to TGF-β1 stimulation. **(b)** Similarly, TGF-β1 stimulation did not change the level of β-catenin in the cytoplasmic fraction from standard levels in WHCO1, WHCO3, WHCO6 and SNO; although there were variations in expression over the 24 hour period. WHCO5 showed a steady increase of β-catenin expression, peaking at 6 hours with a 3.2-fold increase.
*The concentration of β-catenin is expressed as a fold increase (or decrease) relative to (**a**) membrane and (**b**) cytoplasmic standard expression per 5 μg of total protein.

levels WHCO5 showed a steady increase of β-catenin expression in response to TGF-β1 treatment, from 1.4-fold at 0.5 hours, peaking at 6 hours with a 3.2-fold increase.

TGF-β1, together with lithium, did not extensively change the levels of β-catenin expression at the plasma membrane from standard in WHCO3, WHCO5, WHCO6 and SNO (see Figure 3.11.a). However, it should be noted that the WHCO3 cell line showed a steady reduction of β-catenin from the plasma membrane from 0.5 hours to 12 hours of exposure. The WHCO1 cell line differed from the other cell lines by generally expressing a higher concentration of β-catenin expression in this fragment, with levels of expression increasing by over 2-fold at 0.5 and 24 hours and by 1.5-fold at 3 and 12 hours.

Combined TGF-β1 and lithium treatment did not substantially alter β-catenin levels in the cytoplasmic fraction in WHCO1, WHCO3 and WHCO6. The WHCO5 cell line showed an increase of β-catenin concentration to approximately 2.6-fold standard levels from 3 hours to 12 hours of treatment before returning to standard levels (Figure 3.11.b). The SNO cell line also displayed a steady increase of β-catenin expression, increasing from a 1.7-fold at 0.5 hours of exposure and peaking at 6 hours with a 2.6-fold increase.

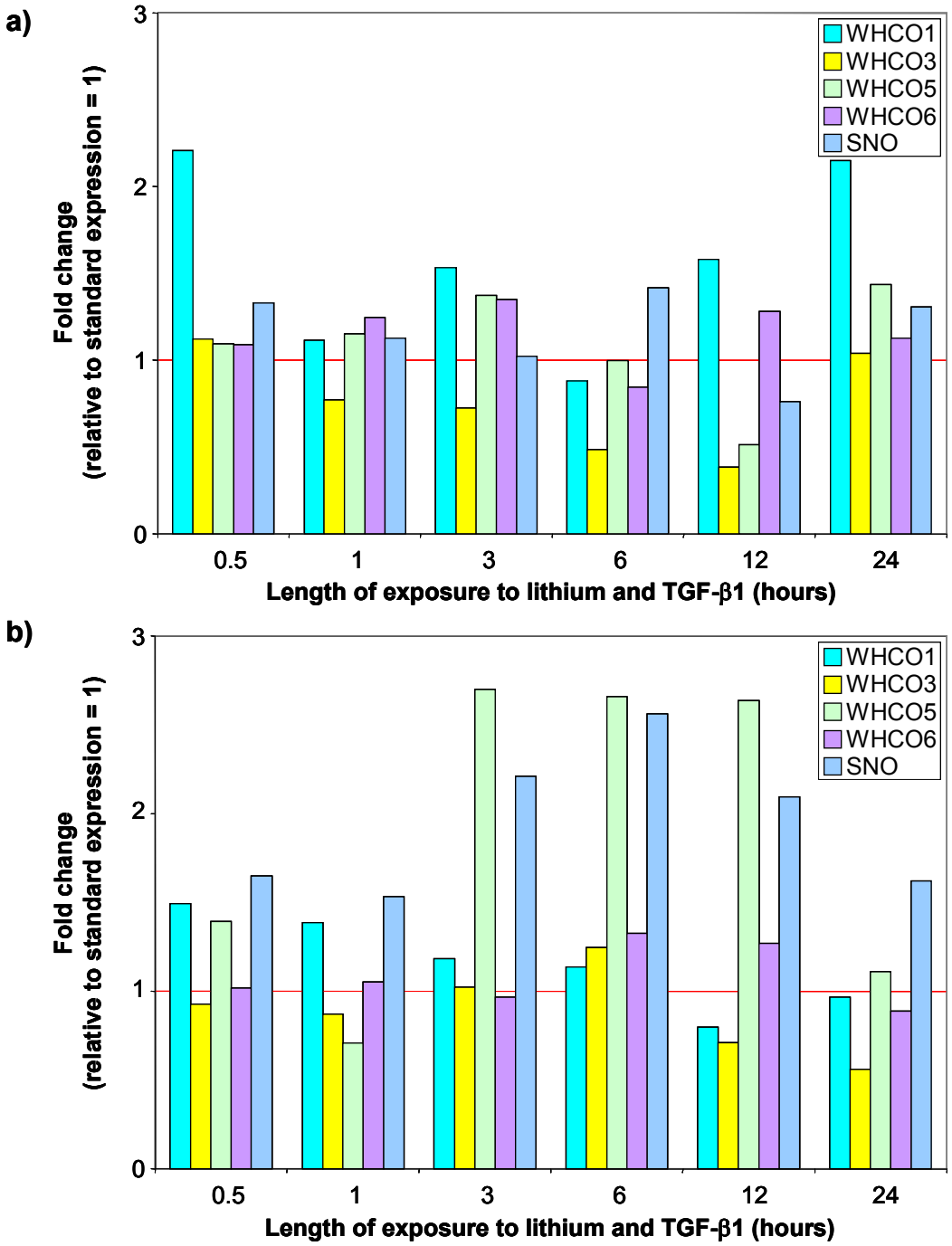


Figure 3.11: The effect of simultaneous lithium and TGF-β1 stimulation on the concentration of β-catenin associated with the plasma membrane and in the cytoplasm. (a) β-catenin concentrations (determined by densitometry of immunoblots) in the plasma membrane fraction did not change from standard expression (= 1, indicated by the red line*) in WHCO5, WHCO6 and SNO subsequent to TGF-β1 and lithium treatment. The WHCO1 cell line displayed increased membrane-associated β-catenin by 2-fold (0.5 and 24 hours) and 1.5-fold (3 and 12 hours), while WHCO3 steadily lost β-catenin from this fraction, returning to standard after 24 hours. **(b)** β-catenin levels in the cytoplasmic fraction did not alter in WHCO1, WHCO3 and WHCO6 in response to concurrent lithium and TGF-β1 exposure. WHCO5 increased β-catenin concentration by over 2.6-fold after 3 to 12 hours of treatment before returning to standard levels. SNO also displayed a steady increase of β-catenin in this fraction over the 24 hour treatment period, peaking at 6 hours with a 2.6-fold increase.
*The concentration of β-catenin is expressed as a fold increase (or decrease) relative to (**a**) membrane and (**b**) cytoplasmic standard expression per 5 μg of total protein.

3.4 Discussion

β-catenin has been implicated in two distinct functions within the cell: adhesion, when associated with the plasma membrane-bound cadherin molecules; and signal transduction, when stabilized in the cytoplasm (refer to Chapter 1.3 and 1.9 for details). Tracking changes in the expression of β-catenin induced by exogenous signals will assist in further understanding the balance that occurs between these two distinct functions of β-catenin in human oesophageal SCC cells. Three major signal transduction pathways, induced by lithium (wnt), EGF and TGF-β1, were assessed in terms of their effect on β-catenin in human oesophageal SCC cell lines. The results of these experiments are interesting and are discussed here.

Lithium is a partial, yet effective, mimic of the Wnt pathway as it acts as a cell permeable, non-competitive inhibitor of GSK-3β, inducing cytoplasmic accumulation of β-catenin (Klein and Melton, 1996; Stambolic *et al*., 1996; Hedgepeth *et al*., 1997; Orford *et al*., 1997; Willert *et al*., 1999; Chen *et al*., 2000; Zhang *et al*., 2003). This has been shown in a number of cell lines, including the mammary epithelial C57MG cells (Willert *et al*., 1999; Chen *et al*., 2000), human transformed epithelial kidney HEK-293 cells (Chen *et al*., 2000), human mammary epithelial SKBR3 carcinoma (Orford *et al*., 1997) and the normal breast epithelial HBL100 cell line (Orford *et al*., 1997). Described in this chapter are experiments which show that administration of lithium to the oesophageal SCC cell lines, resulted in an increase of the cytoplasmic levels in WHCO1, WHCO3 and WHCO5 cells initially (3 hours), but over time (12 and 24 hours) the expression returned to standard levels (Figure 3.2.b). Immunofluorescence further confirmed this increase in WHCO5 which displayed cytoplasmic staining (see Figure 3.8.d). However, no cytoplasmic or nuclear β-catenin was detected by immunofluorescence in WHCO3 or WHCO1, as would be expected from the immunoblot analysis. This may be the result of the cytoplasmic concentration of β-catenin per cell being insufficient to be detected by immunofluorescence, but

the 5 μg of total protein (extracted from several thousand cells) used standardly for the immunoblotting, obviously contained sufficient β-catenin to be detected.

Interestingly, plasma membrane levels initially (3 hours) did not change in response to lithium stimulation, but noticeably increased at 12 hours of exposure (WHCO1, WHCO3 and SNO) before returning to standard levels after 24 hours (except SNO cells, Figure 3.2.a). E-cadherin is known to sequester cytoplasmic β-catenin to the plasma membrane and thus reduce its signalling capacity in various cellular systems (Hinck *et al*., 1994a; Fagotto *et al*., 1996; Sadot *et al*., 1998; Simcha *et al*., 1998; Orsulic *et al*., 1999; Eger *et al*., 2000; Gottardi *et al*., 2001; Stockinger *et al*., 2001). Therefore, the increase of membrane-associated β-catenin noted at 12 hours may be due to the excess cytoplasmic β-catenin that had been stabilized by lithium initially, being sequestered by E-cadherin to the membrane. The level of β-catenin gradually returned to standard concentrations after 24 hours, suggesting that the β-catenin degradation complex returns to its normal level of functioning and is managing to degrade the excess protein induced by this stimulation.

It has long been known that EGF signalling affects intercellular adhesion. For example, HSC-human cutaneous SCC cells, which overexpress EGFR, showed reduced E-cadherin function after 30 minutes of EGF exposure and changed to a more fibroblastic morphology after 3 hours (Fujii *et al*., 1996). Similarly, Shiozaki *et al*. (1995) described modifications to cellular morphology that result in decreased cell-cell adhesion in TE-2R poorly-differentiated oesophageal SCCs in response to EGF stimulation. EGF similarly induces decreased adhesion in the South African WHCO- series of moderately-differentiated oesophageal SCCs used in the present study within 15 minutes of exposure (unpublished observations). The question arises as to how EGF induces these changes to the cadherin-catenin complex. Possible mechanisms by which this could occur are through EGF-induced changes to the expression of the adhesion proteins or by affecting their localization within the cell. Thus, the effect of EGF stimulation on β-catenin concentration and distribution was investigated.

The total concentration of β-catenin was not altered through exposure to EGF over time (see Figure 3.3), suggesting that EGF does not affect the expression of β-catenin at a transcriptional level. This result is similar to those observed by Shiozaki *et al*. (1995) in a single poorly-differentiated oesophageal SCC cell line. The plasma membrane concentration of β-catenin was unaffected by EGF stimulation for either 20 minutes or 3 hours (Figure 3.5.a). However, EGF very briefly induced a large increase in the cytoplasmic pool in the WHCO1 and WHCO3 cell lines (20 minutes, Figure 3.5.b). How does EGF mediate this change? EGF is able to inhibit the activity of GSK-3β through activation of either the MAPK/$p90^{rsk}$ or PI3K/PKB pathways (Stambolic and Woodgett, 1994; Cross *et al*., 1995; Eldar-Finkelman *et al*., 1995; Okano *et al*., 2000; Andl *et al*., 2003). This could allow for the accumulation of β-catenin in the cytoplasm. However, Mizushima *et al*. (2002) stated that EGF alone was insufficient to induce stabilization of cytoplasmic and nuclear β-catenin in the TE-oesophageal SCC cell lines, while Chen *et al*. (2000) similarly demonstrated this in the HEK-293 and C57MG cell lines. The present study shows that the stabilizing effect of EGF on the cytoplasmic accumulation of β-catenin was brief, as the cytoplasmic levels returned to just below standard concentrations after 3 hours of EGF exposure (as can be seen in Figure 3.5.b and 3.8.b). Chen *et al*. (2000) only made their observations after 3 hours of EGF exposure, while Mizushima *et al*. (2002) did not state the duration of exposure to EGF, and thus may have missed the very transient stabilizing effect of EGF. However, it does seem doubtful that this short-lived raise of cytoplasmic β-catenin would be sufficient to increase β-catenin's transcriptional activity in the nucleus. A more stringent test, such as the well characterised β-catenin/Lef/Tcf-TOPFLASH reporter gene assay (Korinek *et al*., 1997), would be required to ascertain this.

TGF-β1 has also been implicated in the dissociation of adherens junctions in late stage tumours (Bakin *et al*., 2000) and to cause alterations to the expression of adherens junction proteins in various epithelial cell lines, such as the HT-144 melanoma, SMMC-7721 hepatocellular and the HK-2 renal proximal epithelial

cell lines (Janji *et al*., 1999; Tian and Phillips, 2002; Xu *et al*., 2003). However, TGF-β1 did not appear to substantially affect the level of plasma membrane-associated β-catenin in any of the oesophageal SCC cell lines, although small increases were noted in the WHCO5 and WHCO1 cell lines (refer to Figure 3.10.a). TGF-β1 signalling has also been reported to increase the level of stable cytoplasmic and nuclear β-catenin in various cell lines (Janji *et al*., 1999; Tian and Phillips, 2002; Xu *et al*., 2003; Masszi *et al*., 2004; Satterwhite and Neufeld, 2004; Edlund *et al*., 2005). In general, the moderately-differentiated oesophageal SCCs did not show any changes in the concentration of the cytoplasmic pool of β-catenin, with the exception of the WHCO5 cell line. This line showed a small yet stable increase of cytoplasmic β-catenin levels over the course of TGF-β1 exposure (maximum 3.2-fold, see Figure 3.10.b). Increased cytoplasmic β-catenin concentrations in response to TGF-β1 stimulation has been attributed to redistribution of the protein from the plasma membrane into the cytoplasm (Janji *et al*., 1999; Weeks *et al*., 2001; Tian and Phillips, 2002). It is however, unlikely that this is occurring in the WHCO5 cell line as the plasma membrane concentration of β-catenin was generally increased in response to TGF-β1 stimulation. TGF-β1 signalling has also been implicated in the downregulation of the β-catenin degradation complex through stimulation of PKB, which inhibits the kinase activity of GSK-3β, or by reducing APC expression (Cheon *et al*., 2004; Edlund *et al*., 2005; Satterwhite and Neufeld, 2004). This may indicate an alternative mechanism underlying this increase in WHCO5, although it remains to be tested.

EGF and TGF-β1 have both been shown to crosstalk with the Wnt signal transduction pathway to alter β-catenin expression and activity in the nucleus in various cell lines (Chen *et al*., 2000; Müller *et al*., 2002; Tuli *et al*., 2003; Lei *et al*., 2004; Zhou *et al*., 2004; Warner *et al*., 2005). It is, therefore, important to discern whether these signals are able to act synergistically to alter the distribution of β-catenin in the moderately-differentiated oesophageal SCC cell lines. Concurrent signals induced by exogenous exposure to EGF and lithium (the Wnt

mimic) led to an enhancement of the cytoplasmic component in the oesophageal SCC cell lines at 3 hours of treatment, and remained elevated after 12 hours of exposure before returning to standard levels by 24 hours (see Figure 3.7.b). This was further confirmed by immunofluorescence as cytoplasmic staining was noted in the SNO cell line (Figure 3.8.h) and cytoplasmic and nuclear staining was found in WHCO5 (Figure 3.8.e). These results mirror those found by Chen *et al*. (2000) who reported that the increase of cytoplasmic levels induced by lithium treatment was enhanced by simultaneous stimulation with EGF in the HEK-293 and C57MG cells. Similarly, Müller *et al*. (2002) found that EGF increases cytoplasmic β-catenin levels in NBT-II rat bladder carcinoma cells, which express a stabilizing mutant of β-catenin, and enhances the transcriptional activity of the β-catenin/Lef complex required for tumourigenic epithelial-to-mesenchymal transition and migration. Interestingly, our results contradict those found by Mizushima *et al*. (2002) who found no synergistic effect of EGF and Wnt-1 stimulation on Tcf-dependent transcription in TE-8 oesophageal SCC cells.

None of these groups, however, directly investigated the effect that simultaneous EGF and lithium signalling have on the plasma membrane-associated β-catenin component. The current study showed that concurrent signals from EGF and lithium resulted in a substantial decrease of membrane-associated β-catenin in the oesophageal SCC cell lines. This suggests that β-catenin is redistributing from the plasma membrane into the cytoplasm under these conditions. How do these signals induce such a shift? Chen *et al*. (2000) demonstrated that the enhancing effect of EGF was due to its activation of the TPA-sensitive isoform of PKC, PKCα. However, they were unable to determine the target of PKCα in this chain of events and speculated that it may be due to PKC's inactivation of GSK-3β. Baulida *et al*. (1999) reported that activation of PKCα by a phorbol ester, PMA, in normal intestinal cells resulted in a shift of β-catenin from the plasma membrane to the nucleus. Thus, rather than aiding the inhibition of GSK-3β as suggested by Chen *et al*. (2000), PKCα may act to disrupt β-catenin's associations with members of the adhesion complex. This would result in β-catenin shifting

from the plasma membrane into the cytoplasm to augment the lithium-induced stable cytoplasmic β-catenin in oesophageal SCCs. As PKCα is a serine kinase, it is most possible that it mediates its effect by inducing a posttranslational modification to β-catenin (or one of its binding partners) through serine phosphorylation. Thus, the following chapter investigates the effect of EGF and/or lithium stimulation on the concentration of serine phosphorylation of β-catenin. In addition, it is crucial to ascertain the tyrosine phosphorylation levels of β-catenin under these conditions as EGFR-mediated tyrosine kinase activity is known to regulate the interactions of β-catenin with members of the adhesion complex and the transcriptional machinery (Hoschuetzky *et al*., 1994; Ochiai *et al*., 1994; Shibamoto *et al*., 1994; Takahashi *et al*., 1997; Hazan and Norton, 1998; Piedra *et al*., 2001). Thus, EGF-mediated tyrosine phosphorylation of β-catenin may serve as an additive mechanism to lithium induced stabilization of β-catenin, by decreasing its affinity for the adhesion complex and increasing its attraction for its signalling role.

Unlike EGF, TGF-β1 did not augment lithium-induced stabilization of β-catenin in the cytoplasmic pool with the possible exception of the SNO cell line (refer to Figure 3.11.b). No change in the concentration of β-catenin in this pool was noted in the WHCO1, WHCO3 or WHCO6 cell lines. An increase was noted in WHCO5 (~2.6-fold) from 3 to 12 hours of exposure, however, this does not reflect synergism between lithium and TGF-β1 stimulation as a similar increase was noted when treating with TGF-β1 alone. The small increase occurring in the SNO cell line (between 1.7- and 2-6-fold) over the 24 hour period of exposure may reflect a low level of synergistic crosstalk between these two exogenous signals, as neither lithium nor TGF-β1 alone increased cytoplasmic β-catenin concentrations in this cell line. This augmentation does not appear to be induced by redistribution of β-catenin from the membrane fraction as the concentration of β-catenin in this pool did not change dramatically from standard expression in response to concurrent lithium and TGF-β1 stimulation in any of the cell lines (see Figure 3.11.a). This increase may reflect the ability of TGF-β1 to

downregulate the activity of the β-catenin degradation complex as discussed for TGF-β1 alone (see above; Cheon *et al*., 2004; Satterwhite and Neufeld, 2004; Edlund *et al*., 2005), while enhancing that induced by lithium, and together these signals reach a threshold which allows for a slight increase of the cytoplasmic concentration of β-catenin.

In conclusion, the data presented in this chapter clearly shows the response of the moderately-differentiated oesophageal SCC cell lines to stimulation from lithium, EGF and TGF-β1 in terms of the distribution of β-catenin. Although small changes in some of the cell lines were noted in response to TGF-β1 stimulation alone, and together with lithium, the general lack of change from standard expression suggests that TGF-β1 does not play a significant role in regulating the expression of β-catenin in this type of tumour cell. However, both lithium and EGF alone were able to induce changes to the concentration of β-catenin present in the membrane and cytoplasm, and were able to synergistically act to elevate, and prolong cytoplasmic stabilization of β-catenin, while simultaneously reducing its membrane expression and possibly, its structural function in these cells. Understanding the mechanism underlying this action thus becomes the substance for the following chapter.

CHAPTER FOUR

How do EGF and Lithium Synergistically Induce the Redistribution of β-catenin in Oesophageal SCC Cell Lines?
- The Role of Phosphorylation -

In the previous chapter, it was found that the combination of exogenous stimulation of the oesophageal SCC cell lines with EGF and a mimic of Wnt, lithium (3 hours), induced a reduction in the concentration of β-catenin expressed at the plasma membrane, whilst prolonging the increase of β-catenin in the cytoplasmic fraction. The question arises as to how these two signals cooperate to induce the redistribution of β-catenin in these cells. It is well known that the adhesion complex is not a static structure but is highly dynamic in nature (Klingelhöfer *et al*., 2003; Perez-Moreno *et al*., 2003; Drees *et al*., 2005; Yamada *et al*., 2005). Posttranslational events, especially through phosphorylation, are known to regulate this complex (Hoschuetzky *et al*., 1994; Kinch *et al*., 1995; Roura *et al*., 1999; Lickert *et al*., 2000). Therefore, it is possible that EGF and lithium act synergistically to alter the phosphorylation status of one or more members of the adhesion complex. β-catenin is an ideal target for this due to the central position it holds in the adhesion complex, as well as it being an excellent phosphoprotein candidate, as it contains a number of tyrosine and serine phosphorylation sites (Peifer *et al*., 1994b; Hu *et al*., 2001; Roura *et al*., 1999).

Tyrosine phosphorylation has been shown to be of particular importance in regulating β-catenin's interactions with members of the adherens junction. Early investigations in v-Src- and Ras-transformed cell lines demonstrated that tyrosine phosphorylation of β-catenin reduced cell-cell adhesion and increased the invasive propensity of these cells (Volberg *et al*., 1991; Matsuyoshi *et al*., 1992; Behrens *et al*., 1993; Hamaguchi *et al*., 1993; Kinch *et al*., 1995; Roura *et al*., 1999; Hu *et al*., 2001). This is due to loss of affinity of β-catenin with E-cadherin (Kinch *et*

al., 1995; Roura *et al*., 1999; Hu *et al*., 2001) and with α-catenin (Takahashi *et al*., 1997; Ozawa and Kemler, 1998a; Hu *et al*., 2001; Piedra *et al*., 2003). Roura *et al*. (1999) demonstrated that phosphorylation of tyrosine residue 654 (Y654), which falls into the twelfth armadillo repeat of β-catenin, was responsible for the reduced association between E-cadherin and β-catenin. Structurally, Y654 forms a hydrogen bond with aspartic acid residue 665 (D665) in the E-cadherin cytoplasmic tail (in Region II, refer back to Figure 1.2), and the addition of a phosphate group to Y654 would result in electrostatic repulsion of E-cadherin D665, reducing the association between the two proteins (Roura *et al*., 1999; Huber and Weis, 2001; Piedra *et al*., 2001). Interestingly, phosphorylation of this residue increases β-catenin's affinity for the TATA-binding protein (TBP) by inducing conformational changes to β-catenin that effectively remove steric hindrance, and thus promotes β-catenin's transcriptional activity (Piedra *et al*., 2001).

The tyrosine residue responsible for regulating the interaction between β-catenin and α-catenin is Y142 (Aberle *et al*., 1996). This residue forms van der Waal forces with several acidic residues in α-catenin (Pokutta and Weis, 2000) and acidification of Y142, through phosphorylation, decreases the attraction between β-catenin and α-catenin (Piedra *et al*., 2003). Phosphorylation of this residue also increases β-catenin's affinity for BCL9-2, which is associated with their translocation away from the plasma membrane into the nucleus (Brembeck *et al*., 2004). It may also play a role in inhibiting β-catenin's association with the degradation complex (Danilkovitch-Miagkova *et al*., 2001). In short, tyrosine phosphorylation appears to reduce β-catenin's role in adhesion and rather promotes its nuclear activity.

EGFR activity is associated with reduced cell-cell adhesion in epithelial cells (Hoschuetzky *et al*., 1994; Ochiai *et al*., 1994; Shibamoto *et al*., 1994; Shiozaki *et al*., 1995; Fujii *et al*., 1996; Takahashi *et al*., 1997; Hazan and Norton, 1998). The activated receptor is well known to associate directly with the cadherin/catenin

complex at the plasma membrane and mediates tyrosine phosphorylation of β-catenin (Hoschuetzky *et al*., 1994; Ochiai *et al*., 1994; Shibamoto *et al*., 1994; Shiozaki *et al*., 1995; Shibata *et al*., 1996; Takahashi *et al*., 1997; Hazan and Norton, 1998; Kuwada *et al*., 1998; Schroeder *et al*., 2002). Many studies refer to Roura *et al*. (1999) as having found that EGFR is able to phosphorylate Y654. However, it should be noted that Roura *et al*. (1999) demonstrated phosphorylation of Y654 through $pp60^{c\text{-}src}$ kinase activation or through treatment with a tyrosine phosphatase inhibitor (sodium orthovanadate), and only suggested EGFR as a likely candidate to efficiently phosphorylate this residue as Y654 falls within its binding domain. Piedra *et al*. (2003) stated that EGFR is able to phosphorylate Y654, but did not show the data for this. In addition, Bonvini *et al*. (2001) showed that the EGFR homologue, erbB2 can successfully phosphorylate Y654. Thus, it is assumed that activated EGFR can phosphorylate this residue reducing the association between E-cadherin and β-catenin.

EGFR-mediated tyrosine phosphorylation of β-catenin can also alter the association between β-catenin and α-catenin. Takahashi *et al*. (1997) demonstrated that EGFR associates with the cadherin/catenin complex and increases tyrosine phosphorylation of β-catenin. This was correlated with a decrease of α-catenin found in the complex, possibly through preventing α-catenin reassociation with the complex. Hazan and Norton (1998) similarly showed that EGFR caused the E-cadherin/β-catenin complex to dissociate from the α-catenin-related, cytoskeletal-associating protein, vinculin, in α-catenin-deficient cells. Ozawa and Kemler (1998a) suggested that the EGFR-mediated phosphorylation of β-catenin may result in its dissociation from α-catenin as EGFR phosphorylates β-catenin in the amino-terminal, α-catenin binding domain. At present there is no evidence that EGFR directly phosphorylates Y142 of β-catenin. However, two kinases, Fer and Fyn, have been shown to effectively phosphorylate Y142, reducing the interaction between β-catenin and α-catenin (Piedra *et al*., 2003). Fer and Fyn associate indirectly with E-cadherin via p120-catenin (Kim and Wong, 1995; Rosato *et al*., 1998; Piedra *et al*., 2003), and it has been shown that Fer is also constitutively bound to EGFR and becomes activated

upon EGF stimulation (Kim and Wong, 1995). Thus, EGFR may indirectly induce phosphorylation of Y142 through activation of Fer.

The role of serine phosphorylation on cell-cell adhesion is not as clearly defined as that of tyrosine phosphorylation. It is known that serine phosphorylation of the cytoplasmic tail of E-cadherin by serine kinases such as GSK-3β and CKII, increases its affinity for binding to β-catenin (Stappert and Kemler, 1994; Lickert *et al*., 2000; Huber and Weis, 2001). However, the effect of serine phosphorylation of β-catenin on the adhesion complex is more ambiguous. Miller and Moon (1997) reported that serine phosphorylation of β-catenin by *Xenopus* GSK-3β increases its ability to bind to the cytoplasmic domain of N-cadherin. In addition, serine phosphorylated forms of the β-catenin-related armadillo protein appear to favour a membrane-association rather than those found in the cytoplasm (Peifer *et al*., 1994b). Bek and Kemler (2002) demonstrated that CKII-mediated phosphorylation of β-catenin on amino-terminal, serine/threonine residues (S29, T102 and T112) increases β-catenin's affinity for α-catenin in HEK-293 cells. They showed that removal of these residues correlated with an increased cytoplasmic localization of β-catenin, and reduced association with the cytoskeleton associated with increased migration. However, Serres *et al*. (1997) showed that hyperphosphorylation of serine residues in β-catenin, induced by serine/threonine phosphatase inhibitors, was associated with β-catenin altering its localization from the membrane to the cytoplasm in keratinocytes. Thus, the effect of serine phosphorylation of β-catenin on its structural role is far from being fully understood.

Chen *et al*. (2000) showed that the augmenting effect of EGF on lithium-induced stabilized, cytoplasmic β-catenin concentration was due to the activation of the serine kinase, PKCα. However, they were unable to identify the target of this kinase. The activation of PKCα has been shown to induce the redistribution of β-catenin from the membrane into the nucleus in normal intestinal cells (Baulida *et al*., 1999). The data presented in the previous chapter suggested that simultaneous

exposure to EGF and lithium (3 hours) resulted in the redistribution of β-catenin from the plasma membrane to the cytoplasm in the oesophageal SCC cell lines. Thus, PKCα may act to disrupt β-catenin's associations with members of the adhesion complex, resulting in a shift from the plasma membrane into the cytoplasm. Determining the concentration of serine phosphorylated, plasma membrane-associated β-catenin will indicate whether this is occurring in these cells under these conditions.

The following investigation revealed that the concentration of tyrosine phosphorylated, plasma membrane-associated β-catenin was increased in the cell lines that responded with the largest augmentation to the cytoplasmic fraction in response to EGF and lithium stimulation. Although the level of serine phosphorylated β-catenin did increase in two of the cell lines, it did not correspond with changes induced by EGF and lithium stimulation. This therefore suggests that tyrosine phosphorylation of β-catenin, but not serine phosphorylation, plays a role in regulating this redistribution induced by EGF and lithium.

4.2 Methods and Materials

4.2.1 Isolation of tyrosine or serine phosphorylated proteins from the plasma membrane-associated fractions of the moderately-differentiated oesophageal SCC cell lines through immunoprecipitation

The plasma membrane-associated protein fraction was extracted from human oesophageal SCC cell lines grown under standard tissue culture conditions, or treated with either EGF (10 ng/ml) alone, or in combination with lithium (10 mM), for 3 hours (refer to Chapter 3.2.2). This fraction (150 μg) was incubated with 4 μl of either monoclonal anti-phosphotyrosine (Sigma) or monoclonal anti-phosphoserine (Sigma) antibodies overnight at 4°C, while mixing.

Protein G-Agarose beads (Sigma; 30 μl per reaction) were washed three times by vortexing gently in 1 ml of cold immunoprecipitation buffer (IP buffer; see Appendix 1.8.1), centrifugating at 12 000 ×g for 30 seconds and discarding the supernatant after each wash. The beads were resuspended in IP buffer (50 μl per reaction) and were added to the antigen/antibody sample. Excess beads were washed off the sides of the tube with IP buffer (30 μl per reaction) and were added to the sample. The sample was allowed to incubate overnight at 4°C while mixing. In addition, a separate control consisting of washed beads, but no antigen was incubated with either anti-phosphotyrosine or anti-phosphoserine antibodies overnight at 4°C, while mixing.

The sample and control were centrifuged at 12 000 ×g for 30 seconds and the supernatant was discarded. The pellet was washed five times by resuspending the pellet in 700 μl IP buffer, centrifugating the sample at 12 000 ×g and discarding the supernatant after each rinse. The last rinse was conducted using 0.1 ×IP buffer. After discarding the supernatant from the last centrifugation, the pellet was resuspended in 40 μl of Laemmli sample buffer and the sample was boiled for 5 minutes. A second control of washed beads alone was also boiled in 40 μl

Laemmli sample buffer. The sample and controls were centrifuged at 12 000 ×g for 30 seconds and the supernatant was transferred to a new eppendorf tube and was stored at -20°C.

4.2.2 Detection and quantification of tyrosine or serine phosphorylated β-catenin through immunoblotting of immunoprecipitates

The tyrosine and serine immunoprecipitated proteins of standard or treated samples were separated by electrophoresis (as performed in Chapter 2.2.3.4), and were immunoblotted for β-catenin as described in Chapter 2.2.3.5. In addition, the controls of Protein G-Agarose beads alone or beads incubated with anti-phosphotyrosine (or anti-phosphoserine), were also electrophoresed and immunoblotted with anti-β-catenin to ensure no cross reaction between the β-catenin antibody and the Protein G from the agarose beads or IgG from the anti-phosphotyrosine (or anti-phosphoserine) was occurring.

Densitometry was used to quantify the intensity of the β-catenin bands exposed in the immunoblot. Note, variations in the loading concentrations were taken into account in subsequent calculations. The concentration of tyrosine (or serine) phosphorylated β-catenin under standard tissue culture conditions was expressed as a percentage of the maximum per 150 μg of total protein used in the immunoprecipitate. The concentration of tyrosine (or serine) phosphorylated β-catenin in the treated samples was expressed as a fold increase (or decrease) with respect to the standard expression in these cell lines (= 1).

4.3 Results

4.3.1 Tyrosine phosphorylation of plasma membrane-associated β-catenin under standard tissue culture conditions

Tyrosine phosphorylation of β-catenin plays an important role in regulating its function in adhesion by altering its interactions with E-cadherin and α-catenin (Kinch *et al*., 1995; Takahashi *et al*., 1997; Ozawa and Kemler, 1998a; Roura *et al*., 1999; Hu *et al*., 2001; Piedra *et al*, 2003). Thus, it is important to establish the basal concentration of tyrosine phosphorylated, plasma membrane-associated β-catenin in the oesophageal SCC cell lines. Plasma membrane-associated β-catenin from the WHCO1 and SNO cell lines grown under standard tissue culture conditions, showed the strongest reaction for tyrosine phosphorylation (see Figure 4.1.a and e, lane 2). This was determined through immunoprecipitating tyrosine phosphorylated proteins from the plasma membrane extract and immunoblotting these for β-catenin. The WHCO3, WHCO5 and WHCO6 showed little, if any, basal expression of tyrosine phosphorylated β-catenin. Quantification of these immunoblots through densitometry, revealed WHCO3 and WHCO5 express a relatively lower concentration tyrosine-phosphorylated β-catenin (51-54%) than WHCO1 (99%) and SNO (100%) under these conditions, as can be seen in Figure 4.2. The immunoblot of the controls of Protein G-agarose beads alone, or beads incubated with anti-phosphotyrosine but no antigen, showed no β-catenin reaction (Figure 4.1.f). This excludes a cross reaction between the anti-β-catenin antibody and the Protein G from the agarose beads or the IgG from the anti-phosphotyrosine antibody (Figure 4.1.f).

4.3.2 Lithium and EGF induce a large increase of tyrosine phosphorylated β-catenin in WHCO3

EGFR is known to induce tyrosine phosphorylation of membrane-associated β-catenin (Hoschuetzky *et al*., 1994; Ochiai *et al*., 1994; Shibamoto *et al*., 1994;

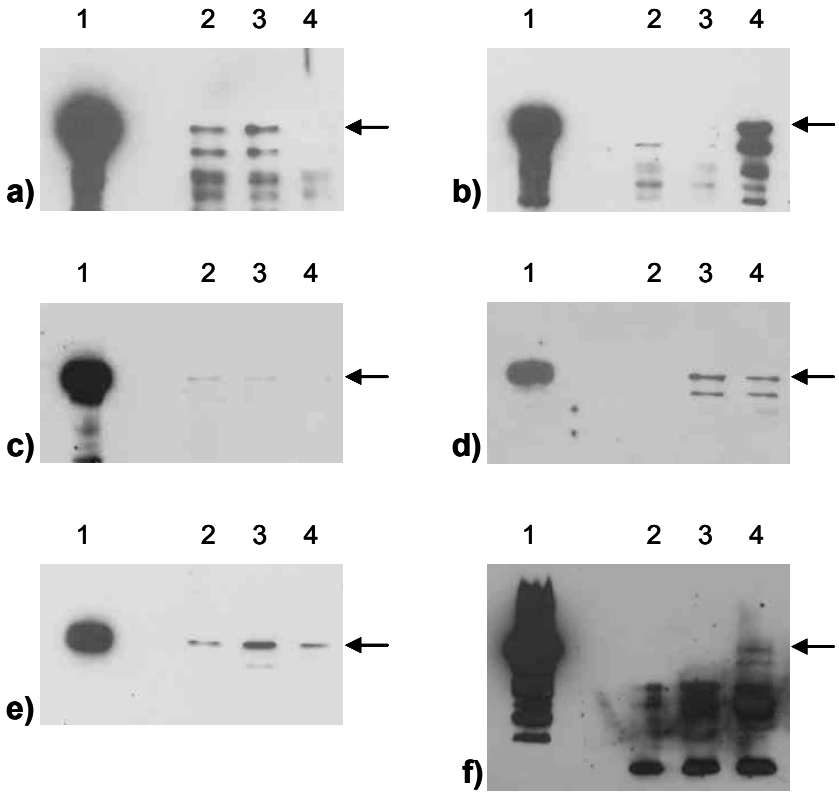


Figure 4.1: The presence of plasma membrane-associated, tyrosine phosphorylated β-catenin in oesophageal SCC cell lines when untreated or exposed to EGF and/or lithium. Tyrosine phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibody (Sigma) from the membrane fractions of oesophageal SCC cell lines grown under standard tissue culture conditions (lane 2) or from those treated with EGF alone (lane 3), or together with lithium (lane 4) for 3 hours. These immunoprecipitated proteins were separated by electrophoresis and immunoblotted for β-catenin. A whole cell extraction from WHCO6 (lane 1) was loaded as a reaction control. WHCO1 **(a)** and WHCO5 **(c)** displayed tyrosine phosphorylated β-catenin (indicated by the arrows) under standard culture conditions as well as when treated with EGF, although this was more prominent in WHCO1 than in WHCO5. WHCO3 **(b)** showed a strong phosphorylation of β-catenin in cultures treated with lithium and EGF, but not by EGF alone. Under standard culture conditions, WHCO6 **(d)** did not display tyrosine phosphorylated β-catenin but did in cultures treated with EGF alone, or together with lithium. The SNO cell line **(e)** showed that β-catenin is tyrosine phosphorylated under all conditions, although more so after exposure to EGF. **(f)** Compared to the tyrosine phosphorylated immunoprecipitated antigen (WHCO1; lane 4), the controls of Protein G-Agarose beads alone (lane 2) and beads incubated with anti-phosphotyrosine but no antigen (lane 3), showed no β-catenin reaction (indicated by the arrow). This excludes cross reactivity between the anti-β-catenin antibody and the Protein G from the agarose beads or the anti-phosphotyrosine IgGs.

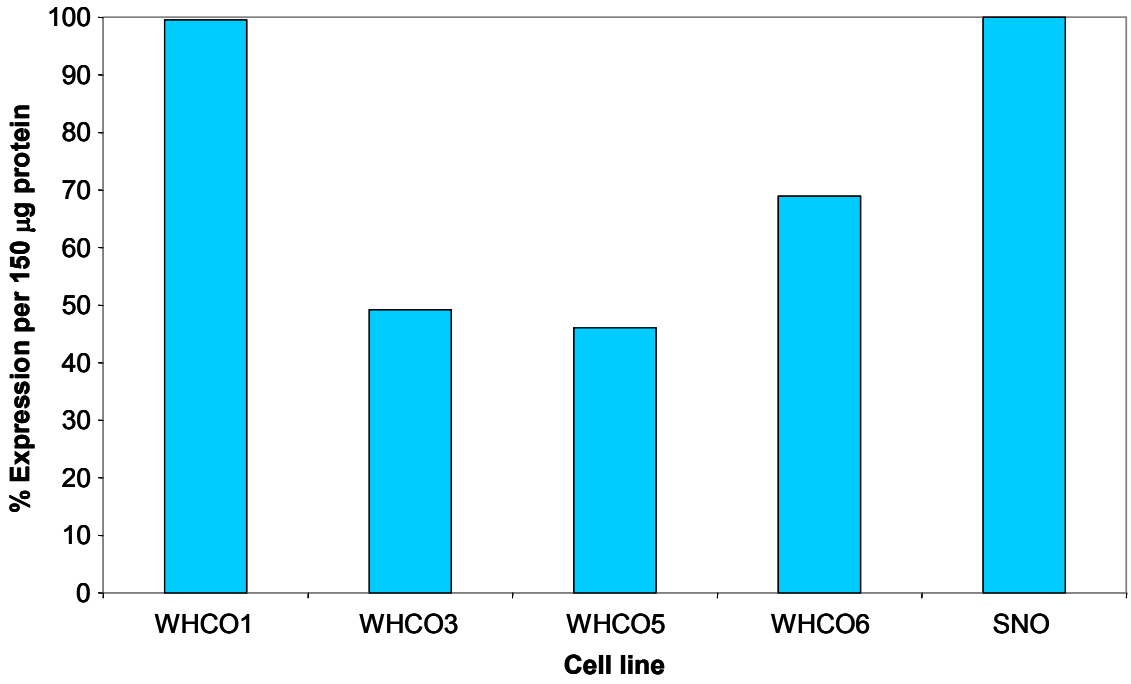


Figure 4.2: The standard level of tyrosine phosphorylated, membrane-associated β-catenin in oesophageal SCC cell lines. Under standard culture conditions, WHCO1 and SNO express high levels of membrane-associated β-catenin that is phosphorylated on tyrosine residues, while WHCO3 and WHCO5 show an approximately 50% lower level of tyrosine phosphorylated β-catenin. The level of tyrosine phosphorylated β-catenin was determined by densitometric analysis of the immunoblots of the phosphotyrosine immunoprecipitates (150 µg of total protein) and is expressed as a percentage of the highest (found in SNO).

Shiozaki *et al*., 1995; Fujii *et al*., 1996; Takahashi *et al*., 1997; Hazan and Norton, 1998). The present investigation set out to determine whether EGF induces a change in the tyrosine phosphorylation status of β-catenin in the moderately-differentiated oesophageal SCC cell lines and the extent to which this occurs. The membrane fractions of cells treated with EGF (3 hours) were subjected to immunoprecipitation for the tyrosine phosphorylated proteins, which were subsequently immunoblotted for β-catenin. Tyrosine phosphorylated β-catenin was detected in all the oesophageal SCC cell lines under these conditions, with the exception of WHCO3 (refer to Figure 4.1, lane 3). When comparing the concentration of tyrosine phosphorylated β-catenin to the basal levels (determined through densitometry of the immunoblots), only WHCO6 and SNO showed elevated concentration levels by 3- and 2.8-fold, respectively, while the other three cell lines remained at approximately standard concentrations (= 1, see Figure 4.3).

Tyrosine phosphorylated β-catenin was strongly detected in the plasma membrane fractions of moderately-differentiated oesophageal SCC cell lines when simultaneously exposed to EGF and lithium (3 hours), with the exception of WHCO1 and WHCO5 (Figure 4.1, lane 4). Quantification through densitometry of the immunoblots, revealed that the WHCO3 cell line showed a large increase in the concentration of tyrosine phosphorylated β-catenin relative to the standard expression (5.2-fold; see Figure 4.3). This was followed by WHCO6 which showed a slightly smaller augmentation of 2.1-fold. WHCO5 and SNO remained at standard expression levels, while WHCO1 displayed a 0.5-fold decrease compared to the basal expression level. This result is interesting as it closely parallels the changes in the cytoplasmic concentration of β-catenin noted under these conditions in the previous chapter (refer back to Figure 3.7). That is, the two cell lines expressing the highest concentration of tyrosine phosphorylated, plasma membrane β-catenin (WHCO3 and WHCO6) showed the largest fold increase in the cytoplasmic component, while the smallest change (WHCO1) showed the lowest tyrosine phosphorylation β-catenin concentration at this time. This suggests that tyrosine phosphorylation may play a pivotal role in inducing the

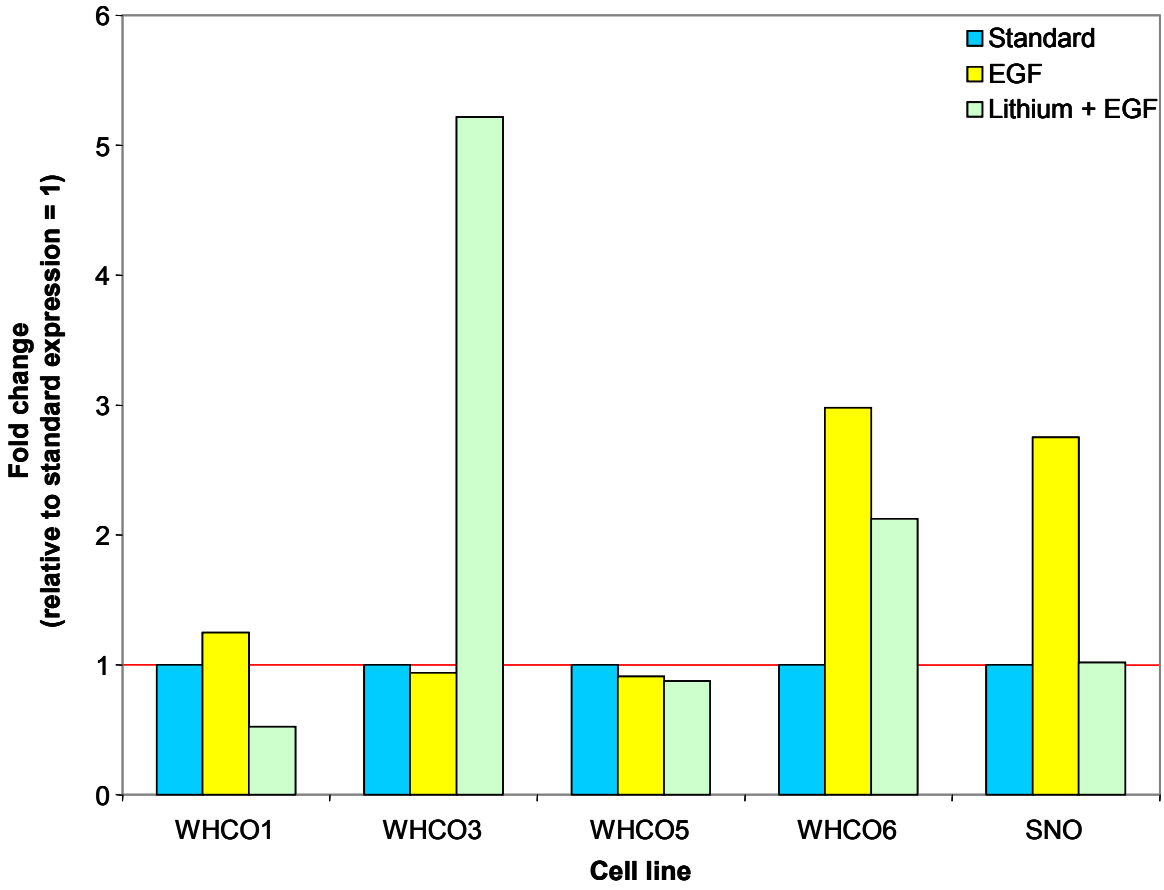


Figure 4.3: The concentration of tyrosine phosphorylated, plasma membrane-associated β-catenin exposed to EGF alone, or concurrently with lithium compared to basal expression. The concentration level of tyrosine phosphorylated β-catenin after treatment with EGF alone (3 hours) increased from standard expression (= 1) in both the WHCO6 and SNO cell lines by 3- and 2.8-fold, respectively, but did not change in the other cell lines. Concurrent exposure to EGF and lithium (3 hours) induced a large increase of tyrosine phosphorylated β-catenin in WHCO3 (5.2-fold) and to a lesser degree in WHCO6 (2.1-fold). WHCO5 and SNO levels remained at standard concentrations, while WHCO1 displayed a slightly decreased level (0.5-fold). The concentration of tyrosine phosphorylated β-catenin in response to EGF stimulation alone, or together with lithium, was assessed through densitometry of the immunoblots and was expressed as a fold change relative to standard concentration of tyrosine phosphorylated β-catenin in these cell lines.

redistribution of β-catenin from the plasma membrane into the cytoplasm seen under these conditions.

4.3.3 The basal concentration of serine phosphorylated membrane-associated β-catenin is generally low in human oesophageal SCCs

The basal concentration of serine phosphorylated β-catenin associated with the plasma membrane was established through immunoblotting serine phosphorylated immunoprecipitated proteins from this fraction for β-catenin. In general, the moderately-differentiated oesophageal SCC cell lines did not show a strong immunoreactivity for serine phosphorylated, membrane-associated β-catenin when grown under standard culturing conditions (see Figure 4.4, lane 2). The SNO cell line however, showed a strong reaction under these conditions. This cell line was shown through semiquantitative densitometric analysis of the immunoblots, to express greater than 80% more serine phosphorylated β-catenin in this fraction than the other four cell lines investigated (Figure 4.5). No cross reactivity occurred between the anti-β-catenin antibody and Protein G from the agarose beads or the anti-phosphoserine IgGs (result not shown).

4.3.4 EGF alone, or together with lithium, increased the concentration of serine phosphorylated, membrane-associated β-catenin only in WHCO5 and WHCO6

The EGF augmentation of the cytoplasmic concentration of β-catenin induced by lithium was suggested by Chen *et al*. (2000) to occur through the activation of PKCα, although they did not identify a target for this kinase. Data from the previous chapter suggests that the adhesion complex may be the target of this kinase. As PKCα is serine kinase, the effect of EGF and/or lithium stimulation on the serine phosphorylated β-catenin concentration was investigated in the oesophageal SCC cell lines. In cells stimulated with EGF alone, or together with lithium (for 3 hours), serine phosphorylated, plasma-membrane associated

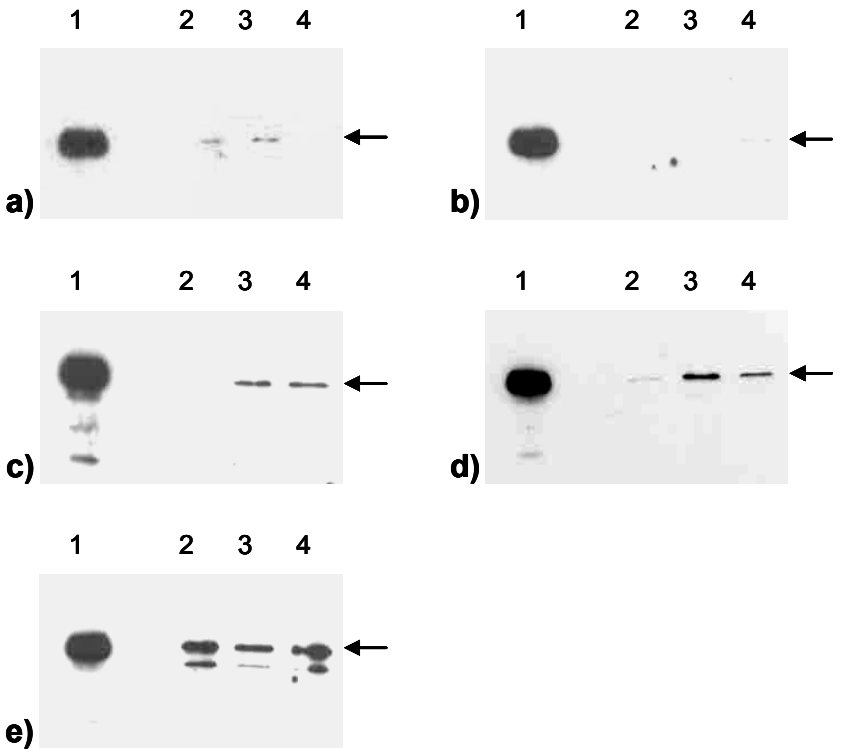


Figure 4.4: Serine phosphorylated, plasma membrane-associated β-catenin identification in human oesophageal SCC cell lines, under standard culturing conditions or when exposed to EGF and/or lithium. All serine phosphorylated proteins were immunoprecipitated with anti-phosphoserine antibody (Sigma) from the membrane fractions of oesophageal SCC cell lines that were either untreated (lane 2), or treated with EGF alone (lane 3) or together with lithium (lane 4) for 3 hours. These immunoprecipitated proteins were separated by electrophoresis and immunoblotted for β-catenin. A whole cell extraction from WHCO6 (lane 1) was loaded as a reaction control. In the WHCO1 cell line **(a)**, plasma membrane-associated β-catenin (indicated by the arrow) was serine phosphorylated under standard tissue culture conditions (lane 2), and when treated with EGF alone (lane 3), but not together with lithium (lane 4). WHCO3 **(b)** only displayed a faint band representing serine phosphorylated β-catenin when treated concurrently with lithium and EGF, but not under standard conditions or when exposed to EGF alone. Exposure to EGF alone, or together with lithium, induced serine phosphorylated β-catenin, but was not at all, or only marginally, phosphorylated under standard tissue culture conditions in WHCO5 **(c)** and WHCO6 **(d)**. β-catenin in SNO **(e)** was strongly phosphorylated on serine residues under all conditions.

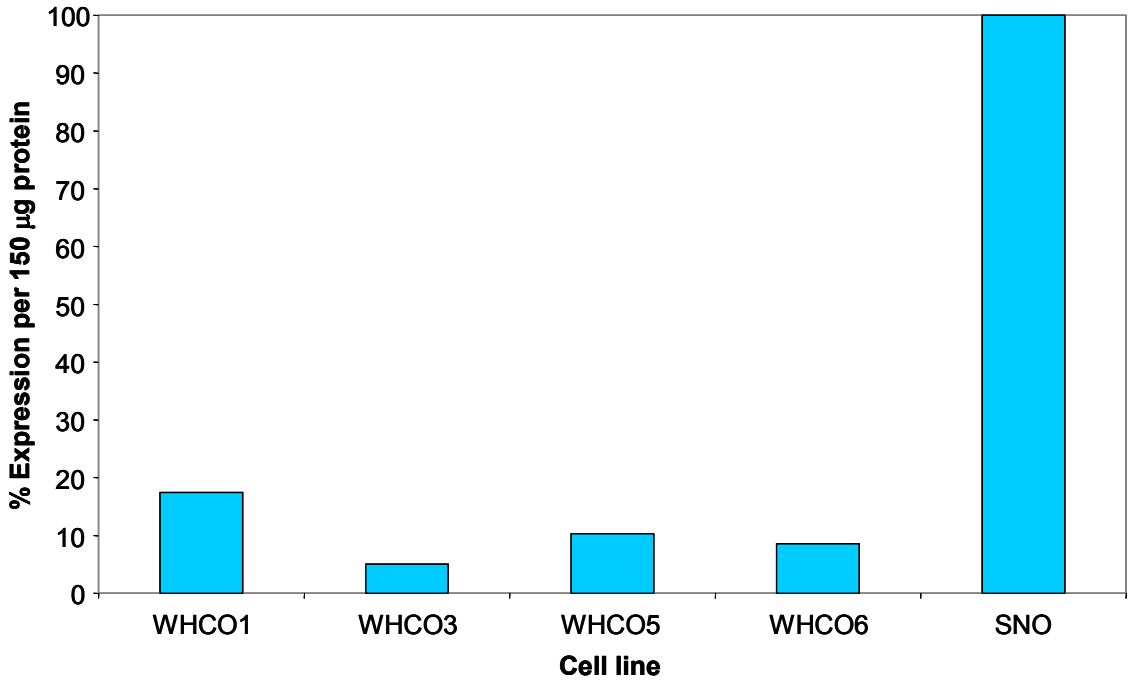


Figure 4.5: The standard concentration of serine phosphorylated, membrane-associated β-catenin in oesophageal SCC cell lines. The majority of the oesophageal SCC cell lines investigated showed a low level of serine phosphorylated β-catenin in the membrane fraction under standard tissue culture conditions. The SNO cell line, however, displayed a much higher concentration of serine phosphorylated β-catenin than the other cell lines, being ~80% greater than the next highest (WHCO1). The level of serine phosphorylated β-catenin was determined through densitometric analysis of the β-catenin immunoblots of the phosphoserine immunoprecipitates (150 μg of total protein) and is expressed as a percentage of the maximum (SNO).

β-catenin was found in the WHCO5, WHCO6 and SNO cell lines (refer to Figure 4.4, lanes 3 and 4). The WHCO1 cell line showed a slight reaction for serine phosphorylated β-catenin after exposure to EGF alone, while none was noted in WHCO3. Conversely, a faint result in WHCO3, but not in WHCO1, occurred in cells concurrently treated with lithium and EGF.

As can be seen in Figure 4.6, EGF alone induced an increase of serine phosphorylated β-catenin in WHCO5 by 3.2-fold, and more notably in WHCO6 by 5.2-fold, when compared to the standard expression (= 1; ascertained through densitometry of the immunoblots). WHCO1 and WHCO3 remained at basal levels, while the SNO cell line showed a small decrease of serine phosphorylated β-catenin. Similarly, concurrent treatment with lithium and EGF resulted in increased serine phosphorylated β-catenin in WHCO5 and WHCO6, although this was slightly lower than with EGF alone (3- and 2.5-fold, respectively). WHCO3 and SNO did not change from standard expression, while the concentration of serine phosphorylated β-catenin in WHCO1 declined slightly.

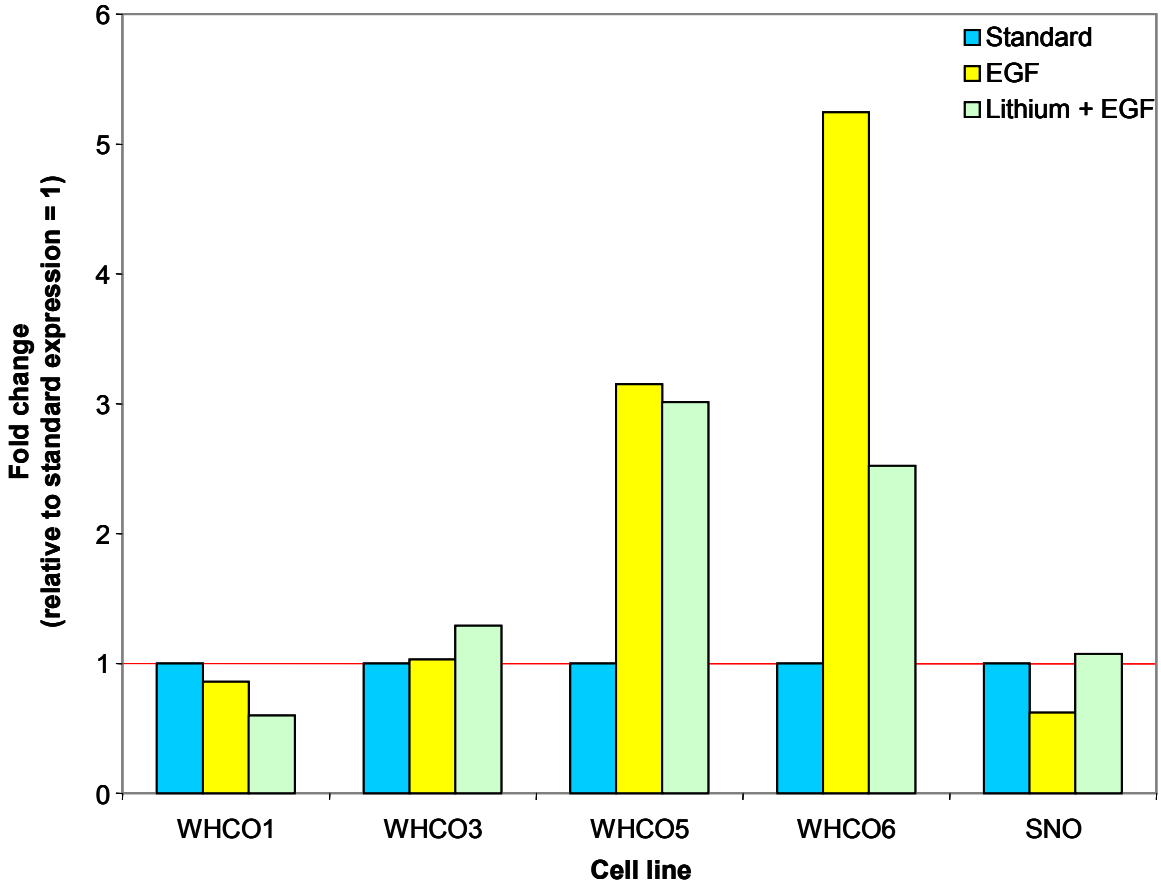


Figure 4.6: EGF and/or lithium treatment induced an increase of serine phosphorylated β-catenin in WHCO5 and WHCO6. In comparison with untreated cells (standard = 1), EGF treatment (3 hours) induced an increase in the concentration of serine phosphorylated, plasma membrane-associated β-catenin only in the WHCO5 (3.2-fold) and WHCO6 (by 5.2-fold) cell lines. SNO displayed a slightly lower than standard phosphorylation level, while WHCO1 and WHCO3 remained unchanged. Concurrent EGF and lithium exposure (3 hours) also induced an increase of serine phosphorylated β-catenin in WHCO5 and WHCO6 (3- and 2.5-fold, respectively). A slight decrease of serine phosphorylated β-catenin was noted in WHCO1, while WHCO3 and SNO remained at standard concentrations. The concentration of serine phosphorylated β-catenin in EGF and/or lithium treated cells, was assessed through densitometry of the immunoblots and was expressed as a fold increase (or decrease) relative to that found under standard tissue culture conditions.

4.4 Discussion

Experiments presented and discussed in Chapter Three showed that simultaneous EGF and lithium stimulation altered the concentration of β-catenin in the membrane and cytoplasmic pools in a manner that suggested a redistribution of β-catenin away from its structural function towards its signalling role in the oesophageal SCC cell lines. It is already known that phosphorylation-induced, posttranslational modification of β-catenin regulates its associations with its various binding partners, including E-cadherin and α-catenin (Hoschuetzky *et al*., 1994; Kinch *et al*., 1995; Roura *et al*., 1999; Lickert *et al*., 2000). Thus, it is most likely that EGF and lithium-induced signals converge to act in such a manner to alter the cell adhesion complex.

Tyrosine phosphorylation of β-catenin plays a particularly important role in regulating its associations with members of the adhesion complex (Kinch *et al*., 1995; Takahashi *et al*., 1997; Ozawa and Kemler, 1998a; Roura *et al*., 1999; Hu *et al*., 2001; Piedra *et al*., 2003). Phosphorylation of Y654 and Y142 have been shown to disrupt the β-catenin's association with E-cadherin and α-catenin, respectively (Roura *et al*., 1999; Piedra *et al*., 2003). Shiozaki *et al*. (1995) showed that under basal culturing conditions, β-catenin was not tyrosine phosphorylated in a poorly-differentiated oesophageal SCC cell line (TE-2R). However, the moderately-differentiated oesophageal SCC cell lines investigated in the present study differ from that described by Shiozaki *et al*. (1995), as the cell lines generally displayed the presence of tyrosine phosphorylated, plasma membrane-associated β-catenin under standard tissue culture conditions. This was especially marked in the WHCO1 and SNO cell lines (Figure 4.2). The strong presence of tyrosine phosphorylated β-catenin in WHCO1 and SNO is interesting as these two cell lines showed the lowest concentration of β-catenin present in the plasma membrane fraction (see Chapter 2, Figure 2.7). Thus, this may imply that these tumour cell lines possess a constitutively active tyrosine kinase that

phosphorylates β-catenin under standard conditions, maintaining a lower level of cell-cell adhesion.

EGFR is a likely candidate as an overactive tyrosine kinase as it is overexpressed in these oesophageal SCC cell lines (Veale and Thornley, 1989). The EGFR is known to associate with the cadherin-adhesion complex (Fukuyama and Shimizu, 1991; Takahashi and Suzuki, 1996) and reduce intercellular adhesion through tyrosine phosphorylation of β-catenin (Hoschuetzky *et al*., 1994; Ochiai *et al*., 1994; Shibamoto *et al*., 1994; Shiozaki *et al*., 1995; Takahashi *et al*., 1997; Hazan and Norton, 1998; Kuwada *et al*., 1998; Schroeder *et al*., 2002). Shiozaki *et al*. (1995) reported that the ectopic stimulation of the poorly-differentiated oesophageal TE-2R cells with EGF induced an increase of tyrosine phosphorylated β-catenin. The ability of EGFR to phosphorylate plasma membrane-associated β-catenin was similarly demonstrated in two of the moderately-differentiated oesophageal SCC cell lines (WHCO6 and SNO) as these lines showed an elevated concentration of tyrosine phosphorylated β-catenin in this fraction (see Figure 4.3). However, the other three cell lines (WHCO1, WHCO3 and WHCO5) did not show an alteration to the concentration of tyrosine phosphorylated β-catenin from standard levels. This may be a consequence of the duration of EGF exposure prior to extraction, as it should be remembered that EGF stimulation for 3 hours did not alter the concentration of β-catenin in either the membrane or the cytoplasmic fractions (refer back to Figure 3.5). This may be due to the activation of the protein tyrosine phosphatase, PTP1B, which reportedly dephosphorylates β-catenin on Y654 (Xu *et al*., 2004). EGF stimulation has been shown to activate PTP1B, leading to a decrease in the level of tyrosine phosphorylated β-catenin, thus acting as a positive feedback regulation for cell adhesion (Xu *et al*., 2004). Thus, if EGF stimulation does indeed increase the concentration of tyrosine phosphorylation of β-catenin in these cell lines, it may already have been reversed by PTP1B after 3 hours, and therefore, was not observed in these cell lines. This is a real possibility as Shiozaki *et al*. (1995) showed that the EGF-induced tyrosine phosphorylation of β-catenin was enhanced by the addition of vanadate, a potent PTP inhibitor.

Müller *et al*. (1999) showed that EGF-induced cell migration in the rat bladder carcinoma NBT-II cell line correlated with the redistribution of β-catenin into the cytoplasm and nucleus and increased tyrosine phosphorylated β-catenin. This group further demonstrated that in NBT-II cells expressing a stabilized mutant of β-catenin (S37/45A), EGF further enhanced the signalling pool of β-catenin, suggesting synergism between EGF stimulation and β-catenin signalling (Müller *et al*., 2002). This result is similar to that obtained in the oesophageal SCC cell lines studies here. EGF stimulation enhanced the cytoplasmic pool of β-catenin stabilized by lithium in these cell lines, suggesting that the phosphotyrosine content of β-catenin under these conditions may also be altered. The effect of simultaneous EGF and lithium exposure (3 hours) on the concentration of tyrosine phosphorylated β-catenin present in the membrane fraction was most interesting. The WHCO3 and WHCO6 cell lines showed a large increase in the concentration of tyrosine phosphorylated β-catenin in this fraction (5.2- and 2.1-fold, respectively, Figure 4.3). Interestingly, these are the cell lines that showed the largest increase to the cytoplasmic β-catenin concentration (refer back to Figure 3.7.b). Furthermore, the WHCO1 cell line, which displayed a decreased concentration of tyrosine phosphorylated β-catenin, was the only cell line that did not show an augmentation to the cytoplasmic β-catenin pool at this length of exposure. However, this cell line did show an increase to the cytoplasmic β-catenin concentrations after 12 hours of exposure, and thus it would be interesting to determine if the level of tyrosine phosphorylated β-catenin would increase at this time. Therefore, from these results it appears that EGF and lithium stimulation alters the phosphotyrosine content of β-catenin in the cadherin-catenin complex, which may alter its affinity for members of the adhesion complex, shifting the protein into the cytoplasm. This has not previously been demonstrated in oesophageal SCC cells.

The redistribution of β-catenin in response to these signals may also be a result of altering the serine phosphorylation of members of the adhesion complex. Chen *et*

al. (2000) described that the EGF-mediated augmentation of cytoplasmic β-catenin concentration induced by lithium in the C57MG and HEK-293 cells occurred as a result of activating the TPA-sensitive, serine kinase, PKCα. However, as discussed previously, they were unable to find a target for this kinase and focused their attention on the role it possibly plays in regulating the β-catenin degradation complex. However, the adhesion complex could be a potential target for PKCα as it has been shown in other studies to play a role in regulating the adhesion complex. For example, activated PKCα in CACO-2 cells associates with the cadherins and results in cadherin-β-catenin dissociation, disrupting the adherens junctions (Malladi *et al*., 2004). In particular, the distribution of β-catenin appears to be affected by PKCα activity at the membrane as Baulida *et al*. (1999) reported that PKCα was able to induce the redistribution of β-catenin away from the plasma membrane into the nucleus in normal intestinal cells. Therefore, the EGF/lithium-induced redistribution of β-catenin noted in the oesophageal SCC cell lines studied here may act through the serine kinase activity of PKCα to disrupt the adhesion complex.

In order to establish if EGF/lithium-induced redistribution of β-catenin is serine kinase-dependent, the effect of EGF and lithium stimulation on the concentration of serine phosphorylated β-catenin was investigated. Under standard culturing conditions, the concentration of serine phosphorylated β-catenin present in the plasma membrane fraction was generally low, with the exception of the SNO cell line (Figure 4.5). This suggests that serine phosphorylation of β-catenin in the adhesion complex remains tightly regulated in these cell lines, possibly through the activity of a serine phosphatase that operates specifically around the adhesion complex. The WHCO5 and WHCO6 cell lines showed an increase in the concentration of serine phosphorylated β-catenin in response to stimulation by EGF alone, or together with lithium (3 hours; Figure 4.6), suggesting that these signals can act to alter the serine phosphorylated β-catenin in the plasma membrane fraction. The WHCO6 cell line showed an increase in the concentration of β-catenin in the cytoplasmic fraction in response to these signals

(refer back to Figure 3.7.b). However, it is debatable whether the increase noted in the serine phosphorylation concentration is related to this increase in cytoplasmic fraction as neither WHCO3 nor SNO, which also displayed increased cytoplasmic β-catenin in response to these signals, showed a change to the concentration of serine phosphorylated β-catenin. Furthermore, although the WHCO5 cell line showed an increase of serine phosphorylated β-catenin under these conditions, it did not show an increase of cytoplasmic β-catenin in response to these signals. This therefore, suggests that serine phosphorylation of β-catenin is unlikely to be playing a role in the EGF and lithium-induced redistribution of β-catenin.

Thus, the present study has shown that increasing the level of tyrosine phosphorylation of β-catenin, but not necessarily the serine phosphorylation, may play a role in inducing the redistribution of β-catenin in response to concurrent stimulation with EGF and lithium in the oesophageal SCC cell lines. However, this does not rule out a possible role for PKCα in inducing the redistribution of β-catenin in response to EGF and lithium stimulation, as it may act on another target within the adhesion complex. For example, it may alter the distribution of E-cadherin, and thus β-catenin as a default (Barbosa *et al*., 2003). Alternatively, it may interfere with GSK-3β-mediated serine/threonine phosphorylation of E-cadherin, reducing its affinity for β-catenin (Lickert *et al*., 2000; Goode *et al*., 1992). This may be further attenuated by lithium-induced inhibition of GSK-3β (Klein and Melton, 1996; Stambolic *et al*., 1996; Zhang *et al*., 2003). This may well form the basis of another study. Therefore, the action of EGF and lithium stimulation on the distribution of β-catenin may occur through inducing multiple effects, which include decreasing the affinity of β-catenin for E-cadherin by increasing the tyrosine phosphorylation of β-catenin and reducing the serine phosphorylation of E-cadherin, while also stabilizing the cytoplasmic fraction through inhibition of GSK-3β-mediated degradation.

CHAPTER FIVE

General Discussion and Conclusions

5.1 The expression of β-catenin in human moderately-differentiated oesophageal SCCs – implications regarding its functions in this type of cancer

Since it first made its appearance in the late 1980's, a large body of evidence has been accumulated to show the significant role β-catenin plays in two distinct areas in the cell. Its main involvement is structural where it plays an essential intermediary role in the adherens junctions by linking the plasma membrane bound cadherin molecules to the actin cytoskeleton (Ozawa *et al*., 1990b; Aberle, *et al*., 1994; Hinck *et al*., 1994b; Oyama *et al*., 1994; Jou *et al*., 1995; Gumbiner, 1996; Pokutta and Weis, 2000; Huber and Weis, 2001). It is due to it occupying this central position that β-catenin is thought to regulate intercellular adhesion (Hinck *et al*., 1994b; Gumbiner, 1996; Takayama *et al*., 1998). The adhesion complex is thought to be a suppressor of invasion and metastasis in many epithelial tumours (Vleminckx *et al*., 1991; Oyama *et al*., 1994; Wijnhoven *et al*., 2000). Therefore, deregulation of intercellular adhesion is an important feature of tumour biology as it potentiates the invasive and metastatic behaviour of the cancer cells (Behrens *et al*., 1989; Frixen *et al*., 1991; Vleminckx *et al*., 1991; Dorudi *et al*., 1993; Birchmeier and Behrens, 1994; Ihara *et al*., 1996; Mbalaviele *et al*., 1996; Shiozaki *et al*., 1996; Perl *et al*., 1998; Wijnhoven *et al*., 2000; Al-Aynati *et al*., 2004).

The second area in which β-catenin is active is in signal transduction and the regulation of gene transcription. A number of genes targeted by β-catenin are potentially oncogenic if overexpressed, good examples being the cell cycle promoters, *c-myc* and *cyclin D1* (He *et al*., 1998; Shtutman *et al*., 1999; Tetsu and McCormick, 1999; also see Table 1.2 for detailed list). Accumulation of free,

unbound β-catenin in the cytoplasm enables translocation of β-catenin into the nucleus, augmenting its transcriptional activity (Behrens *et al*., 1996; Huber *et al*., 1996; Molenaar *et al*., 1996; Korinek *et al*., 1997; Rubinfeld *et al*., 1997). However, the level of cytoplasmic β-catenin is usually tightly regulated by a degradation complex composed of GSK-3β, CKIα, APC and axin, which targets β-catenin for degradation by the ubiquitin-proteasomal pathway (Munemitsu *et al*., 1995; Rubinfeld *et al*., 1996; Aberle *et al*., 1997; Orford *et al*., 1997; Hart *et al*., 1998; Ikeda *et al*., 1998; Kitagawa *et al*., 1999; Liu *et al*., 1999a; Hinoi *et al*., 2000; Liu *et al*., 2002). Deregulation or stabilization of cytoplasmic β-catenin, through mutations or via various signalling pathways (see Chapter 1.8 and 1.9), has been shown to be of great importance in the development of a number of cancers, such as colorectal carcinomas (Ilyas *et al*., 1997; Morin *et al*., 1997; Miyoshi *et al*., 1998; Satoh *et al*., 2000; Jin *et al*., 2003).

It is due to these diverse roles played by β-catenin in cells and tumours that it has become of interest to understanding the development and behaviour of human oesophageal SCCs, especially that of the most poorly understood category – the moderately-differentiated category. This study set out to determine the expression of β-catenin within this category in order to understand the functioning of β-catenin in adhesion and signal transduction/gene transcription in these cells, and thus, the potential role it may play in the development of this type of tumour.

The ability of the components of the adherens junctions to physically associate is imperative in maintaining adhesion and tissue compaction. Truncations of the adhesion proteins can prevent these interactions from occurring. Mutations resulting in a truncated E-cadherin product that has an altered cytoplasmic tail have been reported in lobular breast and gastric carcinomas (Oda *et al*., 1994; de Leeuw *et al*., 1997), but rarely in other types of cancer. In addition, as of yet, no reports of a truncated β-catenin protein, preventing it from binding to E-cadherin, have been described in any tumours regardless of their histological origin. This study showed that in lines derived from moderately-differentiated oesophageal SCCs, the majority of β-catenin localizes to points of cell-cell contact at the

plasma membrane corresponding to the localization of E-cadherin in these cells (Jones and Veale, 2003). This intimates that the association between β-catenin and E-cadherin is not compromised in this type of cancer. This is similar to immunohistochemical studies which showed that the majority of β-catenin was preserved at the plasma membrane in oesophageal SCCs (Sanders *et al*., 1998; Lin *et al*., 2004). Therefore, it is unlikely that adhesion is reduced in this type of tumour due to the inability of E-cadherin and β-catenin to physically associate as a result of an event such as a truncating gene mutation.

Although protein interactions are essential, another important aspect in the maintenance of cell adhesion is the concentration at which the components of the adhesion complex are expressed. Reduced expression of the components of the adhesion complex has been associated with attenuation of adhesion and increased metastasis. For example, Takayama *et al*. (1998) showed that colorectal cancers expressed reduced levels of β-catenin as compared with normal tissue. Several other immunohistochemical studies have reported that β-catenin levels are lower than normal oesophageal mucosa (Nakanishi *et al*., 1997; Sanders *et al*., 1998; De Castro *et al*., 2000; Zhao *et al*., 2003; Lin *et al*., 2004; Zhang *et al*., 2005). Thus, as the moderately-differentiated oesophageal SCC cells used in the present study were derived from already metastasising tumours (and compounded by the supply of, and difficulties in, culturing normal oesophageal squamous cells), it was assumed that the expression of β-catenin in these cell lines was lower than normal tissue. One would expect from the histological grouping of these cell lines into one category (moderately-differentiated), that these cell lines would exhibit similar levels of β-catenin, and thus adhesion. Therefore, the concentration of β-catenin expression *within* this broad histological class was assessed to establish the value of β-catenin to differentiate between members of this category. The total levels of β-catenin detected by radioimmunoassay showed that the cell lines did not differ significantly from each other and thus, β-catenin promised to be a consistent marker for this grade of the disease. However, on investigating the concentration of β-catenin associated with the plasma membrane, there appeared to be large differences in the level of expression at this locality between cell lines

(between 48 and 100%; see Chapter 2.3.4). This indicates that there may be inconsistencies in the level of adhesion, and therefore, one could speculate differences in metastatic potential, occurring between these cell lines that have been traditionally classified as being of similar pathology. However, this would need additional clarification by a cell-cell adhesion assay. Thus, at best, β-catenin expression may be a useful tool for further defining/refining the moderately-differentiated class of oesophageal SCCs into which borderline cases are often placed.

Deregulation and stabilization of β-catenin in the cytoplasm have been associated with the progression of a multitude of cancers, such as hepatocellular carcinoma, ovarian cancer, gastric cancer and in particular, colorectal adenocarcinoma (refer to Table 1.3 for a comprehensive list). These cancers often contain mutations of either the *APC* gene or of the β-catenin gene (*CTNNB1*) itself, which impedes the targeting phosphorylation of β-catenin, freeing the protein from ubiquitination and its subsequent degradation (Rubinfeld *et al*., 1993; Su *et al*., 1993; Ilyas *et al*., 1997; Morin *et al*., 1997). Powell *et al*. (1994) however, showed that the mutation in the *APC* gene, resulting in a product that lacks the β-catenin regulatory domain, is rare in oesophageal SCCs. In addition, it has been demonstrated *in vitro* in these cell lines (Schnugh, 2001), as well as *in vivo* (Sanders *et al*., 1998; De Castro *et al*., 2000; Ninomiya *et al*., 2000; Zhang *et al*., 2005), that oesophageal SCCs lack the mutation in the *CTNNB1* gene that is associated with its stabilization. The results presented in this study further corroborate these findings as very little β-catenin was found in the cytoplasm of the oesophageal SCC cells. This strongly suggests that constitutive stabilization of β-catenin does not play a role in the development of oesophageal SCC.

It can therefore be said with some certainty that under standard *in vitro* conditions, β-catenin's role in moderately-differentiated oesophageal SCC is skewed towards cell-cell adhesion, rather than its nuclear activities, albeit inconsistently, between cell lines. It would be tempting to conclude that β-catenin is a poor molecular marker for this stage of the disease based on these results. However, it must be

remembered that maintenance of the adhesion complex is more complicated than mere protein-protein interactions. A number of signal transduction pathways have been shown to impinge on the function of β-catenin in the adhesion complex, acting to regulate its function and, perhaps as a result, other cellular functions, such as cell-ECM interactions. Therefore, the effect of three important signal transduction pathways induced by Wnt, EGF, and TGF-β1 (both separately and in union) on the expression of β-catenin involved in adhesion was examined.

5.2 The effect of exogenous signals, EGF, TGF-β1 and lithium, on the functions of β-catenin

5.2.1 The Wnt-like stabilization of β-catenin by lithium increased the strength of adhesion

The first pathway of interest to this study was that of the "developmental" pathway, Wnt. The major effect of canonical Wnt signalling is the stabilization of β-catenin in the cytoplasm (see Chapter 1.9.1 for details). The stabilization and accumulation of cytoplasmic β-catenin is known to augment its transcriptional activity in the nucleus. It has already been established that constitutive stabilization of cytoplasmic β-catenin, such as by mutation of *APC* and *CTNNB1*, does not occur in human oesophageal SCCs (Powell *et al*, 1994; Sanders *et al*., 1998; De Castro *et al*., 2000; Ninomiya *et al*., 2000; Schnugh, 2001; Zhang *et al*., 2005). However, stabilization of β-catenin can still occur through activation of the Wnt pathway by exogenous ligands, the wnts, which are secreted from neighbouring cells (Moon *et al*., 2004; Weeraratna, 2005). The wnts that stimulate the canonical Wnt pathway have been shown to be highly transforming in nature (Wong *et al*., 1994; Shimizu *et al*., 1997). Oesophageal carcinomas have been reported to upregulate the expression of one of these wnts, wnt-2 (Vider *et al*., 1996). In addition, oesophageal SCCs have been shown to overexpress a novel wnt receptor, FzE3, which leads to increased nuclear β-catenin (Tanaka *et al*.,

1998). Therefore, stimulation of the Wnt pathway by these factors may be an additional role player in the development of oesophageal SCCs.

Although a large body of information exists as to the role Wnt signalling plays in stabilizing cytoplasmic β-catenin, very little is known about the effect it has on the structural function β-catenin plays at the plasma membrane. This study therefore, focused on the effect that stabilized cytoplasmic β-catenin has on the level of β-catenin present in the adhesion junctions. The wnts are notoriously difficult to isolate (Cadigan and Nusse, 1997) and thus, in order to achieve a Wnt-like stabilization of β-catenin, cells were treated with lithium, a well known mimic of the Wnt signal transduction pathway (Klein and Melton, 1996; Stambolic *et al*., 1996). Lithium acts by inhibiting the kinase activities of GSK-3β, thus preventing the targeting phosphorylation of β-catenin, releasing it from ubiquitination and proteasomal degradation (Harwood *et al*., 1995; Hedgepeth *et al*., 1997; Orford *et al*., 1997; Willert *et al*., 1999; Chen *et al*., 2000; Zhang *et al*., 2003). This results in a pool of stable cytoplasmic β-catenin and increases its transcriptional activities in the nucleus (Chen *et al*., 2000; Playford *et al*., 2000). The inhibitory effect of lithium has previously been shown in a variety of embryos, such as *Xenopus* and *Drosophila* (Klein and Melton, 1996; Pierce and Kimelman, 1995; Stambolic *et al*., 1996), as well as in a number of mammalian cell lines, including mammary (C57MG and HBL100), colorectal cancer (C10) and the kidney (HEK-293) cell lines (Orford *et al*., 1997; Willert *et al*., 1999; Chen *et al*., 2000; Playford *et al*., 2000). However, although these mammalian cell lines are epithelial in origin, breast, colorectal and kidney are glandular epithelia and thus differ considerably from the squamous epithelia found in the oesophagus. Therefore, does lithium effectively stabilize β-catenin in the cytoplasm in human oesophageal SCCs? This is the first study to address this question.

Lithium induced a rapid increase (3 hours) in the levels of cytoplasmic β-catenin in three of the oesophageal SCC cell lines (WHCO1, WHCO3 and WHCO5) demonstrating that it does indeed play an inhibitory role on GSK-3β, as in the breast, colorectal and kidney cell lines (Orford *et al*., 1997; Willert *et al*., 1999;

Chen *et al*., 2000; Playford *et al*., 2000). This established the efficacy of lithium to induce stabilization of cytoplasmic β-catenin in a Wnt-like manner in the oesophageal SCC cell lines. However, it should be noted that in these experiments the stabilization of β-catenin induced by lithium was short-lived since β-catenin was no longer detected in the cytoplasmic fraction after 12 hours. This could occur for a number of reasons. The first, most obviously, being that the β-catenin degradation complex was able to resume its activities to control the level of β-catenin. Alternatively, β-catenin may return to a structural conformation that enables it to resume its more "normal" protein-protein associations within the cell. Gottardi and Gumbiner (2004b) demonstrated in HEK-293T cells that a brief exposure to lithium (under 4 hours) induced conformational changes to free β-catenin that reduced its affinity for E-cadherin, whilst increasing that for the nuclear Tcf protein. However, over longer exposures (greater than 6 hours), lithium was no longer able to influence the β-catenin's binding preference as it associated equally well with E-cadherin and Tcf. This effect of lithium on β-catenin's binding affinities is attractive when considering the effect that lithium produced on the membrane fraction of β-catenin in the oesophageal SCC cell lines studied here.

Short-term lithium stimulation (3 hours) did not alter β-catenin already associated with the plasma membrane in the oesophageal SCC cell lines. However, an increase in the concentration of β-catenin in this fraction was noted after 12 hours (see Chapter 3.3.1). E-cadherin is known to sequester cytoplasmic β-catenin to the plasma membrane in a range of cellular systems such as the colorectal SW480 cancer cell line, the mouse mammary epithelial cell line FosER, the mammary cell lines AtT20 and C57MG, as well as in ES cells (Hinck *et al*,. 1994a; Fagotto *et al*., 1996; Sadot *et al*., 1998; Simcha *et al*., 1998; Orsulic *et al*., 1999; Eger *et al*., 2000; Gottardi *et al*., 2001; Stockinger *et al*., 2001). Thus, it is proposed that the elevation of β-catenin at the plasma membrane noted in the oesophageal SCC cell lines is due to E-cadherin sequestering the excess cytoplasmic β-catenin generated by the lithium signals. However, this could only occur after a longer exposure to

lithium (12 hours), as in the short-term lithium would have restricted the free, stabilized, β-catenin from preferentially associating with the cadherin protein.

The stabilization of β-catenin by a Wnt-like signal does not reduce its adhesive role, which would be expected for increased cell mobility, but rather appears to increase the levels of β-catenin involved in adhesion. This was similarly demonstrated by Hinck *et al*. (1994a) who showed that wnt-1 activity stabilizes the contact between N-cadherin and β-catenin, and consequently increases the strength of adhesion in two mammary cell lines, C57MG and AtT20. Toyofuku *et al*. (2000) also demonstrated that Wnt-stimulation increased the β-catenin-cadherin association in cardiac myocytes and fibroblasts. This strongly indicated that the stabilization of β-catenin by a prolonged Wnt signal may be more important in the formation of cellular adhesion rather than its dissociation. This then could be a crucial factor in the establishment of a tumour cell in distal tissue, after metastasis, leading to the formation of a secondary tumour.

5.2.2 The significance of EGF and TGF-β1 signalling on the role of β-catenin

Both EGF and TGF-β1 have been implicated in inducing alterations to intercellular adhesion. Fujii *et al*. (1996) and Shiozaki *et al*. (1995) both showed that EGF stimulation reduced E-cadherin adhesion, ultimately resulting in alteration of an epithelial cell to a more dedifferentiated, fibroblastic morphology in a human cutaneous SCC and a poorly-differentiated oesophageal SCC cell line, respectively. Similarly, TGF-β1 has been shown to induce dissociation of the adherens junctions, which correlates with changes to cellular morphology in a number of cell types, although this has not been demonstrated in oesophageal SCC (Cui *et al*., 1996; Portella *et al*., 1998; Akhurst and Balmain, 1999; Bakin *et al*., 2000; Ellenrieder *et al*., 2001; Tian and Phillips, 2002; Tian *et al*., 2003; Weeks *et al*., 2001; Xu *et al*., 2003). The EGFR has been shown to be overexpressed in oesophageal SCC (Veale and Thornley, 1989), while the expression of TGF-β1 is increased and its "tumour suppressor" receptor, TβRI, is

downregulated in this type of tumour (Koliopanos *et al*., 2002; Fukai *et al*., 2003; Fukuchi *et al*., 2004). Thus, the question arose as to whether these two growth factors could similarly affect cell-cell adhesion in moderately-differentiated human oesophageal SCC, and if so, how do they bring about these changes. The present study focused on the effect that these growth factors have on the expression and localization of β-catenin.

Similar to the Fujii *et al*. (1996) and Shiozaki *et al*. (1995) reports, a rapid (within 5 minutes of exposure) decrease in cell adhesion was noted in the moderately-differentiated oesophageal SCC cell lines after exposure to EGF (result not shown). This implies that EGF-induced signalling is able to alter the cadherin-catenin complex. Therefore, its effect on β-catenin expression in these cell lines was examined. EGF stimulation did not alter the total concentration of β-catenin (as shown by the RIA) suggesting that it does not affect the expression of β-catenin at a transcriptional level. An extremely brief increase in β-catenin in the cytoplasm was noted after exposure to EGF in two of the cell lines (WHCO1 and WHCO3, see Chapter 3.3.2). Since the concentration of β-catenin associated with the plasma membrane remained unaltered, it is unlikely that the cytoplasmic increase was due to a redistribution of β-catenin within the cell. The cytoplasmic increase could be the result of EGF-mediated inhibition of GSK-3β through the MAPK/$p90^{rsk}$ or PI3K/PKB pathways (Stambolic and Woodgett, 1994; Cross *et al*., 1995; Eldar-Finkelman *et al*., 1995; Okano *et al*., 2000; Andl *et al*., 2003). However, due to the extreme brevity of this increase, it is unlikely that it would be adequate to play a considerable role in activation of gene transcription. This was further compounded by the research conducted in C57MG, HEK-293 (Chen *et al*., 2000) and TE-oesophageal SCCs (Mizushima *et al*., 2002), which demonstrated that EGF inhibition of GSK-3β is insufficient to effectively stabilize cytoplasmic β-catenin and to increase its transcriptional activity.

TGF-β1 has also been implicated in altering the functionality of β-catenin, both in adhesion and in its signalling capacity. TGF-β1 increases the levels of tyrosine

phosphorylated β-catenin resulting in its dissociation from E-cadherin and α-catenin in a renal proximal tumour cell line, HK-2, and in pancreatic carcinoma cells (Tian and Phillips, 2002; Vogelmann *et al*., 2005). It has also been associated with increased cytoplasmic β-catenin, induced by redistribution of the protein, inhibition of GSK-3β or reduced APC expression (Janji *et al*., 1999; Weeks *et al*., 2001; Cheon *et al*., 2004; Satterwhite and Neufeld, 2004; Edlund *et al*., 2005). Under the conditions used in the present study, no considerable alterations to the concentration of β-catenin associated with the plasma membrane, nor in the cytoplasmic pool, were noted in the oesophageal SCC cell lines, with the exception of the WHCO5 cell line, which showed a slight increase in both fractions. This suggests that, in general, TGF-β1 stimulation does not alter the cadherin-catenin complex, nor does it induce β-catenin stabilization and thus, signalling in these cells. However, apart from cell type, it should be noted that the effect of TGF-β1 on β-catenin may be dependent on context and concentration (Tian *et al*., 2003; Masszi *et al*., 2004). For example, cell density has been shown to influence the responsiveness of cells to TGF-β1 (Masszi *et al*., 2004). This was controlled for in the present study as all treatments were conducted in cultures that were in an exponential growth phase. Concentration of TGF-β1 however, may require further examination with respect to altering β-catenin functioning in the oesophageal SCCs. In the current study a single concentration of TGF-β1 that has previously been shown to provide an optimum effect in these cells was used. However, Xu *et al*. (2003) showed that nuclear accumulation of β-catenin in a hepatocellular carcinomas cell line (SMMC-7721), was dependent on the dose of TGF-β1, with a more notable increase of nuclear β-catenin being observed with increasing concentration of TGF-β1. Therefore, it is possible that TGF-β1 may play a role in altering the function of β-catenin, but not at the concentration used in the present investigation. The response observed in the WHCO5 cell line could thus be a consequence of it being slightly more responsive to a lower concentration of TGF-β1 than the other cell lines. Therefore, the effect of different concentrations of TGF-β1 on β-catenin needs to be addressed in future work.

5.2.3 Can EGF/TGF-β1 and Wnt signalling intersect to change β-catenin's functions in the cell?

The activity of a single pathway is often not sufficient to bring about significant change in the cell, which may require the interaction of various signalling pathways. Some growth factors have been shown to induce similar effects to that of Wnt signalling (see Chapter 1.9.5), and thus may intersect with it to augment or enhance its effect. Both EGF and TGF-β1 have been shown to crosstalk with Wnt signalling to alter the expression of β-catenin and its activities in the nucleus (Chen *et al*., 2000; Müller *et al*., 2002; Lei *et al*., 2004; Warner *et al*., 2005). The convergence of EGF and lithium/Wnt signalling pathways was highlighted by Chen *et al*. (2000) who showed that EGF signalling could enhance the accumulation of cytoplasmic β-catenin brought about by the inhibitory action of lithium (3 hours) in C57MG and HEK-293 cells. This accumulation resulted in an increase in the transcriptional activity of β-catenin in the nucleus (assessed by a luciferase assay of the activity of the Lef/Tcf transcriptional factor complex). Müller *et al*. (2002) similarly described EGF enhancement of β-catenin's transcriptional activity in NBT-II cells that express a stabilized mutant form of β-catenin. TGF-β1 has also been shown to crosstalk with Wnt signals to augment Wnt-induced stabilization of β-catenin in the murine embryonic maxillary mesenchymal (MEMM) and gastric adenocarcinoma (AGS) cell lines (Lei *et al*., 2004; Warner *et al*., 2005). Therefore, does convergence of EGF or TGF-β1 signalling with lithium/Wnt have similar effects in oesophageal SCC?

In general, the TGF-β1 and lithium/Wnt signals did not appear to appreciably cooperate with each other to alter the functionality of β-catenin in the oesophageal SCC cell lines. Neither the concentration of β-catenin associated with the plasma membrane, nor in the cytoplasmic fraction, were altered by simultaneous exposure to TGF-β1 and lithium. In contrast, however, the SNO cell line did display a small yet steady increase of β-catenin in the cytoplasm over the 24 hour period of

exposure. As neither TGF-β1, nor lithium, alone could elicit such a response in this cell line, this could be suggestive of a low level of synergism occurring between these signals to enable a threshold capable of inhibiting the β-catenin degradation complex to be reached to stabilize β-catenin in this fraction. However, the significance of this small increase would require further investigation.

EGF, however, clearly displayed a synergism with the Wnt/lithium signal. Concurrent EGF and lithium stimulation induced an elevation in the cytoplasmic pool of β-catenin at 3 hours in the oesophageal SCC cell lines. This is in accordance with the results published by Chen *et al*. (2000) in C57MG and HEK-293 cells. Chen *et al*. (2000) also showed that this increase correlated with an increase in the transcriptional activity of β-catenin after 16 hours of stimulation. Since the concentration of cytoplasmic β-catenin in the oesophageal SCCs remained elevated after 12 hours of exposure, it implies that the increase was sufficiently long enough to enable the upregulation of β-catenin's transcriptional activity, similar to that noted by Chen *et al*. (2000). However, a TOPflash/FOPflash luciferase assay would be needed to confirm this.

Where, however, does this excess β-catenin come from to augment the lithium-induced stabilization of β-catenin? There is considerable crosstalk between the subcellular pools/functions of β-catenin in the cell (reviewed in Bienz, 2004; Harris and Peifer, 2005; Brembeck *et al*., 2006), although researchers tend to separate these functions into two distinct systems. This was particularly evident in the Chen *et al*. (2000) study in which the effect of EGF and lithium on β-catenin in the cytoplasm and nucleus was explored, but excluded the effects these signals have on the concentration of β-catenin localized at the plasma membrane. The current study, therefore, investigated the effect that simultaneous stimulation by these signals has on the balance occurring between β-catenin localized at the plasma membrane and that found in the cytoplasm and nucleus. The result proved to be quite exciting as it was clearly demonstrated that as the concentration of β-

catenin in the cytoplasm rose in response to EGF and lithium stimulation, the concentration of plasma membrane-associated β-catenin decreased (see Chapter 3.3.3). Thus, it is proposed that the augmentation of the lithium-induced stabilization of β-catenin by EGF is due to the protein shifting from its preferred plasma membrane localization into the cytoplasm. This type of signalling, therefore, may indicate a mechanism for increasing the invasive and metastatic behaviour of this type of tumour by decreasing the level of adhesion. However, whether these activities significantly decrease adhesion and increase metastasis needs to be confirmed by cellular adhesion and invasion assays.

5.3 Tyrosine, but not serine, phosphorylated membrane-associated β-catenin increases in oesophageal SCC cells exhibiting altered localization in response to EGF/lithium signalling

How do the EGF and lithium signals converge to induce a shift in the localization of β-catenin? There has recently been a move towards understanding how β-catenin "chooses" between its role in adhesion and signalling and the effect that various signals have on this "decision-making" process. The importance of posttranslational modifications, especially through phosphorylation, have been identified to be key in mediating the affinity that β-catenin shows for its various binding partners. Tyrosine phosphorylation of β-catenin, particularly of Y654 and Y142, have been documented to negatively regulate its role in adhesion (Volberg *et al*., 1991; Matsuyoshi *et al*., 1992; Behrens *et al*., 1993; Hamaguchi *et al*., 1993; Kinch *et al*., 1995; Takahashi *et al*., 1997; Ozawa and Kemler, 1998a; Roura *et al*., 1999; Hu *et al*., 2001; Piedra *et al*., 2003). It may also play a positive role in inducing β-catenin signalling and transcriptional activity (Danilkovitch-Miagkova *et al*., 2001; Piedra *et al*., 2001; Brembeck *et al*., 2004). The effect of serine phosphorylation of β-catenin on its adhesive function is less clear (Peifer *et al*., 1994b; Miller and Moon, 1997; Serres *et al*., 1997; Bek and Kemler, 2002), although its role in regulating β-catenin's cytoplasmic accumulation is extremely

well documented (see Chapter 1.6.1). Due to the importance of phosphorylation on β-catenin's interactions and thus function, the present study set out to explore the effect that the EGF and lithium signal have on the phosphorylation status of β-catenin associated with the plasma membrane.

The EGF-mediated augmentation of lithium induced cytoplasmic β-catenin accumulation in the C57MG/HEK-293 cell lines was shown to involve activation of the serine kinase, PKCα, although its role was yet to be clarified (Chen *et al*., 2000). PKCα has been shown to target the adhesion complex, causing it to dissociate and induce the redistribution of β-catenin to the nucleus in CACO-2 and normal intestinal cells (Baulida *et al*., 1999; Malladi *et al*., 2004). Due to the redistributing effect these signals appear to play on β-catenin's localization in the oesophageal SCC cell lines investigated in the present study, it is possible that PKCα may act to alter the adhesion complex, releasing β-catenin into the cytoplasmic pool. However, the concentration of serine phosphorylated β-catenin was not altered in the cell lines that showed altered distribution in response to the EGF/lithium signal. Therefore, it is unlikely that β-catenin is the target of this EGF/lithium-activated PKCα. This does not rule out the possible role that PKCα may play in inducing the altered localization of β-catenin in these cells, as it may target another member of the adhesion complex. PKCα has been shown to cause E-cadherin to redistribute away from the plasma membrane (Barbosa *et al*., 2003), and thus may result in the internalisation of β-catenin due to their association. Alternatively, E-cadherin may very well be targeted by this kinase to reduce the cadherin/catenin affinity. Lickert *et al*. (2000) demonstrated the importance of serine/threonine phosphorylation of the E-cadherin cytoplasmic tail by GSK-3β in maintaining its association with β-catenin. Gottardi and Gumbiner (2004b) reiterated this as they demonstrated that serine phosphorylation of E-cadherin could overcome the restrictions imposed by Wnt-signalling on the ability of β-catenin to associate with E-cadherin. PKCα has been shown to be able to phosphorylate and deactivate GSK-3β (Goode *et al*., 1992; Murray *et al*., 1999), and thus, EGF/lithium-activation of PKCα may prevent GSK-3β phosphorylation

of E-cadherin required for its interaction with β-catenin. PKCα may also act by physically removing the β-catenin binding site in E-cadherin. Vallorosi *et al*. (2000) showed that E-cadherin was targeted by PKCα in involuting prostate and mammary epithelial cells, resulting in two truncated forms of E-cadherin (a plasma membrane-associated 97 kD form and a cytosolic 35 kD form), most probably elicited by a proteolytic response. The truncated 97 kD E-cadherin removes the β-catenin binding domain and as a consequence the cells dissociate from each other. All these findings could explain how the EGF/lithium-induced activation of PKCα may induce the shift of β-catenin from the membrane into the cytoplasm. However, the role of PKCα in the EGF/lithium pathway in oesophageal SCCs would still need to be ascertained and may well form the basis of another study. This might be done through the use of a PKCα inhibitor, such as Calphostin C, to determine if the EGF/lithium pathway could still elicit β-catenin redistribution.

The role of tyrosine phosphorylation of membrane-associated β-catenin in the EGF/lithium signalling is more apparent. Concurrent stimulation of the oesophageal SCCs by EGF and lithium resulted in an increase of tyrosine phosphorylated β-catenin in the plasma membrane fraction (Figure 4.3). This coincided with the increased concentration of β-catenin in the cytoplasmic fraction noted in response to these signals (Figure 3.7). This is strongly suggestive that tyrosine phosphorylation of β-catenin is a major mechanism through which these signals influence the localization of β-catenin. Tyrosine phosphorylation of β-catenin has previously been associated with reducing its affinity with E-cadherin (Kinch *et al*., 1995; Roura *et al*., 1999; Hu *et al*., 2001). This may explain the prolonged increase of β-catenin noted in the cytoplasmic fraction in these cell lines in response to these signals, as tyrosine phosphorylated β-catenin would be less attractive to the E-cadherin protein, preventing E-cadherin from adequately sequestering the excess β-catenin in the cytoplasm (see above).

The tyrosine phosphorylation of β-catenin is most likely to have been mediated by EGF activation of its receptor as the EGFR is well documented to associate with, and phosphorylate, β-catenin to reduce its affinity for E-cadherin (Hoschuetzky *et al*., 1994; Ochiai *et al*., 1994; Shibamoto *et al*., 1994; Shiozaki *et al*., 1995; Takahashi *et al*., 1997; Hazan and Norton, 1998; Schroeder *et al*., 2002; Piedra *et al*., 2003). However, EGF stimulation alone was not associated with changes in the distribution of β-catenin (Figure 3.5). This suggests that lithium must play a role in regulating the association of β-catenin with the adhesion complex in addition to its stabilization of β-catenin in the cytoplasm. As discussed above, it is improbable that lithium inhibition of GSK-3β affects the phosphorylation of β-catenin to reduce its association with the cadherin adhesion complex. It may, however, act to alter the attractiveness of other proteins competing for β-catenin's association. GSK-3β can phosphorylate E-cadherin to increase its affinity for β-catenin (Lickert *et al*., 2000). Lithium inhibition of this GSK-3-mediated phosphorylation, together with the changes induced by EGFR to β-catenin, may reduce the association/affinity between E-cadherin and β-catenin sufficiently to free β-catenin from the adhesion complex into the cytoplasm (see discussion above). Alternatively, lithium inhibition of GSK-3β may act to increase the appeal of another molecule for β-catenin binding over that of cadherin. For example, the cytoplasmic fraction of a mucin-like glycoprotein, MUC1, binds to β-catenin via a similar motif to that of E-cadherin, competing with E-cadherin for β-catenin, reducing adhesion (Yamamoto *et al*., 1997; Li *et al*., 1998c). This MUC1/β-catenin interaction is associated with an increased concentration of stabilized β-catenin in the cytoplasm and nucleus (Wen *et al*., 2003). GSK-3β downregulates this interaction, whilst EGFR has been shown to increase this association (Li *et al*., 1998c; Li *et al*., 2001b). Thus, lithium may act to inhibit the GSK-3β downregulation of the β-catenin/MUC1 association, while EGFR encourages it, inducing a redistribution of β-catenin. The cytoplasmic tail fragment of MUC1 and β-catenin have previously been shown to associate in the nucleus of the oesophageal SCC cell lines used in the present study (Metcalfe, 2006). Therefore,

an exciting future prospect would be to explore whether EGF and lithium signalling do indeed increase this association in the oesophageal SCC cells.

5.4 In conclusion

The present investigation has demonstrated the importance of β-catenin in cell-cell adhesion in moderately-differentiated human oesophageal SCCs and how various signalling pathways impinge on the function of β-catenin, possibly furthering the development of this type of tumour. This study has highlighted the importance of adopting a more holistic approach when considering the effect of signal transduction pathways on the functionality of β-catenin, rather than investigating these roles in isolation, as the balance between the membrane and cytoplasmic pools of β-catenin are clearly interdependent. Furthermore, the vital importance of posttranslational modification of β-catenin through phosphorylation in influencing the balance of membrane and cytoplasmic β-catenin was exposed. There are, however, many questions that remain to be answered before a more complete picture can be generated of the role of β-catenin in human oesophageal SCC, particularly with respect to cellular adhesion and metastasis.

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APPENDICES

Appendix 1

1.1 Cell lines and Tissue culture

1.1.1 Phosphate Buffered Saline (PBS)

136.9 mM Sodium Chloride
2.68 mM Potassium Chloride
10.1 mM Disodium hydrogen orthophosphate (anhydrous)
1.76 mM Potassium dihydrogen orthophosphate
Make up with distilled water (dH_2O)
Adjusted the pH to 7.2-7.3
Sterilise and store at 4°C

1.1.2 TE - Trypsin in Ethylenediamine-tetraacetic acid (EDTA)

0.02% EDTA in 500 ml PBS
0.1% Trypsin in 1 litre PBS
Mix EDTA:Trypsin (1:1)
Final: 0.01% EDTA/0.05% Trypsin

1.1.3 Trypan Blue (for counting viable cells)

0.4% Trypan Blue
Make up in PBS

1.2 Cellular extractions

1.2.1 Laemmli sample buffer

1.5% Tris.HCl (pH 6.8)

2% SDS

10% Glycerol

5% β-mercaptoethanol

Make up in dH_2O

Store at 4°C

1.2.2 Phenyl-methyl-sulphonyl fluoride (PMSF) stock solution

20 mM PMSF powder (Merck)

Make up with Methanol

Store at 4°C

1.2.3 PMSF/Aprotinin stock solution

0.5% PMSF stock solution (see 1.2.2)

1% Aprotinin (Trasylol® Bayer)

Make up in PBS

Store at 4°C

1.2.4 Hypotonic buffer

20 mM	Tris.HCl (pH 7.4-7.5)
25 mM	Sodium fluoride
1 mM	EDTA

Make up to volume with dH_2O

Add protease inhibitor mixture (just prior to use)

5 µl/ml	PMSF stock solution (see 1.2.1)
10 µl/ml	Aprotinin (Trasylol® Bayer)
1 µl/ml	Pepstatin A (Sigma)
0.5 µl/ml	Leupeptin (Sigma)

Store at 4°C

1.3 Protein Estimation

1.3.1 2 μg/μl Bovine Serum Albumin (BSA) solution

For cytoplasmic fractions:

0.2% BSA (Merck)

In hypotonic buffer (see 1.2.4)

Store at 4°C

For plasma membrane fractions:

0.2% BSA

In a 0.1% Sodium dodecyl sulphate (SDS) in 20 mM Tris.HCl (pH 7.4) solution

Store at 4°C

For whole cell extracts

0.2% BSA

In Laemmli sample buffer (see 1.2.1)

Store at 4°C

1.3.2 Trichloroacetic Acid (TCA)

7.5% TCA

Make up to volume with dH_2O

1.3.3 0.25% Coomasie Blue Stain

0.25% Coomasie blue powder

50% Methanol

10% Acetic acid

Make up to volume with dH_2O

Store at room temperature

1.3.4 Destain

12% Ethanol

10% Acetic acid

Make up in dH_2O

1.3.5 Elution solution

67% Methanol

32% dH_2O

1% concentrated Ammonia

Store at room temperature

1.4 SDS-PAGE

1.4.1 10% Polyacrylamide Gel

Separating gel:

10% Acrylamide

25% of a 1.5 M Tris.HCl (pH 8.8) separating buffer

4% of a 0.17 M SDS solution

4% of a 0.16 M *NN*'-Methylenebisacrylamide solution

Make up to volume with dH_2O

Just prior to pouring add:

1.8% of a 0.05 M ammonium persulphate (APS) solution

0.25% *N*,*N*,*N'*,*N'*–tetramethylene-diamine (TEMED)

Allow to set for approximately 20-30 minutes

Note, a thin layer of 0.1% SDS in dH_2O was poured on top of gel to assist polymerisation

Stacking gel:

5% Acrylamide

25% of 0.5 M Tris.HCl (pH 6.8) stacking buffer

4% of 0.17 M SDS-solution

4% of 0.16 M *NN*'-Methylenebisacrylamide solution

Make up to volume with dH_2O

Just prior to pouring add:

1.8% of 0.05 M APS

0.2% TEMED

Allow to set for approximately 15 minutes

1.4.2 Electrophoresis Running Buffer for SDS-PAGE

25 mM Tris

0.2 M Glycine

3.5 mM SDS

Make up separately in dH_2O

Mix together

Adjust to pH 8.3 with HCl

Store at room temperature

1.4.3 Destain for SDS-PAGE

10% Acetic acid

10% Methanol or ethanol

Make up in dH_2O

1.5 Immunoblot

1.5.1 Western Blot Transfer Buffer

0.3% Tris

1.41% Glycine

20% Methanol

Dissolve in dH_2O

Adjust to pH 8.3 with HCl

Store at 4°C

1.5.2 Tris Buffered Saline (TBS)

0.606% Tris.HCl (pH 7.8)

0.86% Sodium Chloride

0.0294% Calcium Chloride (dihydrate)

Make up with dH_2O

Store at 4°C

1.5.3 Blocking buffer (BLOTTO)

0.606% Tris.HCl (pH 7.8)

0.86% Sodium Chloride

0.0294% Calcium Chloride (dihydrate)

5% Non-fat milk powder

0.05% Triton X-100

Make up with dH_2O

Store at 4°C

1.5.4 Working Solution

Mix equal volumes of:

Luminol (SuperSignal® West Pico Chemiluminescent kit, Pierce)

Peroxide (SuperSignal® West Pico Chemiluminescent kit, Pierce)

Note, the total volume depended on the size of nitrocellulose being treated

1.5.5 Developer: D19B

6.4 M	Metol
0.6 M	Sodium Sulphite (anhydrous)
80 mM	Hydroquinone or quinol
0.45 M	Sodium carbonate (anhydrous)
34 mM	Potassium bromide

Make up with dH_2O

1.5.6 Fixer

0.8 M	Sodium thiosulphate
0.2 M	Sodium or Potassium metabisulphite

Make up with warm dH_2O

1.5.7 Fast green stain

0.1%	Fast green
45%	Methanol
10%	Acetic acid

Make up in dH_2O

Destain as for SDS-PAGE

1.6 Immunofluorescence

1.6.1 4% Paraformaldehyde

Solution A:

166 ml of a 0.14 M sodium dihydrogen orthophosphate (anhydrous) solution

34 ml of a 0.63 M sodium hydroxide solution

Solution B:

4% Paraformaldehyde

Dissolve in Solution A

Heat to approximately 80°C, whilst stirring, until clear

Cool and adjust pH to between 7.2-7.4

Filter and store at 4°C

1.6.2 Elvanol

7.5 g Glycerol

With continual but slow stirring add:

3.0 g Polyvinyl Alcohol (grade 51-05)

Mix well

Add slowly down the side of the tube to avoid clumping of the polyvinyl alcohol

7.5 ml dH_2O

Stir for 24 hours at room temperature

Add

15 ml 0.1 M Tris.HCl (pH 8.5)

Continue stirring in a 50°C water bath for 48 hours

Clear by centrifugation (1500 $\times$g) for 30 minutes at room temperature

Separate supernatant into aliquots, seal and store at 4°C

1.7 Radioimmunoassay (RIA)

1.7.1 4% Paraformaldehyde in PBS

Solution A:

83 ml of a 0.14 M sodium dihydrogen orthophosphate (anhydrous) solution

17 ml of a 0.65 M sodium hydroxide solution

Solution B:

4% Paraformaldehyde

Dissolve in Solution A

Heat to approximately 80°C whilst stirring, until clear

Make up to 200 ml with 2 × PBS

Cool and adjust the pH to between 7.2-7.4

Filter before use and stored at 4°C

1.8 Immunoprecipitation

1.8.1 Immunoprecipitation buffer

0.24% Tris.HCl (pH 8.0)

0.5% NP-20

0.9% Sodium Chloride

Make up with dH_2O

Appendix 2

2.1 Estimation of the Total Protein Content of Cells

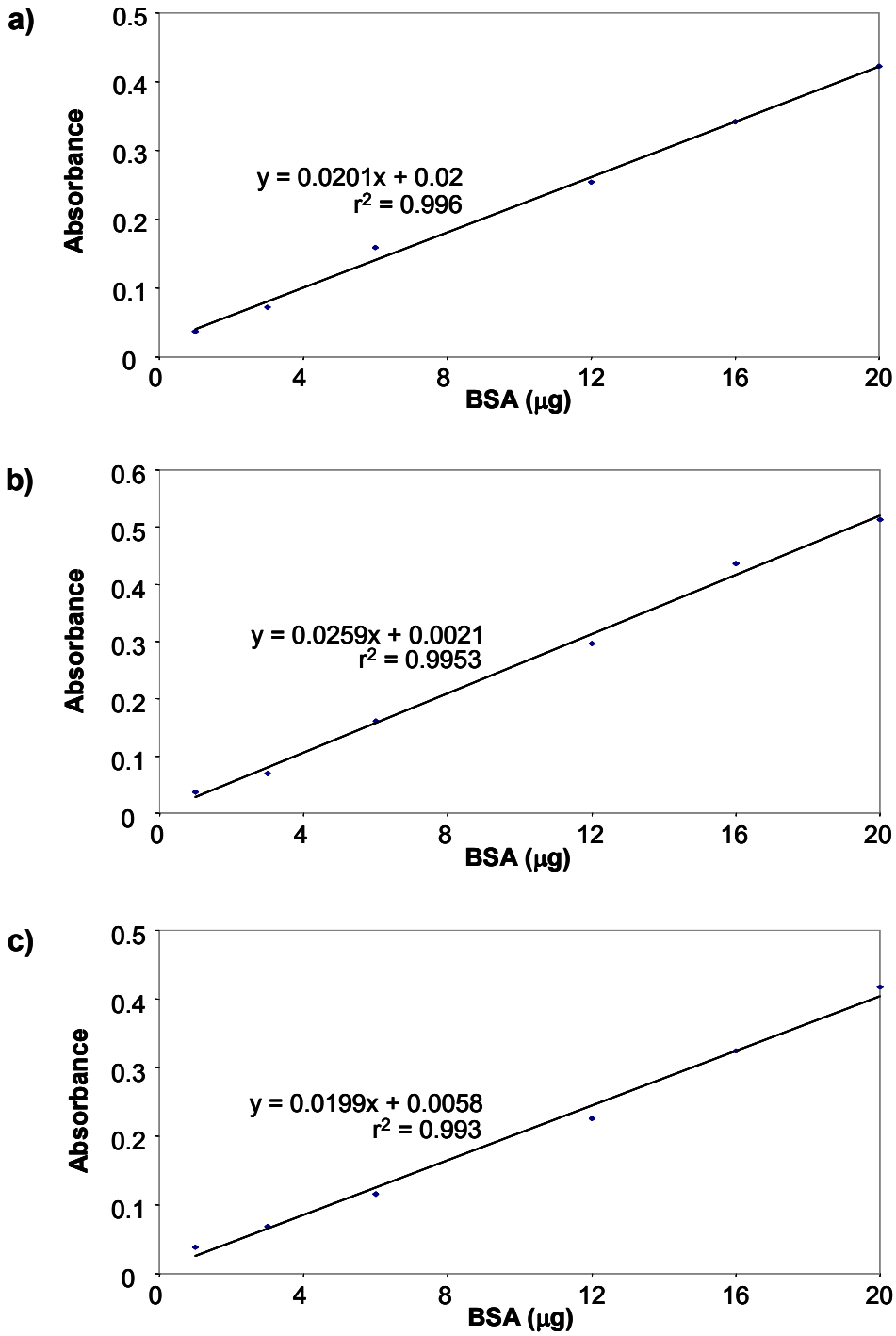

Figure A: BSA standard curve for protein estimation. The absorbance of 1, 3, 6, 12, 16 and 20 µg BSA were used to construct a standard curve for protein estimation of **a)** the whole cell, **b)** the plasma membrane and **c)** the cytoplasmic extracts. The concentrations of the total protein of the extracts were calculated from these BSA standard curves (y = 0.0201x + 0.0202, r^2 = 0.9834 for the whole cell extracts; y = 0.025x + 0.024, r^2 = 0.999 for the plasma membrane extracts; y = 0.020x + 0.006, r^2 = 0.993 for the cytoplasmic extracts).

Appendix 3

3.1 Determination of Molecular Weight (Mr) for SDS-PAGE

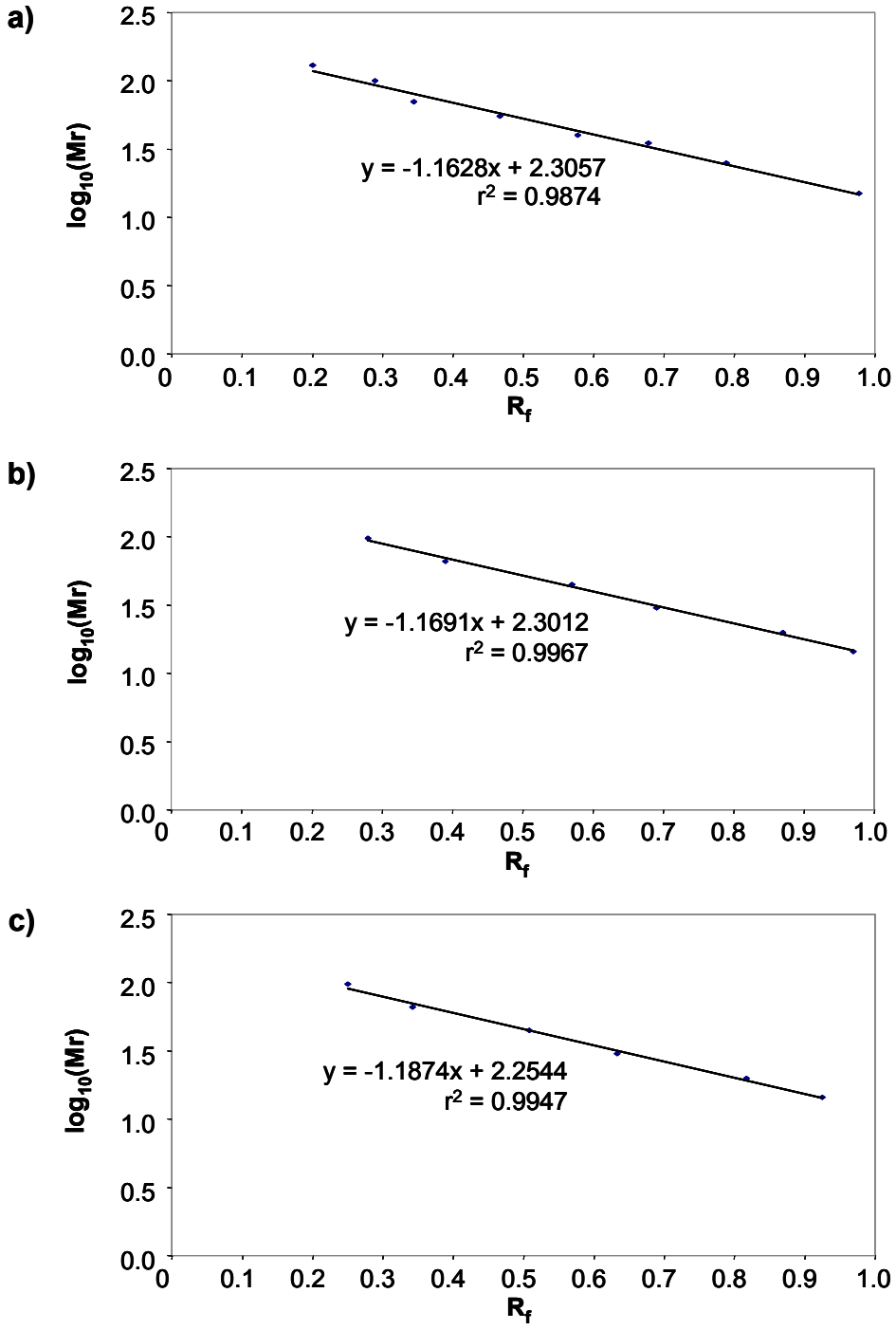

Figure B: Calibration curves for SDS-PAGE. R_f values were plotted against $\log_{10}$(Mr) to construct a calibration curve of the SDS-PAGE for the **a)** whole cell ($y = -1.16x + 2.31$, $r^2 = 0.987$), **b)** membrane ($y = -1.17x + 2.30$, $r^2 = 0.997$) and **c)** cytoplasmic extracts ($y = -1.19x + 2.25$, $r^2 = 0.995$). These curves were used to calculate the position of the 94 kD β-catenin band.

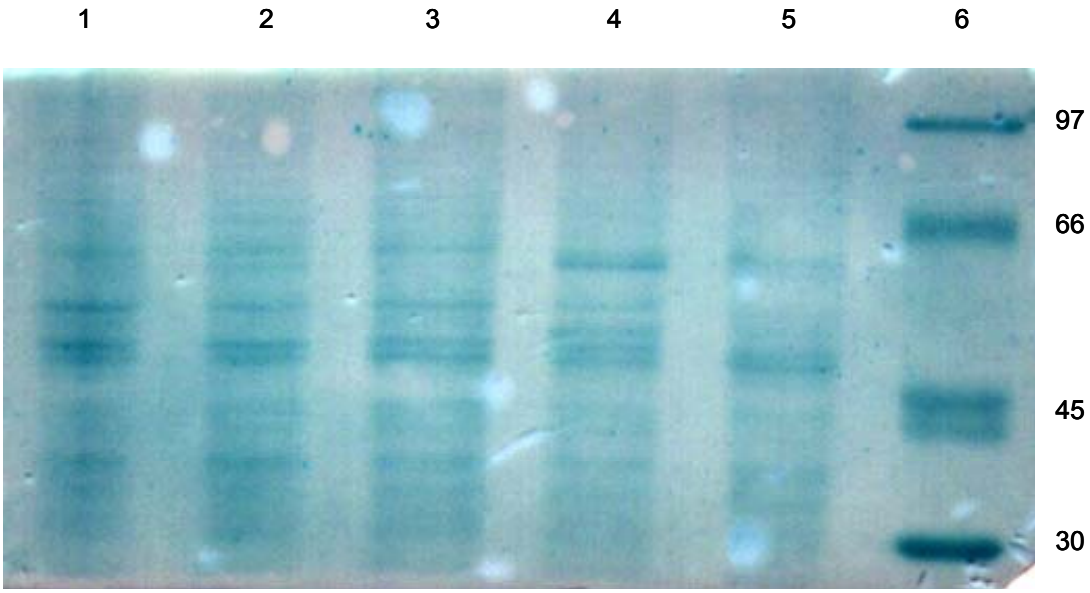

Figure C: Effective protein transfer of polypeptides to the nitrocellulose membrane. Successful protein transfer from the SDS-PAGE to nitrocellulose membrane for immunoblotting was shown by Fast green staining of the nitrocellulose membrane. Lanes 1-5 represents WHCO1, WHCO3, WHCO5, WHCO6 and SNO respectively. Lane 6 represents the molecular weight marker proteins (97, 66 45 and 30 kD).

Appendix 4

4.1 Statistical Results of Radioimmunoassay of Cells under Standard Tissue Culture Conditions

Table A: General ANOVA of the Total Level of β-catenin Expression under Standard Conditions

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Cell Line	4	7730144.	42	7104115.	1.08812	0.374744
Control	1	431127E3	42	7104115.	60.68699	0.000000
Cell Line and Control	4	5587617.	42	7104115.	0.78653	0.540439

Indicates significant difference

Table B: Tukey's HSD: Possibilities for Post Hoc Tests showing the Interaction between Experiment (E) and Control (C)

	CO1 (E) (7801.7)	CO1 (C) (1125.8)	CO3 (E) (5280.6)	CO3 (C) (523.1)	CO5 (E) (7617.4)	CO5 (C) (464.5)	CO6 (E) (7977.4)	CO6 (C) (1066.8)	SNO (E) (4452.1)	SNO (C) (830.5)
CO1* (E)		0.003275	0.886416	0.003473	1.000000	0.003144	1.000000	0.002936	0.745075	0.019725
CO1 (C)	0.003275		0.316312	0.999998	0.013032	0.999995	0.002366	1.000000	0.752365	1.000000
CO3 (E)	0.886416	0.316312		0.162110	0.924753	0.150881	0.840803	0.298102	0.999988	0.373853
CO3 (C)	0.003473	0.999998	0.162110		0.004749	1.000000	0.002579	0.999999	0.547819	1.000000
CO5 (E)	1.000000	0.013032	0.924753	0.004749		0.004299	1.000000	0.011829	0.800335	0.025585
CO5 (C)	0.003144	0.999995	0.150881	1.000000	0.004299		0.002337	0.999998	0.527367	1.000000
CO6 (E)	1.000000	0.002366	0.840803	0.002579	1.000000	0.002337		0.002114	0.687820	0.015325
CO6 (C)	0.002936	1.000000	0.298102	0.999999	0.011829	0.999998	0.002114		0.733768	1.000000
SNO (E)	0.745075	0.752365	0.999988	0.547819	0.800335	0.527367	0.687820	0.733768		0.655112
SNO (C)	0.019725	1.000000	0.373853	1.000000	0.025585	1.000000	0.015325	1.000000	0.655112	

Denotes a significant difference

*The WHCO- cell lines have been abbreviated to CO-

4.2 Statistical Results of Radioimmunoassay of Cells under EGF Treatment

Table C: ANCOVA of the Total Level of β-catenin Expression under EGF Treatment (Controlling for Cell Line)

Effect	df Effect	MS effect	df Error	MS Error	F	p-level
Cell Line	4	946628E2	208	116401E2	8.132451	0.000004

Denotes a significant difference

Table D: Tukey's HSD test: Possibilities for Post Hoc Tests of p-values between Cell Lines

Cell Line	WHCO1 (6122.55)	WHCO3 (7607.53)	WHCO5 (7690.18)	WHCO6 (5258.11)	SNO (4340.72)
WHCO1		0.242309	0.217555	0.798438	0.099984
WHCO3	0.242309		0.999962	0.016111	0.000049
WHCO5	0.217555	0.999962		0.014664	0.000053
WHCO6	0.798438	0.016111	0.014664		0.743243
SNO	0.099984	0.000049	0.000053	0.743243	

Denotes a significant difference

Table E: ANCOVA of the Total Level of β-catenin Expression under EGF Treatment (Controlling for Time)

Effect	df Effect	MS effect	df Error	MS Error	F	p-level
Time	7	210054E2	205	121860E2	1.723737	0.105046

Appendix 5

5.1 An α-actin loading control to enable comparisons between immunoblots

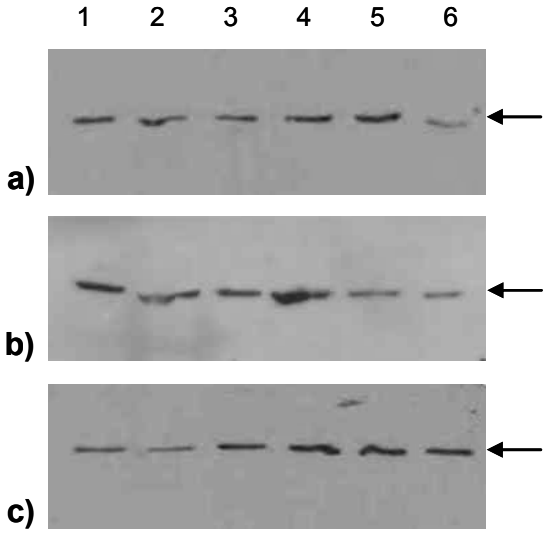

Figure D: An α-actin loading control to enable comparisons between immunoblots. α-actin (42 kD; indicated by the arrows) was identified all five oesophageal SCC cell lines grown under **(a)** standard tissue culture conditions, as well as in cell cultures treated with **(b)** EGF alone (10 ng/ml), or **(c)** together with lithium (10mM) for 3 hours. **(a)** The bands are of equal strength, indicating equivalent loading and transferral of protein. Lanes 1-5 represent WHCO1; WHCO3; WHCO5; WHCO6 and SNO, respectively. A control sample of SNO is indicated in lane 6. **(b)** and **(c)** Similarly, α-actin bands of equal strength were identified in the treated oesophageal SCC cell lines when compared to an untreated control sample (WHCO5, lane 6). This indicates that treatment does not affect protein concentration, enabling comparisons between the immunoblots to be conducted.