DECLARATION

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

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Belinda Catherine Bezuidenhout

29th day of September 2008

“I can do all things in Christ who strengthens me”

- Philippians 4:13

ABSTRACT

Axin, in combination with adenomatous polyposis coli (APC), forms the scaffold of the β-catenin degradation-targeting complex. Intricate interactions within this complex lead to the phosphorylation and subsequent ubiquitination of β-catenin, followed by proteosome-mediated degradation. Low density lipoprotein receptor-related protein 5 (LRP5), the canonical Wnt coreceptor, is able to function in axin inhibition, by recruiting it to the plasma membrane in response to canonical Wnt/β-catenin signalling. This study investigates the influences of each of these proteins within the context of human oesophageal squamous cell carcinoma (HOSCC). Western immunoblotting, and subsequent densitometric analysis of whole cell protein lysates revealed the presence of differing levels of β-catenin, axin and APC in five oesophageal carcinoma cell lines. Furthermore, both β-catenin and axin were able to associate with the plasma membrane, and, along with APC, were shown to localize to the nucleus. Immunofluorescence experiments confirmed this distribution pattern for β-catenin. APC and β-catenin were co-immunoprecipitated, displaying the interaction between components of the degradation-targeting complex. Relatively high levels of axin were detected at the plasma membrane, and so studies on LRP5 were commenced. LRP5 was detected in all five cell lines, and unexpectedly low levels of LRP5 at the plasma membrane were confirmed by indirect immunofluorescence. In response to EGF (10 ng/ml) treatment, there were a number of differences observed in the different cell lines, but average levels of LRP5 “peaked” at 3 hours. Further investigation revealed that each cell line had an almost unique response to EGF treatment when it came to axin plasma membrane-localization. These results confirm the influence of growth factors on HOSCC, as well as indicate that axin and its role in oesophageal tumourogenesis must, therefore, be influenced by additional intermediates not assayed in this study.

CONFERENCE

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α	alpha
APC	adenomatous polyposis coli
APS	ammonium persulfate
asef	APC-stimulated guanine nucleotide exchange factor
axin	axis inhibitor
AXIN1	axin gene
β	beta
Bis	*N'*, *N'*,-methylenebis-acrylamide
BME	β-mercaptoethanol
bp	base pair
BSA	bovine serum albumin
β-TrCP	beta transducin-repeat-containing protein
ºC	degrees Celsius
C-terminal	carboxy-terminal
Ca	calcium
Cadherins	Ca^{2+}-dependent cell-cell adhesion molecules
CKI	casein kinase one
CKII	casein kinase two
cm	centimeter(s)
C/N	cytoplasmic/nuclear fraction
CO_2	carbon dioxide
CRM1	chromosome maintenance region 1
CTNNB1	β-catenin gene
Cul 1	Cullin 1
DEP	dishevelled, Egl-10 and pleckstrin homology
dH_2O	distilled water
DIX	dishevelled homologous

Dkk1/2	Dickkopf 1/2
DMEM	Dulbecco’s modified Eagle’s medium
DNA	deoxyribonucleic acid
DTT	dithiolthreitol
Dvl	dishevelled
ε	epsilon
E-cadherin	epithelial-cadherin
EC	extracellular
ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERC	endosomal retention components
Erk	extracellular-signal-regulated kinase
Ex	exon
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FRAT	frequently rearranged in advanced T-cell lymphomas
Fz	frizzled
γ	gamma
g	grams
× g	gravitational units
GBP	GSK3β-binding protein
GEF	guanine nucleotide exchange factor
GSK3β	glycogen synthase kinase 3 beta
HCl	hydrochloric acid
HEK	human embryonic kidney
HIPK2	homeodomain-interacting protein kinase-2
HMG	high mobility group
HOSCC	human oesophageal squamous cell carcinoma

h	hour(s)
HRP	horseradish peroxidase
IC	intracellular
IgG	immunoglobulin class G
in	inch
IP	immunoprecipitation
JNK	c-jun N-terminal kinase
Kb	kilobase
kDa	kilodaltons
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
lb	pounds
Lef	lymphocyte enhancing binding factor
Li	lithium
LRP5/6	low density lipoprotein receptor-related protein 5 or 6
µ	micro
µg	microgram(s)
µl	microlitre(s)
M	molar
mA	milliampere(s)
MAPK	mitogen activated protein kinase
MCR	mutational cluster region
ME	plasma membrane-associated protein extract
MEKK	MAPK/ERK kinase kinase
Mesd	mesoderm development
min	minutes
mg	milligram(s)
ml	millilitre(s)
mM	millimolar
mRNA	messenger RNA

MW	molecular weight
N-cadherin	neuronal cadherin
N/NH_2-terminal	amino-terminal
nm	nanometre(s)
Nonidet P-40	non-ionic detergent P-40
%	percent
Φ	phi
$p90^{RSK}$	ribosomal protein S6 kinase, 90-kD
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDZ	PSD-95/Discs large/ZO-1 domain
PMSF	phenyl-methyl-sulfonyl-fluoride
PP2A	protein phosphatase 2A
PPARγ2	peroxisome proliferator-activated receptor gamma 2
PPPSP motif	proline-proline-proline-serine-proline motif
PSD-95	post-synaptic density 95
RAP	receptor-associated protein
RGS	regulators of G-protein signalling
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
RTK	receptor tyrosine kinase
RXRα	retinoid X receptor alpha
SAMP repeats	serine-alanine-methionine-proline repeats
SCF	SKP1, CUL1 and F-protein
sdH_2O	sterile distilled water
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

Ser	serine
Siah1	seven in absentia, Drosophila, homologue of, 1
SIP	Siah1-interacting protein
Skp1	S-phase kinase-associated protein 1A
TβR-I	TGF-β type I receptor
TCA	trichloroacetic acid
Tcf	T-cell factor
TE	trypsin/EDTA
TEMED	*N,N,N'N'*-tetramethylenediamine
Thr	threonine
TGFβ	transforming growth factor beta
Tris	tris(hydroxymethyl)aminomethane
Tyr	tyrosine
TZD	troglitazone
Ub	ubiquitin
UTC	untreated control
UV	ultraviolet
V	volts
WCE	whole cell extract
WHCO	Wits human carcinoma of the oesophagus
Wnt	Wingless-type
ZO-1	zonula occludens

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

Adhesion and adhesion-based signalling are important and strictly regulated contributors in cell proliferation and migration, both in development and metastasis (Siegfried and Perrimon, 1994). In order to elucidate the extent to which these contribute to tumour biology, it is necessary to study the critical components of both adhesion and adhesion-based signalling. The current dissertation attempts to shed some light on the intricate processes occurring in one particular adhesion/signalling pathway during oesophageal tumourogenesis.

1.1. Cancer – a multifaceted disease

Cancer is a multifaceted disease that may be initiated by abnormalities such as mutations (single or multiple) or epigenetic effects. As a result of these abnormalities, over- or under-expression of proteins frequently occurs and is responsible for the development of the hyperproliferative state observed in cells comprising the tumour (Korinek *et al*., 1997; Miyoshi *et al*., 1998). Growth factors and their receptors exhibit a plethora of effects on their target cells including cell growth, differentiation, proliferation and migration (Frey *et al*., 2004; reviewed in Howell, 2004). In addition, aberrant signalling by several pathways involved in growth and development, as well as adhesion/anti-adhesion events contribute to the cancerous state (Christofori, 2003). However, in order to best understand these events they have commonly been studied within the context of development, where there are typically few complicating abnormalities.

1.2. Development

Development is a complex process by which a single fertilized egg becomes a multicellular organism with numerous different functional tissues. An integral part of development is differentiation – the process by which the cellular diversity observed in a fully developed organism is achieved. It is this intricate process that allows cells of a single origin to diverge in morphology and function, ultimately creating a multicellular organism.

Following fertilization of an egg, the single cell undergoes rapid sequential mitotic divisions (Gilbert, 1991), resulting in the production of a sphere known as a blastula. By the 32-cell stage, dramatic cell migration starts to take place within the developing embryo, and cells are rearranged within the embryo in a process known as gastrulation. Three layers are produced as a result of gastrulation – the ectoderm, mesoderm, and endoderm (Gilbert, 1991). Once these three layers have been established, organogenesis occurs and the organism enters the final stages of development.

Since cells are undergoing such dramatic rearrangement during gastrulation, and because cells within a blastula adhere to one another and to the extracellular matrix, points of cell adhesion must be dynamic and strictly regulated. It has been observed that during development, adhesion/anti-adhesion events are critical to the ability of cells to migrate within the embryo (Wolpert *et al*., 1999). Within adult tissues however, this type of cell migration rarely takes place under normal conditions, but these events have been observed in carcinogenesis during the process of metastasis (Behrens *et al*., 1992). In order for malignant tumour cells to acquire the ability to invade surrounding tissue or to metastasize to a secondary site, complete or partial loss of both cell-cell (Lallemand *et al*., 2003) and cell-matrix interactions must occur

(DeClerck *et al*., 2004). Thus the adhesive/anti-adhesive interactions between cells within a tissue are critical for migration within any context.

1.3. Cell Adhesion

Two main components of cell adhesion are cell-matrix and cell-cell adherens junctions (Mandai *et al*., 1999). Cell-matrix adherens junctions involve a set of molecules known as the integrins – members of this large group of molecules exist in heterodimeric complexes, each with an α- and a β-subunit. These single transmembrane glycoproteins bind to a number of components of the extracellular matrix (ECM) as well as to other cell surface proteins (Kerrigan *et al*., 1998), and serve to provide a link between the cytoskeleton and the extracellular matrix. This is achieved by the interaction of the cytoplasmic tail of the integrins with the actin cytoskeleton of the cell, via numerous cytoplasmic actin-binding proteins (Hynes, 1992). These cell/ECM junctions, however, are peripheral to the focus of this study.

1.3.1. Cell-cell adherens junctions

Cell-cell adherens junctions function to join adjacent cells together by linking their actin cytoskeletal components. This is accomplished by a group of single transmembrane proteins known as cadherins (Yap *et al*., 1997). The extracellular domains of two cadherin molecules from adjacent cells interact by homophilic binding (Yap *et al*., 1997). The intracellular domains of cadherins are linked to a group of peripheral cytoplasmic proteins known as catenins (α, β, and γ) (Figure 1.1), which are, in turn, linked to actin-binding proteins such as vinculin and alpha-actinin (Drees *et al*., 2005).

β- and γ-catenin (plakoglobin) both bind to the intracellular domain of epithelial (E)-cadherin, as well as to actin-binding protein, α-catenin. Thus α-catenin is not able to

directly bind the intracellular domain of E-cadherin, but interacts with it indirectly via β-catenin or its homologue γ-catenin (Aberle *et al*., 1994; Drees *et al*., 2005; Yamada *et al*., 2005; Weis and Nelson, 2006).

α-Catenin is also able to associate with other cytoplasmic proteins via specific binding domains – a necessary interaction as α-catenin is unable to bind both actin and β-catenin simultaneously (Drees *et al*., 2005; Yamada *et al*., 2005; Weis and Nelson, 2006). Vinculin, a cytoskeletal-associated protein, is able to bind a number of different cytoplasmic proteins including actin microfilaments and α-catenin (Weiss *et al*., 1998), thereby forming an essential component of the adherens junction (Figure 1.1). Because of this interaction with both actin and α-catenin, when associated with α-catenin, vinculin may be responsible for the recruitment of actin to the adherens junctions (Watabe-Uchida *et al*., 1998).

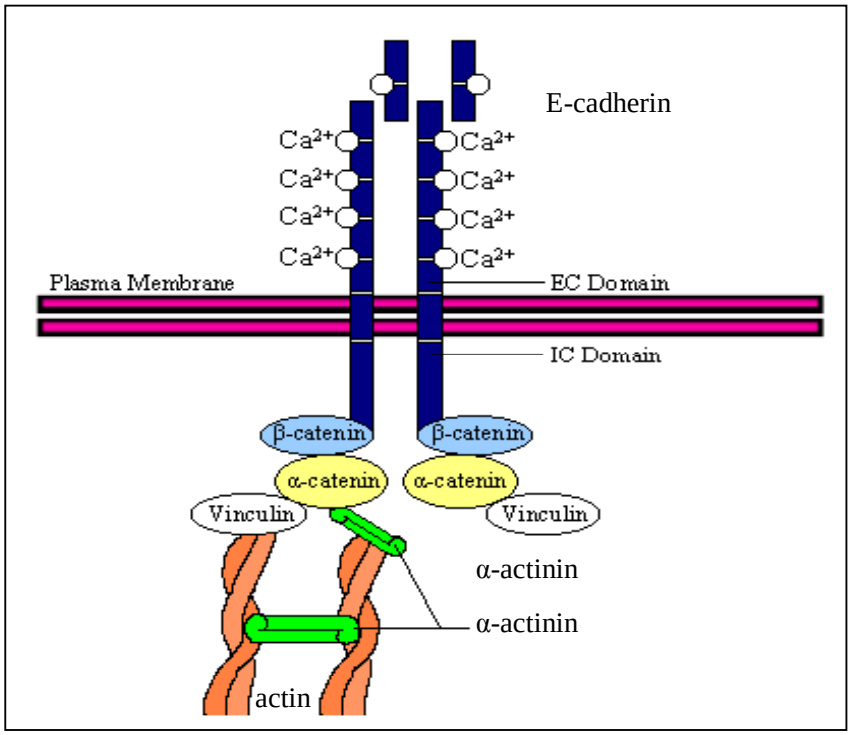

Figure 1.1. Schematic representation of the structure of a typical β-catenin cell-cell adherens junction. This multiprotein complex of specialized interactions functions to link the actin cytoskeletons of adjacent cells together (modified from Kobielak and Fuchs, 2004).
EC – extracellular domain, IC – intracelluler

α-Catenin also associates with the actin binding protein, α-actinin. This cytoplasmic protein also forms a component of the adherens junction and binds via α-catenin. It has been shown that this interaction is dependent on α-catenin being bound to the cadherin/β-catenin complex (Knudsen *et al*., 1995). This association, like that of α-catenin and vinculin, is responsible for linking the cadherin/catenin complex to the actin cytoskeleton.

Interestingly, it has been shown that the β-catenin/γ-catenin component of adherens junctions is involved not only in cell adhesion, but also in signal transduction pathways (Gumbiner and McCrea, 1993; Gumbiner, 1995; Peifer, 1995) (Figure 1.2). For the purpose of this study, the focus will be on the role played by β-catenin within the cell signal transduction context.

1.4. The canonical β-catenin/Wnt signalling pathway

The canonical β-catenin/Wnt signalling pathway was discovered through its role in development, and to date it has been implicated in several events in embryogenesis including cell division, differentiation, morphogenesis and migration (Siegfried and Perrimon, 1994).

Upon stimulation of a cell by one of the class 1 Wnts (includes Wnt1, Wnt3a, Wnt8) (reviewed in Miller, 2001) a series of events takes place ultimately resulting in the translocation of β-catenin to the nucleus. As both Frizzled (Fz) and low density lipoprotein receptor (LDLR)-related protein (LRP) 5/6 are required for binding of Wnt1 and Wnt3a (Pinson *et al*., 2000; Tamai *et al*., 2000; Liu *et al*., 2005), this specific transduction event requires the initial formation of a ternary complex between Wnt, Fz and LRP5/6 at the cell surface (Amit *et al*., 2002). Having been activated by multiple phosphorylation events by casein kinase I or II (CKI/CKII), dishevelled (Dvl) transduces the signal to the cytoplasm in response to the activation

of the Fz/LRP5/6 complex. Dvl and CKIε together form an interaction with axin (Kishida *et al*., 2001), which induces the maximal activation of the Wnt signalling pathway. This interaction has been postulated to stimulate FRAT (frequently rearranged in advanced T-cell lymphomas), a glycogen synthase kinase 3β (GSK3β) binding protein (GBP), to form a complex with the central PSD-95/Discs large/ZO-1 domain (PDZ) domain of Dvl, and it is the formation of this complex that induces the dissociation of GSK3β from axin, thereby preventing GSK3β-dependent phosphorylation of β-catenin (Figure 1.2). Free cytoplasmic β-catenin is then able to translocate into the nucleus where it acts as a coactivator of transcription with T-cell factor (Tcf, also known as lymphocyte enhancing binding factor or Lef) (Behrens *et al*., 1996). This complex initiates transcription of numerous target genes, including cyclin D1 and c-myc (He *et al*., 1998; Tetsu and McKormick, 1999).

In addition to its role in development, the Wnt/β-catenin signal transduction pathway has been implicated in carcinogenesis (Morin *et al*., 1997; Wang *et al*., 2006; Zeng *et al*., 2006). Since the Wnt signal is no longer secreted in normal adult tissues, events such as mutation that lead to the aberrant and inappropriate expression of Wnt or any of the members of the signalling cascade could lead to uncontrolled cell proliferation, migration or a change in cell morphology, all of which are associated with carcinogenesis. Thus it has been implicated in invasion and metastasis in numerous different tumours (reviewed in Morin, 1999).

Therefore, in normal adult tissues:

In the absence of Wnt, a complex containing GSK3β, axin and tumour suppressor adenomatous polyposis coli (APC) binds cytosolic β-catenin (Figure 1.2). Casein kinase I, which is linked to this complex via diversin, is responsible for the initial phosphorylation of β-catenin at the Ser45 residue (Amit *et al*., 2002). This reaction primes β-catenin for phosphorylation by GSK3β on residues Thr41, Ser37 and Ser33, creating a docking site for beta-transducin repeat containing protein (β-TrCP)/E3RS

(Amit *et al*., 2002). The association between GSK3β and axin enhances GSK3β's ability to phosphorylate β-catenin (Ikeda *et al*., 1998). GSK3β also stabilizes axin by phosphorylation (Figure 1.2). Phosphorylated β-catenin then forms a complex with β-TrCP, an F-box protein in the E3 ubiquitin ligase complex, which tags it for proteosome-mediated degradation by ubiquitination (Figure 1.3) (Aberle *et al*., 1997).

β-Catenin can also be regulated in the canonical Wnt pathway in a GSK3β-independent manner by the recruitment of axin to the cell membrane by LRP5/6 upon Wnt signalling (Mao *et al*., 2001; Kawana and Kypta, 2003). Thus, β-catenin is no longer degraded, and cytoplasmic accumulation and subsequent nuclear translocation thereof occurs, leading to active gene transcription (Figure 2.2).

As is typical of most signalling pathways, the canonical Wnt/β-catenin pathway is also subject to inhibition by extracellular factors, one of the best described of which is the Dickkopf (Dkk) family of proteins, particularly Dkk1 (Figure 1.2) (Brott and Sokol, 2002; Mao *et al*., 2002; Mao and Niehrs, 2003). These proteins form interactions with the extracellular domains of LRP5/6, resulting in the inhibition of Wnt-induced Fz-LRP5/6 complex formation and, therefore, the inhibition of the canonical Wnt/β-catenin signalling pathway (Semënov *et* al, 2001; Mao and Niehrs, 2003).

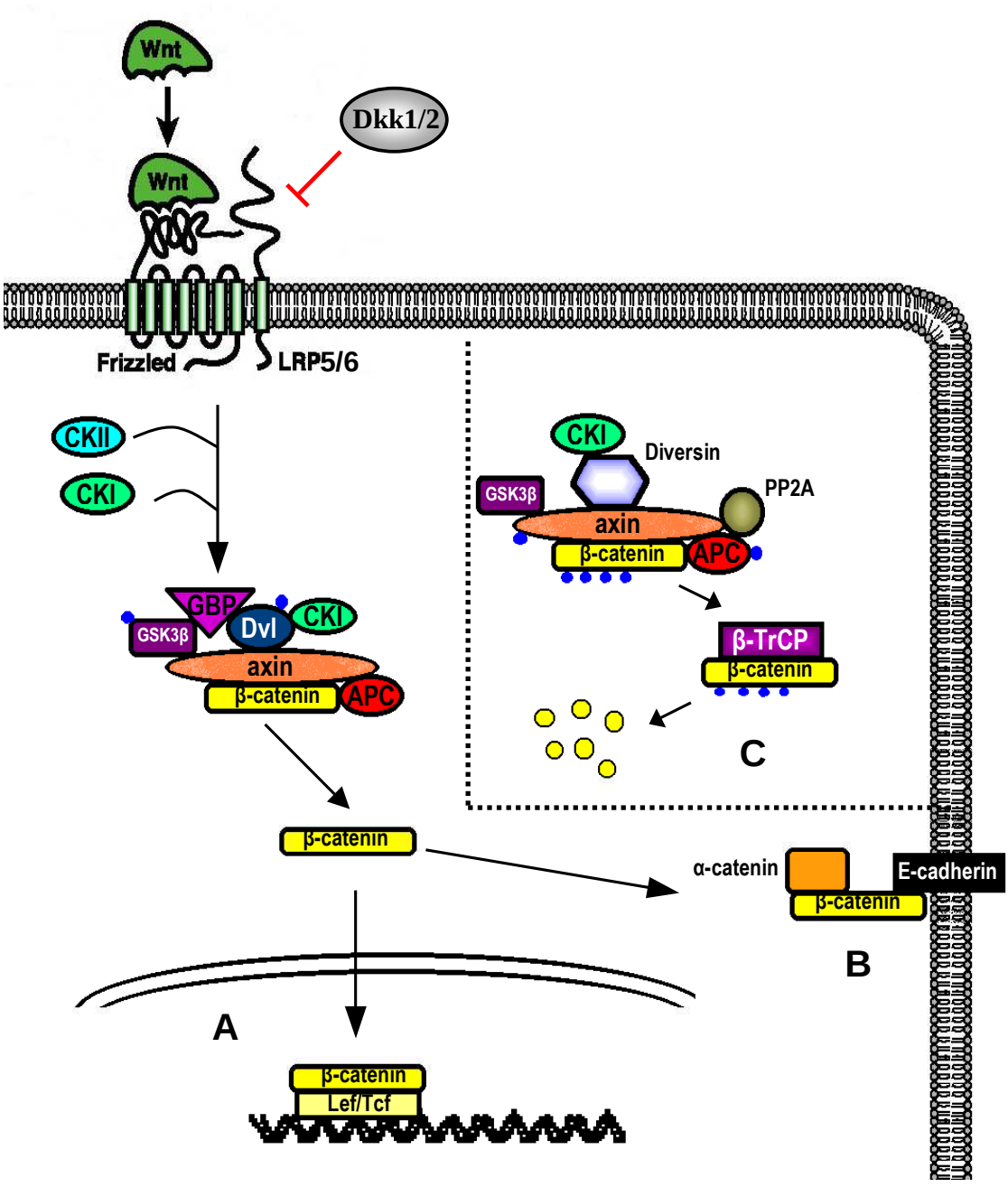

Figure 1.2. Schematic representation of the canonical Wnt/β-catenin signal transduction pathway. Many specialized interactions with β-catenin result in the formation of multiprotein complexes critical to the Wnt/β-catenin signalling pathway giving rise to transcription (A), or α-catenin/β-catenin adherens junctions (B) (not the focus of this study), or the inhibition thereof (C) by the degradation of β-catenin (modified from Roberts and Gores, 2005). Blue circles - phosphorylation.

1.5. β-Catenin degradation

The canonical degradation of β-catenin has been the central focus of numerous different studies. However, recently, pathways other than the canonical pathway have been implicated in the degradation of β-catenin (Matsuzawa and Reed, 2001; Xiao *et al.*, 2003; Sharma *et al.*, 2004). In the case of stimulation by troglitazone (TZD), a member of a family of insulin sensitizers, peroxisome proliferator-activated receptor γ2 (PPARγ2) is activated, and is able to both inhibit GSK3β and to degrade β-catenin in an as yet unidentified pathway (Sharma *et al.*, 2004).

β-Catenin has also been shown to interact with retinoid X receptor α (RXRα), in the absence of ligand binding (Xiao *et al.*, 2003). Agonists of RXR stimulate the degradation of β-catenin via RXRα by enhancing their interaction with one another, and interestingly, no change occurs in this effect when cells are targeted by GSK3β inhibitors or in cancer cell lines with APC or p53 mutations (Xiao *et al.*, 2003). This suggests a novel β-catenin degradation pathway involving retinoids. However, since the ability of PPAR to initiate the transactivation of target genes is dependent on its dimerization with RXR, there appears to be a possibility that these two novel pathways may indeed contribute to a single, as yet unidentified pathway.

Yet another more extensively researched β-catenin degradation pathway is that stimulated by genotoxic stress. A degradation complex forms containing Siah-1 (seven in absentia, Drosophila, homologue of, 1), Ebi and Siah-1 interacting protein (SIP) (Matsuzawa and Reed, 2001). Siah-1 binds to Ebi via SIP, and expression of Siah-1 is induced by p53. Ebi, like β-TrCP, is an F-box protein that is able to directly associate with the N-terminal domain of β-catenin. An interesting observation is that Ebi is able to bind unphosphorylated β-catenin as well as phosphorylated β-catenin, suggesting that the association is independent of the phosphorylation state of β-catenin (Matsuzawa and Reed, 2001).

The canonical Wnt signalling pathway degrades β-catenin in a highly complex manner involving the interactions of several proteins (Figure 1.2). The minimal typical β-catenin degradation-targeting complex includes the scaffolding protein axin, the tumour suppressor protein, APC, CKI, protein phosphatase 2A (PP2A), GSK3β and β-catenin (Xing *et al*., 2003) (Figure 1.3). Interactions between the components of this complex prime β-catenin for ubiquitination and degradation.

1.6. Specific interactions within the β-catenin degradation complex

In the context of the canonical signalling pathway, once β-catenin has been phosphorylated and released from the degradation-targeting complex, it associates with β-TrCP via its phosphorylated Ser33 and Ser37 residues. β-TrCP binds to the six base consensus sequence DSGϕXS, where ϕ represents a hydrophobic amino acid (Winston *et al*., 1999). Binding of β-TrCP allows β-catenin to associate with the $SCF^{\beta\text{-}TrCP}$-ubiquitin-ligase machinery, which includes S-phase kinase-associated protein 1A (Skp1) and Cullin 1 (Cul 1) (an E3 ubiquitin ligase). The interaction of these components results in the ubiquitination of β-catenin, targeting it for proteosome-mediated degradation (Figure 1.3).

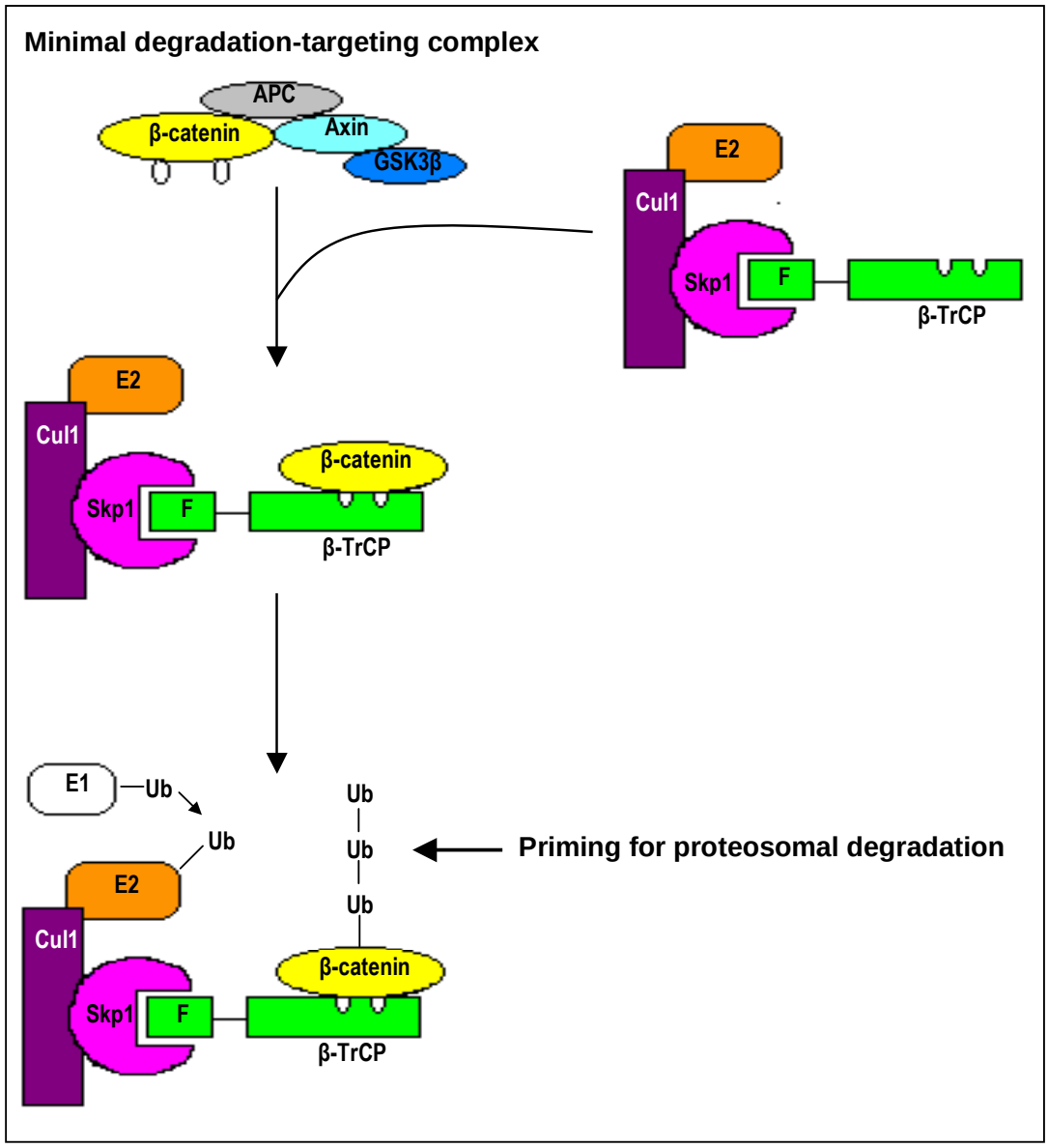

Figure 1.3. Priming of β-catenin for destruction via ubiquitination by the SCF$^{\beta\text{-TrCP}}$-ubiquitin-ligase complex in the canonical Wnt pathway. Phosphorylated β-catenin associates directly with the F-box protein, β-TrCP, and hence is linked to the ubiquitin-ligase machinery (E1/E2), which primes it for proteosome-mediated degradation (modified from Winston *et al*., 1999).

1.7. Effects of growth factors on adhesion/signalling events

Growth factors are a class of small, secreted molecules responsible for the induction of numerous cellular functions by binding specific receptors on the cell surface. Responses typically include cellular proliferation and migration, as well as differentiation (Alberts *et al*., 1994; reviewed in Frey *et al*., 2004). In addition to their roles in cell signalling, growth factors are also involved in the regulation of the dynamic adherens junctions and desmosomes (Lorch *et al*., 2004), allowing for changes in adhesion/anti-adhesion events, and thus for cell migration which is central to invasion and metastasis.

Typically, different growth factors are specific for different tissues, for example, epidermal growth factor (EGF) is known to specifically activate cellular responses in epithelial cells (Kingston *et al*., 2003). In the light of this, since human oesophageal squamous cell carcinoma is derived from epithelial cells, and since it is known to overexpress epidermal growth factor receptor (EGFR) (Veale and Thornley, 1989), it is critical to include EGFR signalling in this analysis of human oesophageal squamous cell carcinoma.

1.8. EGFR signalling

Epidermal growth factor receptor is a monomeric protein capable of forming homo- or heterodimers upon ligand binding. This allows the transduction of several different signals within the cell. To date, EGFR signalling has been shown to stimulate a number of pathways including extracellular-signal-regulated kinase (Erk) (Sanchez-Esteban *et al*., 2004), c-jun N-terminal kinase (JNK) and phospholipase C, and these pathways ultimately lead to increased cell motility, cell migration and even differentiation (Sanchez-Esteban *et al*., 2004).

Because of the vast array of intracellular molecules affected by EGFR signalling, it is interesting, although unsurprising, that members of the Wnt signalling pathway are also affected by this signalling event (Zhimin *et al*., 2003). Eldar-Finkelman *et al*. (1995) showed that a well-established component of the β-catenin degradation-targeting complex, GSK3β, is inhibited in response to EGFR signalling. This occurs via the activation of the specific GSK3β inhibitor, $p90^{RSK}$, by mitogen activated protein kinase (MAPK) signalling (Eldar-Finkelman *et al*., 2005), which, as mentioned above, is indirectly initiated in response to EGF stimulation.

EGFR signalling has also been shown to lead to an increase in tyrosine phosphorylation of β-catenin, thereby dissociating it from the cell-cell adherens junctions (Piedra *et al*., 2003), and so it affects the levels of stabilized β-catenin within the cells specifically when treatment with EGF is coupled with that of lithium (Jones and Veale, 2003).

Yet another study confirmed that a deficiency in APC led to an increase in EGFR responsiveness in intestinal enterocytes (Moran *et al*., 2004), thereby creating a positive feedback loop in which cells deficient of APC not only are unable to downregulate β-catenin in the degradation-targeting complex, but also, there is an observed decrease in the amount of plasma membrane-bound β-catenin due to the increased response to EGFR signalling. Thus it can be seen that there is a significant amount of "cross-talk" between these two pathways, and it is one of the aims of this study to try to understand these multiple inputs.

1.9. Tumour development

The formation of a tumour from a single altered cell is a complex and multistep process, and is usually not due to a single factor. In a process known as hyperplasia, a single altered cell undergoes hyperproliferation to produce a mass of cells with a

normal appearance, within a tissue (Farmer and Walker, 1985). This state of hyperproliferation is the result of disruption of cell cycle control which is usually due to mutations in cell cycle regulatory genes/proteins e.g. the tumour suppressor gene p53 (Bosari *et al*., 1995; Levine, 1997), or it could be due to an alteration in one or more signal transduction pathways, e.g. the Ras pathway (Horada *et al*., 2007).

Following hyperplasia, the cells may enter a state of dysplasia. This involves many genetic alterations within the cells, resulting in a change in their appearance. The cells no longer resemble each other or those in the surrounding normal tissue (Farmer and Walker, 1985), and their state of anomalous division increases even further. This may result in the mass of cells becoming disorganized within the tissue.

Dysplastic cells may then undergo further alterations. The cells now constitute a large volume of the tissue, but no invasion has taken place. These cells appear to regress to an almost undifferentiated state (Farmer and Walker, 1985) and are now referred to as anaplastic, and although they are not malignant, they are often treated as such.

Anaplastic cells have the potential to become malignant. If this occurs, these cells will cross the basal lamina and invade the surrounding tissues. Some cells may even detach from the primary tumour as single cells or in clumps and migrate into the blood or lymph vessels (Franks and Teich, 1997). This allows them to be transported to a secondary site, where they will attach to form a secondary tumour. This process is known as metastasis, and once a tumour has reached this state, it is said to be malignant.

Certain tumours will never progress to the point of invasion of surrounding tissues and will never metastasize to sites of secondary tumour formation. These tumours are said to be benign, and are usually not harmful to the organism. Because of this, they are not classified as cancer (Alberts *et al*., 1994).

Not all cancers develop as a result of genetic alterations. There are other non-genetic mechanisms of carcinogenesis, known as epigenesis (Franks and Teich, 1997). Epigenesis can result from alterations in protein molecules that interact with DNA or it may be the result of altered DNA methylation states (Deng *et al*., 2004). Even though this state is not due to genetic aberrations, it is inherited with as great a frequency as genetic modifications.

Of course, regardless of where cells are within the process of tumourogenesis, or how they came to be there, a central event in the process is always cell adhesion, and, therefore, understanding the importance of adhesion and adhesion-based signalling are critical in studies of this nature.

1.10. Wnt signalling and tumour formation

Since the Wnt signalling pathway is so critical for the maintenance of cell cycle regulation and response to cellular stress, via p53 and p21, it is critical that it is maintained in a normal fashion. Alterations to any of the components may be detrimental and result in long-term consequences, such as carcinogenesis.

One example of such a component is β-catenin. It is an integral part of the Wnt pathway and there are numerous deleterious mutations that may occur in this protein. One group of mutations characteristic of the β-catenin gene (*CTNNB1*) encompasses all mutations affecting the amino-terminal of β-catenin which disrupt any potential interactions with APC (Morin *et al*., 1997). This allows β-catenin to signal continuously as it is not being degraded. Thus cells are stimulated to proliferate, perhaps in an uncontrolled manner. Mutations in β-catenin have been found in a multitude of different tumours including hepatocellular carcinomas, intestinal-type gastric cancers, sporadic medulloblastomas, gastric carcinomas, pulmonary blastomas, melanomas and many others (Rubinfeld *et al*., 1997a; de la

Coste *et al*., 1998; Miyoshi *et al*., 1998; Zurawel *et al*., 1998; Park *et al*., 1999; Sekine *et al*., 2003; Wang *et al*., 2006).

A critical component of the β-catenin degradation-targeting complex, APC, has been shown to be altered in a number of human solid tumours. The promoter region of the APC gene is typically hypermethylated in these tumours. Promoter methylation induces a conformational change in the chromatin, which in turn interferes with the binding of the transcription factor CCAAT-binding factor (Deng *et al*., 2004), thereby preventing the transcription of APC, and thus β-catenin cannot be degraded by this pathway in these tumours. Tumours that have been shown to possess a hypermethylated promoter region of APC include prostate cancer, hepatoblastoma, colorectal tumours, renal cancers and several others (Smith *et al*., 1994; Kurahashi *et al*., 1995; Hoque *et al*., 2004; Jerónimo *et al*., 2004).

It is curious that although it is such a critical component of the Wnt signalling pathway, there have been no reports of mutations of GSK3β in human solid tumours. In colorectal tumours, no mutations or deletions were seen in the GSK3β gene (Sparks *et al*., 1998). It is possible that mutations or deletions within this gene are simply incompatible with life as it is such a critical component in a number of pathways, although the same could be argued from the other perspective – that is, that if a single mutation were to occur, it may be sufficient for tumourogenesis.

Mutations have been detected in the scaffolding protein axin, within hepatoblastomas, hepatocellular carcinomas and colorectal cancer cells (Satoh *et al*., 2000; Taniguchi *et al*., 2002). It was found that the missense mutation rate within a certain range of colorectal cancer tissues was 11%, and it was thus suggested that a lack or deficiency of axin may contribute to the formation of colorectal tumours (Jin *et al*., 2003). Deletions of the AXIN1 gene have been reported in sporadic medulloblastomas (Dahmen *et al*., 2001), and loss of the AXIN1 locus, but no mutations, has been observed in adenocarcinomas of the gastro-oesophageal junction (Koppert *et al*., 2004). It has

also been noted by Nakajima *et al*. (2003) that reduced axin expression is possibly associated with tumour progression in human oesophageal squamous cell carcinoma (HOSCC).

The β-catenin degradation pathway is the central focus of this dissertation. Thus axin, a central component of the degradation-targeting complex, and a critical molecule in the crossroads of stabilization, degradation and translocation of β-catenin, becomes the candidate of choice for study.

1.11. Human oesophageal squamous cell carcinoma (HOSCC)

HOSCC is a malignant cancer of increasing frequency within the world, and it is one of the leading causes of cancer-related deaths worldwide. With a 5-year survival rate of 6% (Earlam and Cunha-Melo, 1980), HOSCC is typically associated with a poor prognosis (Morgan *et al*., 1999). However, it should be noted that in Sweden, since the 1980s, the use of adjuvant chemo- and radiotherapy has significantly increased the 5-year survival rate (Sundelöf *et al*., 2002). Geographically, there is an unusual distribution of the disease, which is found at very high frequencies in certain parts of China, France, South Africa, Iran, Uruguay and Italy (reviewed in Stoner and Gupta, 2001). In Southern Africa, in particular, an escalating number of cases of oesophageal carcinoma are being reported, with particular prevalence in the Shangaan population in Mozambique and the South Africans living in the Transkei (Marasas *et al*., 1988).

Risk factors contributing to the development of oesophageal squamous cell carcinoma are outlined in Table 1.1. Within the South African population, it has been noted that poor dietary intake (infrequent intake of fruit and vegetables, as well as a change of staple diet from sorghum to maize) (Isaacson, 2005), excessive alcohol consumption and the use of unrefined tobacco (De Stephani *et al*., 1993) are central to the prevalence of HOSCC in a number of cases (Brown *et al*., 2001). Fungal, and, therefore, aflatoxin contaminations of food sources are also well-described players in

oesophageal carcinogenesis (Marasas *et al.*, 1988; Horn and Dorner, 1999; Peraica *et al*., 1999).

HOSCC is a well-known metastatic and invasive disease of the epithelium lining the oesophagus. Aspects of adhesion are involved in the processes of invasion and metastasis, because it is during these processes that cell migration is taking place. Since much is known about the clinical/pathological properties of this cancer, yet little is known about its invasive and metastatic mechanisms on a molecular level, for reasons expounded above, research surrounding the adhesive properties and adhesion-based signalling in this particular cancer is crucial.

Table 1.1. Known risk factors for HOSCC (Stoner and Gupta, 2001)

Tobacco
Alcohol
Salt-pickled, salt-cured and mouldy foods
N-nitrosamine carcinogens (from multiple sources)
Vitamin (A, E, etc.) and trace element (zinc, selenium) deficiencies
Hot beverages
Fungal invasion of oesophageal tissues
Human papilloma virus (HPV) serotypes 16 and 18
Heritable susceptibility genes

1.12. Components of the β-catenin degradation-targeting complex and their influence on cell function

Although β-catenin's influence on the cell has already been extensively discussed, it must be noted that other components of the degradation-targeting complex have additional roles in cell function. APC is thought to interact with APC-stimulated guanine nucleotide exchange factor (Asef GEF), and together they are involved in upward

migration of intestinal epithelial cells from the crypt to the tip of the villus (Kawasaki *et al*., 2003). In addition, APC has been shown to block cell cycle progression from G_0 to S phase, thereby acting as a tumour suppressor (Baeg *et al*., 1995). Axin, on the other hand, was discovered for its role in development, and was so named because of its ability to inhibit axis duplication – axis inhibitor (Zeng *et al*., 1997). Furthermore, axin has been shown to be a multifaceted signalling protein by virtue of the fact that it is able to function in numerous different pathways, including those stimulated by canonical Wnts and transforming growth factor beta (TGFβ) (Furuhashi *et al*., 2001), as well as being able to act as the major scaffolding protein in JNK signalling (Zhang *et al*., 2000; Luo *et al.*, 2003). It has also been shown to be involved in p53-dependent apoptosis (Rui *et al*., 2004). Thus axin is a key component in a wide variety of cellular functions.

1.12.1. Axin as a scaffold for the degradation-targeting complex

Axin is a 900 amino acid scaffolding protein involved in the degradation of β-catenin, that exists as one of two isoforms, the details of which are addressed in Chapter 2. Axin continuously shuttles between the nucleus and the cytoplasm, and the nuclear export thereof is dependent on the chromosome maintenance region 1 (CRM1) nuclear receptor (Cong and Varmus, 2004). It has been speculated that axin may be required for the axin-induced cytoplasmic shift of β-catenin, and thus may function to anchor β-catenin in the cytoplasm, but it has been further suggested that axin may act as a molecular chaperone for the nuclear export of β-catenin. In complex with APC, the axin-dependent nuclear export of β-catenin is accelerated (Cong and Varmus, 2004).

As it acts as the main scaffolding protein for the β-catenin degradation-targeting complex, it can be said that axin is an essential component of the pathway, since, without it, β-catenin would not be able to be primed for ubiquitination by GSK3β, as axin is the only component of the complex with binding sites available for all the

other components (Figure 1.4), and is the only component that is able to bind directly to GSK3β. Thus any alterations to axin would surely disrupt the β-catenin degradative potential of this complex.

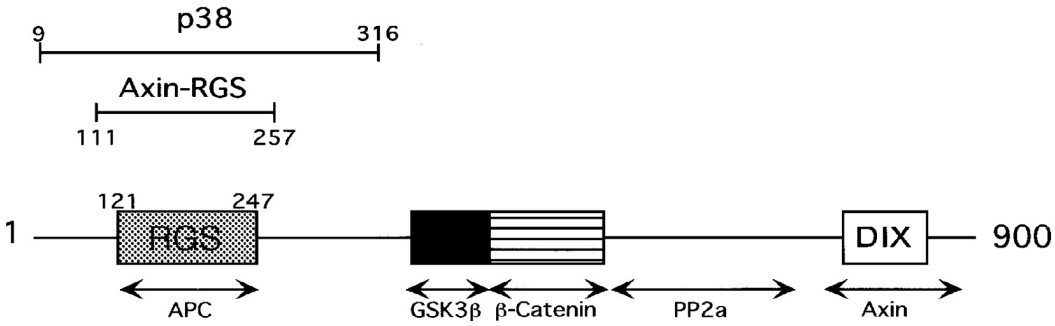

Figure 1.4. Schematic representation of the binding domains of axin. There are a number of different binding domains, including a homodimerization domain to which another axin molecule may bind. The binding sites for all critical members of the β-catenin degradation-targeting complex have been indicated by double-headed arrows. However, it should be noted that although CKI is involved in the complex, it cannot bind directly to it (Spink *et al.*, 2000). Two different isoforms are produced as a result of alternative splicing with isoform a representing the entire transcript, but isoform b lacking the putative PP2A binding domain.

1.13. Objectives of this study

1. To establish the presence of the major components of the degradation-targeting complex (β-catenin, axin and APC) in each of five HOSCC cell lines and to establish their distribution in the plasma membrane, nucleus or the cytoplasm.

2. To analyze the interaction between β-catenin and APC in HOSCC cell lines.

3. To identify LRP5 in WHCO1, WHCO3, WHCO5, WHCO6 and SNO cell lines.

4. To analyze the effect of EGF treatment on axin and LRP5 expression levels, as well as to determine its effect on axin's ability to associate with the plasma membrane.

CHAPTER 2: PRESENCE, SUBCELLULAR LOCALIZATION AND INTERACTION OF COMPONENTS OF THE β-CATENIN DEGRADATION-TARGETING COMPLEX IN HOSCC CELL LINES

2.1. Introduction

2.1.1. Targeting of β-catenin for degradation

As previously mentioned, in the absence of canonical Wnt signalling, cytoplasmic β-catenin is typically targeted for degradation by interacting with a specific complex of proteins. Axin, the primary scaffolding protein within this context, binds β-catenin, APC and GSK3β, and brings them into close proximity with each other, allowing GSK3β to phosphorylate several members of the complex (Ikeda *et al*., 1998) (Figure 1.2). Another important player in this process is CKIε, which is linked to the complex via diversin (Schwarz-Romond *et al*., 2002). It is this kinase that is responsible for the phosphorylation of β-catenin on Ser45, thereby priming β-catenin for sequential phosphorylation by GSK3β on residues Thr41, Ser37 and Ser33, creating a docking site for β-TrCP/E3RS (Amit *et al*., 2002). This binding event leads to β-catenin being targeted for degradation by ubiquitination (Aberle *et al*., 1997) (Figure 1.2).

2.1.2. Specific interactions within the β-catenin degradation-targeting complex

Numerous proteins comprise the complex targeting β-catenin for degradation. Specific interactions of these components of the β-catenin degradation-targeting complex are necessary for phosphorylation (Ikeda *et al*., 1998; Schwartz-Romond *et al*., 2002), and, therefore, ubiquitination and subsequent proteosomal degradation of β-catenin. Since there are many different interactions occurring within this complex, and because axin acts as a primary scaffolding protein in this context, multiple

binding sites in the axin protein, required for interactions within the destruction complex have been identified. These sites include those for APC, GSK3β, Dvl, CKI and for β-catenin itself (Figure 2.1) (reviewed in Salahshor and Woodgett, 2005). The interaction of these various domains with their respective binding partners results in the formation of a large multiprotein complex, the primary function of which is mentioned above.

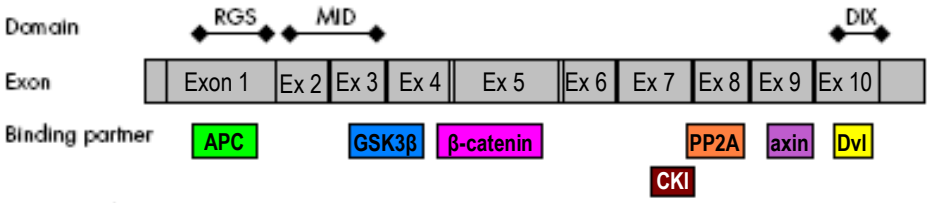

Figure 2.1. Gene diagram of axin, showing binding domains for components of the β-catenin degradation pathway (adapted from Salahshor and Woodgett, 2005). The full transcript represents isoform-a, whereas exon 8 is spliced out in isoform-b, removing the PP2A binding domain.

2.1.3. Subcellular localization of axin, β-catenin and APC

Although axin, β-catenin and APC typically interact within the cytoplasm, different signals and interactions render them able to localize both to the nucleus and plasma membrane (Näthke *et al*., 1996; Neufeld and White, 1997; Kikuchi, 2000; Kawana and Kypta, 2003; Cong and Varmus, 2004). Moreover, these sites of subcellular localization determine the function of each component at a given time (Figure 2.2). β-catenin is a well-known element of the cell-cell adherens junction system where it interacts with E-cadherin at the plasma membrane. It is also required to translocate to the nucleus upon Wnt signalling (Kikuchi, 2000).

Axin itself is also able to shuttle between the cytoplasm and the nucleus (Cong and Varmus, 2004), and is sequestered to the plasma membrane by LRP5/6 upon Wnt signalling (Kawana and Kypta, 2003) (Figure 2.2). These observations are critical to the understanding of axin's function in a cellular environment because, in addition to its role as a scaffolding protein within the β-catenin degradation-targeting complex, this multifaceted protein has also been implicated in the nuclear export of β-catenin (Cong and Varmus, 2004). Its documented role in c-Jun NH_2-terminal kinase (JNK) signalling (Zhang *et al*., 1999; Luo *et al*., 2003) as well its roles in the activation of Smad3 in response to TGFβ signalling, (Furuhashi *et al*., 2001), and in p53-dependent apoptosis (Rui *et al*., 2004), show an impressive array of intracellular interactions that make axin a major signalling molecule.

Axin and β-catenin perform multiple different functions, as does APC, which has multiple roles in the cell, from degradation of β-catenin, to a potential role in mitosis (Neufeld and White, 1997; Green *et al*., 2005) (Figure 2.2). Its ability to interact with the plus ends of microtubules results in its translocation to the plasma membrane (Munemitsu *et al*., 1994; Smith *et al*., 1994), particularly to prominent plasma membrane protrusions in response to wounding (Näthke *et al*., 1996) (Figure 2.2). In an additional study to those conducted on APC at the plasma membrane, it was shown that APC is able to colocalize with rRNA in the nucleus (Neufeld and White, 1997). Interestingly, like axin, APC is also able to bind to β-catenin in the nucleus and shuttle it to the cytoplasm (Neufeld *et al*., 2000; Hamada and Bienz, 2004). Although APC and β-catenin have been shown to interact within the nucleus in the absence of axin (Neufeld *et al*., 2000; Hamada and Bienz, 2004), it has also been reported that the degradation potential of the degradation-targeting complex is reliant on axin-dependent phosphorylation of APC (Rubinfeld *et al*., 2001; Xing *et al*., 2003). Thus, it can be seen that specific interactions of axin, β-catenin and APC with other cellular components dictate their cellular functions.

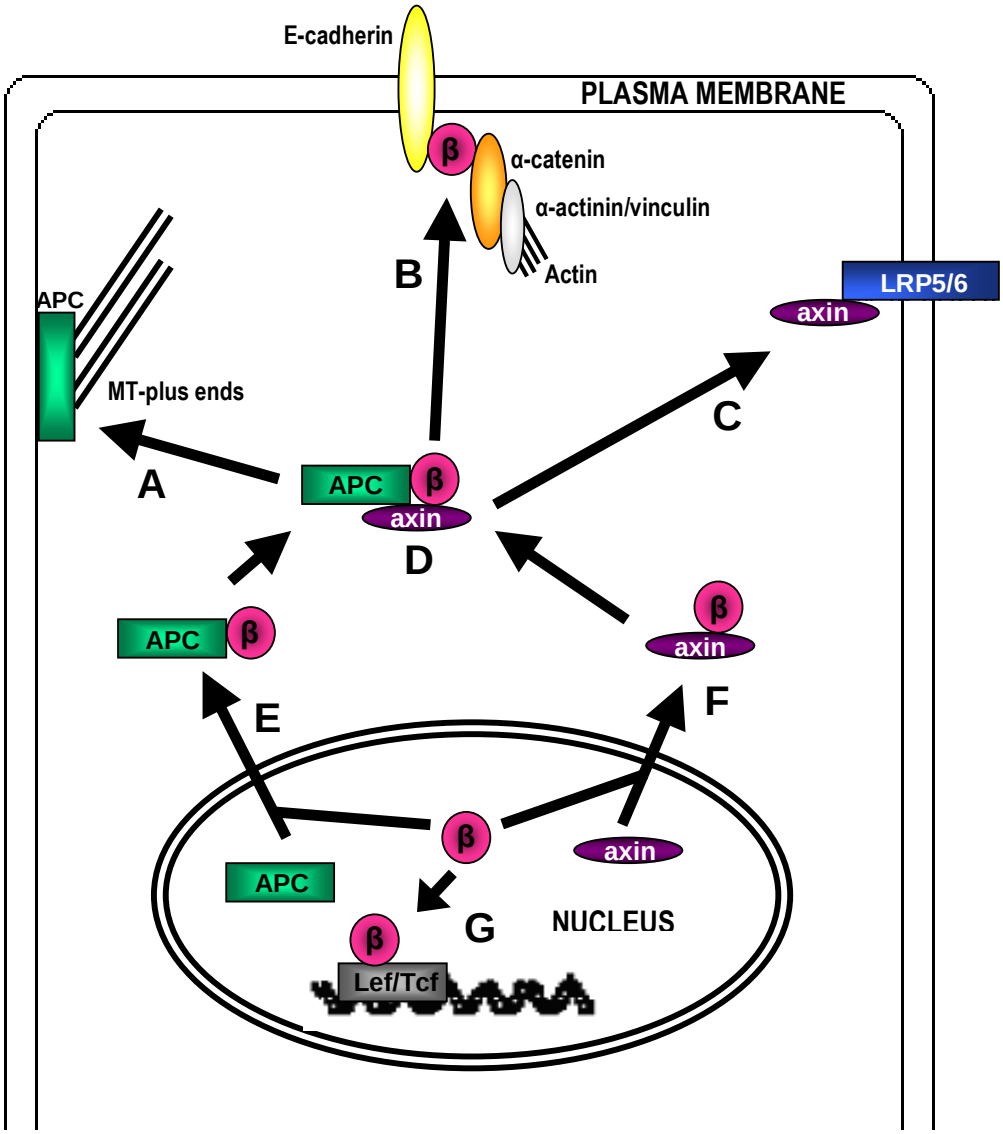

Figure 2.2. Subcellular distribution of components of the β-catenin degradation-targeting complex. Subcellular localization of each component is determined by its selection of binding partner(s). Translocation to the nucleus or plasma membrane is dependent on specific protein-protein interactions. *Note: β = β-catenin. (A) APC forms an association with MT-plus ends, resulting in its translocation to the plasma membrane. (B) β-catenin acts as an integral component of the cell-cell adherens junctions. (C) Axin is recruited to the plasma membrane by LRP5/6 during Wnt signalling. (D) Axin, APC and β-catenin interact in the degradation-targeting complex in the cytoplasm. (E) APC may bind to β-catenin in the nucleus and shuttle it to the cytoplasm. (F) Axin, too, may bind to β-catenin in the nucleus and shuttle it to the cytoplasm. (G) β-catenin forms an interaction with Lef/Tcf in the nucleus leading to active gene transcription.

2.1.4. β-catenin, axin and APC in cancer

In recent years, several studies have reported that β-catenin, axin and/or APC are mutated in a wide range of human cancers including prostate cancers, melanomas, pancreatic adenocarcinomas, colorectal carcinomas, hepatocellular carcinomas, and endometrial carcinomas, amongst others (Nishisho *et al*., 1991; Powell *et al*., 1992; Rubinfeld *et al*., 1997b; de La Coste *et al*., 1998; Fukuchi *et al*., 1998; Miyoshi *et al*., 1998; Voeller *et al*., 1998; Satoh *et al*., 2000; Taniguchi *et al*., 2002; Zeng *et al*., 2006). These mutations typically lead to stabilization of cytoplasmic β-catenin resulting in its translocation to the nucleus. This leads to augmented β-catenin-dependent gene transcription and hence increased cellular proliferation and cell migration (Barker *et al*., 2000; Cheon *et al*., 2002).

These observed alterations leading specifically to the above changes have been attributed to mutations at specific points within the genes encoding each protein. The β-catenin gene, *CTNNB1*, contains a 200bp region in exon 3, which has particular "predisposition" for mutation (Rubinfeld *et al*., 1997b; Fukuchi *et al*., 1998; Miyoshi *et al*., 1998; Palacios and Gamallo, 1998). APC is typically altered by one of three mechanisms: promoter hypermethylation, deletion of one or both alleles, or a mutation within the mutational cluster region (MCR) of exon 15 (reviewed in Aust *et al*., 2002). These in turn may lead to modifications in the protein, at an expression and/or functional level (Deng *et al*., 2004; Hoque *et al*., 2004; Jerónimo *et al*., 2004). In contrast, axin does not appear to have a specific mutational "hotspot" region. It has been reported to be mutated in several different ways – a nonsense mutation was detected in 1 of 29 ovarian endometrioid carcinomas (Wu *et al*., 2001), large deletions (12% of *AXIN1* gene) were found in ~7% of medulloblastomas (Dahmen *et al*., 2001), and 7 missense mutations were detected in a study of 73 hepatocellular carcinomas and 27 hepatoblastomas (Taniguchi *et al*., 2002). It has also been noted

by Nakajima *et al*., (2003) that reduced axin expression is possibly associated with tumour progression in human oesophageal squamous cell carcinoma.

Exon 3 of β-catenin has already been extensively examined by polymerase chain reaction (PCR) within these five HOSCC cell lines, and no mutations were observed (Schnugh, 2001). It has also been reported that no mutations resulting in stabilized β-catenin, or non-functional APC have been found in HOSCCs (Powell *et al*., 1994; Ninomiya *et al*., 2000). In the light of these data, axin became a prime candidate for the current study, as changes affecting its presence and/or subcellular location could result in greatly altered signalling potential within HOSCC. It was thus deemed critically important to examine the interactions of these components within the five HOSCC cell lines in order to ascertain the ability of these cells to degrade β-catenin.

2.1.5. Objective

To determine the presence, subcellular distribution and interactions between members of the β-catenin degradation-targeting complex in WHCO1, WHCO3, WHCO5, WHCO6 and SNO HOSCC cell lines.

2.2. Materials and Methods

2.2.1. Tissue culture

The five human oesophageal squamous cell carcinoma lines WHCO1, WHCO3, WHCO5, WHCO6 (Veale and Thornley, 1989) and SNO (Bey *et al*., 1976) are laboratory stock cell lines derived from moderately differentiated oesophageal tumours. Cell cultures were maintained in 3:1 medium (Highveld Biologicals) (Appendix 6.1.1) supplemented with 10% foetal calf serum (Highveld Biologicals). The cultures were maintained in 10 cm culture dishes (Corning) at a temperature of 37ºC in air with a CO_2 level of 5%.

Cells were grown to a confluency level of ~80%. The medium was aspirated and cells were washed with 2 ml of sterile single phosphate buffered saline (1xPBS) (Appendix 6.1.2). Thereafter, cells were incubated at 37˚C for approximately 5 minutes in 2 ml Trypsin (Gibco BRL)/ethylenediaminetetra-acetic acid (EDTA) (BDH Laboratories) (TE) (Appendix 6.1.3), or until all cells had detached. A fraction of the trypsinized cells were resuspended in fresh culture medium and culturing continued as described above.

2.2.2. Whole cell protein extraction

Cells were passaged to ~80% confluency in 10 cm culture dishes, washed 3 times with 1xPBS, scraped off into 1 ml 1xPBS, and centrifuged in a Tomy HF-120 centrifuge (1145 × g, 5 min). The supernatant was removed and 100 µl – 150 µl single Laemmli lysis buffer (Appendix 6.2.1.b) was added to the pellet, and vortexed. Samples were then incubated in a boiling water bath for 5 minutes, and centrifuged (4ºC, 8800 × g, 20 min) (Eppendorf Centrifuge 5413). All extracts were stored at -20ºC.

2.2.3. Cytoplasmic/membrane and nuclear extraction

Extractions were performed on all cell lines, at approximately 80% confluency, using the CelLytic™ NuCLEAR™ Extraction Kit from Sigma. Cells were rinsed thrice with 1xPBS, and scraped off into 1 ml 1xPBS, and centrifuged at (1145 × g, 5 min) (Tomy HF-120 centrifuge). The supernatant was discarded and 500 µl kit hypotonic lysis buffer (containing 5 µL 0.1 M dithiolthreitol (DTT), 5 µl protease inhibitor cocktail) was added to the pellet. The pellet was gently resuspended, and allowed to swell for 17 minutes on ice. IGEPAL (30 µl) was added followed by vigorous vortexing for 10 seconds. Samples were briefly centrifuged (8800 × g) (SORVAL® MC (12V)), and the supernatant was decanted and stored at -70ºC (cytoplasmic/membrane fraction). Nuclear extraction buffer (70 µl) was added to the pellet and samples were placed on a shaker on ice (30 min), followed by centrifugation (20 000 × g, 5 min) (Medifriger-BL). The supernatant containing nuclear proteins was decanted and stored at -70ºC.

2.2.4. Cytoplasmic/nuclear and plasma membrane extraction

Confluent cells (~80%) were rinsed twice with 1xPBS and once with PBS/phenyl-methyl-sulfonyl-fluoride (PMSF)/Trazylol (Appendix 6.2.2), scraped off in 1 ml PBS/PMSF/Trazylol. Samples were briefly centrifuged (1145 × g) (Tomy HF-120 centrifuge), and the supernatant was discarded. Hypotonic buffer (500 µl) (Appendix 6.2.3) with protease inhibitors (Appendix 6.2.3.a) was added to the pellet and frozen overnight at -70ºC. Samples were thawed on ice and homogenized with a Dounce homogenizer and all the homogenate was transferred to an ultracentrifuge tube. Samples were ultracentrifuged (100 000 × g, 30 min) (Beckman L7-65 Ultracentrifuge). The supernatant containing the nuclear/cytoplasmic fraction was decanted and stored at -70ºC. Sodium dodecyl sulfate (SDS) (500 µl, 0.1% (w/v), 20mM Tris-HCl, pH 7.4) was added to the pellet and allowed to resuspend overnight.

Once fully resuspended, samples were transferred to sterile Eppendorf tubes and stored at -70ºC.

2.2.5. Triton-X-100 protein extraction

Proteins were extracted according to a modified version of the protocol by Giancotti and Ruoslahti (1990). Cells were grown to ~80% confluency in 10cm culture dishes, washed 3 times with PBS/Trazylol/PMSF, scraped off in a small amount of PBS/Trazylol/PMSF and transferred to an Eppendorf tube. Samples were centrifuged for 2 minutes in the Tomy HF-120 centrifuge at 1145 × g. The supernatant was removed and 100 µl – 150 µl of extraction buffer (Appendix 6.2.4) was added to the pellet, followed by vortexing. The samples were then incubated on ice for 2 hours, followed by centrifugation (4ºC, 8800 × g, 10 min) (Eppendorf Centrifuge 5413). The supernatant was decanted and stored at -70ºC.

2.2.6. Protein estimation on extracted samples

Protein estimations were carried out according to the method employed by Bramhall *et al.* (1969).

Watmann filter paper was subjected to a dehydration series (20 min in distilled water, and 5 minutes in 95% ethanol, 100 % ethanol and 100% acetone, respectively). The filter paper was air-dried under a fume hood. Standard amounts of bovine serum albumin (BSA) (1 µg, 3 µg, 6 µg, 12 µg, 16 µg and 20 µg, in the relevant buffer) or sample (5 µl) were dotted onto the paper, allowed to air-dry, fixed onto the filter paper by incubation in trichloroacetic acid (TCA, 7.5% w/v, 40 min), stained in Coomassie Brilliant Blue stain (Appendix 6.2.5) (5 min), after which the Coomassie was discarded. The filter paper with the fixed proteins was placed in fresh Coomassie for 45 minutes, followed by destaining (Appendix 6.2.6) for 1½ hours. The paper was

then dried and the stained circles were cut out, placed in tubes containing 5ml of elution solution (Appendix 6.2.7) and placed in the dark overnight. The absorbance at 596nm was recorded after approximately 8 hours and a standard curve was constructed in order to estimate the amount of protein present in each sample (Appendix 6.2.8).

2.2.7. Sample preparation for SDS-PAGE

Nuclear and Triton-X-100 extractions were prepared for SDS-polyacrylamide gel electrophoresis (PAGE) by a 1:1 dilution with double lysis buffer (Appendix 6.2.1.a), followed by incubation in a boiling water bath for 5 minutes.

Enough plasma membrane-associated protein extract from each cell line to contain 250 μg of protein was frozen at -70°C and then lyophilized in an Edwards Freeze Dryer for 8-9 hours. Lyophilized samples were resuspended in β-mercaptoethanol (BME) water (Appendix 6.2.9), followed by incubation in a boiling water bath for 5 minutes and stored at -70°C.

2.2.8. Co-immunoprecipitation

Co-immunoprecipitation was conducted using Triton-X-100 protein extracts previously obtained. Anti-APC (2 μl, Santa Cruz) was added to enough of each sample to contain 70 μg of protein. The solution was made up to a total volume of 600μl using IP buffer (Appendix 6.2.10) and incubated on a rotor overnight at 4ºC. Protein G Sepharose beads were washed with IP buffer and added to the protein/antibody complex. IP buffer (70 μl) was added to the beads and they were tapped gently for resuspension. The bead/IP buffer mixture (30 μl) was added to the protein/antibody complex and the sample was again incubated overnight on a rotor at

4ºC. Following the incubation, the sample was centrifuged (12000 × g, 30 sec) (SORVAL® MC (12V), the supernatant was removed and IP buffer (700 µl) was added. The process was repeated twice more, with a final wash in 0.1% IP buffer, and double lysis buffer (50 µl) was added to the pellet. The sample was incubated in a boiling water bath for five minutes and centrifuged (4ºC, 8800 × g, 10 min) (Eppendorf Centrifuge 5413). Samples were stored at -70˚C. Immunoprecipitates were subsequently used in western immunoblotting.

2.2.9. SDS-PAGE

Discontinuous SDS-PAGE was performed following the protocol described by Laemmli (1970). The gel was composed of two components – a 10% separating gel (Appendix 6.3.1) and a 5% stacking gel (Appendix 6.3.2). After polymerization of these two components between one glass and one silicon plate (Mighty Small™, dual gel caster, Hoeffer), samples were loaded into the wells. Low molecular weight marker (2.5 µl) (PageRuler™ Prestained Protein Ladder, Fermentas) was loaded in a separate lane and proteins were separated on the gel (1 h, 25mA, 400V), in running buffer (Appendix 6.3.3). The gel was stained in Coomassie Brilliant Blue stain (0.25% w/v, 1 h), then destained (Appendix 6.3.4) until clear, in order to view the separation of the proteins within the gel. A calibration curve of the log of the distance migrated by each of the standard proteins was constructed (Appendix 6.3.5), and the bands corresponding to the respective molecular weights of the aforementioned proteins were determined.

2.2.10. Antibodies

All antibodies were diluted in 1xPBS unless otherwise stated.

Rabbit polyclonal anti-β-catenin antibody (Sigma: C2206)

Concentration for: a) western immunoblotting = 1:5000

b) indirect immunofluorescence = 1:500

Goat polyclonal anti-axin antibody (Santa Cruz: sc-8568)

Concentration for western immunoblotting = 1:400

Rabbit polyclonal anti-APC antibody (Santa Cruz: sc-896)

Concentration for western immunoblotting = 1:250 in Blotto (Appendix 6.4.2)

Mouse monoclonal anti-E-cadherin antibody (R&D Systems)

Concentration for western immunoblotting = 1:300

Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibody (Sigma A9917)

Concentration for western immunoblotting = 1:4500

HRP-conjugated goat anti-rabbit antibody (Sigma)

Concentration for western immunoblotting with:

a) anti-β-catenin = 1:10000
b) anti-APC = 1:15000.

HRP-conjugated rabbit anti-goat (BioYeda)

Concentration for western immunoblotting = 1:20000

Flourescein isothiocyanate (FITC)-conjugated goat anti-rabbit (Cappel)

Concentration for indirect immunofluorescence = 1:100

2.2.11. Western immunoblotting

Western immunoblotting was performed according to the protocol described by Towbin *et al.* (1979).

A 10% SDS-PAGE gel was run as above, the gel was cut within the relevant range to contain the protein of interest and the proteins were transferred onto nitrocellulose for 3 hours in western blot transfer buffer (Appendix 6.4.1) using a Biorad Criterion™ Blotter. The nitrocellulose membrane was washed three times with 1xPBS, and

placed in blocking buffer (Appendix 6.4.2). The membrane was incubated in primary antibody and then subjected to six 5-minute washes with 1xPBS. The membrane was subsequently transferred to secondary antibody (horseradish peroxidase–conjugated) and incubated in the dark for 1 hour. Again the membrane was subjected to six 5-minute washes followed by a 6 minute incubation in SuperSignal® West Pico Chemiluminescent Substrate (Pierce). Film was exposed to the membrane, developed (Appendix 6.4.3) for 5 minutes and fixed (Appendix 6.4.4) for 5 minutes, to allow for visualization of the bands produced. Since the reactions for all antibodies were shown to be specific, blots were cropped as has become the practice in numerous international journals e.g. EMBO J., Cell, J. Biol. Chem, J. Cell Biol.

2.2.12. Densitometric analysis

Densitometric analysis was performed on western immunoblots in order to quantitatively determine a relative level of expression of each protein. Densitometry was used for two reasons – firstly, it gives a semi-quantitative analysis of the bands produced and, secondly, since it is well known that the film exposure of a western blot is not visually linear, Labworks^{TM} Image Acquisition and Analysis software (Labworks version 4.5) a program which operates specifically on scanned images and regularizes the background, was used, thereby providing the basis for comparison.

2.2.13. Indirect immunofluorescence

Cells were grown to ~50% confluency and the medium aspirated. The cells were washed with 1xPBS (2 ml), followed by incubation in TE in a CO_2 incubator (2 ml, 37ºC, 10 min). The cell/TE mixture was removed from the dish and placed in a mixing bottle with medium (8 ml). The mixture (20 µl) was removed and mixed with 0.4% trypan blue (20 µl) (Appendix 6.5.1) and this mixture was placed on a haemocytometer for counting. Enough of the cell/TE/medium mixture was added to a

clean coverslip to ensure that approximately 350000 cells were present. The cells and the coverslips were placed back into the incubator (24 h) to allow attachment to the coverslip to occur. The medium was once again aspirated and the cells were washed 5 times with 1xPBS, followed by fixation onto the coverslip with 4% paraformaldehyde (30 min) (Appendix 6.5.2). The cells were washed 5 times with 1xPBS and permeabilized with Triton-X-100 (0.25%, 10 min). The Triton-X-100 was removed from the cells and they were washed twice with 1xPBS, after which the coverslips were rinsed with dH_2O and allowed to air-dry. Circles were drawn onto the coverslips using a DAKO pen and rehydration with 1xPBS was performed over a period of 30 minutes. Rabbit anti-β-catenin antibody (20 µl of 1:500) was added to the experimental well, 1xPBS (20 µl) was added to the negative control well, and coverslips were maintained in a moist environment for 1 hour. The cells were subjected to five 1-minute washes with 1xPBS, followed by incubation in Fluorescein-conjugated goat anti-rabbit antibody (20 µl, 1:100 in the dark for 1 h). Cells were again subjected to washes (1xPBS, five 1-min) and 8 µl of VECTASHIELD® HardSet™ Mounting Medium (Vector Laboratories) was added to each well. The coverslips were mounted onto slides and visualized using the Zeiss confocal laser scanning microscope (FITC: excitation wavelength = 490 nm, emission wavelength = 525 nm).

2.3. Results

2.3.1. All HOSCC cell lines express axin, β-catenin and APC

Separation of equal amounts of whole cell lysates (~5 μg, as determined by protein estimation) by reducing SDS-PAGE revealed the presence of a number of bands within the regions corresponding to β-catenin (~92 kDa, pink arrow), axin (~125-130 kDa, purple arrow) and APC (~310 kDa, green arrow) (Figure 2.3). These findings hinted that polypeptides with the appropriate molecular weights were present within each cell line. However, in order to confirm the presence of each, western immunoblotting was employed using antibodies specific for each protein. The presence of β-catenin, APC and both isoforms of axin were confirmed (Figures 2.4-2.6).

Densitometric analysis of the respective β-catenin whole cell lysate western immunoblots revealed different levels of expression across the cell lines (Figure 2.4). The highest level was observed within the WHCO3 cell line and the lowest in SNO. However, although there is a noticeable difference between the five cell lines, expression levels varied by only ~38%, with the exception of SNO, where there was substantially less β-catenin present overall compared to the other cell lines.

This was contrasted by both isoform-a of axin, and APC, where WHCO6 and SNO cell lines demonstrated relatively high levels of expression, whereas cell lines WHCO1, WHCO3 and WHCO5 showed the lowest levels of expression overall (Figures 2.5, 2.6). For APC, there was an almost 84% difference in the expression levels between the cell lines exhibiting highest and lowest levels of expression and for axin isoform-a there was ~68% difference. This was considered to be a marked difference which demonstrates that there is a great similarity between the patterns of expression of APC and isoform-a of axin. Isoform-b of axin showed comparatively similar levels of expression across all cell lines, with a difference of only ~18% across all cell lines.

Because of the similar pattern of expression observed in APC and axin isoform-a, it appeared that, potentially, the integrity of the degradation-targeting complex was maintained within these cell lines, as APC and axin bind each other with a ratio of 1:1 (Spink *et al*., 2000; Lee *et al*., 2003) (Figure 1.3). Thus it was necessary to determine the relative levels of expression of all three proteins at the plasma membrane and in the nucleus. However, because of the relationship observed between APC and axin isoform-a within the whole cell lysates, it was assumed that within the context of the whole cell lysates, the APC/axin interaction was intact and, therefore, β-catenin became the primary focus of this part of the study.

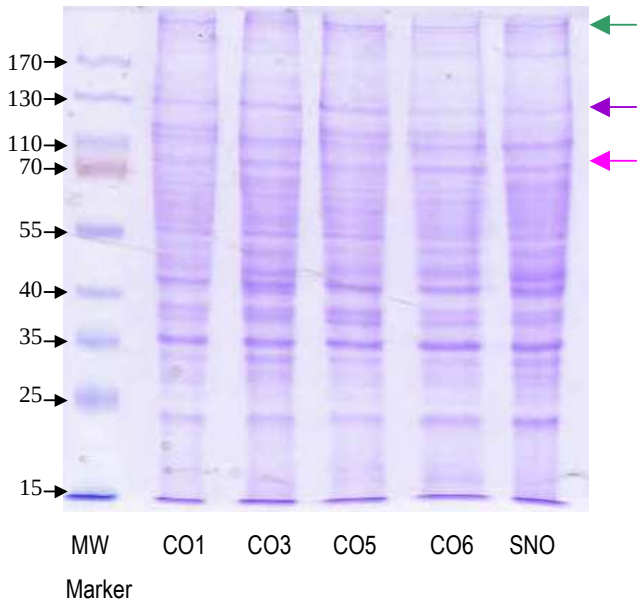

Figure 2.3. Electrophoresis of whole cell lysates on a 10% SDS-PAGE gel. Whole cell lysates were obtained from each of the five cell lines and separated on a 10% reducing SDS-PAGE gel. A prestained low molecular weight protein marker was loaded in the first lane (LabAid™) and the size corresponding to each band is indicated by an arrow next to the gel. A uniform level of staining was observed across all the cell lines reflecting the fact that the same amount of protein (~5 µg) was loaded in all lanes.
*Note: CO1=WHCO1, CO3=WHCO3, CO5=WHCO5, CO6=WHCO6

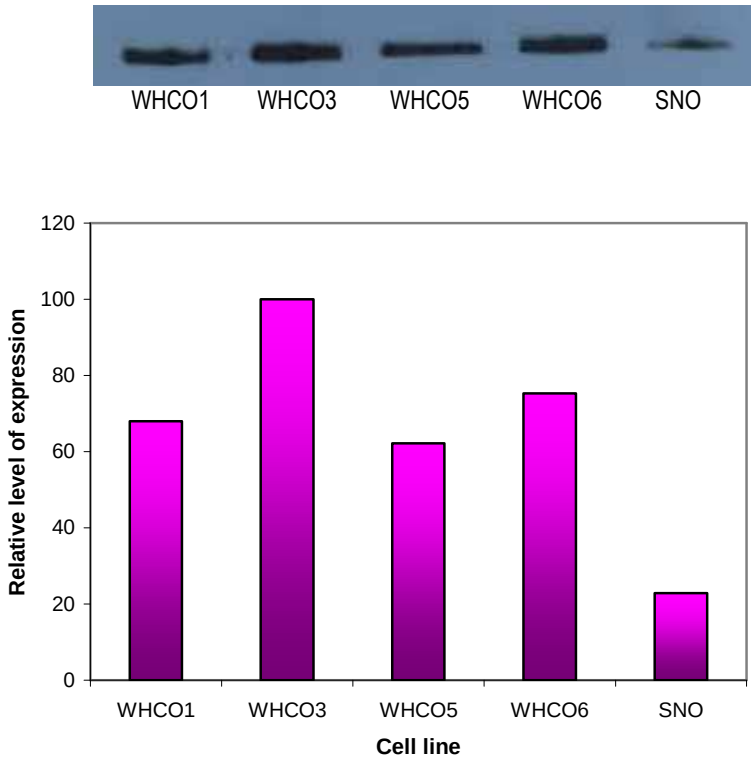

Figure 2.4. β-catenin is expressed by HOSCC cell lines. Western immunoblotting of whole cell lysates and densitometric analysis thereof, confirm the presence of β-catenin at varying levels across the five HOSCC cell lines.

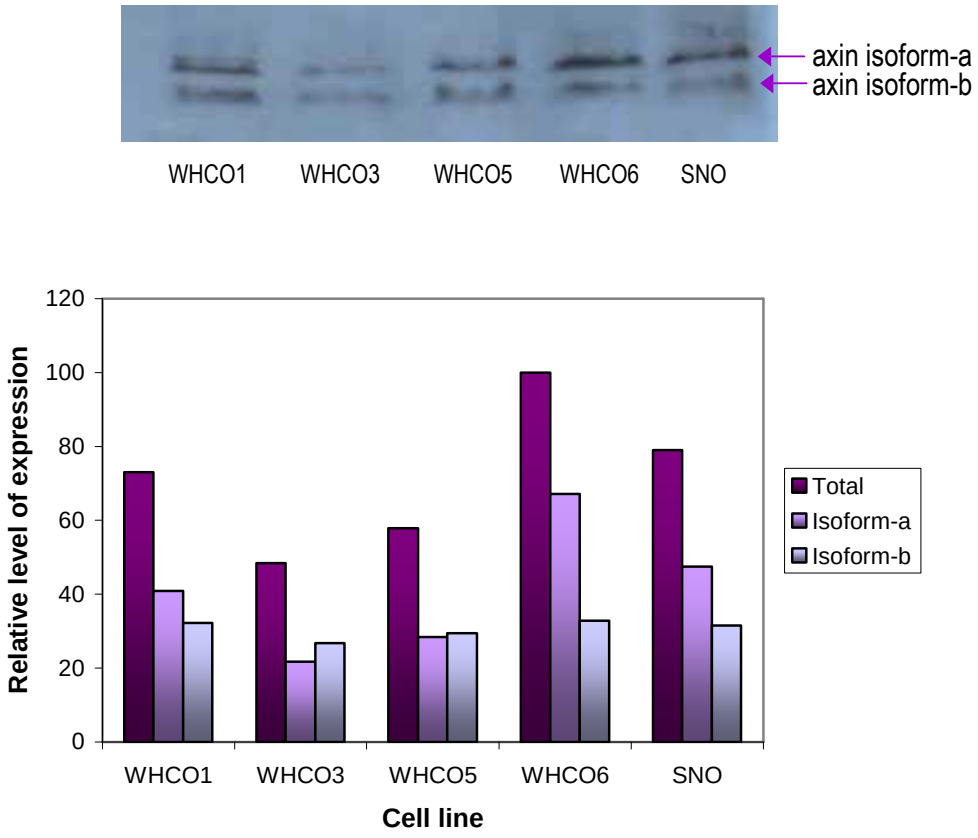

Figure 2.5. All cell lines express both isoform-a and isoform-b of axin. Western immunoblotting of whole cell lysates and densitometric analysis thereof, revealed the presence of both isoform-a and isoform-b of axin within the five HOSCC cell lines.

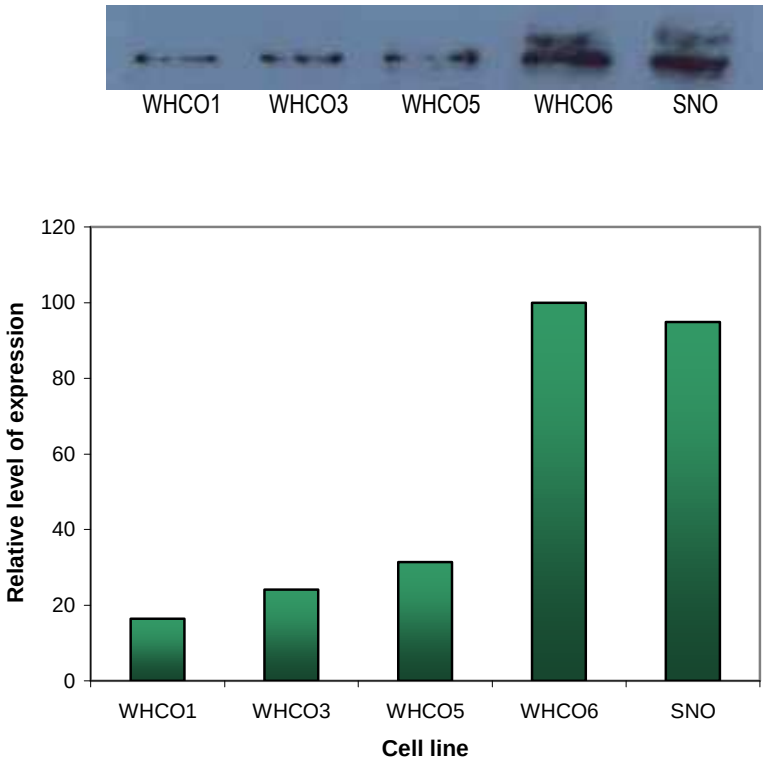

Figure 2.6. APC is expressed by HOSCC cell lines. Western immunoblotting of whole cell lysates and densitometric analysis thereof, verified the presence of APC within the five HOSCC cell lines. Observation of the doublet band in WHCO6 and SNO can be attributed to alternative cleavage possibly by caspases, leading to the production of a lower molecular weight polypeptide (Browne *et al*., 1998).

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2.3.2. Distribution of axin, β-catenin and APC in HOSCC

Following the confirmation of the presence of each of the components of the degradation-targeting complex within each of the five HOSCC cell lines, it was imperative to determine their patterns of subcellular distribution (Figure 2.2). Plasma membrane-associated and nuclear protein extractions were performed on each cell line as described previously (Section 2.2.3 and 2.2.4) to assess the relative distribution of APC, β-catenin, and axin relative to the anticipated distribution described in Figure 2.2. Equal amounts of these extracts (~5 µg), as determined by protein estimation (Bramhall *et al.*, 1969), were separated by SDS-PAGE and bands corresponding to the correct molecular weight ranges were observed upon staining (β-catenin: ~92 kDa, pink arrow; axin: 125-130 kDa, purple arrow; and APC: ~310 kDa, green arrow) (Figure 2.7).

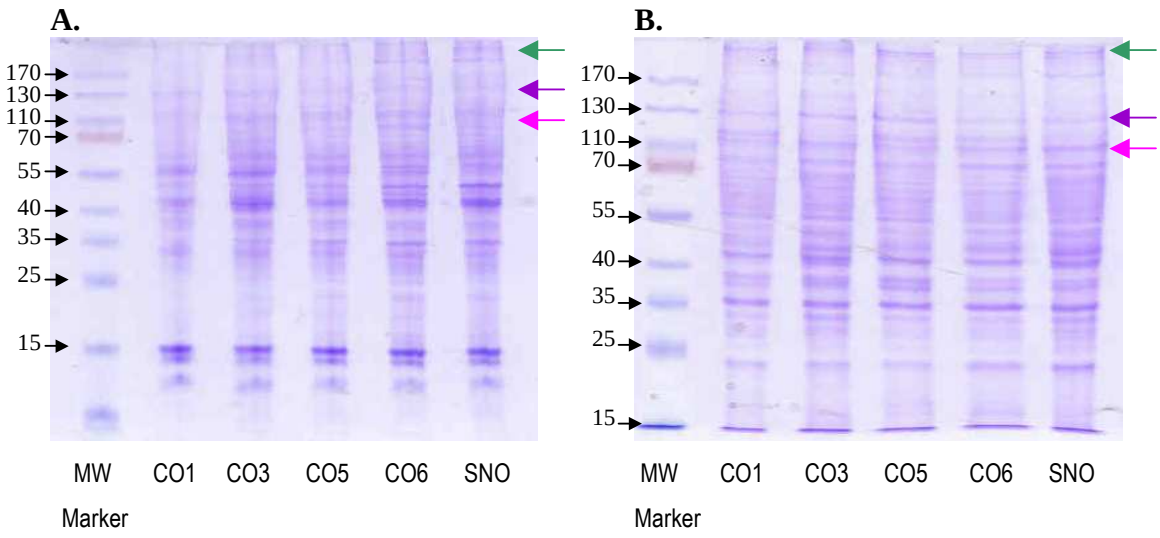


Figure 2.7. Electrophoresis of plasma membrane-associated and nuclear extracts on 10% reducing SDS-PAGE gels. (A) Plasma membrane-associated and (B) nuclear protein extracts obtained from all HOSCC cell lines and separated on 10% reducing SDS-PAGE gels. A prestained low molecular weight protein marker was loaded in the first lane (LabAid™) and the corresponding size of each band is indicated by an arrow next to the lane. A uniform level of staining was observed across all the cell lines reflecting the fact that the same amount of protein (~5 µg) was loaded in all lanes.
*Note: CO1=WHCO1, CO3=WHCO3, CO5=WHCO5, CO6=WHCO6

Despite the presence of appropriately sized polypeptides in each of the extracts, it was necessary to confirm the presence of each protein component by western immunoblotting. Densitometric analysis of western immunoblots again revealed different levels of expression of each protein within the subcellular components of the cells (Figures 2.8-2.14).

APC was not present in levels high enough to be detected in plasma membrane-associated protein extracts (Figure 2.10). This could be explained by virtue of the fact that APC typically only localizes to the plasma membrane when associated with microtubule plus ends (Munemitsu *et al*., 1994; Smith *et al*., 1994; Näthke *et al*., 1996) and although numerous reports have shown this interaction to occur, it is possible that the association is not occurring at particularly high levels within these cells. In support of this, it must be noted that in several cases visualization of this association required APC overexpression (Munemitsu *et al*., 1994; Smith *et al*., 1994; Langford *et al*., 2006).

Both axin and β-catenin were shown to be associated with the plasma membrane (Figures 2.8, 2.9). While isoform-a of axin appeared to be associated with the plasma membrane at lower levels overall, isoform-b was expressed at relatively high levels at the plasma membrane in WHCO1, WHCO3 and WHCO5. It is curious that in WHCO6 and SNO cell lines there are higher levels of expression of isoform-a than isoform-b, as unlike the other three cell lines, this pattern of expression mirrors that of the whole cell lysates. Thus Dvl, the protein thought to transport axin to the plasma membrane (Cliffe *et al*., 2003), must, for some reason, show differing affinities for the two isoforms in the different cell lines. Alternatively, this observed difference might be attributed to the alternate functional levels of another molecule upstream to Dvl.

In contrast to the overall levels of expression, β-catenin levels at the plasma membrane were shown to be highest in WHCO1 and lowest in WHCO6 (Figure 2.8).

This result is curious as the pattern of expression was not the same as that of E-cadherin, which paradoxically showed the highest level of expression in WHCO6 and the lowest in WHCO1 and WHCO5, both overall and at the plasma membrane (Figures 2.10.b, 2.11).

A lower molecular weight β-catenin degradation product reportedly produced by caspase-3 or -7 cleavage (Brancolini *et al*., 1997; Steinhusen *et al*., 2000; Ling *et al*., 2001) was observed in some of the cell lines (Figure 2.8). Nuclear expression of β-catenin appeared relatively uniform across all lines, with a slightly higher level of expression observed in WHCO3 (Figure 2.12). Although APC and β-catenin are known to interact within the nucleus (Henderson, 2000; Rosin-Arbesfeld *et al*., 2000), the expression levels of APC in the nucleus across the cell lines did not mirror those of β-catenin (Figure 2.14). This observation may be explained by the ability of APC and β-catenin to associate with other molecules within the nucleus (Behrens *et al*., 1996; Neufeld and White, 1997; He *et al.*, 1998) (see discussion).

The pattern of subcellular distribution of β-catenin was further confirmed by indirect immunofluorescence (Figure 2.15). Using WHCO5 as an example, indirect immunofluorescence confirmed the formation of β-catenin aggregates at the plasma membrane, where it is known to bind to E-cadherin in a 1:1 molar ratio in the adherens junctions (Aberle *et al*., 1994; Adams *et al*., 1998; Pokutta and Weis, 2000). Cytoplasmic and nuclear staining were also observed across all cell lines with clear exclusion from the nucleoli. These data are consistent with previous reports (Dahan, 2005; Metcalfe, 2006) and confirm the data obtained by western immunoblotting. Thus it appears that although there is active β-catenin signalling within these five cell lines, there is also sequestration thereof both to the plasma membrane and cytoplasm. Therefore, some sort of equilibrium must have been established between β-catenin signalling and β-catenin degradation in HOSCC.

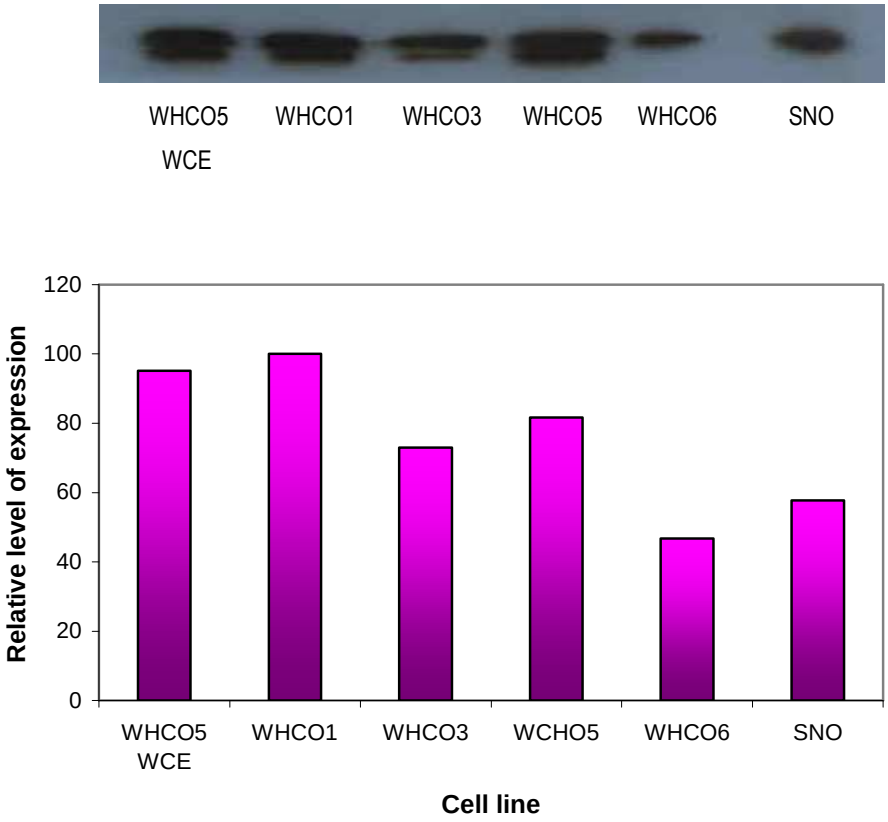


Figure 2.8. β-catenin is present at the plasma membrane. β-catenin-specific western immunoblotting revealed the presence of β-catenin at the plasma membrane in varying levels across the five HOSCC cell lines. A whole cell lysate (WCE) was included as a positive control.

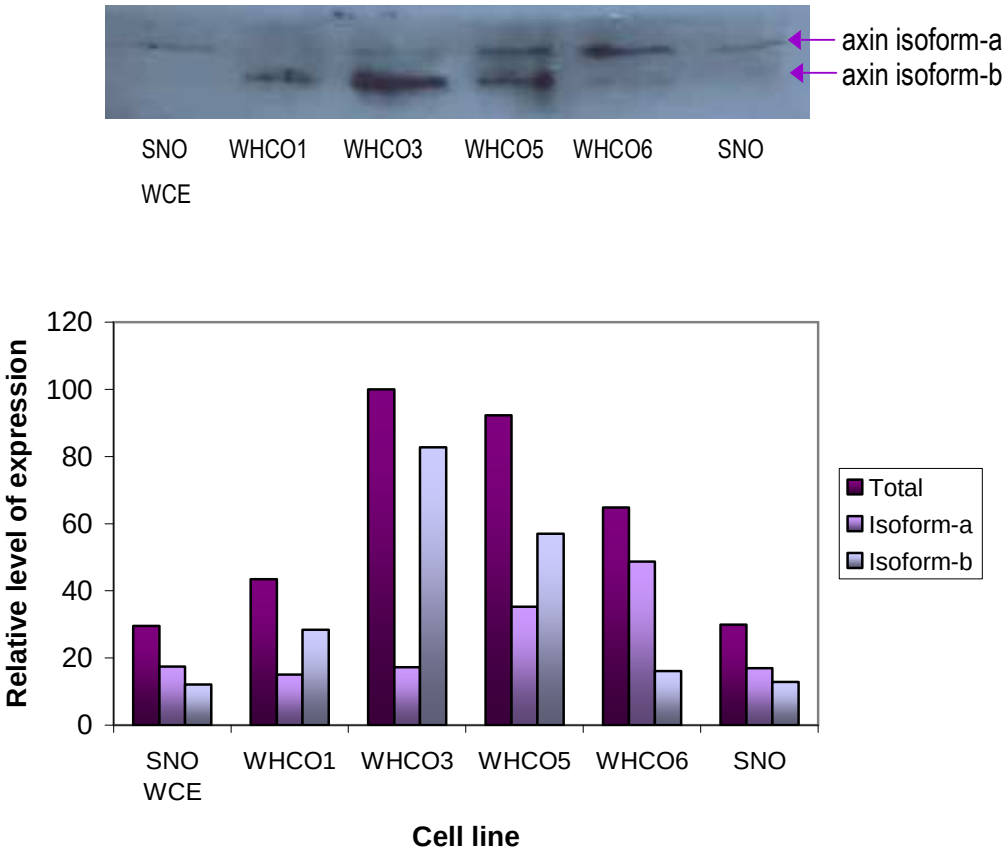


Figure 2.9. Axin is located at the plasma membrane of HOSCC cells. Using axin-specific western immunoblotting, it was observed that both isoforms of axin localize to the plasma membrane in HOSCC cell lines WHCO1, WHCO3, WHCO5, WHCO6 and SNO. A whole cell lysate (WCE) was included as a positive control.

A.

B.

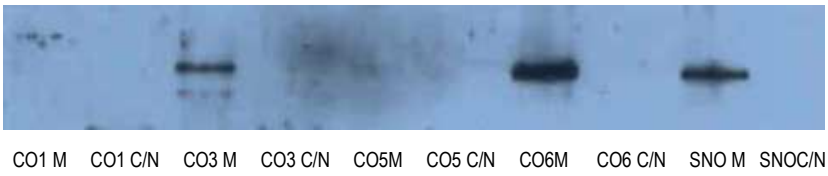


Figure 2.10. APC is not present at the plasma membrane in HOSCC cell lines (A) APC-specific immunoblot, where no bands indicating the presence of APC were obtained in plasma membrane-associated protein extracts, WHCO5 WCE = whole cell lysate positive control (B) E-cadherin control confirming the accuracy of the plasma membrane-associated protein extraction. Although a band was not produced for cell lines WHCO1 and WHCO5, it was confirmed by a western immunoblot on whole cell lysates with the same E-cadherin-specific antibody that these two cell lines do indeed express a lower level of E-cadherin overall when compared to the other cell lines (Figure 2.11). Absence of bands indicates that the proteins were not expressed at high enough levels to be detected by western immunoblotting.
*Note CO1 M = WHCO1 membrane fraction, CO1 C/N = WHCO1 cytoplasmic/nuclear fraction etc.

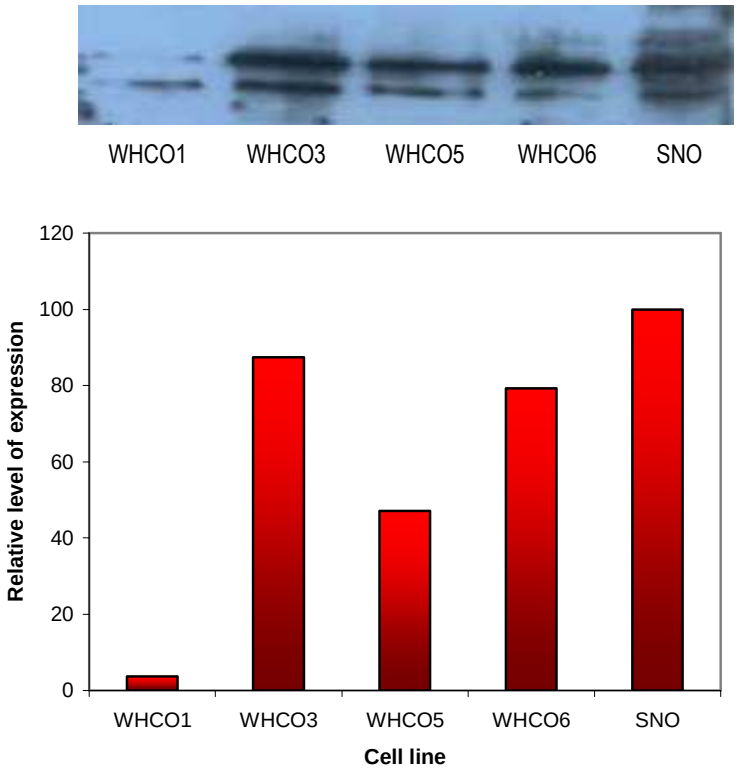


Figure 2.11. E-cadherin expression in HOSCC cell lines. Whole cell lysates were electrophoresed on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Western immunoblotting with anti-E-cadherin, followed by densitometric analysis revealed the presence of E-cadherin in varying levels across the five HOSCC cell lines.

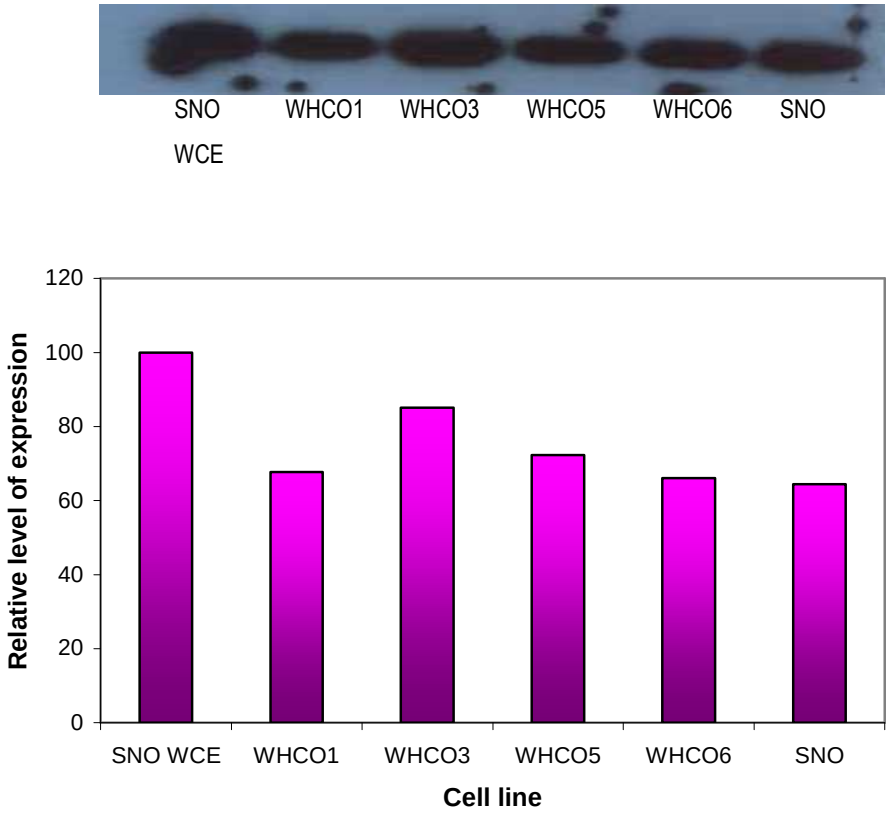


Figure 2.12. β-catenin is expressed in the nucleus of HOSCC cell lines. A β-catenin-specific western immunoblot performed on nuclear extracts confirmed its presence in the nucleus. A whole cell lysate (WCE) was loaded onto the gel as a positive control.

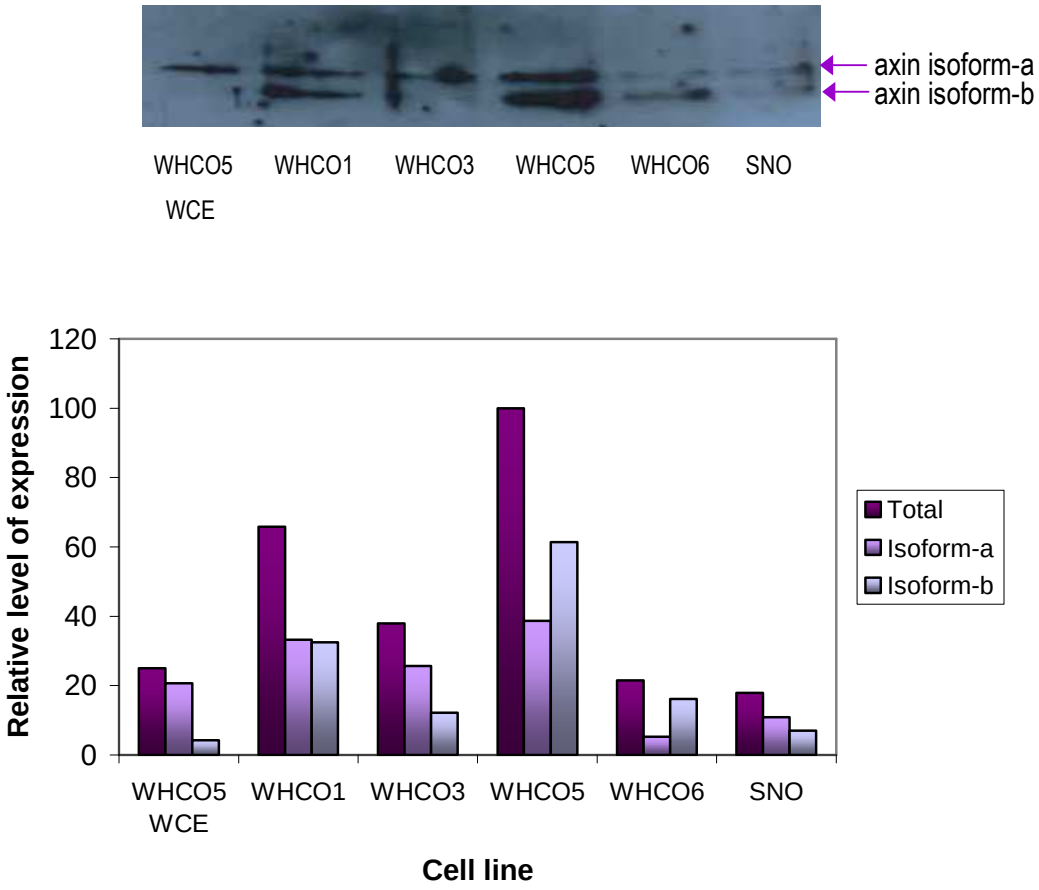


Figure 2.13. Confirmation of the presence of axin in the nucleus. Axin-specific immunoblotting performed on nuclear lysates showed the presence of both isoforms within the nucleus. A whole cell lysate (WCE) was loaded onto the gel to act as a positive control.

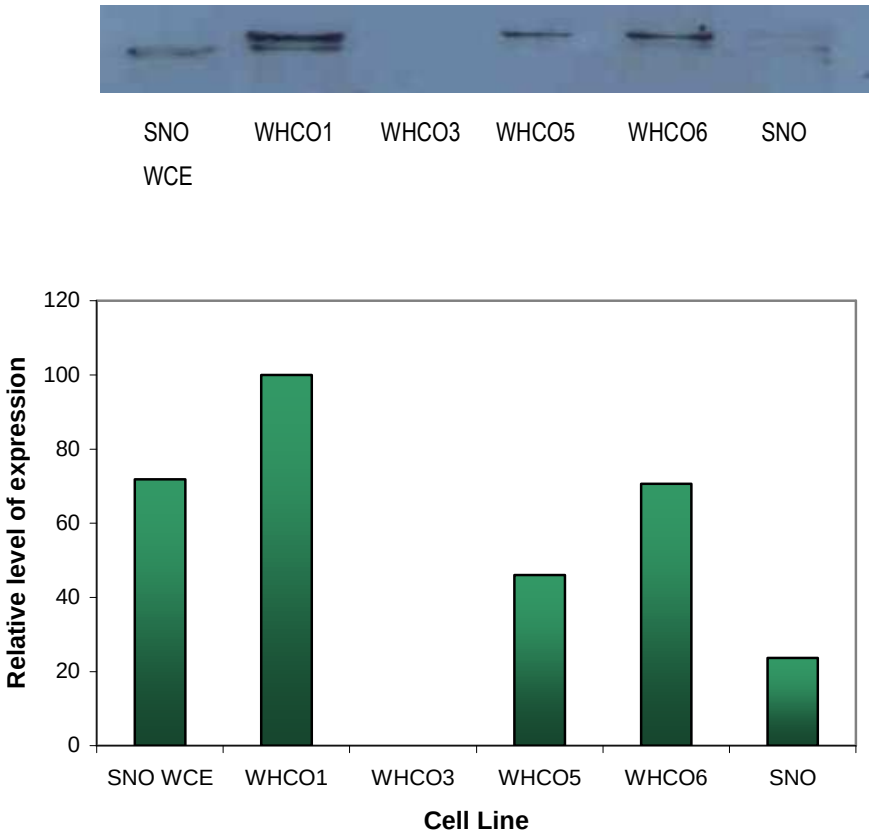


Figure 2.14. APC localizes to the nucleus in most HOSCC cell lines. APC-specific western immunoblotting performed on nuclear protein extracts confirmed the presence of APC in the nucleus. A whole cell lysate (WCE) was loaded onto the gel as a positive control.

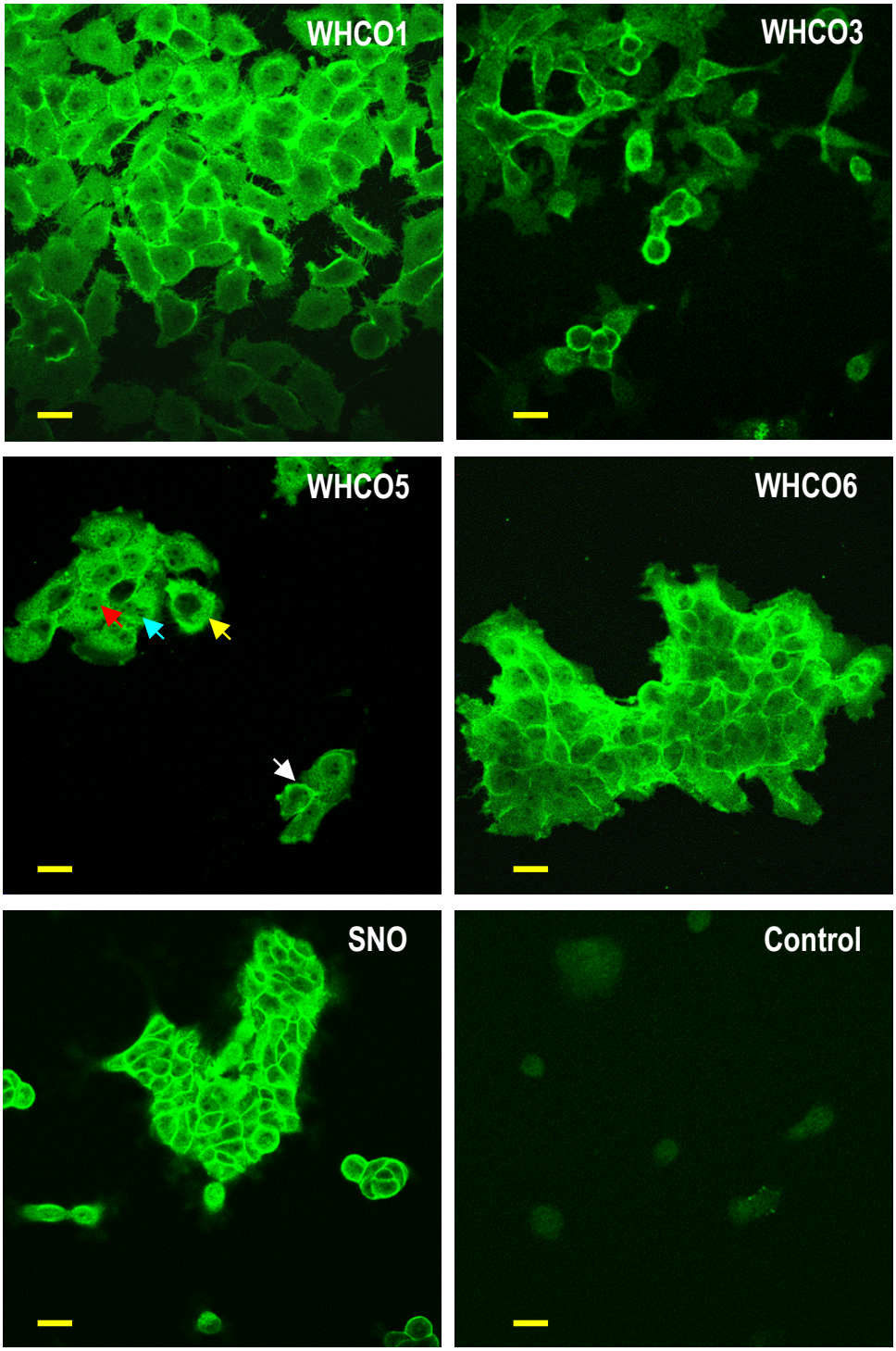


Figure 2.15. Subcellular localization of β-catenin within HOSCC cell lines. β-catenin was shown to be localized to the nucleus (red arrow), cytoplasm (yellow arrow) and plasma membrane (white arrow) within each of the five cell lines, but was excluded from the nucleolus, particularly in WHCO5 (turquoise arrow). Yellow bar = 25μm

2.3.3. β-catenin interacts with APC in HOSCC cell lines

Although it was demonstrated (above) that these proteins are expressed in the five cell lines and are able to localize to the same subcellular compartments, it needed to be ascertained whether an interaction between β-catenin and APC did indeed occur in HOSCC. Using anti-APC antibody, co-immunoprecipitation was performed on WHCO1 and WHCO6 cell lines. These lines were specifically chosen as representatives since APC was seen to be more highly expressed in the nuclear fractions of these two lines. WHCO1 and WHCO6 APC immunoprecipitates were electrophoresed and subjected to western immunoblotting, confirming interactions between β-catenin and APC within these cell lines (Figure 2.16, pink arrow).

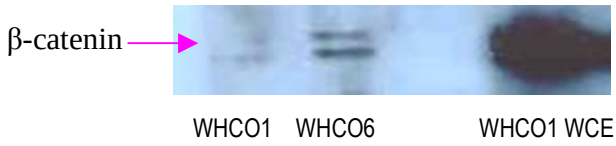


Figure 2.16. β-catenin and APC interact in HOSCC cell lines. The separation of WHCO1 and WHCO6 APC immunoprecipitates on a 10% SDS-PAGE gel, followed by western immunoblotting with anti-β-catenin revealed a band at approximately 92 kDa, thereby confirming the interaction of β-catenin with APC.

2.4. Discussion

Deregulated β-catenin turnover is a common event in tumour development and progression (Satoh *et al*., 2000; Taniguchi *et al*., 2002; Zeng *et al*., 2006). Since axin and APC proteins are key players in the regulation of β-catenin turnover and because it is known that β-catenin is able to signal aberrantly in the presence of altered/mutated axin or APC, the central focus of this component of the study was to detect APC, axin and β-catenin in these HOSCC cell lines, identify their subcellular distribution patterns and detect interactions between APC and β-catenin.

A 92 kDa polypeptide was obtained for β-catenin – a result consistent with those published in the literature (Alami *et al*., 2003). On occasion, a lower molecular weight β-catenin degradation product, reportedly produced by caspase-3 or -7 cleavage (Brancolini *et al*., 1997; Steinhusen *et al*., 2000; Ling *et al*., 2001), was observed in some of the cell lines. Overall, β-catenin displayed a varied level of expression across the different cell lines. With regard to the plasma membrane-associated protein extraction, β-catenin showed higher levels of expression in WHCO1 and WHCO5 than other cell lines, even though, overall, the lines with the highest levels of expression were WHCO3 and WHCO6. Given the context in which β-catenin finds itself at the plasma membrane – as an integral component of the cell-cell adherens junctions (Aberle *et al*., 1994) – this observation can possibly be explained by virtue of the fact that other proteins within the adherens junctions (e.g. E-cadherin) may well be recruiting β-catenin with a higher affinity in these two cell lines than in the others. However, analysis of the levels of E-cadherin expression at the plasma membrane in itself yielded interesting results as it appears that WHCO1 and WHCO5 express the lowest levels of E-cadherin overall (Figure 2.16). It, therefore, appears that another component of the adherens junctions e.g. α-catenin (Aberle *et al*., 1994), must be responsible for the high level of recruitment of β-catenin to the plasma membrane.

Contrary to the observed pattern of expression in plasma membrane-associated protein extracts, β-catenin expression in the nucleus appears relatively uniform across all cell lines, with a slightly higher level observed in WHCO3. Because of the great differences observed in overall expression, as well as plasma membrane-associated expression, the ability of the cells to degrade β-catenin must still be intact for there to be such uniformity in the nuclear expression levels. Therefore there is potentially a variation in the efficiency by which the different cell lines target β-catenin for ubiquitination and subsequent degradation.

Axin exists within the cells as one of two isoforms, isoform-a and isoform-b, which are produced by alternative splicing of the mRNA transcript. Isoform-a is the protein derived from the entire Axin1 transcript, whereas isoform-b lacks the polypeptide fragment produced by exon 8 (which is spliced out) (reviewed in Salahshor and Woodgett, 2005). Although these splice variants are somewhat different, many of the functions are still conserved across both isoforms, as both are able to bind β-catenin, APC and GSK3β. A putative PP2A binding domain exists within a portion of exon 8 and this may lead to a divergence of function between the two isoforms (reviewed in Salahshor and Woodgett, 2005).

The molecular weights obtained for axin isoform-a (~130 kDa) and isoform-b (~110 kDa), as well as APC (~310 kDa) correspond to those published previously (Tominaga *et al*., 1998; Salahshor and Woodgett, 2005). From the analysis of the whole cell lysate western immunoblots of axin and APC it appears that there is a similarity in their expression patterns, at least in terms of APC and axin isoform-a. The binding between the two in the scaffolding complex occurs at a ratio of 1:1 (Spink *et al*., 2000; Lee *et al*., 2003) and hence the similarity in expression levels observed is not entirely surprising, although there is not as marked a difference in axin as in APC. Isoform-b of axin was shown to have fairly uniform expression levels (max of ~6% difference) across the cell lines. The varying levels of alternative splice variants hints

that the rate at which exon 8 is spliced out of certain axin molecules must be strictly regulated, producing approximately equal levels of isoform-b across all cell lines. However, if this is the model by which production of the splice variants occurs, overall production of the axin transcript must vary across all the lines in order to obtain the differences observed in axin isoform-a expression levels.

APC was expressed in the nucleus at the highest level in WHCO1 and WHCO6 cell lines, whereas little, if any, could be detected in fractions of equal protein concentration from SNO and WHCO3. Immunoprecipitation of non-reducing whole cell homogenates of WHCO1 and WHCO6 indicate that the observed interaction between β-catenin and APC could simply be as a result of APC binding β-catenin within the nucleus, independent of axin. Such evidence initially seems to indicate this and the additional observation that β-catenin levels in the nucleus are also relatively high seems to substantiate this. But WHCO1 expressed ~30% more APC in the nucleus than WHCO6. Thus, the predominant interaction cannot be nuclear because if WHCO1 displayed the highest level of nuclear expression for APC and the levels of β-catenin were very similar for both WHCO1 and WHCO6 overall, then this association would surely have been shown to occur at a notably higher level within WHCO1 than in WHCO6. However, if anything, WHCO6 appeared to have a higher level of association between the two proteins than WHCO1. Thus it can be speculated that because it follows the same pattern as is shown for the APC whole cell lysates, this interaction occurs predominantly within the cytoplasm rather than in the nucleus. More importantly, APC binding of β-catenin in the cytoplasm (in the absence of axin) is only a transient interaction and so it is highly likely that this interaction is found within the context of the β-catenin degradation-targeting complex. Bearing in mind that this particular method of detection allows only a “snap shot” of the interactions to be taken, in order to more fully confirm this interaction, titration assays would have to be performed over a given period of time, such that the complete picture may be seen.

Furthermore, these results indicate that the predominant interaction of β-catenin in the nucleus is not with APC. Since β-catenin is known to associate with Tcf to form an active transcriptional activator, it is therefore possible that the predominant interaction occurring in the nucleus is not β-catenin/APC, but rather, β-catenin/Tcf, since binding of APC and Tcf by β-catenin are mutually exclusive events (Neufeld *et al*., 2000; Hamada and Bienz, 2004).

The links between β-catenin, APC and axin, and carcinogenesis have been well established over the years (Taniguchi *et al*., 2002; Salahshor and Woodgett, 2005; Zeng *et al*., 2006). Alterations to any of these three proteins are characteristic of many cancers. Large deletions in the *AXIN1* gene have been shown to occur in medulloblastomas (Dahmen *et al*., 2001), however, since the correctly-sized doublet band was obtained for axin in all five HOSCC cell lines in this study, this cannot be the case as a large deletion in the gene would lead to an equally large deletion in the protein.

It has been well established that the alterations observed in APC due to mutations are associated with colorectal tumorigenesis (Nishisho *et al*., 1991; Powell *et al*., 1992; Kawasaki *et al*., 2003). Reports have shown that truncating mutations in APC render it unable to bind β-catenin and target it for degradation (Sadot *et al*., 2002), but obviously this is not the case when looking at HOSCC, as APC's ability to bind β-catenin was clearly demonstrated by co-immunoprecipitation experiments. In the same way, it can be assumed that the APC-binding domain of β-catenin must be intact and functional in HOSCC.

Together these data present the intriguing possibility that the functional capacity of the β-catenin degradation-targeting complex remains intact, despite the obvious ability of β-catenin to translocate to the nucleus. Previous studies on β-catenin, APC and axin in

oesophageal squamous cell carcinoma (Powell *et al*., 1994; Ninomiya *et al*., 2000; Nakajima *et al*., 2003), failed to demonstrate any alterations in axin, β-catenin exon 3 or the mutational cluster region (MCR) of APC. However, Nakajima *et al.* (2003) correlated reduced axin expression with tumour progression. In the light of their results and the results obtained in the current study, it can be speculated that although axin and APC appear to be expressed at reasonable levels overall, it may be that in the case of axin, where the relatively high levels observed at the plasma membrane are most likely due to sequestration upon Wnt signalling, a decreased amount of cytoplasmic axin would be available for interactions within the degradation-targeting complex. The next chapter is aimed at elucidating if this is indeed the case within the context of oesophageal squamous cell carcinoma.

CHAPTER 3: AXIN, LRP5 AND THE EFFECT OF EFGR STIMULATION IN HOSCC CELLS

3.1. Introduction

3.1.1. Low density lipoprotein receptor-related proteins (LRPs)

The LRP family consists of eight single-pass transmembrane receptor proteins – LRP1-6, LRP8 and LRP10. Although each member is able to bind numerous different ligands and, therefore, initiate several different cellular processes, all LRPs display three conserved structural elements (Howell and Herz, 2001; Strickland and Raganathan, 2003). All LRPs are comprised of an extracellular LDLR ligand-binding domain, a single transmembrane-spanning unit and a cytoplasmic tail. Typically, members of this family of proteins are able to perform endocytic functions within the cell and, because of their ability to bind and endocytose a vast number of different ligands, they are responsible for diverse biological functions e.g. lipid metabolism, homeostasis of proteases and their inhibitors and activation of lysosomal enzymes (Howell and Herz, 2001; Strickland and Raganathan, 2003). Although this family of proteins was discovered because of its endocytic function and structural similarity to the LDLR family, there remain two nonendocytic LRPs with high identity – LRP5 and LRP6 (Brown *et al*., 1998). In recent years (reviewed in Grey *et al*., 2004; Hay *et al*., 2005) it has been established that LRPs may also play a role in signal transduction that is separable from their endocytic function. Intrinsic to the current study is the ability of LRP5 and LRP6 to transduce canonical Wnt signals.

3.1.2. LRP5

LRP5 (also known as LRP7) is a 179 kDa single transmembrane protein, which functions as a coreceptor for Wnt signalling (Hay *et al*., 2005) and shares 71% identity

with another member of the LRP family, LRP6 (Brown *et al*., 1998). Both proteins are able to form part of the Wnt coreceptor complex (Tamai *et al*., 2000; Tamai *et al*., 2004), binding Wnt and Frizzled proteins simultaneously, thereby allowing the signal to be transduced across the plasma membrane. Wnt1 and LRP5 have previously been shown to interact at the membrane surface to synergistically activate LEF-1-dependent transcription (Tamai *et al*., 2000; Mao *et al*., 2001) – an interaction that is inhibited by the high-affinity ligand dickkopf-1 (Dkk1) (Semënov *et al*., 2001).

LRP5 and LRP6 both consist of an intracellular domain with several proline-proline-proline-serine-proline (PPPSP) motifs (Figure 3.1), some of which contain phosphorylation sites important for the recruitment of axin (Davidson *et al*., 2005). The extracellular domain consists of four YWTD repeats, four reiterated EGF-like domains and three LDLR type A domains (Figure 3.1). This composition of the extracellular domain is crucial for its interactions with chaperone protein Mesd, inhibitor protein Dkk1, inhibitor/activator protein Dickkopf-2 and the canonical Wnts (Tamai *et al*., 2000; Brott and Sokol, 2002; Hsieh *et al*., 2003; Mao and Niehrs, 2003; Li *et al*., 2005).

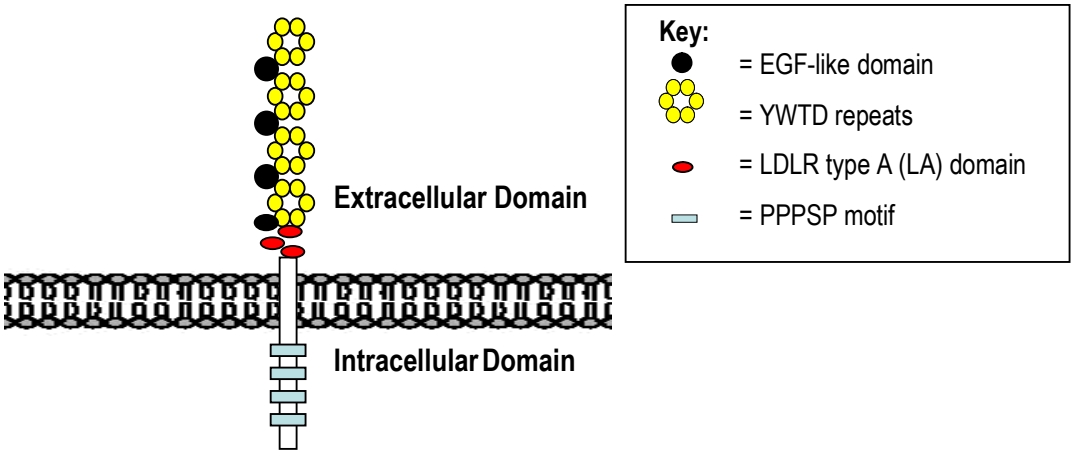


Figure 3.1. Schematic representation of the LRP5/6 proteins and their functional domains. Comprised of numerous different components, these transmembrane proteins are able to bind a plethora of different molecules, allowing either activation or inhibition of canonical Wnt signals (modified from He *et al*., 2004).

3.1.3. Recruitment of axin to LRP5 upon Wnt signalling

As mentioned previously (Section 1.5), LRP5 may recruit axin to the plasma membrane to enhance β-catenin signalling. This interaction has been shown to be related to Wnt signalling, as treatment of cells with Wnt3a led to an increase in the levels of axin at the plasma membrane (Mao *et al*., 2001). Thus it is probable that, upon Wnt signalling, axin is not only dissociated from the degradation-targeting complex, but also is subsequently translocated to the plasma membrane in order to associate with LRP5/6. It has been proposed by Cliffe *et al*. (2003), that this translocation event is mediated by Dvl. Mao *et al*. (2001) showed that the interaction between axin and LRP5/6 is dependent on phosphorylation of the cytoplasmic domain of LRP5/6 at multiple sites. More specifically, the interaction between axin and LRP5 has been localized to the five reiterated PPPSP motifs within the cytoplasmic tail of LRP5. It has been demonstrated that in order for axin to be recruited to this cytoplasmic tail of LRP5, obligatory phosphorylation of two sites present in all PPPSP motifs, site I (within the PPPSP motif) and site II (3 amino acids downstream from PPPSP), is required. These sites have been shown to be specifically phosphorylated by a dual kinase system – GSK3β is responsible for phosphorylation of site I, and CKIγ (a membrane-bound member of the CKI family) phosphorylates site II (Zeng *et al*., 2005). CKIγ has also been shown to be necessary for the phosphorylation of two additional sites termed clusters I and II, which are unrelated to the PPPSP motifs, but have been reported to be equally necessary for axin recruitment upon Wnt signalling (Davidson *et al*., 2005).

Moreover, it has been shown previously that upon EGFR signalling, the kinase activity of GSK3β is inhibited by the activation of a specific inhibitor, $p90^{RSK}$, via MAPK signalling (Eldar-Finkelman *et al*., 1995). Since GSK3β plays a significant role in LRP5 phosphorylation and, therefore, recruitment of axin to the plasma membrane upon Wnt signalling, it is possible that the EGFR signalling cascade may indeed act as a negative feedback loop in the canonical Wnt signalling pathway.

3.1.4. LRP5 in disease

Although LRP5 is known to be expressed in multiple different tissue types including the heart, liver, pancreas, small intestine, ovaries, thyroid, adrenal gland, colon, trachea, eye, and skeleton (Kim *et al*., 1998), it is interesting to note that the phenotypic consequences of its mutations appear to affect very few tissues (Gong *et al*., 2001; Hoang *et al*., 2003). It has been proposed that the reason for this observation is that in most cell lines, there is a likely functional overlap in the Wnt signalling pathway between LRP5 and LRP6 (Pinson *et al*., 2000). There is a great similarity in function between members of the LRP family. Thus any dramatic mutations in most cell/tissue types may well be masked by the overlap in function observed between members of the LRP family.

Within the skeletal context, LRP5 mutations typically result in one of two extreme outcomes. Loss of function mutations result in low bone mass diseases and are associated with autosomal recessive osteoporosis-pseudoglioma syndrome (Gong *et al*., 2001). These mutations usually result in the production of premature stop codons or frameshift mutations that lead to a lack of production of full length LRP5.

The second class of mutations characteristic of bone disorders is that of missense mutations. These mutations lead to diseases characterized by increased bone density, including endosteal hyperostosis, Van Buchem disease, autosomal dominant osteosclerosis and osteopetrosis type I (Boyden *et al*., 2002; Little *et al*., 2002; Van Wesenbeeck *et al*., 2003; Zhang *et al*., 2004). In particular, the G171V mutation, located in the first YWTD repeat of the extracellular domain, has been shown to act by diminishing LRP5's capacity to bind to chaperone protein mesoderm development (Mesd), therefore, decreasing the number of LRP5 receptors at the plasma membrane. It has been postulated, however, that in the presence of autocrine Wnt stimulation and paracrine Dkk1 production, these cells would still be able to maintain Wnt signalling

(Zhang *et al*., 2004). Thus it can be seen that LRP5 functioning is critical in the development and maintenance of skeletal elements.

3.1.5. Epidermal growth factor receptor (EGFR) signalling

There has been no link established between LRP5/6 and EGFR signalling as yet. However, due to the nature of the Wnt signals transduced by LRP5/6 (which lead to increased cellular proliferation, amongst other things) (Siegfried and Perrimon, 1994), it is highly likely that there should be some degree of cross-talk since EGFR overexpression renders cells more sensitive to mitogenic factors. Thus it became a necessary component of this study to examine the effects of EGFR signalling on LRP5 and axin, and the close association of these two proteins at the plasma membrane.

EGF is a small extracellular signalling protein of 53 amino acids that is able to stimulate a multitude of different processes within the cell(s), including proliferation (Andl *et al*., 2003; Rescan *et al*., 2005), morphological differentiation (Hillborn *et al*., 1997), cell motility (Chen *et al*., 1994; Andl *et al*., 2003). It does this via the specific interaction with EGFR, a member of the class I receptor tyrosine kinases (RTKs) which also includes ErbB2, ErbB3 and ErbB4 (Coussens *et al*., 1985; Kraus *et al*., 1989; Plowman *et al*., 1993). Typically, the members of this class of RTKs are characterized by the presence of 2 cysteine-rich repeat sequences in the extracellular domain of the monomers (Ullrich and Schlessinger, 1990). Because of its ability to not only form homodimers, but also heterodimers upon ligand binding, EGFR stimulates a vast number of signalling pathways within the cell. These cellular responses include activation of the Erk and JNK pathways, as well as phospholipase C and, therefore, turnover of phosphatidylinositol (Meisenhelder *et al*., 1989; Koch *et al*., 1991), β-catenin tyrosine phosphorylation (Hoschuetzky, *et al*., 1994) and, therefore, disassembly of adherens junctions, potentially leading to cytoplasmic accumulation of β-catenin. Since this “cross-talk” between the EGFR and Wnt signalling pathways

exists, it seems imperative to analyze the effect of EGFR signalling on LRP5 recruitment of axin to the plasma membrane, in order to determine the full extent of the effect of EGFR signalling on members of the canonical Wnt signal transduction pathway.

3.1.6. EGF receptor recycling and degradation

Upon EGF-stimulation of cells expressing EGFR, receptors clustered in clathrin-coated pits are rapidly internalized and targeted either for degradation or recycling (reviewed in Holbrook *et al*., 1999). This occurs via a multistep pathway beginning with the maturation of the clathrin-coated pits into clathrin-coated vesicles. Thereafter, these vesicles fuse with the early endosome (reviewed in Alwan *et al*., 2003). At this point, receptors are sorted into free and ligand-bound groups. Free receptors diffuse into the tubular endosomal compartment (t), which breaks off to become a post-sorting recycling compartment (r), subsequently recycling them to the cell surface (Figure 3.2.a). In contrast, ligand-bound receptors are confined to the vesicular endosomal compartment (v) by selective retention by endosomal retention components (ERCs) (Figure 3.2). These early endosomes then mature into late endosomes (d) and fuse with pre-existing lysosomes, leading to lysosomal degradation of EGFRs (reviewed in Hendriks *et al*., 2003).

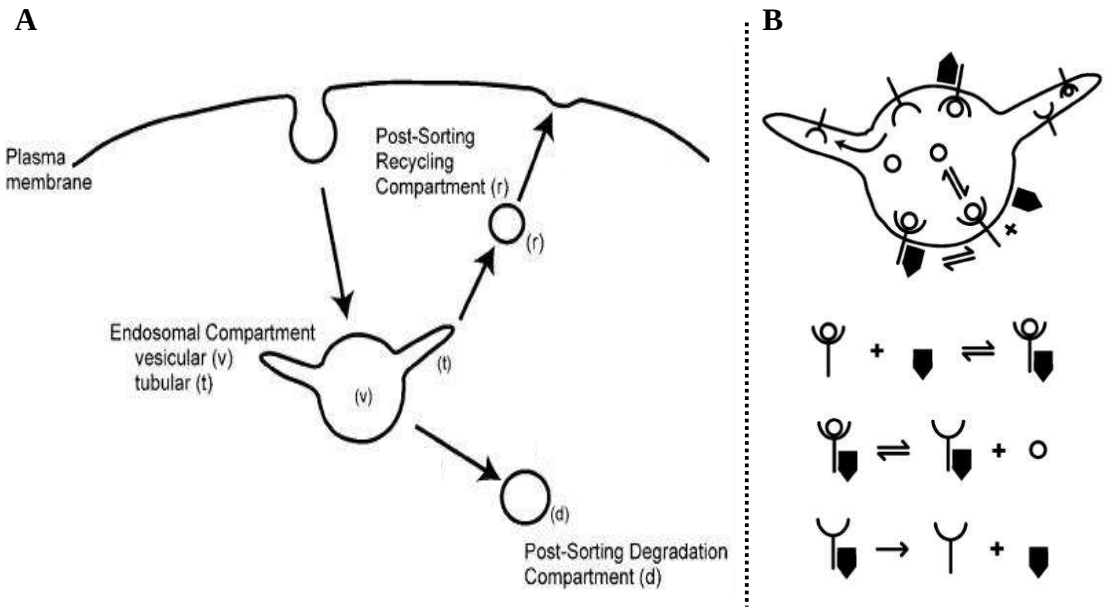


Figure 3.2. Endosomal sorting of EGF receptors. (A) A model proposed by French and Lauffenberger (1996): The EGFR-presenting cell contains four separable compartments: endosomal vesicle, endosomal tubule, recycling and degradation components. Upon receptor internalisation, receptors are either sorted into recycling compartments or are selectively retained in the central vesicle, ultimately leading to degradation. (B) Occupied EGFRs (represented by Y-shape) are specifically retained within the vesicular endosomal compartment by interaction with ERCs (solid pentagons). Free receptors are able to diffuse into the tubular compartment for recycling (adapted from Hendriks *et al*., 2003).

3.1.7. EGFR signalling and cancer

Since EGFR signalling is able to induce such a variety of cellular changes, it can easily be seen how even the smallest change to the receptor may be detrimental to cell function. Indeed, EGFR has been shown to be overexpressed in a number of human solid tumours including non-small cell lung carcinoma, metaplastic breast carcinomas, oral and oropharynx squamous cell carcinomas and oesophageal squamous cell carcinoma (Selvaggi *et al*., 2004; Reis-Filho *et al*., 2005; Hanawa *et al*., 2006; Salam *et al*., 2006; Laimer *et al*., 2007). More specifically, Veale and Thornley (1989)

reported the overexpression of EGFR receptors in the five HOSCC cell lines used in the current study. Thus, given the context of this study, it is essential to discover the extent of the effect of this overexpression on LRP5 and axin post EGF-treatment in the five oesophageal squamous carcinoma cell lines.

3.1.8. Objectives

To determine the presence and subcellular localization of LRP5 in WHCO1, WHCO3, WHCO5, WHCO6 and SNO cell lines.

To establish the response of axin and LRP5 to EGFR signalling, as well as to determine the ability of axin to localize to the plasma membrane with LRP5 post-EGF treatment.

3.2. Materials and methods

3.2.1. Tissue culture

Cells were cultured as described previously (Section 2.2.1). HEK293 cells (Graham *et al*., 1977) were kindly donated by Dr. Clem Penny (Medical School, University of the Witwatersrand) and used as a control cell population.

3.2.2. EGF treatment of cells

Cells were passaged to a confluency level of between 60% and 80%. Medium was aspirated and cells were rinsed with 1xPBS. Fresh medium with a total EGF concentration of 10ng/ml (Sigma) was added to the cells and they were incubated for a period of 30 minutes, 3 hours, 9 hours or 24 hours, followed by whole cell protein extraction (Section 2.2.2).

3.2.3. Whole cell lysate preparation

Whole cell lysates were prepared from cells as described in Section 2.2.2.

3.2.4. Plasma membrane-associated protein extraction

Following 3-hour EGF treatments on all five HOSCC cell lines, plasma membrane-associated protein extracts were performed as described in Section 2.2.4.

3.2.5. Protein estimation on extracted samples

Protein concentration of extracted samples was determined as mentioned previously (Section 2.2.6), with BSA in either single lysis buffer for whole cell lysates, or 0.1% SDS in 20 mM Tris-HCl (pH 7.4) for plasma membrane-associated protein extracts.

3.2.6. Sample preparation for SDS-PAGE and western immunoblotting

As described previously in Section 2.2.7, plasma membrane-associated protein extracts containing 250 μg protein were lyophilized and the pellets were resuspended in BME water, incubated in a boiling water bath for 5 minutes and stored at -70°C.

3.2.7. SDS-PAGE

Prepared samples were separated on 10% SDS-PAGE gels, the preparation and use of which may be found in Section 2.9.

3.2.8. Antibodies

All antibodies were diluted with 1xPBS unless otherwise stated.
Goat polyclonal anti-axin antibody (Santa Cruz: sc-8568)
Concentration for western immunoblotting = 1:400
Goat polyclonal anti-LRP5 antibody (Santa Cruz: sc-21389)
Concentration for western immunoblotting = 1:6000 in Blotto
Concentration for indirect immunofluorescence = 1:50
Rabbit polyclonal anti-actin antibody (Sigma: A2066)
Concentration for western immunoblotting = 1:2000
HRP-conjugated goat anti-rabbit antibody (Sigma)
Concentration for western immunoblotting = 1:7500

HRP-conjugated rabbit anti-goat (BioYeda)

Concentration for western immunoblotting with anti-axin = 1:20000

Concentration for western immunoblotting with anti-LRP5 = 1:13500

FITC-conjugated rabbit anti-goat (Sigma 045K4818)

(kindly donated by Dr. M. Ntwasa, Fly Lab, University of the Witwatersrand)

Concentration for indirect immunofluorescence = 1:1000

3.2.9. Western immunoblotting

Western immunoblotting was performed as described previously (Section 2.11), with antibodies and concentrations stated above.

3.2.10. Densitometric analysis of western immunoblots

Densitometric analysis was performed on western immunoblots using Labworks™ Image Acquisition and Analysis software (Labworks version 4.5). For more details, refer to Section 2.2.12.

3.2.11. Indirect immunofluorescence

Indirect immunofluorescence was performed as described previously (Section 2.2.13), with minor alterations. Cells were not subcultured on coverslips, but rather cultured on 10 cm dishes. Cells were then fixed onto the dish with paraformaldehyde (4%, 30 min), and a slide was cut from the bottom of the dish. Thereafter, the slide was rinsed 5 times with 1xPBS, followed by a further fixation step (10 min). Thereafter, the procedure was identical, except for the antibody used (Section 3.2.3).

3.3. Results

3.3.1. LRP5 is present in all HOSCC cell lines and localizes to the plasma membrane

It was deemed likely that LRP5 was present in both whole cell and plasma membrane-associated protein extracts because a band representing a polypeptide of the correct molecular weight was observed when these samples were separated by SDS-PAGE (Figure 3.3, blue arrows). A doublet band was obtained for LRP5 by western immunoblotting with an antibody specific for LRP5 (Figure 3.4). The lower and upper molecular weight bands were approximately 110 kDa and 130 kDa, respectively. When consulting the literature several discrepancies were noted concerning the actual molecular weight reported for LRP5 and, curiously, when Neth *et al*. (2006) used this same antibody, a band corresponding to a molecular weight of ~85 kDa was obtained. This will be discussed in more detail in Section 3.4.

In order to confirm that the molecular weights observed in this study were not simply due to a truncation peculiar to oesophageal squamous cell carcinoma, an additional control using HEK293 cells was included (Cong *et al.*, 2004) and the same banding pattern was observed (Figure 3.4.a). Thus it appears that LRP5 exists as two different variants.

The overall levels of expression of LRP5 were observed to be markedly different across all cell lines, with the highest overall level being expressed by WHCO5 and the lowest by WHCO3. Interestingly, the expression pattern of the two variants did not follow the same trend as that of the overall expression levels across the five oesophageal cell lines. The higher molecular weight band of LRP5 showed the highest level of expression in WHCO1 and the lowest in WHCO6, but this level of expression did not correlate with the pattern of expression observed for the lower molecular

weight band which showed its highest level of expression in WHCO6 and its lowest in WHCO3 (Figure 3.4.a). Thus it would appear that a molecule upstream of LRP5, which determines the levels of each variant produced, must also display different levels of activity across all HOSCC cell lines.

The levels of LRP5 expression at the plasma membrane were subsequently determined in order to ascertain whether this unexpected molecular mass would affect its localization to the plasma membrane (Figure 3.4.b). Densitometric analysis revealed that although the protein was expressed at different levels across the cell lines, there appeared to be a distinctly low level of LRP5 at the plasma membrane when compared with the whole cell control. In order to confirm this observation, indirect immunofluorescence was employed.

Indirect immunofluorescence revealed an unexpected perinuclear pattern of staining within the five HOSCC cell lines, since LRP5, being a single transmembrane protein, was considered likely to be found in substantial amounts at the plasma membrane. However, in contrast to the oesophageal cell lines, the HEK293 cells exhibited a strong plasma membrane-staining pattern (Figure 3.5). This data thus further confirms the observations in the western immunoblots that indicated a lower level of expression of LRP5 at the plasma membrane than overall in each oesophageal cell line.

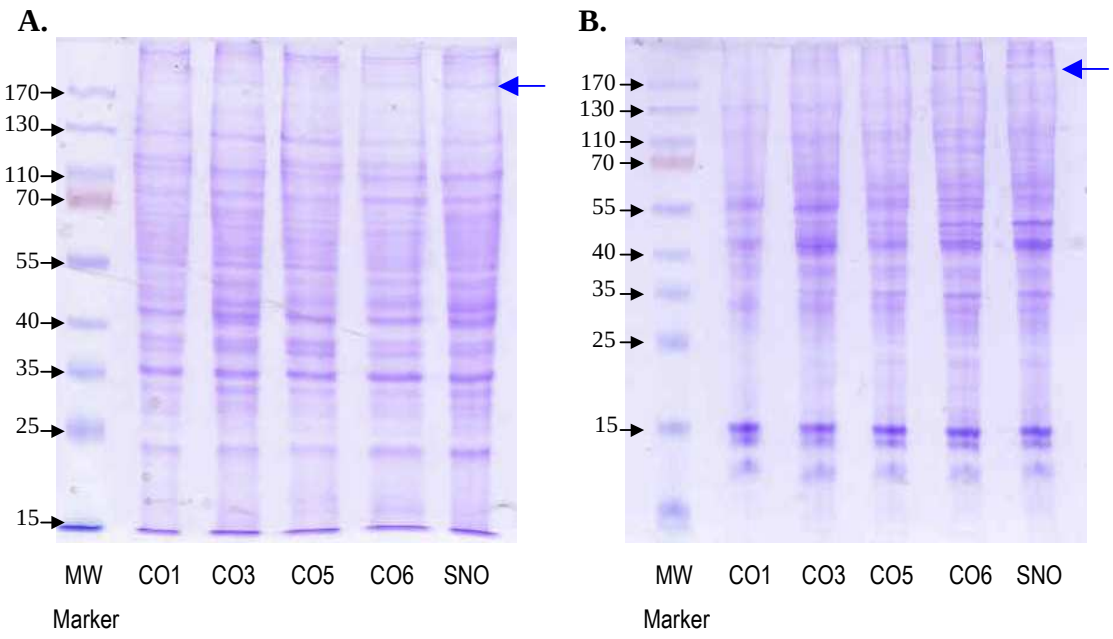
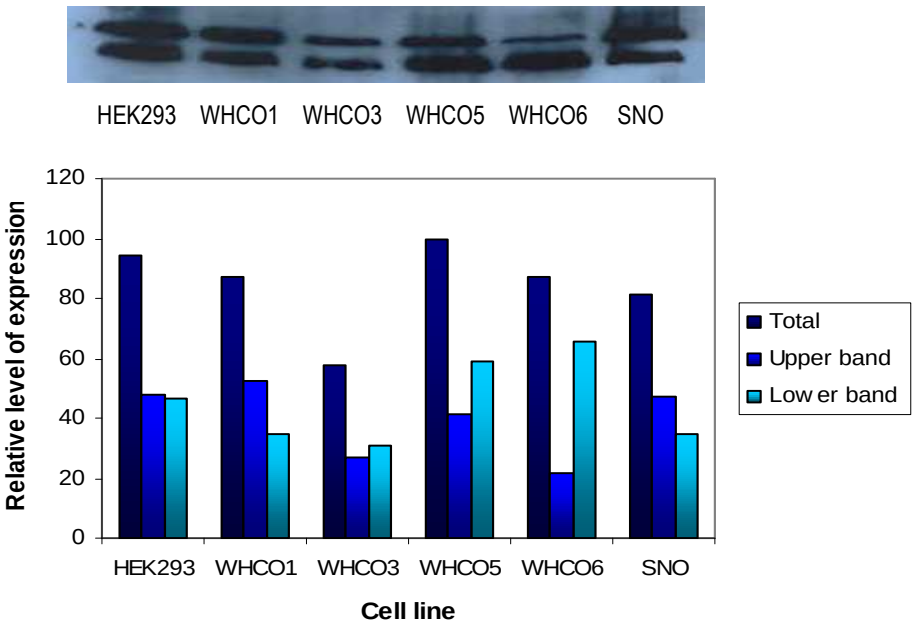


Figure 3.3. Separation of protein extracts by SDS-polyacrylamide gel electrophoresis (A) Whole cell lysates and (B) plasma membrane-associated protein extracts were separated on 10% reducing SDS-PAGE gels. Samples were prepared under reducing conditions in the presence of β-mercaptoethanol and equal amounts of protein were loaded across all lanes (~5 µg), indicated by a uniform level of staining observed.
*Note: CO1 = WHCO1, CO3 = WHCO3, CO5 = WHCO5, CO6 = WHCO6.

A.



B.

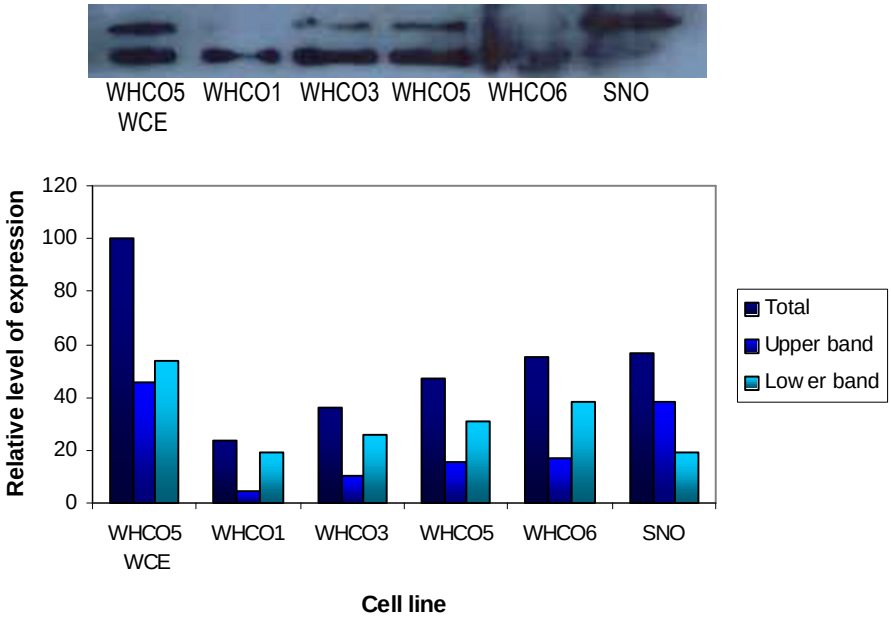


Figure 3.4. Presence and subcellular localization of LRP5. Western immunoblots and densitometry of (A) whole cell lysates and (B) plasma membrane-associated protein extracts revealed a doublet band corresponding to LRP5. The differences observed in expression can be attributed to each cell line expressing a unique level of LRP5.

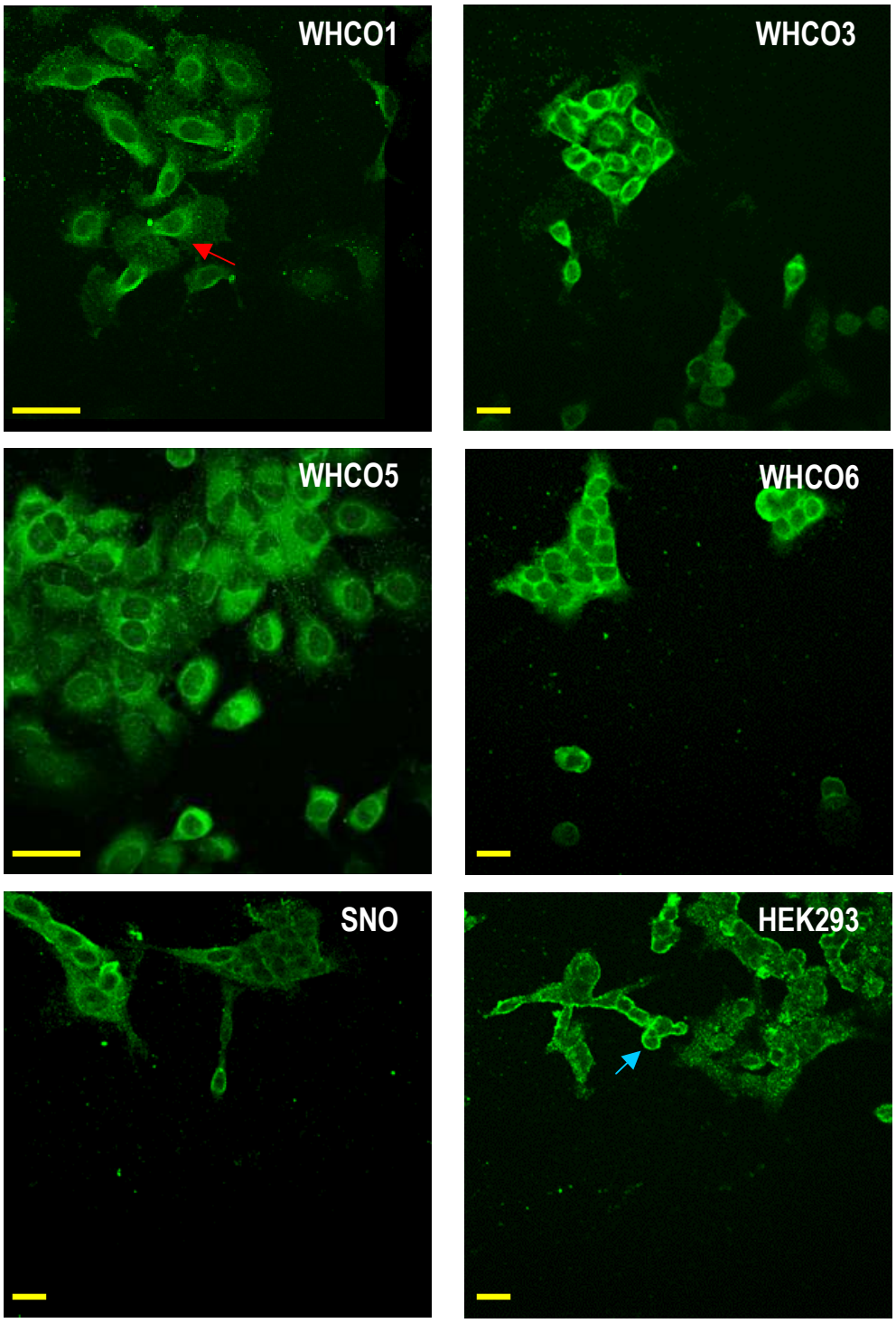


Figure 3.5. (Legend over page)

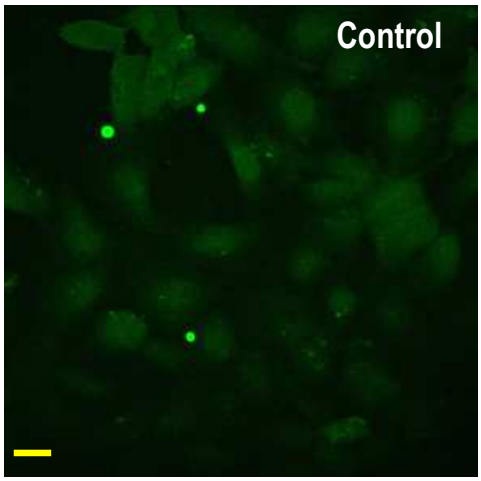


Figure 3.5. Confirmation of the subcellular localization of LRP5. The presence of LRP5 was confirmed within the five HOSCC cell lines, with a distinct perinuclear staining pattern (red arrow). HEK293 cells were used as a positive control and a plasma membrane-staining pattern was observed in distinct contrast to the HOSCC cell lines (blue arrow). A negative control with only FITC-anti-goat was included. Yellow bar = 25μm.

3.3.2. Response of LRP5 to EGF treatment

Specific responses of LRP5 and axin to EGFR signalling were determined by treatment of cells with 10 ng/ml EGF. Whole cell lysates were obtained over a period of 24 hours at specific intervals, separated by SDS-PAGE, and transferred to nitrocellulose for western immunoblotting with the specific antibody (Figure 3.6).

The overall effect of EGF was observed to vary greatly across cell lines (Figures 3.6, 3.7). A peak in expression of LRP5 was observed in all cell lines except for WHCO1, where a steady decrease in LRP5 levels occurred over time. WHCO3, WHCO5 and WHCO6 cell lines all demonstrated a peak in expression at 3 hours, while SNO peaked somewhat earlier at 30 minutes. WHCO3 and WHCO5 displayed the greatest change over time, with the peak in overall expression at 3 hours being nearly ~80% greater than the lowest level. All other cell lines by comparison exhibited an overall change of 49% - 56%. It should be noted that WHCO1, WHCO3 and SNO lines showed a marked decrease (45%, 70% and 12%, respectively) in expression levels between the untreated control and the 24-hour treatment. WHCO6 by comparison showed an increase of ~29%, but although WHCO5 showed an increase, it was only by 3%. Thus it appears that the response of LRP5 to EGF stimulation has a time component, however, this time component varies across the five HOSCC cell lines.

LRP5 western immunoblots and densitometric analysis also revealed greatly varied responses to EGFR signalling across all cell lines at a variant level (Figure 3.6) when compared to the untreated control (UTC). Although the overall trend has already been discussed, the change in expression of each variant over time often did not mirror those of the overall pattern. Though there was an observed peak in the WHCO6 line at 3 hours overall, the higher molecular weight variant displayed its highest level of expression at 30 minutes – an observation identical to the SNO cell line. Important to note at this stage is that each cell line appears to predominantly express one of the two

variants. In WHCO1, WHCO3 and WHCO5 cell lines high levels of the lower molecular weight variant and comparatively low levels of the higher molecular weight variant were observed. The difference between the upper and lower molecular weight variants in WHCO3, however, was not as substantial as in the other two cell lines. In direct contrast cell lines WHCO6 and SNO displayed a higher level of expression of the higher molecular weight variant. This diverse array of results will be further discussed in Section 3.4.

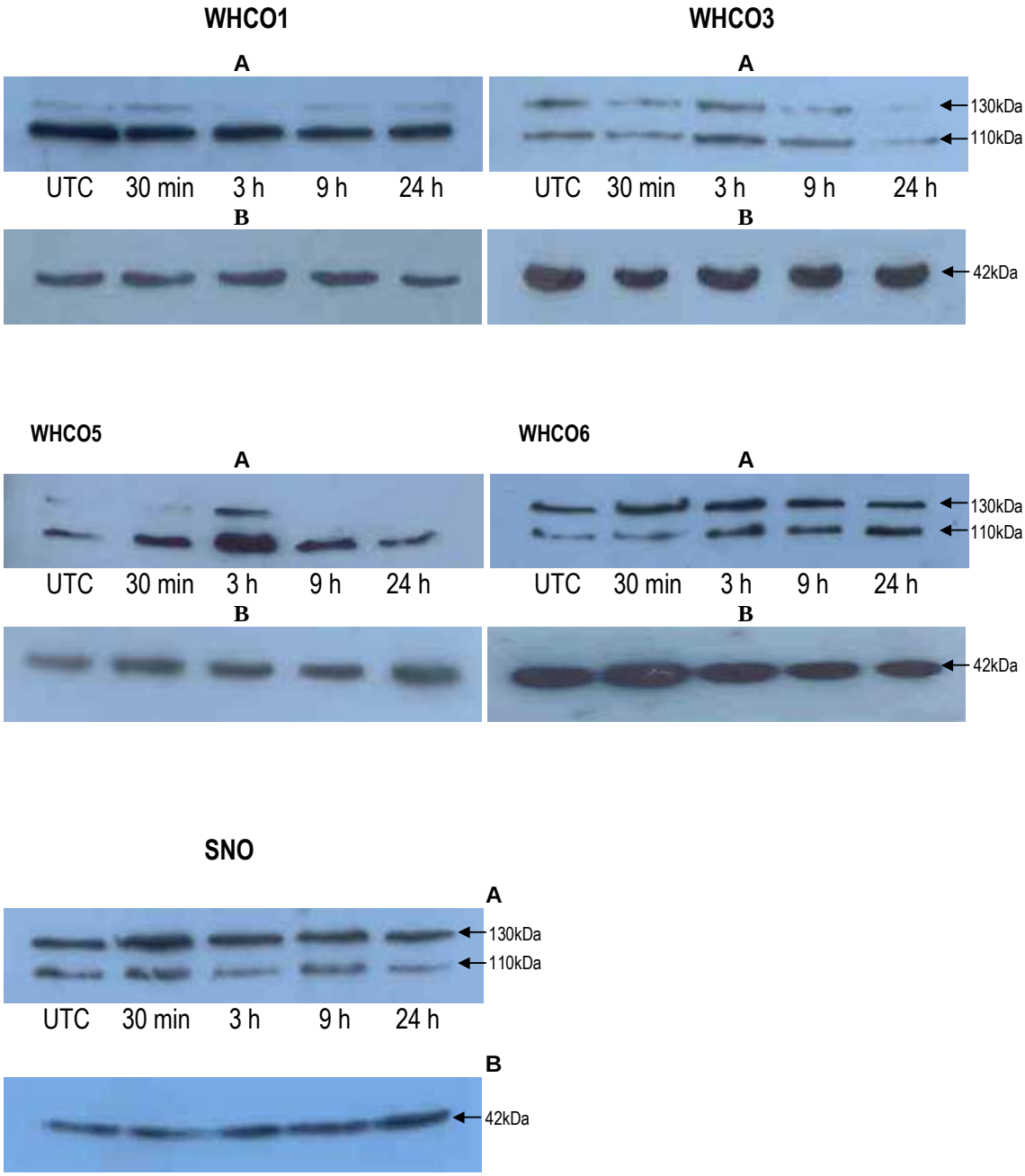


Figure 3.6. Response of LRP5 to EGF treatment in HOSCC cell lines. (A) LRP5-specific western immunoblots of EGF-treated samples confirmed the presence of both variants of LRP5, with a noticeable change in expression levels in response to EGFR signalling. (B) Actin-specific western immunoblots were used as internal loading controls, to confirm the uniform level of protein loading on the gels.

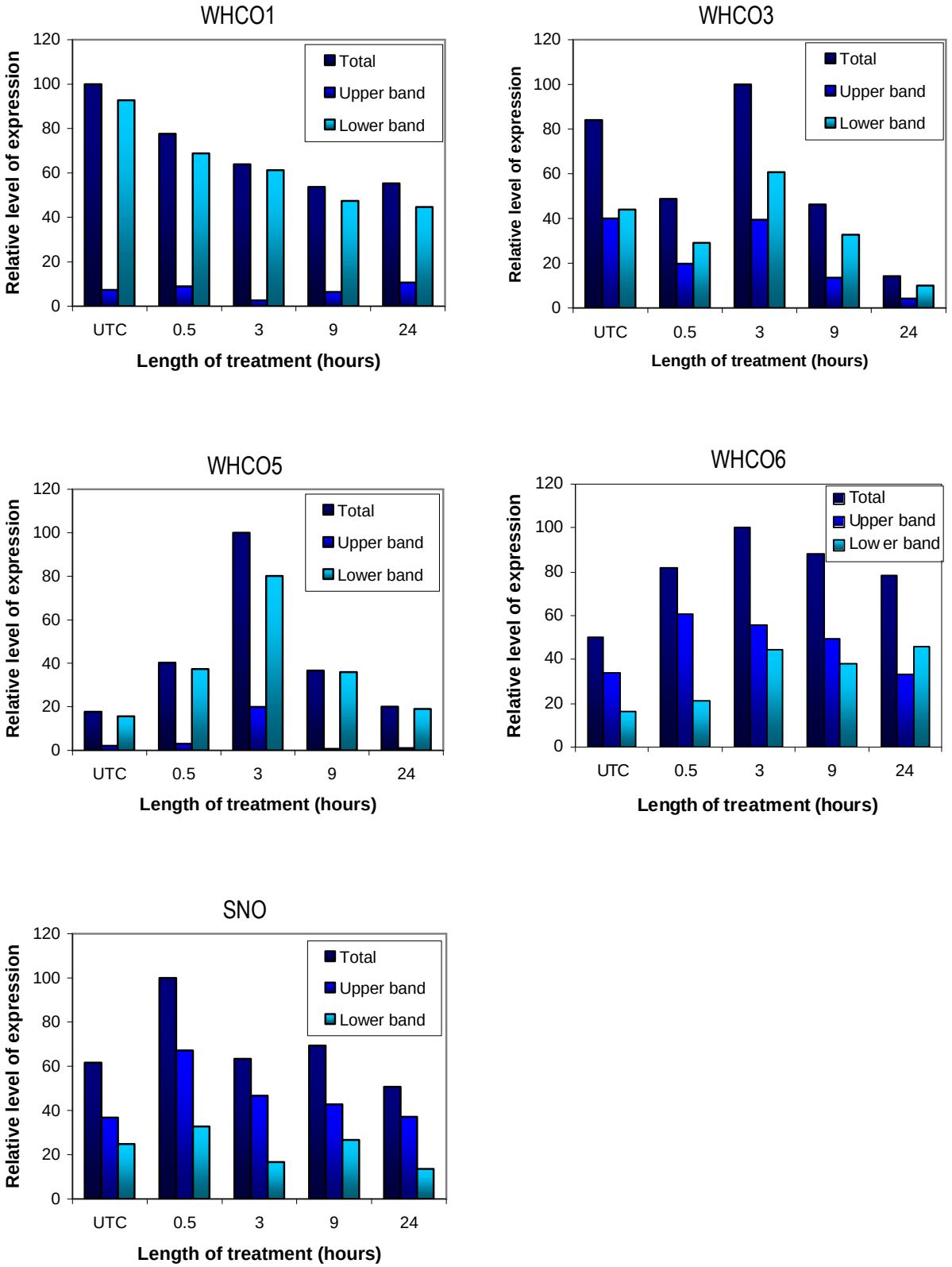


Figure 3.7. LRP5 response to EGF treatment of HOSCC cell lines. Densitometric analysis of western immunoblots obtained previously (Figure 3.6) revealed that LRP5 expression in response to EGFR signalling was reasonably varied across cell lines, with a distinct peak in overall expression at 3 hours in WHCO3, WHCO5 and WHCO6.

3.3.3. Response of axin to EGF treatment

Densitometric analysis of axin western immunoblots (Figure 3.8) for each cell line showed an axin response to EGFR signalling which varied greatly across all cell lines (Figure 3.9). Overall, EGF stimulation of the cells induced a peak in protein expression at some point within the 24-hour treatment period for all cell lines except for WHCO3, in which a steady decrease in axin levels was observed. All cell lines displayed a marked change in total axin expression over time (Figures 3.8, 3.9). WHCO1 and WHCO3 both showed a decrease in expression between the untreated control and the 24-hour treatment. This was contrasted by cell lines WHCO5, WHCO6 and SNO, in which rather substantial increases were observed over time (16%, 31% and 23%, respectively).

Isoform expression levels were also affected by EGF stimulation, though on occasion these patterns were different from that of the overall levels (Figures 3.8, 3.9). Where WHCO1 and SNO cell lines displayed a peak at 30 minutes, both isoforms followed the same expression pattern in SNO, with a slightly higher level of isoform-a being expressed than isoform-b. Although in the case of WHCO1, isoform-b peaked in the untreated control, isoform-a mirrored the overall expression pattern and, like in SNO, was expressed at a slightly higher level than isoform-b overall. A WHCO5 peak in expression in isoform-a occurred at 3 hours. Just as in the overall pattern, however, isoform-b peaked earlier at 30 minutes. Isoform-a in WHCO3 exhibited a peak at 3 hours, compared to isoform-b that did not peak, but rather declined over time. Curiously, WHCO6 showed very similar expression patterns overall as well as for each isoform. These results were very interesting, displaying a vast array of cellular responses across the five cell lines.

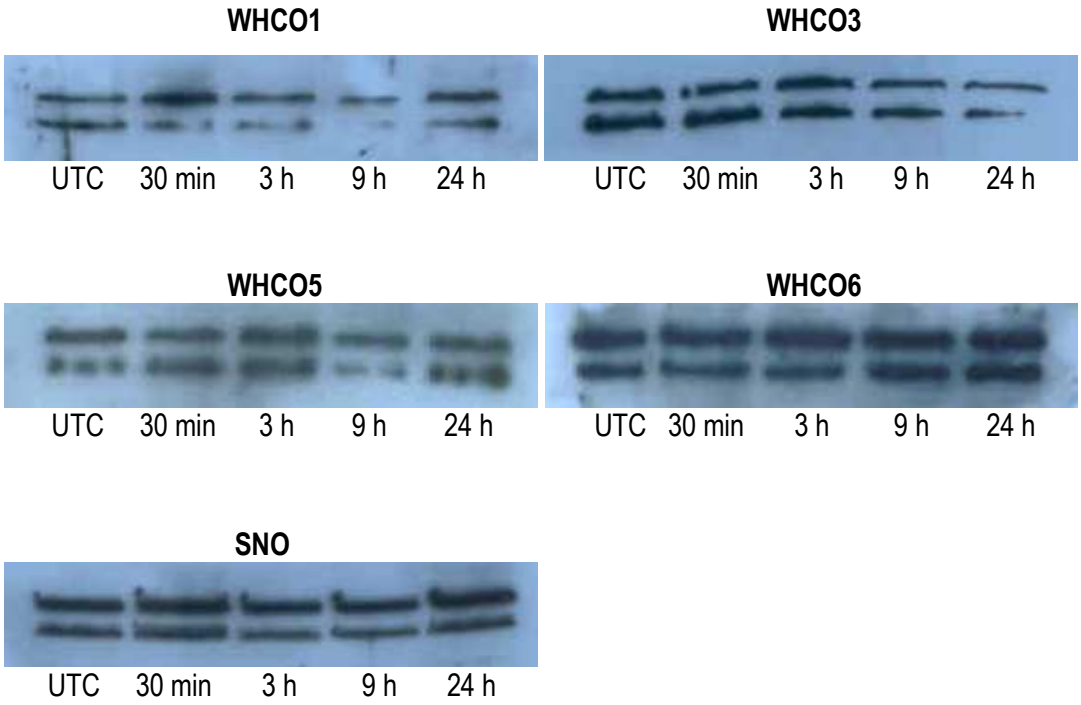


Figure 3.8. Response of axin to EGF treatment in HOSCC cell lines. Following treatment of cells at 30 min, 3 h, 9 h and 24 h, it appears that there is no definite trend in axin expression levels in response to EGFR signalling.

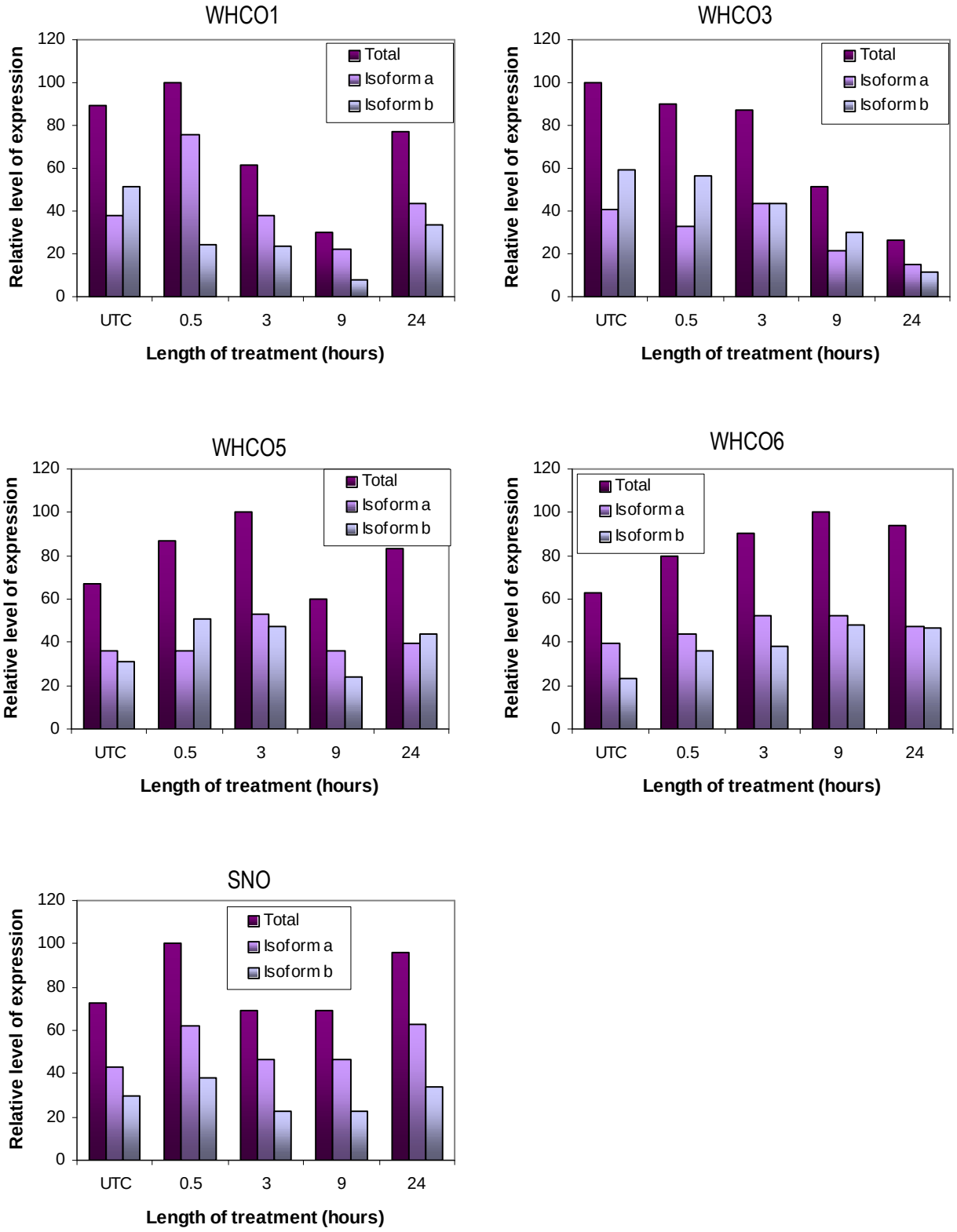


Figure 3.9. The response of axin to EGFR signalling. Densitometric analysis of previously obtained western immunoblots (Figure 3.8) revealed the varied responses of axin to EGF treatment across the five oesophageal carcinoma cell lines.

3.3.4. Plasma membrane-localization of axin in response to EGFR signalling

Since the overall trend in LRP5 response to EGF treatment was observed to have a peak at 3 hours in 60% of the lines observed (Figures 3.6, 3.7), it was decided that in order to determine whether EGFR signalling affects axin localization with LRP5 at the plasma membrane, this time period would be most appropriate for treatment. This is because at this time the effect of growth factor on LRP5 would be maximal and, therefore, the greatest impact on the interaction between the two proteins would be likely to be observed at this point. Because it is accepted that GSK3β activity is inhibited in response to EGF treatment (Eldar-Finkelman *et al*., 1995) and because axin's recruitment to LRP5 is dependent on GSK3β phosphorylation of LRP5 (Zeng *et al*., 2005), it was of interest to determine if EGFR signalling did indeed have an effect on axin localization to the plasma membrane.

Plasma membrane-associated protein extracts were analyzed by western immunoblotting and desitometric analysis (Figure 3.10), revealing a marked decrease (>10%) in isoform-b expression levels in WHCO3 and WHCO6 in response to EGF treatment. However, only WHCO6 showed a marked decrease in the level of isoform-a. WHCO5 did show a small change in expression, but since the changes were less than 10%, they were not considered a marked increase/decrease.

Unexpectedly, WHCO1 and SNO cell lines showed a marked increase in the levels of both isoforms of axin at the plasma membrane in response to EGFR signalling. With an increase of ~54% in isoform-a and an increase of ~56% in isoform-b observed in SNO, it appears that this association is either altered or greatly diminished in this cell line. Since axin is able to associate with other molecules at the plasma membrane - the TGFβ receptor I (TβR-I)/Smad3/Smad7 complex (Furuhashi *et al*., 2001; Liu *et al*., 2006), it is likely, that in these cell lines, this is the predominant interaction being observed. Alternatively, there exists the possibility that there is an alteration in the

LRP5 phosphorylation motifs that renders them able to be phosphorylated by CKIγ alone, thus allowing for recruitment of axin in the absence of functional GSK3β.

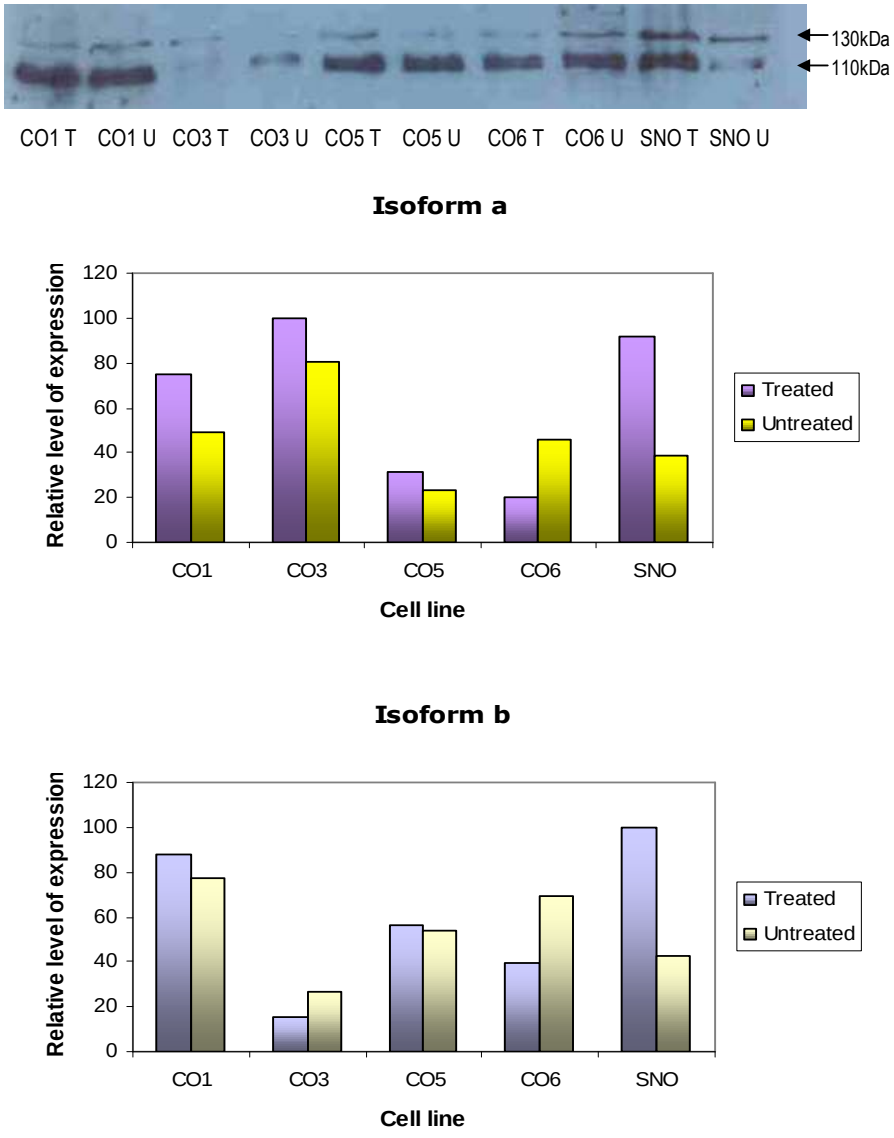


Figure 3.10. Plasma membrane-localization of axin in response to EGFR signalling. Axin levels at the plasma membrane were analyzed in response to EGF treatment of cells after 3 hours. Interesting to note is that there are different responses not only across cell lines, but also between isoforms. Given that film exposure of a western immunoblot is not visually linear, slight differences may appear between the blot itself and the densitometric analysis seen above. *Note CO1 T = WHCO1 Treated, CO1 U = WHCO1 untreated, etc.

3.4. Discussion

The canonical Wnt signalling pathway is commonly deregulated in human solid tumours (Satoh *et al*., 2000; Taniguchi *et al*., 2002; Zeng *et al*., 2006), and of particular interest to this study is human oesophageal squamous cell carcinoma. In addition, since the recruitment of axin to LRP5 at the plasma membrane is an integral component of canonical Wnt signalling (Mao *et al*., 2001; Davidson *et al*., 2005; Zeng *et al*., 2005; Swiatek *et al*., 2006), analysis thereof, both in EGF-stimulated and normal conditions in HOSCC, constitutes the prime focus of this part of the study.

Analysis of whole cell lysates revealed two specifically reacting polypeptides representing LRP5 that did not correspond to previously published molecular weights. According to the sequence given for LRP5 in Entrez (NP_002326), most antibody producers have estimated the molecular weight to be ~179 kDa. However, only Abcam ab38311 (datasheet) actually produced a band corresponding to this molecular weight. Novus Biologicals NB100-1886 (datasheet) and Everest Biotech EB06771 (datasheet, 2007) detected a protein of ~26 kDa. Another report (in which the same Santa Cruz antibody was used as in this report) showed it to be ~85 kDa (Neth *et al*., 2006). Upon analysis of a HEK293 whole cell lysate control, it was revealed that this observed molecular weight was not due to an alteration characteristic of oesophageal squamous cell carcinoma, but rather, that these two polypeptides actually represent LRP5. The production of these two variants must be at the RNA level – either at the point of transcription or post-transcription, as the very same doublet band was observed in plasma membrane-associated extracts, albeit at a substantially lower level. This means that at least some of the transcripts may be processed correctly and delivered to the plasma membrane by the two chaperone proteins, Mesd and receptor associated protein (RAP). Furthermore, research by Pospisil *et al*. (2006) confirmed the presence of two different isoforms of LRP5 in colon cell lines and patient tissues, where one isoform represented the entire transcript, but the other was lacking the polypeptide

region encoded by exon 5. Thus it seems likely that LRP5 may exist as two different isoforms and both of these are expressed within all five HOSCC cell lines.

An unusual pattern of subcellular localization was revealed by immunofluorescence analysis using the antibody specific for LRP5. Strong perinuclear staining was observed in all the HOSCC cell lines, whereas a distinct plasma membrane-staining pattern was observed in the HEK293 control. As mentioned above, correct folding and translocation of LRP5 to the plasma membrane of the cell requires the action of both Mesd and RAP. If one or both of these chaperone proteins becomes defective within a cell, LRP5 will either not be translocated to the plasma membrane or transport will occur at a substantially lower level of efficiency. This is a possible explanation for the observed results – LRP5 may well be confined to the endoplasmic reticulum in these cells. Moreover, Cong *et al*. (2004) reported a similar perinuclear staining pattern for LRP6 expression. Upon analysis of Mesd in that particular study, it was discovered that it harboured a mutation that rendered it incapable of folding or delivering LRP6 in a normal fashion. Once expression of full-length Mesd was accomplished, strong plasma membrane staining was observed for LRP6 (Figure 3.11). Thus it appears unlikely that the problem exists within LRP5 itself, but rather that there may be an alteration to one or both chaperone proteins. Further investigation will have to be done in the future to analyze Mesd and RAP within the HOSCC cell lines, but such studies were not the major focus of this project.

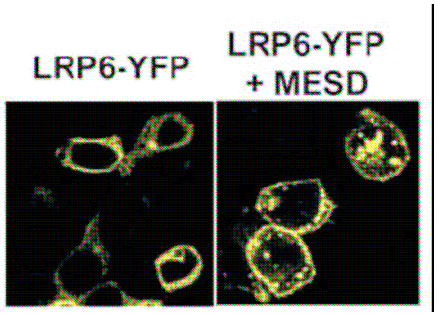


Figure 3.11. Subcellular localization of LRP6 in human 293T cells in the absence and presence of chaperone protein Mesd. LRP6 was tagged with yellow fluorescent protein and cells were visualized by fluorescence microscopy (Cong *et al*, 2004).

A number of interesting results were produced upon EGF stimulation of cells and subsequent analysis of axin and LRP5 expression levels. Overall, each cell line showed a peak in expression in response to EGF stimulation, with one exception for each of axin and LRP5. One may have expected a trend across cell lines because they are all derived from moderately differentiated tumours, however, it should be noted that variation amongst tumours is able to occur even within those of the same pathological grading.

Epidermal growth factor stimulation increased the levels of LRP5 in four of five cell lines used in this study. Although all cell lines are derived from moderately differentiated tumours, no two cell lines were identical in their responses to EGF at a variant level. Curiously, EGFR signalling tended to stimulate each cell line differentially, with predominantly either the lower (WHCO1, WHCO3 and WHCO5) or the higher molecular weight variant (WHCO6 and SNO) being expressed. Thus it appears that growth factor signalling may be able to indirectly amplify the ability of each HOSCC cell line to express one variant above the other. To date, it does not appear that this is a commonly observed EGF-response, however, EGFR signalling has been shown to demonstrate a preference for one isoform of ErbB3 receptor binding protein 1 (Ebp1) above another at a functional level (Liu *et al*., 2006), thus it cannot be

discounted that predominance of one variant based on structure may occur. This is further confirmed by virtue of the fact that the two variants frequently displayed peaks in expression at different time intervals. Thus it is highly likely that many of the variations observed between cell lines are due to an indirect effect of EGF.

Furthermore, since each cell line is so dramatically different, it is possible that they exhibit different rates of EGFR turnover. Due to the nature of EGF receptor cycling, there are numerous points of regulation (Hendriks *et al.*, 2003), and each of these points may function at a slightly different level in each cell line. This is important to note, as variations between cell lines when looking at LRP5 and axin may be attributed to a difference in EGFR cycling.

Axin, too, displayed an increase in expression following EGF stimulation of cells and, as was the case with LRP5, EGFR signalling appeared to stimulate a predominant expression of either isoform-a or isoform-b. Even though the expression levels seemed to be influenced by EGF in so many different ways, it still remained possible that there would be a change in the subcellular location of axin in response to EGF treatment because of the inhibition of GSK3β via an EGF-stimulated pathway (Eldar-Finkelman *et al.*, 1995). Therefore, it was imperative to determine the effect of EGFR signalling on the level of plasma membrane-associated axin.

LRP5 expression appeared to peak at 3 hours overall, in contrast to axin, where responses varied tremendously across the five cell lines. Although there were some discrepancies when it came to the levels of the two variants, this observation did occur in 60% of tested cell lines, therefore it was assumed to be a trend. Taking this into account when determining the effect of EGFR signalling on the level of axin at the plasma membrane, treatment time was set at 3 hours. As expected there was a marked decrease in axin levels (>10%) at the plasma membrane in both WHCO3 and WHCO6

cell lines. However, WHCO5 did not show any substantial changes in expression and WHCO1 and SNO cell lines showed dramatically marked increases in axin levels at the plasma membrane in response to EGFR signalling. This hints that in WHCO3 and WHCO6, the predominant association of axin with the plasma membrane occurs via an interaction with LRP5. This means that, upon EGF treatment and subsequent GSK3β inactivation (Eldar-Finkelman *et al*., 1995), axin was removed from the plasma membrane, because the binding sites on LRP5 were no longer primed for interaction with axin (Zeng *et al*., 2005). Just as a similar pattern was observed in LRP5 expression over time in response to EGF treatment, WHCO1 and SNO cell lines shared a similar, unexpected result – an increase of axin at the plasma membrane in response to EGFR signalling. In WHCO1 and SNO cell lines, therefore, it would appear that axin becomes associated with the plasma membrane via another route. This could potentially occur via an interaction with the TβR-I/Smad3/Smad7 signalling complex (Furuhashi *et al*., 2001; Liu *et al*., 2006). It is also feasible that there exist alterations to LRP5 in these two cell lines that render it able to recruit axin because of phosphorylation by CKIγ alone, upon EGF stimulation of the cells. In WHCO5, however, no marked increase or decrease was observed. This points to the possibility that WHCO5 is able to maintain axin levels at the plasma membrane in some sort of equilibrium between its interaction with LRP5 and its interaction with the TβR-I/Smad3/Smad7 signalling complex (Furuhashi *et al*., 2001; Liu *et al*., 2006). In order to further study this unexpected result, the intracellular domain of LRP5 would need to be sequenced and analyzed to determine whether there are any alterations present therein within cell lines WHCO1 and SNO.

It appears that HOSCCs harbour an alteration in at least one cellular component responsible for the processing and shuttling of LRP5 to the plasma membrane. This is because of a very low level of plasma membrane-associated LRP5 in comparison to that found in whole cell lysates, along with a strong perinuclear staining pattern hinting that it is still confined to the endoplasmic reticulum. The implications for HOSCC are,

therefore, that, since β-catenin may still translocate to the nucleus, perhaps Wnt signalling is not the predominant form of β-catenin regulation. In fact, since it is known that β-catenin signalling may be amplified by EGFR signalling (Eldar-Finkelman *et al.*, 1995) and since all cell lines used in this study are known to overexpress EGFR (Veale and Thornley, 1989), a likely explanation is that β-catenin upregulation in HOSCC is predominantly by EGFR signalling. Moreover, since there appeared to be an increase in the amount of axin at the plasma membrane in response to EGF stimulation in two cell lines, it is possible that in certain HOSCCs, this response may be augmented by the recruitment of axin to the plasma membrane, preventing it from partaking in β-catenin down-regulation.

CHAPTER 4: GENERAL DISCUSSION

The role of β-catenin as a significant player in both signalling and adhesion events has been well established in developmental and cancerous contexts (Siegfried and Perrimon, 1994). Numerous human solid tumours have been reported to harbour mutations or aberrant signalling capacities in one or more of the vast numbers of regulatory proteins responsible for keeping β-catenin signalling in check (Korinek *et al*., 1997; Morin *et al*., 1997; Zeng *et al*., 2006). Of particular interest to this study is the fact that the aforementioned abnormalities occur within the group of epithelially derived carcinomas (de la Coste *et al*., 1998; Miyoshi *et al*., 1998; Ueda *et al*., 2001; Zeng *et al*., 2006). Indeed, β-catenin cannot be ignored as an integral player in the intricate process of carcinogenesis. However, although great emphasis has been placed on β-catenin in the past, it cannot function as a separate entity, and so it must be considered as one component of a multiprotein signalling pathway.

4.1. β-catenin signalling as an integral component of HOSCC

The canonical Wnt/β-catenin signalling pathway is the inducer of numerous cellular responses including cell cycle progression, cell proliferation, morphogenesis and migration (Siegfried and Perrimon, 1994). It does so by interacting with transcription factors in the nucleus, resulting in the transcription of several different target genes including c-myc and cyclin D1 (He *et al*., 1998; Tetsu and McKormick, 1999). Anomalous signalling by this pathway results in unchecked cell proliferation and, to date, this particular behaviour has been reported repeatedly as a main contributor in the process of carcinogenesis (de la Coste *et al*., 1998; Miyoshi *et al*., 1998; Ueda *et al*., 2001; Zeng *et al*., 2006).

It appears that oesophageal squamous cell carcinoma may also be affected by abnormal β-catenin signalling. Although this study showed no substantial differences in expression across cell lines, β-catenin retains the ability to translocate to the nucleus in each of the five cell lines, thereby indicating that inappropriate signalling and, by implication, dysfunctional regulation of β-catenin degradation occur within this context. It could be ruled out that this behaviour, which is atypical of adult tissues, is due to mutations in β-catenin exon 3 (a common feature of human cancers) (de la Coste *et al*., 1998; Miyoshi *et al*., 1998) because previous analyses conducted on these very cell lines by PCR revealed nothing abnormal (Schnugh, 2001). Thus is was deemed necessary to focus attention on members of the degradation-targeting complex, since it was obvious that something far more complex was taking place in these cells.

4.2. The role of axin and APC in β-catenin degradation

The synergistic actions of axin and APC allow for the phosphorylation, and subsequent release of β-catenin for ubiquitination and degradation. Work by Xing *et al*. (2003) showed that APC is involved as a significant scaffolding protein within the degradation-targeting complex. It is recruited to the complex by axin via an interaction between its serine-alanine-methionine-proline (SAMP) repeats and the axin regulators of G-protein signalling (RGS) domain. These two proteins then recruit β-catenin to the complex and, once phosphorylated, the 20 amino acid repeats of APC compete with axin for binding of β-catenin, while the 15 amino acid repeats remain bound to β-catenin. Thus β-catenin is displaced from axin and, following dephosphorylation of the 15 amino acid repeats of APC, is released for interaction with β-TrCP (Amit *et al*., 2002). By this token, it is obvious that the functional capacity of the degradation-targeting complex is dependent on the presence of both axin and APC.

In terms of HOSCC, it appears that the functional capacity of the complex remains intact because the overall levels of expression of axin isoform-a and APC appeared to

mirror one another across the five cell lines studied (Figure 2.5, 2.6). Since it is known that these two proteins display a binding ratio of 1:1 (Spink *et al.*, 2000; Lee *et al.*, 2003), this observation is not unexpected, yet raises further questions about the ability of these cells to degrade β-catenin. If, as was shown (Figure 2.16), APC maintains the ability to interact with β-catenin in HOSCC, and if the functional capacity of the complex is dependent on the binding of at least β-catenin, APC, axin and GSK3β, then it appears that the ability of the cells to degrade β-catenin is not compromised. However, although able to degrade β-catenin, it is highly likely that the rate of turnover is somewhat diminished. This then implies that axin and APC may be predominantly preoccupied with other activities within the cell. Thus their levels of expression within or at different subcellular compartments were investigated. Although analysis of APC proved somewhat inconclusive with respect to its subcellular sequestration, western immunoblot analysis of extracts derived from the plasma membrane showed that axin is expressed at relatively high levels at the plasma membrane of HOSCC cells. Given that axin is typically recruited to the plasma membrane by LRP5/6 in response to stimulation of the canonical Wnt/β-catenin signalling pathway (Mao *et al.*, 2001; Davidson *et al.*, 2005; Swiatek *et al.*, 2006) and, since this interaction renders it unable to bind β-catenin within the degradation targeting complex, it was deemed likely that the diminished capacity of these cell lines to degrade β-catenin may be due to the inappropriate sequestration of axin.

4.3. Axin, LRP5 and the effect of EGFR signalling

The association between axin and LRP5 at the plasma membrane was established only relatively recently (Mao *et al.*, 2001; Davidson *et al.*, 2005; Swiatek *et al.*, 2006). However, it was this particular event that became one of the main focuses of this study. It was established that both proteins are expressed by HOSCC cell lines (Figure 2.5; 3.4.a), and that both are able to associate with the plasma membrane (Figure 2.9; 3.4.b). However, LRP5 molecular weights were dramatically different to those previously published (Abcam ab38311 (datasheet); Novus Biologicals NB100-1886

(datasheet), Neth *et al*., 2006; Everest Biotech EB06771 (datasheet, 2007)), and the levels of LRP5 at the plasma membrane were reduced by comparison to the overall levels expressed by each cell line. Additional experimental data confirmed that LRP5 was indeed inappropriately expressed in a perinuclear region of each cell line, suggesting that it was restricted to the endoplasmic reticulum. This data is consistent with a previous report by Cong *et al*. (2004), in which it was discovered that a chaperone protein for LRP5, Mesd, was dysfunctional and therefore unable to fold LRP5 correctly, or to transport it to the plasma membrane. Furthermore, it was speculated then, that at least one of the chaperones for LRP5, is functionally abnormal in an oesophageal context and this opens the door for future studies on these particular proteins.

Epidermal growth factor is known to amplify β-catenin signalling (Eldar-Finkelman *et al*., 1995) in cells of an epithelial nature and since it is known that these five cell lines overexpress EGFR (Veale and Thornley, 1989), it was imperative to determine whether there was a noticeable change in the expression of LRP5 and axin in response to EGF stimulation of the cells. From the data obtained, it is evident that LRP5 expression is most greatly affected 3 hours after stimulation in 60% of treated cell lines, however, axin's overall expression level displayed numerous different reactions across the five cell lines. Thus we chose to use LRP5's "peaking time" in further experiments, as the greatest difference in axin's level at the plasma membrane should be observed when the greatest difference in LRP5 occurs. Thus we continued to develop a model for plasma membrane-associated axin response to EGF stimulation of the cells.

4.4. Levels of axin at the plasma membrane in response to EGF stimulation

The proposed model was that if EGFR signalling inhibits the function of GSK3β (Eldar-Finkelman *et al*., 1995), then the appropriate sites on LRP5 would no longer

become phosphorylated, thus axin would no longer be recruited to LRP5, leaving it free to perform one of any number of activities (see plan outlined in Figure 4.1). Once released from LRP5, axin is then free to:

1. Form part of a positive feedback loop in which it forms a homodimer and, through the binding of MAPK/ERK kinase kinase (MEKK) 1 or 4, amplifies the JNK signal produced by EGFR signalling (Zhang *et al*., 1999; Luo *et al*., 2003).
2. Recycle to the plasma membrane to form a complex with TβR1 and Smad3, aiding in signal transduction (Furuhashi *et al*., 2001).
3. Form a multimeric complex including both p53 and homeodomain-interacting protein kinase-2 (HIPK2), which is responsible for binding and phosphorylating Ser46 of p53 in an ultraviolet (UV)-induced p53-dependent apoptotic context (Rui *et al*., 2004).

The possibilities for axin in response to EGF-stimulation are, therefore, numerous. However, upon analysis of data obtained from EGF-treated plasma membrane-associated protein extracts, it could be seen that, although the expected result of a decrease of axin at the plasma membrane in response to EGF stimulation occurred in two of the five cell lines, there was little or no change in one cell line, and two cell lines displayed a marked increase in axin at the plasma membrane following treatment. This implies that in WHCO3 and WHCO6 cell lines, the predominant interaction responsible for sequestration of axin at the plasma membrane was that of axin/LRP5. However, in WHCO1 and SNO cell lines, which displayed a marked increase in axin at the plasma membrane, it is likely that the predominant interaction is occurring between axin and TβR1/Smad3, although the possibility of an alteration to LRP5 which renders it phosphorylatable by CKIγ alone, or by a functionally overlapping kinase of GSK3β, cannot be ruled out. WHCO5 alone appeared to be almost unaffected, introducing the possibility that this particular cell line naturally exhibits a balance between axin's interaction with LRP5 and the TβR1/Smad3 signalling complex (Furuhashi *et al*., 2001; Liu *et al*., 2006).

Of course some of these differences may be attributed to the fact that although all five cell lines are derived from moderately differentiated oesophageal squamous cell carcinomas, there are indeed differences on an individual basis. This indicates that perhaps there should be subgroups introduced within each histopathological grading currently observed, in order to better categorize tumours.

Because of its many different functional capacities within the cell, numerous additional studies will need to be conducted in order to elucidate the specific response of axin to EGFR signalling, in order that it may be determined whether it is involved in a positive feedback loop, Smad signalling or p53-dependent apoptosis, or a combination of all three (Figure 4.1).

It seems that the more this extraordinary protein is studied, the more functions are discovered. It would be interesting for future research to include studies on the upstream triggers of each set of axin binding partners, and where and how they are able to form components of "cross-talk". Thus, it is possible that in future years different functional domains of this multifaceted protein may become the target of numerous therapeutic agents.

4.5. β-catenin, APC, axin and LRP5: implications for HOSCC

Within the broader context of tumour biology it should be noted that β-catenin and APC have been the primary points of focus in most tumours, with less emphasis placed on other players within the receptor or degradation-targeting complexes. While β-catenin was shown to be able to signal inappropriately in these cells, as is typical of many human cancers (Siegfried and Perrimon, 1994), the outcomes of the current study appear to be unique in many ways.

While oesophagus and colon are both components of the gastrointestinal tract, no peculiar truncations or alterations in APC's subcellular distribution were observed in HOSCC, unlike in colon carcinomas (Powell *et al*., 1992). Naturally it is tempting to consider the possibility of promoter hypermethylation, which is known to occur in breast, prostate, renal and bladder cancers (Dulaimi *et al*., 2004a; Dulaimi *et al*., 2004b; Hoque *et al*., 2004; Topaloglu *et al*., 2004), especially since cancers of these organs are frequently epithelially-derived. However, it is highly unlikely that this is the case in HOSCC, since promoter hypermethylation leads to gene inactivation and levels of APC were not so substantially diminished in the five cell lines that gene inactivation could have occurred. It therefore appears that the mechanism by which inappropriate β-catenin signalling occurs is not via altered APC, especially since the two are able to interact within HOSCC.

Given its role in the transduction of canonical Wnt signals, LRP5 is an obvious candidate for study within a tumour biology context. However, very few studies have been conducted on LRP5 within this context. It has been shown to be particularly prominent and involved in the pathogenesis of bone diseases such as high-grade osteosarcoma (Hoang, *et al*., 2003) and autosomal recessive osteoporosis-pseudoglioma syndrome (Gong *et al*., 2001). It appears that it is only recently that the importance of LRP5/6 is being realized in cancers. One study in colon carcinoma showed the production of two splice variants, but there was no study included on subcellular distribution that may change as a result of the production of these two variants (Pospisil *et al*., 2006). Recent work by Björklund *et al*. (2007) showed that LRP5 harboured an internal truncation in 86% of primary and 100% of secondary hyperparathyroid tumours rendering it unable to be inhibited by Dkk1. Of course, since hyperparathyroid tumours and osteosarcoma are tumours of a very different nature to HOSCC, it is unlikely that these tumours would share similar characteristics with HOSCC. However, as previously discussed (see Section 3.4), two splice variants were also obtained in HOSCC – a result similar to that described in colon carcinoma. The implications for HOSCC, therefore, are that while there may be a similarity to one

other carcinoma, there is a distinct lack of information with which to compare the results obtained in the current study. Furthermore, it appears that this is the first study on LRP5 in HOSCC.

Studies on axin revealed the presence of both isoforms, as well as confirmed their ability to localize to different subcellular compartments. The only unusual observation was axin's high level of association with the plasma membrane. The report by Nakajima *et al*. (2003) describing a direct correlation between tumour progression and decreased axin levels was interesting although unrelated to the nature of the current study, as all cell lines used here were derived from tumours of the same pathological grading. It thus appears that HOSCC may be unique in nature, for when compared to other carcinomas, which harbour at least one type of mutation e.g., ovarian endometrioid adenocarcinomas (Wu *et al*., 2001) or hepatocellular carcinomas (Satoh *et al*., 2000; Taniguchi *et al*., 2002), there were no dramatic alterations to axin in moderately differentiated oesophageal SCCs. Research surrounding axin's response to EGF stimulation in HOSCC appears to be novel, since much research has been done on β-catenin, but very little attention has been paid to other members of this signalling pathway.

All in all, these data point towards a unique passage to malignancy for oesophageal SCC. While no remarkable alterations were found in axin, APC or β-catenin, other than axin's high level of association with the plasma membrane, LRP5 displayed certain atypical features, which may, in turn, affect other proteins in the cell, specifically axin. Thus, inappropriate signalling by β-catenin in HOSCC cell lines may be more complicated than previously thought.

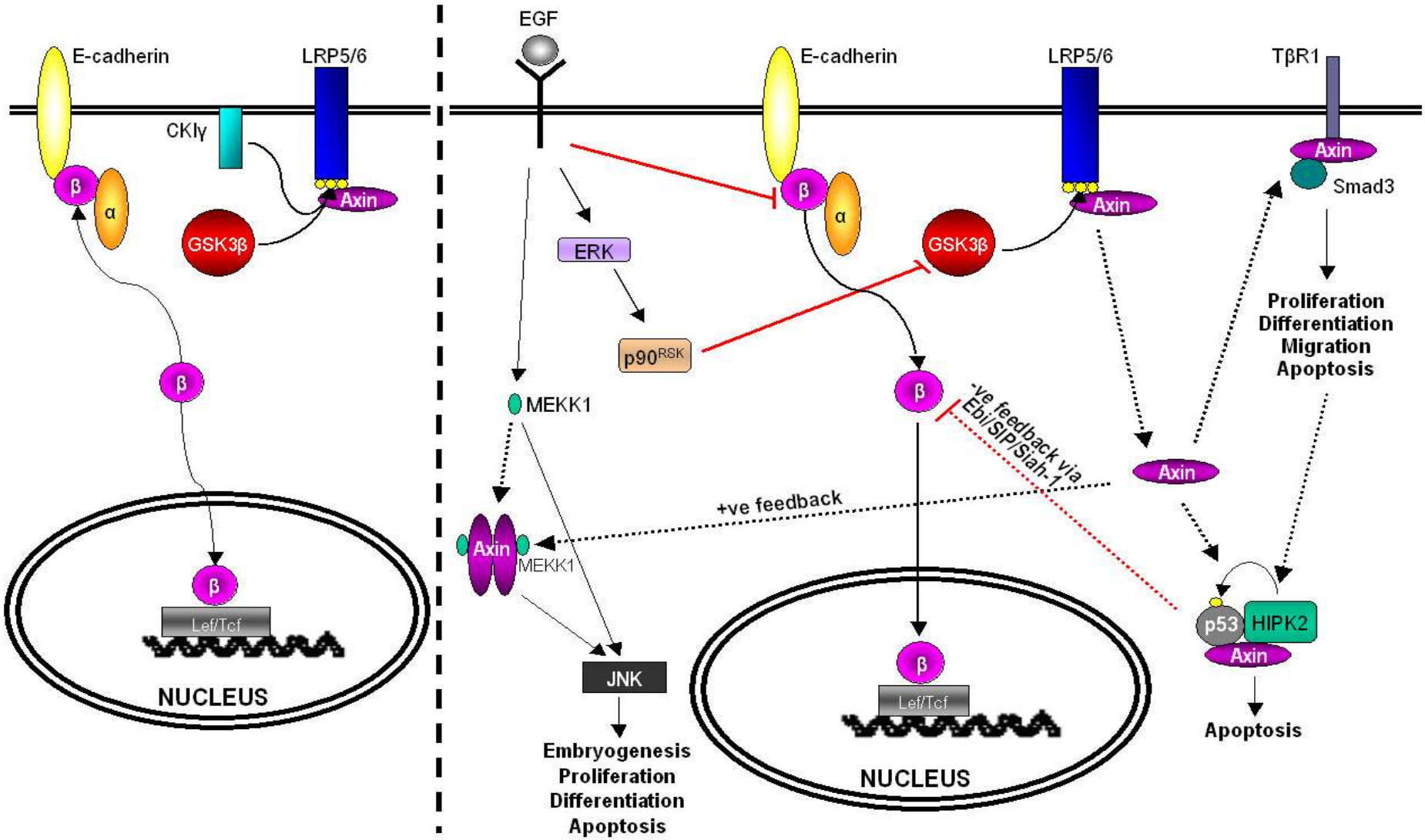


Figure 4.1. Schematic representation of axin's potential responses to EGFR signalling. If GSK3β is inactivated by EGFR signalling, then it is likely axin will no longer be able to be recruited to LRP5 and thus it is free to partake in numerous different pathways including potential negative and positive feedback loops.

4.6. Conclusion

The equilibrium between β-catenin signalling, degradation and adhesive roles is strictly maintained within normal human adult tissues. However, disruption of this equilibrium by events such as mutations in regulatory proteins or "cross-talk" with other pathways e.g. EGFR signalling, is a common component of many different human solid tumours. The data obtained in the current study on HOSCC cell lines indicate that:

1. The degradation-targeting complex remains functionally intact, despite β-catenin's ability to translocate to the nucleus.
2. This is possibly due to the inappropriate sequestration of axin by other cellular proteins.
3. Since there appears to be alteration in at least one cellular component responsible for the processing and shuttling of LRP5 to the plasma membrane, it is unlikely that the main binding partner of axin within all the cell lines is LRP5.

Thus it may be concluded that, although possible, it is unlikely that the canonical Wnt signalling pathway remains the predominant form of β-catenin regulation in these cell lines. Since it is known that β-catenin signalling may be amplified by EGFR signalling (Eldar-Finkelman *et al*., 1995) and, since all cell lines used in this study are known to overexpress EGFR (Veale and Thornley, 1989), a likely explanation is that β-catenin upregulation in HOSCC is predominantly due to EGFR signalling. However, it should also be noted that there exist more than one pathway by which β-catenin may be degraded within the cell and further studies may focus on such pathways which include the novel PPARγ2-dependent pathway (Sharma *et al*., 2004), the retinoid pathway (Xiao *et al*., 2003) and the more extensively studied Ebi/SIP/Siah-1 degradation complex (Matsuzawa and Reed, 2001).

Of course one cannot discount the possibility that the Ebi/SIP/Siah-1 degradation complex may be initiated by yet another potential negative feedback loop involving axin. This is feasible because axin binds to and forms a ternary complex with HIPK2 and p53, leading to HIPK2-dependent phosphorylation of p53 on Ser46 (Rui *et al*., 2004). The result of this phosphorylation event is then that p53-dependent transcriptional activity as well as apoptosis increases (D'Orazi *et al*., 2002; Hofmann *et al*., 2002). This is important in the context of Siah-1, since expression of Siah-1 is p53-dependent (Matsuzawa and Reed, 2001). Thus it is possible that the decreased amount of axin available for the targeting of β-catenin for degradation may indeed be initiated by EGFR signalling-dependent inhibition of the typical degradation-targeting complex as well as interaction between LRP5 and axin, thereby introducing axin to function in a negative feedback loop, driving p53-dependent transcription of the Siah-1 gene, thus leading to a downregulation of β-catenin via an indirect pathway (Figure 4.1). Furthermore, because axin is involved in TGFβ signal transduction, which may lead to induction of p53, this may form an even bigger, more complicated feedback loop.

In conclusion, axin can be seen as a central component of signal transduction events within many different cellular contexts. Its role in oesophageal squamous cell carcinoma has already been established somewhat by Nakajima *et al*. (2003), who correlated decreased levels of axin with tumour progression, however, this current study attempted to elucidate the molecular interactions and processes occurring in moderately differentiated tumours. Within the same pathological grading (here moderately differentiated), it appears that not only axin, but also its numerous binding partners, in particular LRP5, may exhibit decreased levels of expression, mutations, or even inappropriate subcellular sequestration. All in all the implication for HOSCC is that axin, with its numerous binding domains, is an important regulatory protein, and thus may become an equally important drug target for the regulation of aberrant β-catenin/EGFR signalling in the future.

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CHAPTER 6: APPENDIX

6.1. Tissue culture

6.1.1. Tissue culture medium

6.1.1.a. DMEM Solution

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

6.1.1.b. Hams F12 Medium Solution

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

Mix:

3 volumes DMEM medium solution : 1 volume Hams F12 medium solution
Filter sterilize
Store at 4˚C

6.1.2. 1x Phosphate buffered saline

136.9 mM NaCl

2.86 mM KCl

10.1 mM $Na_2HPO_4 \cdot 12H_2O$

1.76 mM KH_2PO_4

Adjust pH to 7.2-7.3 and make up to final volume with dH_2O.

Sterilize the solution for 20 minutes at 151 lb/in^2 and store at 4˚C.

6.1.3. Trypsin:EDTA (ethylenediaminetetra-acetic acid) solution

0.004% EDTA in 1×PBS

0.01% Trypsin in 1×PBS

Mix EDTA:Trypsin (1:1)

Store at 4ºC.

6.2. Preparation of samples for SDS-PAGE

6.2.1. Laemmli lysis buffer

6.2.1.a. Double lysis buffer

4% SDS

20% Glycerol

10% β-mercaptoethanol

0.125 M Tris-HCl, pH 6.8

Make up to final volume with dH_2O.

6.2.1.b. Single lysis buffer

Dilute double lysis buffer at a ratio of 1:1 using dH_2O.

6.2.2. PBS/PMSF/Trazylol solution

0.5% PMSF stock solution (20mM in Methanol)
1% Trazylol
Make up to final volume using 1×PBS.

6.2.3. Hypotonic lysis buffer

20 mM Tris-HCl, pH 7.4-7.5
25 mM Sodium fluoride (NaF)
1 mM EDTA
Make up to final volume with dH_2O.

6.2.3.a. Hypotonic lysis buffer with protease inhibitors

0.1 mM PMSF
1% Trazylol
0.1% Pepstatin
0.05% Leupeptin
Make up to final volume with hypotonic buffer.

6.2.4. Triton-X 100 Extraction Buffer

0.5% Triton-X 100
50 mM Tris
150 mM NaCl
1 mM $CaCl_2$
1 mM $MgCl_2$
0.01% Aprotinin
Make up to final volume with dH_2O.
Store at 4ºC.

6.2.5. Coomassie Brilliant Blue stain (0.25%)

0.25% Coomassie Brilliant Blue powder
50% Methanol
10% Acetic Acid
Make up to final volume with dH_2O.

6.2.6. Destain

12% Ethanol
10% Acetic Acid
Make up to final volume with dH_2O.

6.2.7. Elution solution

67.4% Methanol
31.6% Distilled H_2O
1% Ammonia

6.2.8. Example of a standard curve derived from protein estimation

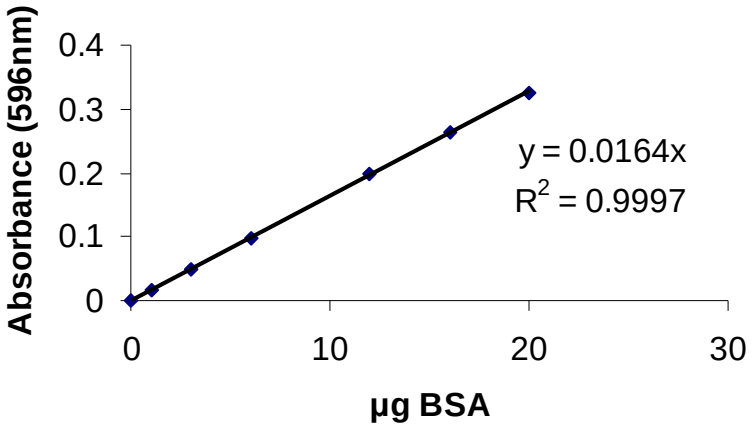

Figure 6.1. Standard curve derived from the protein estimation representing the relationship between the number of µg of protein, and the relative absorbance at 596nm. The absorbance of six fixed amounts of BSA were used to construct a standard curve. The relative concentrations of each of the protein extracts from the five cell lines were estimated using this curve by measuring their absorbances at 596nm. The equation of the curve was used to calculate the number of µg of protein present in each of the samples, and hence the concentration thereof. R^2 = linear regression analysis.

6.2.9. BME (β-mercaptoethanol) water

10% Glycerol
5% β-mercaptoethanol
Make up to final volume with dH_2O

6.2.10. IP (immunoprecipitation) buffer

20mM Tris (pH 8.0)
0.5% Nonidet P-40
0.9% NaCl
Make up to final volume with dH_2O

6.3. SDS-PAGE

6.3.1. Separating gel (10%)

10% Acrylamide

375 mM Tris-HCl, pH 8.8

0.1% NN'-methylenebisacrylamide

0.2% SDS

Make up to final volume with distilled H_2O

Add 0.1125% ammonium persulfate (APS) and 0.25% TEMED just before use.

6.3.2. Stacking gel (5%)

5% Acrylamide

125 mM Tris-HCl, pH6.8

0.1% NN'-methylenebisacrylamide

0.2% SDS

Make up to final volume with distilled H_2O

Add 0.1125% ammonium persulfate (APS) and 0.2% TEMED just before use.

6.3.3. SDS-PAGE running buffer

3.5 mM SDS

52 mM Tris-HCl, pH8.3

192.5 mM Glycine

Make up to final volume with dH_2O.

6.3.4. Destain solution

10% acetic acid

10% methanol

Make up to a final volume with dH_2O

6.3.5. Example of calibration curve

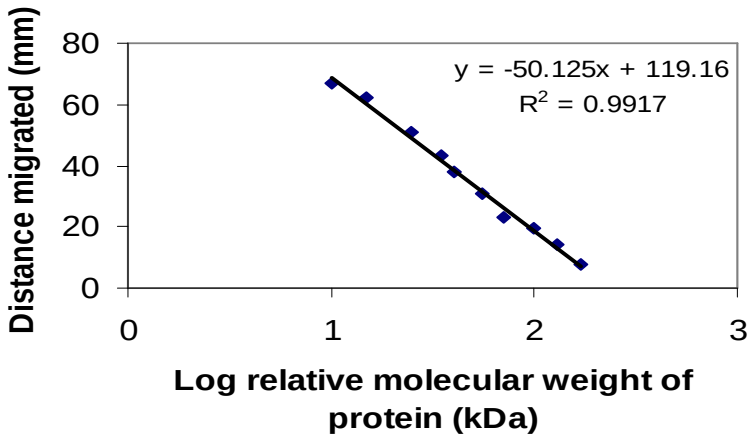

Figure 6.2. Example of a Standard SDS-PAGE calibration curve. Proteins of known molecular weights (x-axis) migrated unique distances within the gel (y-axis). The equation calculated from the curve was used to determine if the band of the desired size was present in a given sample. R^2 = linear regression analysis.

6.4. Western immunoblotting

6.4.1. Transfer buffer

25 mM Tris-HCl, pH 8.3

20% Methanol

188 mM Glycine

Make up to final volume with distilled H_2O.

6.4.2. Blocking buffer (Blotto)

50 mM Tris-HCl, pH 7.8

147 mM Sodium chloride (NaCl)

2 mM Calcium chloride dihydrate ($CaCl_2 \cdot H_2O$)

5% Non-fat milk powder

0.05% Triton-X 100

Make up to final volume using dH_2O.

Store at 4ºC.

6.4.3. Developer

6.4 M Metol

0.6 M Sodium sulfate (anhydrous)

80 mM Hydroquinone or quinol

0.45 M Sodium carbonate (anhydrous)

34 mM Potassium bromide

Make up to final volume dH_2O in the dark.

Store in the dark.

6.4.4. Fixer

0.8 M Sodium trisulfate

0.2 M Sodium/potassium metasulfate

Make up to final volume with warm dH_2O.

Store in the dark.

6.5. Indirect immunofluorescence

6.5.1. 0.4% Trypan blue

0.4% Trypan blue powder

Make up to final volume with 1×PBS

6.5.2. 4% Paraformaldehyde

4% Paraformaldehyde

116 mM Sodium dihydrogen orthophosphate (anhydrous)

107 mM Sodium hydroxide

Make up to final volume with dH_2O.

Heat to 80˚C, stir until clear, cool and adjust pH to 7.2-7.3.

Filter and store at 4˚C.