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á-Catenin expression in oesophageal squamous cell carcinoma

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2006-03-23
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CHAPTER 1

INTRODUCTION

1.1 CELL ADHESION AND CANCER

Cell adhesion plays a crucial role in the maintenance of a wide variety of cellular processes and hence the breakdown of cell adhesion may have devastating implications for the cell (Hollande *et al.,* 2002). Cancer cells, are cells that have been set free from proliferative and survival controls. These cells arise through the stepwise accumulation of discrete genetic changes (mutations) that affect growth control, differentiation and survival of a cell (Mandard *et al.,* 2000). These mutations may alter the expression of the mutated gene or its product, resulting in the circumvention of normal cell controls that regulate cell division and the state of differentiation (Nicolson, 1991). Cells require mutations in multiple genes in order to become metastatic. An example of this is colorectal cancer, which needs at least seven genetic events to occur, in a specific order, for the tumour to become metastatic (Kinzler and Vogelstein, 1996).

The highest cause of death amongst cancer patients is metastatic disease (Fidler and Hart, 1982). Metastasis is the propensity of cells from malignant neoplasms to disseminate from their primary site to distant organs and there develop into metastases or secondary tumours (Fidler and Hart, 1982). This implies that metastasis may be an ordered series of events. The metastatic process may essentially be split into eight events, namely: 1. Tumour penetration of the basement membrane and invades the interstitial stroma, 2. Penetration of the basement membrane of the blood or lymph vessels, 3. Evasion of host immunological defence mechanisms, 4. Arrest in a capillary bed, 5. Adherence to the basement membrane of the capillary bed, 6. Extravasate, 7. Respond to microenvironment and 8. Secondary tumour cell proliferation (Nicolson, 1991, Beavon, 2000, Cairns *et al.,* 2003). Central to many of the predicted steps of metastasis is a loss or alteration of cellular adhesion (Beavon, 2000).

1.2 CELL ADHESION AND ITS ROLE IN METASTASIS

To achieve some of the characteristics of metastasis, it is important that there is a change in cellular adhesion (Cairns *et al.,* 2003). Loss of cell adhesion is particularly important in cancers involving epithelial cells, due to the strong dependence on cell adhesion displayed by these cells. Epithelial cells are robustly attached to one another and to the basal lamina, a loss of adhesion in these cells would therefore be crucial to tumour development (Raven and Johnson, 1996). There are various different adhesion structures found in cells. It is essential that these cell adhesions are maintained.

1.2.1 Cell adhesive interactions

There are five distinct types of adhesion involving cell-cell or cell-basement membrane interactions, found in mammalian epithelial cells. They are 1. Tight junctions, 2. Adherens junctions, 3. Desmosomes, 4. Gap junctions and 5. Hemidesmosomes (Perez-Moreno *et al.,* 2003). The major components of these different types of adhesions are the cadherins, integrins, selectins and immunoglobulin super family of adhesion molecules (Hynes, 1999). The tight junctions form a continuous layer around epithelial cells near the apical surface of the cells (Hollande *et al.,* 2002). Tight junctions serve as a barrier to regulate the diffusion of molecules across the epithelium (Hollande *et* al., 2002). The gap junctions form links between cells where the plasma membranes of two cells run in close proximity (2-4 nm) to each other (Alberts *et al*., 1998). Desmosomes and hemidesmosomes form the major cell attachment sites for intermediate filaments, attaching cell to cell and cell to basement membrane respectively (Legan *et al.,* 1992, Green and Jones 1996). Desmosomes form focal adhesions around epithelial cells, which directly mediate cell-cell contacts and serve an important role in the maintenance of tissue architecture (Huber, 2003). Desmosomes are primarily made up of cadherin molecules (Perez-Moreno *et al.,* 2003, Green and Jones, 1996). The hemidesmosomes anchor the epithelial cells at the basal surface to the basement membrane (Borradori and Sonnenberg, 1999). They consist primarily of integrin molecules (Borradori and Sonnenberg, 1999). The adherens junctions attach the actin bundles of the cytoskeleton of

one cell to those of another. The adherens junctions are made up of cadherin and catenin proteins (Nieset *et al.*, 1997). Adherens junctions are important in epithelial cells, and therefore warrant closer examination into their structure and function.

1.2.2 Adherens junctions composition and role in epithelial cell integrity

Adherens junctions are intercellular structures, which are particularly prominent in epithelial cells (Nieset *et al.*, 1997). The adherens junctions function in cell-cell adhesion. They appear in regions where two cells are close to and parallel to each other, and form intracellular plaques into which actin filaments insert (Nieset *et al*., 1997). Adherens junctions form a belt around the epithelial cells near the apical surface, just below the tight junctions, thus forming a linear cell adhesion "zipper" (Figure 1.1) (Alberts *et al*., 1998).

Epithelial cells are polar in that they have a basal and an apical surface. These surfaces are different reflecting the cells internal organisation (Nieset *et al.*, 1997). If molecules are allowed to pass between the cells instead of through them, then the internal polarised organisation of the cell may be affected. Therefore it is important that the adherens junctions formation and degradation is regulated (Wijnhoven *et al*., 1999). It has been found that adherens junctions are lost at the invading front of cancer cells, which have a high metastatic ability (Hirohashi and Kanai, 2003).

Adherens junctions are primarily made up of cadherins and catenins (Figure 1.1). The cadherin family of adhesion molecules has been split into ten subclasses, with E-cadherin (epithelial) constituting the principle cadherin component of epithelial adherens junctions (Wijhoven *et al.*, 2000).

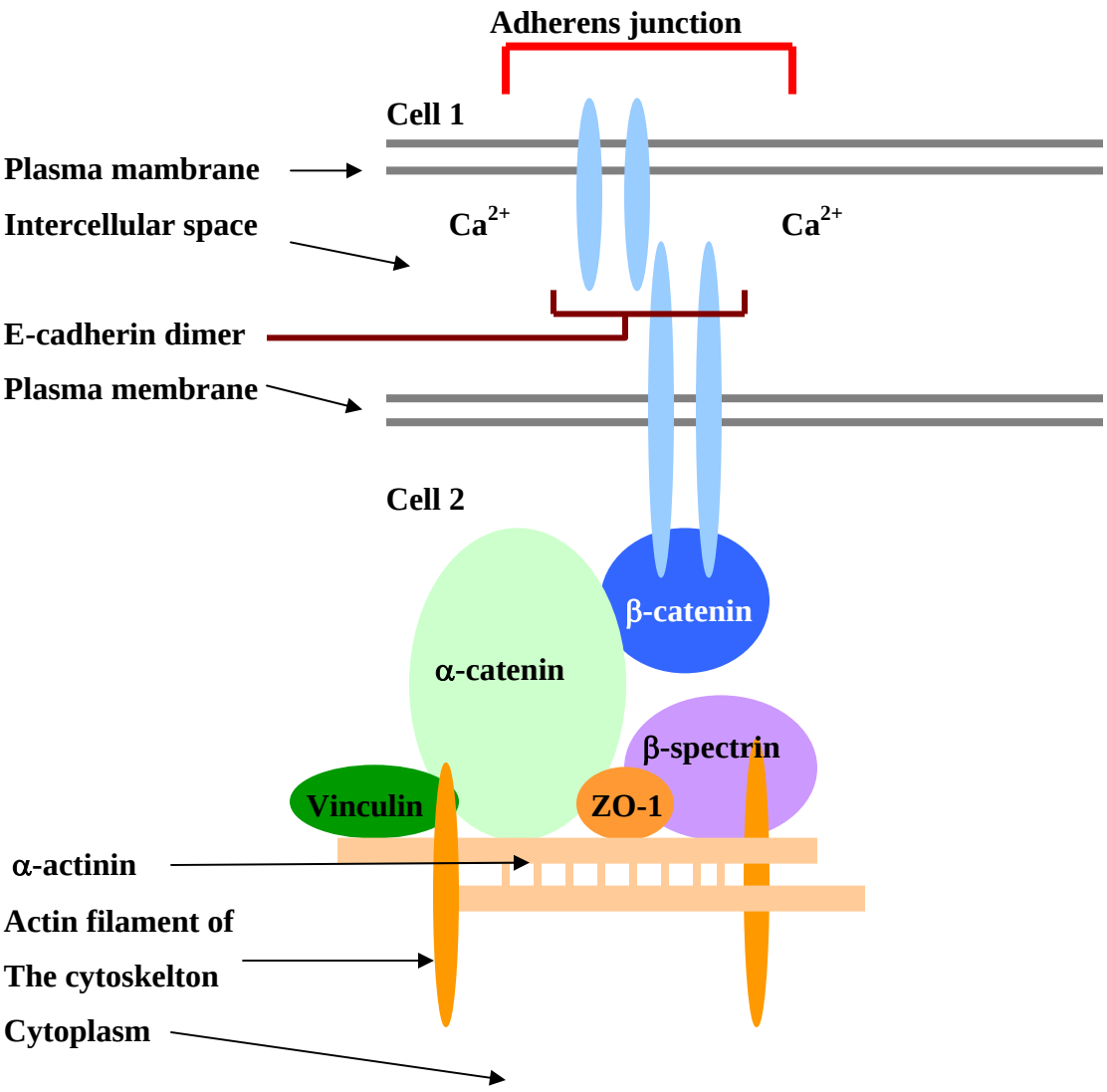


Figure 1.1: Representation of an epithelial cell adherens junction (Hynes, 1999; Nieset et al., 1997, Pradhan et al., 2001; Wijhoven et al., 2000). In the presence of calcium ions (Ca^{2+}) the extracelullar tails of the two E-cadherin molecules from one cell bind to the two extracelullar E-cadherin tails from another cell. The cytoplasmic domain of E-cadherin binds to the armadillo repeat region of β-catenin. This complex is then attached to α-catenin. α-catenin binds to α-actinin, vinculin, ZO-1, β-spectrin and to the α-actin filaments of the cytoskeleton (Nagafuchi, 2001, Hirohashi and Kanai, 2003).

Cadherin is a calcium-dependent glycoprotein, which binds through a homophilic mechanism and is a principle mediator of cell-cell adhesion (Hiroya and Masayuki 1997, Woodfield *et al.*, 2001). Cadherins are transmembrane proteins possessing an extracellular calcium-binding domain and a highly conserved intracellular domain (Hiroya and Masayuki 1997). The extracellular portion of classical cadherins consists of five ectodomains, which bind calcium and adopt a rod-like template for homophilic interactions with E-cadherin molecules on the surface of neighbouring cells (Perez-Moreno *et al.*, 2003). The intracellular portion of E-cadherin is linked to the actin based cytoskeleton (Johnson *et al.*, 1993) (Figure 1.1). E-cadherin plays a critical role in maintaining epithelial tissue architecture, through the formation and maintenance of adherens junctions (Nieset *et al*., 1997, Ikeguchi *et al.*, 2001). For adherens junctions to be formed, the cytoplasmic domain of E-cadherin must be attached to the cytoskeleton. This attachment occurs via the interactions of the cytoplasmic domains of E-cadherin with a group of intracellular proteins, the catenins (Figure 1.1) (Hunter *et al.*, Nieset *et al*., 1997).

The catenins were first identified as peripheral cytoplasmic proteins in association with E-cadherin (Alberts *et al*., 1994). Two main types of catenins have been found in adherens junctions, namely α-catenin and β-catenin (Johnson *et al*., 1993). β-catenin binds to the cytoplasmic tail of E-cadherin in a mutually exclusive manner (Figure 1.1). This binding interaction occurs via β-catenin's armadillo repeat region (central region of β-catenin), and amino acids 832-862 of the cytoplasmic domain of E-cadherin (Huber *et al.*, 1997, Rubinfeld *et al*., 1995, Sacco *et al*., 1995). The binding of β-catenin to E-cadherin is enhanced by serine/threonine phosphorylation of β-catenin (Nelson and Nusse 2004). The β-catenin/E-cadherin complex is linked to the actin cytoskeleton via α-catenin.

Strong intercellular adherens junctions depend on the linkage of the cadherin/catenin complex to the actin cytoskeleton via α-catenin (Janssens *et al*., 2001). α-catenin can interact directly with the actin cytoskeleton or indirectly via adaptor molecules, such as vinculin, α-actinin, β-spectrin or ZO-1 (Figure 1.1) (Pradhan *et al.*, 2001, Giannini *et al.*,

2000). The central and c-terminal domains of α-catenin, associate with α-actin in (Giannini *et al*., 2000). This interaction is augmented by the interaction of α-catenin's amino-terminal with β-spectrin and other molecules such as ZO-1, vinculin and α-actinin (Figure 1.1) (Pradhan *et al*., 2001, Giannini *et al.,* 2000). α-catenin therefore, plays an important role in the formation of the adherens junctions. Closer examination of α-catenin is merited to shed light on the manner in which it fulfils its role in adherens junction formation.

1.2.3 α-catenin's interaction with the adherens junction components

In the adherens junctions, α-catenin associates indirectly with E-cadherin through its interaction with β-catenin. One of α-catenin's functions is the modulation of cell adhesive strength (Nieset *et al.,* 1997). This function is brought about through α-catenin linking the E-cadherin/β-catenin complex to the actin cytoskeleton (Figure 1.1). The link between α-catenin and β-catenin is essential for the formation and maintenance of the adherens junctions (Nieset *et al*., 1997). It is therefore essential that the link between α-catenin and the actin cytoskeleton be formed correctly. The binding domains on α-catenin responsible for its interaction with β-catenin and the actin cytoskeleton have all been identified. The amino acids of α-catenin, that are sufficient for the binding of it to α-actinin, are amino acids 325-394 (Nieset et al., 1997). The α-catenin binding site on α-actinin has been located in the region amino acids 479-529 of α-actinin (Nieset *et al*., 1997). With respect to the α-catenin/β-catenin interaction, the α-catenin binding site in β-catenin has been narrowed down to the amino-terminal region near the first armadillo repeat, specifically, a peptide spanning amino acids 120-150 of β-catenin (a different portion to that which interacts with E-cadherin) (Aberle *et al*., 1994, Aberle *et al*., 1996, Nieset *et al*., 1997). This region, near the first armadillo repeat of β-catenin, which forms the interaction with α-catenin has been shown to contain mostly hydrophobic amino acids (Huber *et al.,* 1997). α-catenin binds specifically to this region of β-catenin. α-catenin's β-catenin binding site has been localised to the region amino acids 97-148 of α-catenin (Yang *et al.,* 2001).

The interaction between α-catenin and the actin cytoskeleton only occurs if α-catenin is attached to β-catenin (Nieset *et al*., 1997). This highlights the danger of the components of the adherens junctions accumulating mutations, which would result in their inability to perform their normal functions. This is important in epithelial cells, since they are anchorage dependent for growth and differentiation (Hynes, 1999). Cell adhesion molecules such as α-catenin and β-catenin have a duel function, in that they function as adhesion molecules and as signal transduction molecules. β-catenin forms part of the Wnt pathway (Seidensticker and Berherns, 2000). The Wnt pathway activates genes, which results in an increase in cell proliferation and a decrease in cell adhesion (Seidensticker and Berherns, 2000). It is therefore necessary to examine these effects on cell adhesion and the degradation of β-catenin. On this background it will be possible to highlight α-catenin's signal transduction role and how it interlinks with these pathways bringing about the changes in cell adhesion and proliferation observed in tumour cells.

1.3 β-CATENIN'S ROLE IN THE WNT PATHWAY

The function of β-catenin depends on its subcellular localisation (Ling *et al.*, 2001). β-catenin may be found in three locations in cells, namely: at the plasma membrane (as part of the adherens junctions), in the cytoplasm [either free or attached to the adenomatosus polyposis coli (APC) molecule] or in the nucleus [attached to the lymphoid enhancer factor/T cell factor (Lef/Tcf)] (Kikuchi, 2000). In normal epithelial cells the cytoplasmic and nuclear levels of β-catenin are low, with a high level of bound β-catenin found in the plasma membrane (Seidensticker and Berherns, 2000). This bond plasma membrane β-catenin forms part of the adherens junctions. Free cytoplasmic β-catenin is normally broken down by the glycogen synthase kinase 3 β (GSK-3β), APC and axin/conductin complex (Figure 1.2).

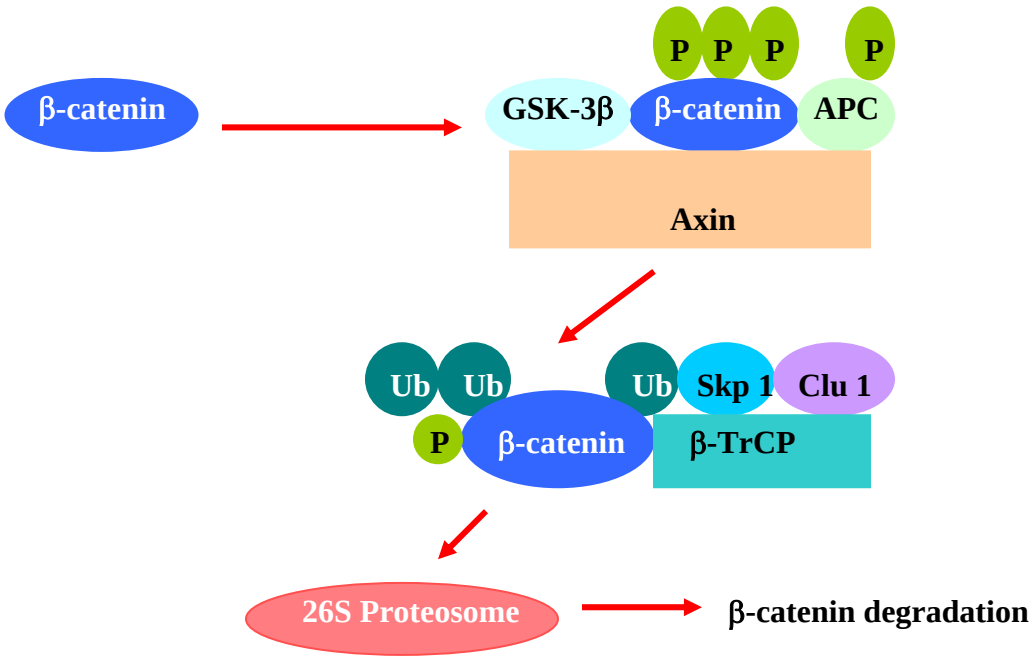


Figure 1.2: β-catenin degradation (Kikuchi., 2000; Novak and Dedha,r 1999; Seidensticker and Berherns, 2000). Free cytoplasmic β-catenin binds to the APC/axin/GSK-3β complex. β-catenin is then phosphorylated by GSK-3β. The ubiquitin-ligase (βTrCP), the ubiquitin activating enzyme (Skp1) and the ubiquitin-conjugating enzyme (Clu1) associate with β-catenin, resulting in its ubiquitination (Ub) (Kikuch, 2000). Ubiquitinated β-catenin is then targeted for degradation by the 26S proteosome (Orford et al., 1997).

Free cytoplasmic β-catenin, binds to the APC/axin/GSK-3β complex. Once associated with the axin/GSK-3β/APC complex, GSK-3β phosphorylates β-catenin at highly conserved residues at the amino terminal (Ninomiya *et al*., 2000). The ubiquitination complex consisting of a ubiquitin-ligase (βTrCP), ubiquitin activating enzyme (Skp I) and ubiquitin-conjugating enzyme (Clu I) is then formed (Figure 1.2). Once the ubiquitin complex has been formed, it tags β-catenin at lysine residues with multiple copies of a small protein, ubiquitin (Easwaran *et al*., 1999, Liu *et al*., 1999). The 26S proteosome then degrades the ubiquitinated β-catenin (Liu *et al*., 1999).

The degradation of β-catenin may be prevented by the Wnt pathway (Figure 1.3) (Seidensticker and Berherns, 2000, Rubinfeld *et al.*, 1997). Wnt is the mammalian homolog of the *Drosophila* Wingless (Wg) glycoprotein (Wnt-wingless pathway) (Beavon, 2000). This pathway regulates organ development, cell proliferation, morphology, motility and cell fate (Kikuchi, 2000). Wnt acts on cells in a paracrine fashion. Wnt prevents the destruction of β-catenin, which can then translocate to and enter the nucleus (Figure 1.3). Activation of Wnt-1 signalling leads to a constitutive increase in the regulation of cytosolic β-catenin, leaving plasma membrane associated β-catenin unaffected (Chen *et al*., 2001). Excessive amounts of β-catenin in the nucleus can result in an increase in cell proliferation, by the binding of β-catenin to Lef/Tcf, resulting in the transcription of genes involved in cell proliferation and adhesion (e.g. cyclin D1) (Figure 1.3). Tcf's are sequence-specific DNA binding proteins, which apparently have no classical transcription domain of their own (Seidensticker and Berherns, 2000). Tcf can mediate the repression of its target genes (in the absence of Wnt signalling), or it can act as a transcription activator (in the presence of Wnt signalling). Lef/Tcf require β-catenin as a transcription co-activator (Novak and Dedhar, 1999). The β-catenin/Tcf complex can be regarded as bipartite transcription factors, in which the DNA binding and transcription functions are controlled by two separate proteins (Seidensticker and Berherns, 2000).

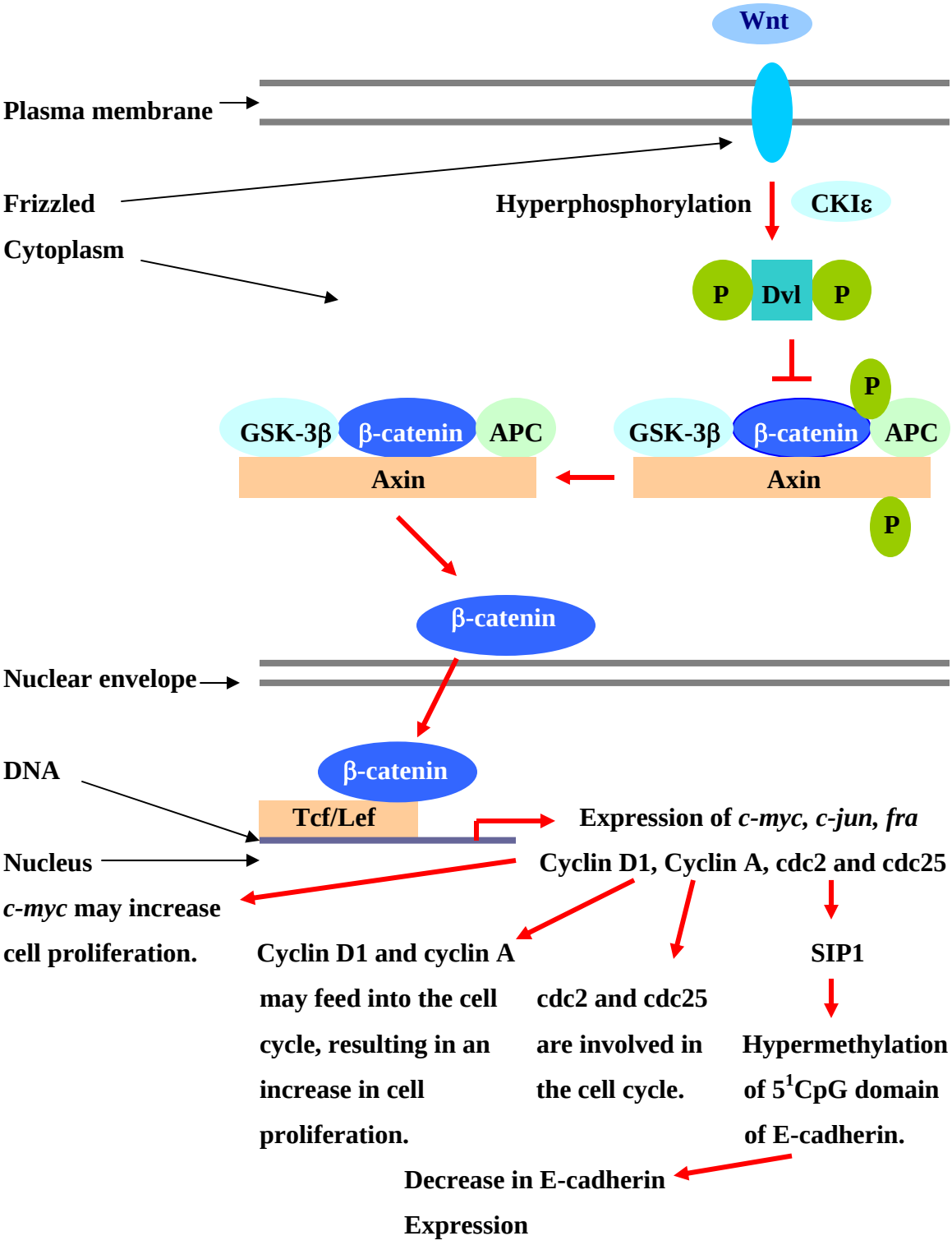


Figure 1.3: The Wnt pathway (Julius et al., 2000, Kikuchi, 2000, Liu et al., 1999, Novak and Dedhar, 1999, Seidensticker and Berherns, 2000). See next page for legend…

Wnt binds to the membrane receptor frizzled (Novak and Dedhar 1999). This then recruits dishevelled (Dvl), resulting in its hyperphosphorylation by Casein Kinase Iε (CKIε). The hyperphosphorylated Dvl, then blocks axin's function and inhibits GSK-3β. This results in the dissociation of the β-catenin destruction complex. β-catenin then translocates to the nucleus and enters via its armadillo repeats (Ikeguchi et al., 2001). Once inside the nucleus, it binds to Lef/Tcf, and acts as a transcription factor. This results in the transcription of the following genes: c-myc, c-jun, fra, cyclin D1, cyclin A, cdc2 and cdc25. Cyclin D1, cyclin A, cdc2 and cdc25 all feed into the cell cycle and may result in an increase in cell proliferation. SIP1 (Smad-interacting, multi-zinc finger protein I) hyper-methylates a domain in the promoter of E-cadherin resulting in a decrease in E-cadherin expression.

The genes activated by β-catenin-Tcf play key roles in the process of cell proliferation. Cyclin D1 is active in the G1 (Gap 1) phase of the cell cycle. An excess of cyclin D1 in the cell stimulates the activity of cdk4. The cdk4-cyclin D1 complex results in the cell progression from the G1 phase to the S phase of the cell cycle (Morgan *et al*., 1999). This may result in the loss of cell cycle control at the G1/S checkpoint, resulting in uncontrolled cell proliferation (Morgan *et al*., 1999).

β-catenin-Tcf/Lef activated genes could increase cell proliferation and decrease cell adhesion as follows. The E-cadherin gene is one of the genes regulated by the β-catenin/Tcf complex. The β-catenin/Tcf complex down regulates the expression of E-cadherin (Kallakury *et al.,* 2001). This decrease in the levels of E-cadherin may result in a decrease in the levels of adherens junctions. Importantly, it was found that a decrease in α-catenin levels correlated with a decrease in E-cadherin levels (Kallakury *et al*., 2001). The decrease in E-cadherin and α-catenin levels, may lead to a further decrease in the levels of adherens junctions. These changes in cell-cell adhesion and proliferation have been implicated in the formation of a tumour and/or a tumours progression to the metastatic phenotype. The levels of β-catenin-Tcf/Lef in a cell are regulated by the level of soluble β-catenin (Omer *et al*., 1999). It is therefore important that the levels of free β-catenin are carefully regulated. β-catenin may be regulated by the sequestration of it, by α-catenin (Giannini *et al.,* 2000). It is therefore crucial that α-catenin is degraded in the correct manor in order to prevent any aberrant molecules disrupting the α-catenin/β-catenin interaction.

1.4 α-CATENIN PROCESSING

Proteins have set lifetimes, resulting in cells being continuously busy synthesising or degrading them (Voet and Voet, 1995). It is also important that proteins involved in signalling are degraded once they have completed their signalling role, so the same pathway is not continually reactivated. There are numerous systems for the degradation of proteins. β-catenin's degradation system has been well studied, and was mentioned in

the previous section (section 1.3). However the details of the processing of α-catenin are as yet not clear. Takahashi and co-workers (2000) discovered that free cytoplasmic α-catenin was actively degraded within 5 hours after protein synthesis. This indicates that α-catenin may have a high turn over rate. α-catenin has been found to contain a PEST sequence, suggesting that it may be targeted by calpains or the proteasome degradation system (Takahashi *et al.,* 2000). However, neither of these systems could be implicated in the degradation of α-catenin (Takahashi *et al*., 2000). Experimental observations have indicated that α-catenin may be degraded by a ubiquitous proteolytic system (e.g. lysosomes) or an as yet uncharacterised protein degradation system (Takahashi *et al*., 2000). Never the less, cytoplasmic α-catenin, plays an important role in sequestering β-catenin and regulating its signalling abilities.

1.5 α-CATENINS REGULATION OF β-CATENIN LEVELS

It is important that signal transduction pathways are closely regulated, so as to prevent aberrant signalling. As shown in section 1.3, aberrant activation of the Wnt pathway, may result in an increase in cell proliferation (Seidensticker and Berherns, 2000). For this reason it is important that the levels of β-catenin are tightly regulated. As demonstrated in section 1.2.3, α-catenin may sequester β-catenin at the adherens junctions. Once α-catenin binds β-catenin, β-catenin is prevented from entering the cytoplasm thus standing the chance of participating in the Wnt pathway. α-catenins regulation of β-catenin was highlighted in colon cancer (CC) cell lines lacking α-catenin expression. These cells showed an increase in β-catenin/Tcf dependent transcription (Giannini *et al*., 2000).

α-catenin may also regulate the levels of unbound β-catenin in the cytoplasm. α-catenin in the cytoplasm may sequester β-catenin (Giannini *et al*., 2000). Over expression of α-catenin resulted in α-catenin binding to cytoplasmic β-catenin and disrupting β-catenin mediated Wnt signalling (Nagafuchi, 2001). The sequestration of β-catenin by α-catenin may pose another mechanism, were by β-catenins' signalling could be regulated. Due to

the deleterious outcomes of the inappropriate activation of signalling pathways, such as the Wnt pathway, there are several mechanisms in place to keep these pathways in check (Seidensticker and Berherns, 2000). Cancer cells often exhibit a breakdown of these control systems, especially when the oncogenes involved, are growth factors, which require signal transduction to excert their effect on cell processes (e.g. cell growth).

1.6 GROWTH FACTORS AND THEIR EFFECTS ON CELL ADHESION AND SIGNALLING

Growth factors are extracellular signalling proteins, which regulate cell processes such as cell growth, proliferation, differentiation and survival (Alberts *et al.*, 1998). Growth factors may be tissue specific, an example being epidermal growth factor (EGF), which is found to act on epithelial cells (Kingston *et al.*, 2003). EGF may bring about a wide range of changes in the cell including cytoskeletal reorganisation and a decrease in cellular proliferation (Solic and Davies, 1997). Therefore aberrant signalling by EGF may hold important implications for cell adhesion.

Due to the large numbers of adherens junctions found in epithelial cells and the fact that EGF specifically acts on these cells, epithelial cells are an important tissue to study. The epidermal growth factor receptor (EGFR) is a transmembrane protein receptor with tyrosine kinase activity (Andl *et al.*, 2003). Signalling via the EGF pathway may result in a plethora of cellular changes, ranging from cytoskeletal reorganisation to cell rounding (Figure 1.4) (Solic and Davies, 1997). A key outcome of EGF signalling is a decrease in cellular adhesion (Solic and Davies, 1997).

Tumour progression has largely been associated with changes in the expression of various molecules involved in cell adhesion and growth (Nicolson, 1991). Growth factors and their receptors are not immune to alterations through genetic mutation. Changes in growth factor receptors have been frequently observed in a large number of tumours (Shiozaki *et al.*, 1995). In particular, EGF has been found to be frequently altered or over expressed in malignancies of squamous cell origin (Andl, 2003).

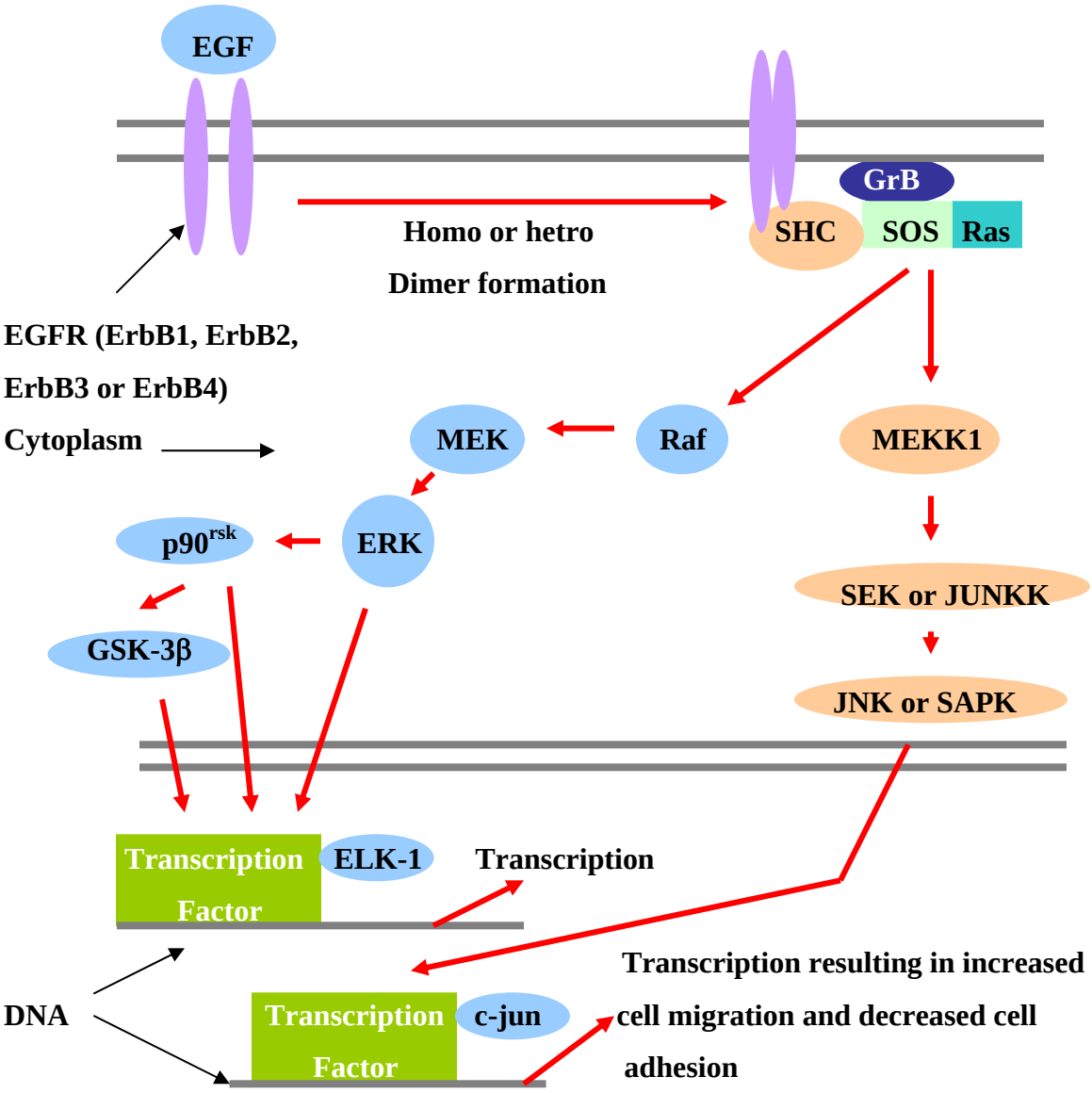


Figure 1.4: The EGF pathway (Andl et al., 2003, Lu et al., 2001, Hoschuetzhy, Aberle and Kemler, 1994). EGF binds to EGFR resulting in the EGFR forming homo or hetro dimers with other EGFR family members. The receptor is autophosphorylated resulting in its activation (Lu et al., 2001, Shiozaki et al., 1995). Adaptor molecules (SHC and GrB) and a guanine nucleotide releasing factor (Sos) are recruited to the dimerized EGFR (Andl et al., 2003). Sos is brought into close proximity with Ras, allowing for its activation. The activated Ras results in the activation of the Erk and Jnk signalling pathways. The activated Erk and Jnk pathways ultimately result in increased cell proliferation, increased cell migration and decreased cell adhesion (Andl et al., 2003).

The EGFR has been found to be overexpressed in premalignant oesophageal squamous dysplastic lesions and in oesophageal squamous cell carcinomas (OSCC) (Andl, 2003, Veale and Thornley, 1989). Patients with OSCC, which over express EGFR, have been found to show poor clinical outcome (Shiozaki *et al.*, 1995). This suggests that EGF may play a role in the malignant process. EGF signalling could bring about some of the alterations (e.g. decrease in cell adhesion) in tumour cells, which contributes to the metastatic phenotype. This, therefore, warrants a closer look into the mechanisms by which EGF alters cell adhesion.

1.6.1 The effects of EGF on cell adhesion

Changes in the number of growth factor receptors have been frequently observed in a large number of tumours (Veale and Thornley, 1989, Hazan and Norton 1998). Alterations in EGF and EGFR have been found in malignancies of squamous cell origin (Andl, 2003). The increased levels of EGFR observed in these malignancies were shown to decrease cell adhesion (Shiozaki *et al.*, 1995 Hazan and Norton, 1998). This decrease in cell adhesion could not be attributed to changes in the expression levels of any of the adherens junction components (Hazan and Norton, 1998 and Shiozaki *et al.*, 1995). This suggests that changes in the levels of cell adhesion could be due to the uncoupling of the adherens junctions. The cell localisation (e.g. plasma membrane) of β-catenin has been shown by Jones and Veale (2003) to be un-altered upon EGF treatment. This suggests that the link between α-catenin and β-catenin may be uncoupled under the influence of EGF, resulting in the reduction in cell adhesion.

1.7 CHANGES IN ADHESIVE PROPERTIES AND SIGNALLING FUNCTIONS OF ADHESIVE MOLECULES

Over the past decade it has been noted that a fundamental role of cell-adhesion receptors is the transduction of signals (Hynes, 1999). Signalling by adhesion receptors has become of such importance that an entire conference in 2003 was devoted to dealing with this

dynamic and rapidly developing field of research (Cell junctions and signal transduction, 42nd meeting of the American Society for Cell Biology, San Francisco, December 2003).

A key example of this is β-catenins signalling via the Wnt pathway (section 1.3). β-catenin plays a key role in the induction of certain genes, which may result in an increase in cell proliferation (section 1.3). An increase in the levels/expression of β-catenin has been linked to an increase in tumour grade and invasiveness (Mialhe *et al*., 1997). However, in order for the cell to complete the cell cycle it must pass certain checkpoints, such as the G1 and G2 (Gap 1 and 2) checkpoints. It has been discovered in OSCC, that genetic changes occur, which disrupt the control of the cell cycle at the G1 checkpoint (Mandard *et al.,* 2000). One of the requirements to pass from the G1 checkpoint into the S phase (synthesis phase) is anchorage control. In this regard, it has been found that there is a change in adhesion and cytoskeletal interactions of transformed epithelial cells (Kinch *et al*., 1995). These changes in adhesion properties of transformed epithelial cells could be linked to changes in α-catenin, which may affect its expression or functionality.

It was observed that non-adherent and invasive carcinoma cells, not expressing α-catenin recovered their adhesiveness upon the exogeneous expression of α-catenin in these cells (Aberle *et al*., 1996). The expression levels of α-catenin, has been found to be frequently reduced in several tumours (Giannini *et al*., 2000, Vermeulen *et al*., 1999). α-catenin has also been found to harbour mutations in a large range of tumours (Giannini *et al.,* 2000, Vermeulen *et al.*, 1999). This serves to further highlight the importance of α-catenin in maintaining the integrity of cell adhesion. Epithelial cells, due to their nature contain large numbers of adherens junctions, making them an ideal cell type in which to study the components of the adherens junctions. OSCC has been found to contain increased levels of EGFR (Andl *et al.,* 2003, Veale and Thornley, 1989). Abnormally high levels of OSCC occur in South Africa, making it a disease of both national and international importance.

1.8 OESOPHAGEAL CARCINOMA: A PROBLEM OF ENORMOUS PROPORTIONS IN SOUTH AFRICA

The oesophagus begins at the circopharyngeus (upper oesophageal sphincter) and ends at the diaphragmatic hiatus (lower oesophageal sphincter) (Gore *et al.,* 1997). It is a muscular tube lined mainly by stratified squamous epithelium (Gore *et al.,* 1997). The oesophagus has no serosa, which helps limit the spread of lacerations or cancers. Oesophageal cancer occurs in two forms, namely squamous cell- and adenocarcinoma (Powell *et al*., 1994; Sharma, 1999). These two variants of oesophageal cancer have different etiological and pathological characteristics. Barrett's oesophagous is a complication of chronic gastro-oesophageal reflux disease, which is a premalignant condition and may be the cause of adenocarcinoma (Sharma, 1999). Oesophageal carcinoma is a slow-growing solid tumour, which usually occurs late in life (between the ages of 45-85 years) (Gore *et al.,* 1997). This arises from an accumulation of genetic changes, which are similar to those occurring in colorectal neoplasms (Powell *et al*., 1994). OSCC is a highly malignant disease, which normally has a very poor prognosis (Morgan *et al*., 1999).

OSCC develops as a result of a sequence of histopathological changes that typically involves oesophagitis, atrophy, mild to severe displasia, carcinoma *in situ* and finally a metastatic tumour (Mandard *et al.,* 2000). In 1997, OSCC was found to account for over 95% of oesophageal malignancies (Gore *et al*., 1997). Males show a higher incidence of OSCC then females. In the male population, the highest incidence of OSCC occurs amongst Black males, followed by Coloured, Asian and White males (http://www.cansa.co.za/registry_oesophageal.asp). Black females show the highest rate of OSCC cases amongst the female population (http://www.cansa.co.za/registry_oesophageal.asp). OSCC also shows striking variations in its geographical distribution (Gore *et al.,* 1997).

In the non endemic parts of the world the incidence rates range from 2.5 to 5 for men and 1.5 to 2.5 for women per 100 000 population (Gore *et al*., 1997). This differs quite

drastically to the endemic parts of the world, where the incidence rates often exceed 100 per 100 000 population (Gore *et al*., 1997). The Transkei region of South Africa, exhibits the highest incidence rate of OSCC in the world (Lu, 2000).

The localised geographical clustering exhibited by OSCC has been the focus of numerous epidemiological studies. It has been found that the cause of OSCC may largely be attributed to environmental rather than hereditary factors (Gore *et al*., 1997). Two main factors in the development of OSCC are alcohol and tobacco consumption (Launoy *et al*., 1997, Saeki *et al*., 2000). The consumption of alcohol and or tobacco, resulted in an attributable risk of 48% and 44% respectively (Powell *et al*., 1994, Saeki *et al*., 2000). This risk depended on the duration of consumption and former consumption had a lesser effect (Launoy *et al*., 1997). Due to the incidence rate and poor prognosis of OSCC patients, research into the biology of OSCC, to gain a better understanding of the disease is crucial.

1.9 OBJECTIVES OF THIS INVESTIGATION

1. To isolate the α-catenin gene from the WHCO1, WHCO3, WHCO5, WHCO6 and SNO OSCC lines and the DLD 1 α- CC cell line.
2. To determine whether the α-catenin gene isolated from the five OSCC and CC cell lines contains any mutations in the vinculin, ZO-1 and α-actin binding sites.
3. Determine the localisation of α-catenin in the OSCC and CC cell lines and estimate the relative levels of α-catenin expression in the plasma membrane and cytoplasm/nucleus of these cell lines.
4. Treat the OSCC cell lines with EGF and determine the effects, which EGF may have on the relative levels of α-catenin expression in these cell lines.
5. To over express α-catenin in the WHCO5 OSCC cell line in order to determine the effects, which over expression of α-catenin may have on signalling pathways and cell adhesion.

CHAPTER 2

INVESTIGATION OF THE α-CATENIN GENE IN OSCC

2.1 INTRODUCTION

Cell adhesion plays an important role in a wide variety of cell processes (Nagafuchi *et al.,* 1991). These cell processes amongst others, include, embryogenesis, morphogenesis, epitheliod morphology and polarization of epithelial cells (Nagafuchi *et al.,* 1991). The important role played by cell adhesion in the above mentioned processes also means, that a loss or breakdown of cell adhesion may hold dire implications in for the cell. One of the diseases, which may occur as a result of a loss or breakdown of cell adhesion, is cancer (Beavon, 2000). It has been speculated that a decrease in cell adhesion is a crucial step in the metastatic process (Beavon, 2000).

Epithelial cells are particularly dependent on cell adhesion. In order to form strong attachments to neighbouring cells, maintain their polarized state and tissue morphology (Nagafuchi *et al.,* 1991). One of the key cell-cell adhesions found in epithelial cells is adherens junctions. The adherens junctions are predominantly made up of cadherens and catenins proteins (Johnson *et al.,* 1993). The cadherens are linked via catenins to the actin cytoskeleton (Johnson *et al*., 1993). α-catenin links the E-cadherin/β-catenin complex to the actin cytoskeleton (Figure 1.1). This link formed by α-catenin, is essential to the adherens junction formation and results in a strong cell-cell adhesion being formed (Vanpoucke *et al.,* 2004).

α-catenin has an open reading frame of 2721 bp, which codes for a 906 amino acid protein (Figure 2.1) (Hirohashi *et al*., 2000, Nagafuchi *et al.,* 1991, Rimm *et al.*, 1994).

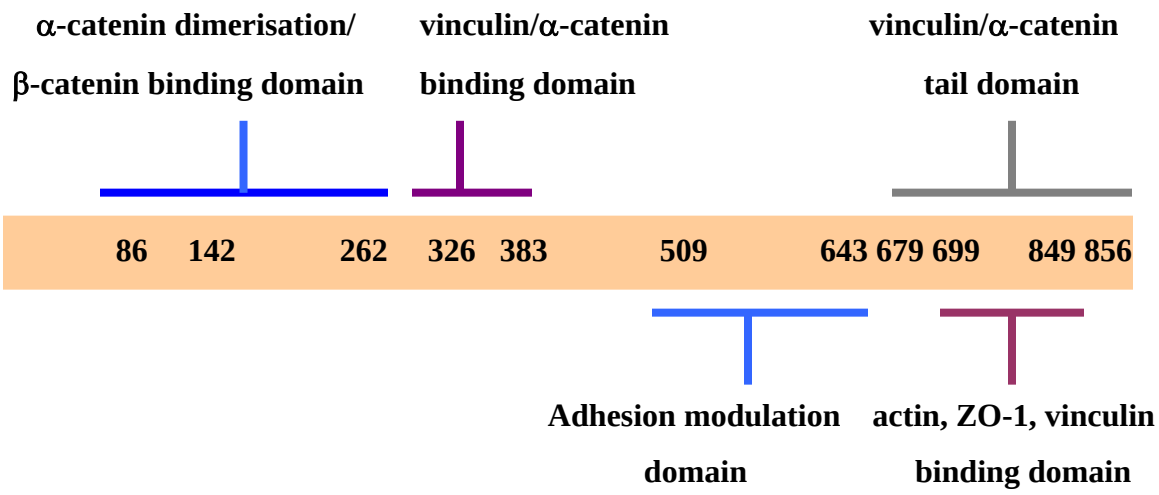


Figure 2.1: Schematic representation of α-catenin amino acid sequence indicating the various binding sites located on it (Yang et al., 2001). This schematic representation depicts the position of the β-catenin, vinculin, actin and ZO-1 binding domains and the adhesion modulation domain (Yang et al., 2001).

α-catenin is coded by the CTNNA1 gene, located on chromosome 5q31 (Tuhkanen *et al.*, 2004). Three tissue specific isoforms of α-catenin have been found, namely α-E-catenin (epithelial), α-N-catenin (neural) and α-T-catenin (testis) (Vanpoucke *et al.*, 2002). α-E-catenin is the form of α-catenin found in epithelial cells (Vanpoucke *et al.*, 2002). The regions of α-catenin responsible for its interaction with the adherens junction components and the actin cytoskeleton have all been mapped (Figure 2.1) (Yang *et al.*, 2001). The β-catenin binding domain is located near the amino terminus of α-catenin (Yang *et al.*, 2001). The binding domains of actin and the associated cytoskeletal proteins (e.g. vinculin, ZO-1 and actin) are located towards the carboxy terminal of α-catenin (Figure 2.1) (Yang *et al.*, 2001).

α-catenin has been found to harbour a large number of mutations in various types of cancers (Candidus *et al.*, 1996, Oda *et al*., 1993, Hirohashi and Kanai 2003). Alterations in α-catenin which affect its functionality could have detrimental effects on the adherens junction formation and so doing contribute to the formation of disease such as cancer (Hirohashi and Kanai, 2003). OSCC is a cancer of epithelial origin (Mandard *et al.*, 2000). This means that there are a large number of adherens junctions found in the oesophageal cells, attaching neighbouring cells to one another and maintaining tissue integrity. It would therefore be important to determine if the α-catenin gene contained in OSCC cell lines harbours any mutations.

2.2 OBJECTIVES

1. To isolate the α-catenin gene from the WHCO1, WHCO3, WHCO5, WHCO6 and SNO OSCC and the DLD 1 α- CC cell lines.

2. To determine whether the α-catenin gene isolated from the OSCC and CC cell lines contains any mutations in the vinculin, ZO-1 and α-actin binding sites.

2.3 METHODOLOGY

2.3.1 Tissue culture

The following five South African moderately differentiated human OSCC cell lines, WHCO1, WHCO3, WHCO5, and WHCO6 (Veale and Thornley, 1989) and SNO (Bey *et al*., 1976) were obtained from the Cell Biology Laboratory, School of Molecular Cell Biology, University of the Witwatersrand. The DLD 1 α- human CC cell line was kindly supplied by Dr. Robert, M. Kypta, from the Medical Research Council Laboratory for Molecular Cell Biology, University College London. The DLD 1 α- cell line lacks expression of α-catenin and was developed from the DLD 1 CC cell line by Dr. D. L. Dexter in 1977 (Matsubara and Ozawa, 2001).

The cell lines were maintained using Dulbecco's modification of Eagles medium:Hams F12 (3:1), supplemented with 10% fetal calf serum (Sigma) (Giannini *et al*., 2000, Nieset, 1997, Veale and Thornley, 1989). The cell lines were grown in a 37^0 C incubator, where the atmosphere was maintained at 5% carbon dioxide (CO_2) in air.

2.3.2 Extraction of RNA

The TRIzol® reagent (GibcoBRL®) is a mono-phasic solution of phenol and guanidine isothiocynate, which can be utilized for the isolation of total RNA from cells (GibcoBRL®). TRIzol® maintains RNA integrity while disrupting and dissolving cell components (GibcoBRL®). RNA was extracted from the following OSCC and CC cell lines: WHCO1, WHCO3, WHCO5, WHCO6, SNO and DLD 1 α-. A dish of confluent cells (70-90%) was taken and washed twice with 2 ml of PBS (appendix 1.1). The cells were removed from the dish and centrifuged in a Tomy Fuge (Seicko). 1 ml of TRIzol® reagent was added to the tube and incubated at room temperature for 5 minutes. Chloroform was used to remove proteins and DNA from the extractions (appendix 1.2). RNA was precipitated by adding isopropyl alcohol (4^0 C) and centrifuging at 12 000 x g and 4^0 C for 10 minutes. 1 ml of 75% ethanol (4^0 C) was added and centrifuged for 5

minutes at 7 500 x g and 4^0 C. The extracted RNA was resuspended in distilled tissue culture water (dH_2O) (SIGMA). All centrifugation steps were carried out using a MC 12V Sorvall Bench top centrifuge (Du Pont). An aliquot of the extractions was separated on a 1% agarose gel (methods 2.3.3)

2.3.3 Visualisation of extracted RNA

A 1% agarose gel was set up (White SCi) (appendix 1.3). Once the gel had set, it was placed inside a Mimi Submarine Agarose gel unit and covered with 1x TAE buffer (appendix 1.4). Ethidium bromide (EtBr) was added to the anode trough and the samples loaded. The samples contained 2 µl of extracted RNA and 4 µl of loading buffer (appendix 1.5). In a separate well a 1 kb+ molecular weight marker (Roche) was loaded, to be used for molecule size determination. Only the most appropriate sizes of the 1 kb+ molecular weight marker were indicated next to the gel.

The gel was run at 70 Volts and 400 mA for one hour. The gel was then viewed using an ultraviolet trans illuminator (Bachofer, Laboratoriumsgerate). An image of the gel was captured using a Direct Screen Instant Polaroid camera (Polaroid) or the BioDoc-It™ System (UVP).

2.3.4 Reverse transcriptase polymerase chain reaction (RT-PCR)

A two-step RT-PCR was performed on the RNA extracted from the WHCO1, WHCO3, WHCO5, WHCO6 and SNO OSCC and DLD 1 α- CC cell lines.

2.3.4.1 Step one, complementary DNA synthesis

Approximately 50 ng of extracted RNA was combined with Random Primers (Roche) and incubated at 70^o C for 5 min. The reaction mixture was added and incubated at 37^o C for 1 hour (appendix 1.6). The MMLV-RT enzyme, was inactivated by heating to 90^o C for 5 min (Rimm *et al.*, 1994).

The phenol-chloroform DNA purification technique is a widely employed DNA extraction technique. Phenol denatures proteins efficiently and any traces of phenol are removed by the inclusion of a chloroform step (Sambrook *et al.,* 1989). A standard phenol-chloroform extraction was performed to extract the cDNA (appendix 1.2). Three times the volume of 100% ethanol and one-tenth of the volume of 3M sodium acetate pH 5.2, was added to the aqueous phase and incubated at -20^0 C for 2 hours (appendix 1.7). The aqueous phase was centrifuged at 12000 x g and 4^0 C for 20 minutes. The pellet was air dried and resuspended in the appropriate volume of dH_2O (SIGMA). All of the centrifugation steps were performed using a Sorvall® MC12V bench top centrifuge (Du Pont). To determine the success of the cDNA synthesis and the phenol-chloroform extraction, an aliquot was separated on a 1% agarose gel (methods 2.3.3).

2.3.4.2 Step two, PCR

The cDNA sequence of α-catenin was amplified using PCR. This region was amplified in order to determine the presence of sequence mutations. In order to amplify the desired area of the α-catenin cDNA sequence contained in the various cell lines, three amplifications were set up utilizing the following sets of primers:

Amplification 1 [α-catenin's 5' terminus (5' term)] (Qiagen):

Forward primer aCAT F1: 5^1 GCGGTCGACCAGCTAGCCGCAG 3^1.

Reverse primer aCAT a3: 5^1 CACCTGTTCCGCAATCTTCG 3^1 (Oda *et al*., 1993).

Amplification 2 [α-catenin's 3' terminus (3' term)] (Qiagen):

Forward primer aCAT 4: 5^1 GAGTGTAATGCTGTCCGCCAGG 3^1.

Reverse primer aCAT p5: 5^1 CGAACGTCGACTGATATTCAGG 3^I.

Amplification 3 [α-catenin's vinculin, ZO-1 and actin binding domains (VZA)] (Qiagen):

Forward primer aCAT s3: 5^1 TCAGAAGCAGGACGAGCGT 3^1 (Oda *et al*., 1993).

Reverse primer aCATp2: 5^1 AAATTCACCACCTGGACTGG 3^1.

The 5^1 term and 3^1 term amplicons, were designed so that there sequebces overlapped to a small degree. A 50 µl reaction mixture was set up and thirty cycles consisting of: 95^o C for 1 minute, 55^o C for 1 minute and 72^o C for 1.5 minutes, were performed (appendix 1.8) (Rimm *et al.*, 1994). The Expand high Fidelity^{plus} PCR System *taq* polymerase (Roche) was used due to its high fidelity in order to minimise errors made by the *taq* polymerase. An aliquot of the fragments produced from the amplifications, were then separated on a 1% agarose gel (methods 2.3.3).

2.3.5 PCR fragment purification

The fragments produced from the VZA amplification (obtained above) were purified using the QIAquick® PCR Purification kit (Qiagen). The fragments were purified in order to remove the enzyme, PCR buffer and mineral oil used in the PCR, so that they did not interfere with the sequencing of the PCR fragments. In order to obtain a higher yield of purified fragments, two identical amplifications were performed per cell line, and once completed, combined (methods 2.3.4.2).

Five times the volume of buffer PB (appendix 1.10) (Qiagen) was added to the PCR reaction mixtures and mixed by briefly vortexing. The samples were then applied to the Qiagen QIAquick® PCR Purification column and centrifuged for 1 min. The flow-through was discarded, the column washed by applying 0.75 ml of buffer PE (appendix 1.11) (Qiagen) and centrifuged for 1 min. The flow-through was discarded and the column re-centrifuged for 1 min. The DNA was eluted by adding 30 µl of dH_2O (Sigma) to the column, incubating at room temperature for 1 min and then centrifuged for 1 min. An aliquot of the purified fragment was separated on a 1% agarose gel. All centrifugation steps were performed at 12 000 x g in a Sorvall MC12V bench top centrifuge (Du Pont).

2.3.6 DNA sequencing

The purified α-catenin fragment (VZA) (obtained above) contained the vinculin, ZO-1 and actin binding sites. This fragment was sequenced in both directions, to ascertain if

there were any mutations contained in this region of α-catenin (nucleotide 2097-2547) (Yang, 2001). The fragments were sequenced in both directions so as to verify the presence of mutations. This bi-directional sequencing would ensure that any mutations arising due to incorrect amplification could be identified. The purified α-catenin fragment obtained from each of the OSCC cell lines was sent to Inqaba Biotechnology for sequenceing. The sequence obtained was then aligned to the α-catenin cDNA sequence using a Basic Local Alignment Search Tool (BLAST) (www.ncbi.nih.nlm.gov/BLAST/) (appendix 1.12-1.16). The BLAST identity numbers were: WHCO1: 1095076474-13232-211923534671.BLASTQ1, WHCO3: 1095408023-15282-194268213219.BLASTQ4, WHCO5: 1095408362-23109-53397524142.BLASTQ4, WHCO6: 1095061154-26209-150951175314.BLASTQ4 and SNO: 1095408746-20108-165043016537.BLASTQ1.

2.4 RESULTS

The success of the TRIzol RNA extraction technique was determined by separating an aliquot of the extracted RNA on a 1% agarose gel (Figure 2.2). Figure 2.2 indicates that RNA was successfully extracted from the OSCC and CC cell lines. The RNA extraction from the WHCO3 and WHCO6 cell lines yielded less RNAThis could be seen by the presence of the 18S and 28S ribosomal RNA (rRNA) subunits, on a light background smear (Wilson and Walker, 1996). The 18S and 28S rRNA subunits were represented by bands at 0.65 kb and 1.5 kb (Voet and Voet, 1995, Jiang *et al.*, 1997) (Figure 2.2). This indicated that the RNA extraction from the OSCC and CC cell lines was of a good quality. The light background smear indicated that only minimal amounts of RNA degradation had occurred during the extraction. The same concentration of from each cell line was used to produce cDNA. The cDNA produced in the first step of the RT-PCR (Figure 2.3) was generally of a high quality. This was evidenced by the size and intensity of the smears present in Figure 2.3. The cDNA produced contained fragments in the size range of 0.2-12 kb (kilo base pairs).

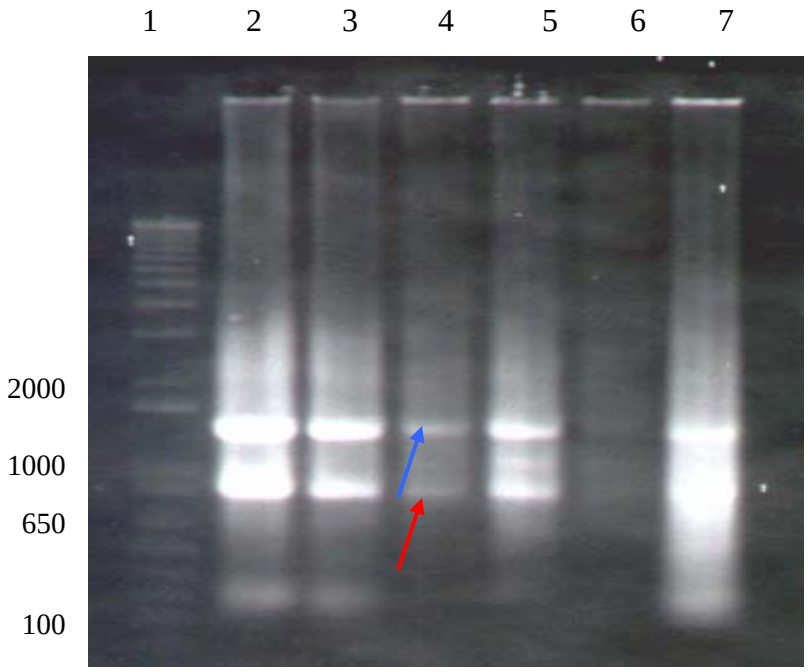


Figure 2.2: Separation of RNA extracted from the OSCC and CC cell lines. RNA was extracted from the following cell lines (lanes indicated above the gel): DLD 1 α- (lane 2), WHCO1 (lane 3), WHCO3 (lane 4), WHCO5 (lane 5), WHCO6 (lane 6) and SNO (lane 7). A 1 kb+ molecular weight marker (Roche) was loaded in lane 1. Two bands of approximately 0.65 kb (red arrow) and 1.5 kb (blue arrow) were present in lanes 2-7.

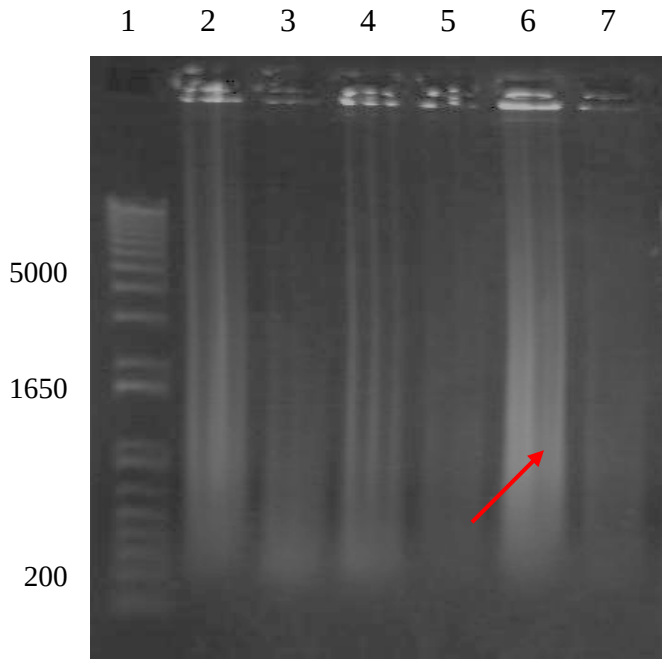


Figure 2.3: Separation of cDNA produced by RT utilising RNA extracted from the OSCC and CC cell lines. cDNA produced from the following cell lines was separated on the gel: DLD 1 α- (lane 2), WHCO1 (lane 3), WHCO3 (lane 4), WHCO5 (lane5), WHCO6 (lane 6) and SNO (lane 7) (lanes indicated above the wells). A 1 kb+ molecular weight marker (Roche) was run in lane 1. A smear in the size range of 0.2-5 kb was produced in lanes 2, 3, 4, 5, 6 and 7 (red arrow).

The size of the α-catenin fragment and the limitations of the DNA sequencing procedure, meant that three different amplifications of α-catenin had to be performed in order to obtain the desired fragments. The first two amplifications (5' term and 3' term) produced fragments, which spanned the entire α-catenin cDNA sequence. The 5' term and 3' term amplifications, amplified regions of α-catenin which slightly overlapped each other.

The third amplification (VZA) was performed to amplify the last 916 nucleotides α-catenin, so as to sequence this portion of the molecule. This amplification produced a shorter fragment so as to increase the specificity of the sequencing reaction. The 5' terminus of the α-catenin (5' term amplification) (from the start codon to nucleotide 2091) was successfully amplified using mRNA as template obtained from the WHCO1, WHCO3, WHCO5, WHCO6 and SNO cell lines (Figure 2.4). As could be seen in Figure 2.4, there were very fewnon-specific fragments produced for all of the amplifications.

The 3' terminus of α-catenin (3' term amplification) (the last 1773 nucleotides of the open reading frame of α-catenin) (a 1.7 kb amplicon) was successfully amplified using mRNA obtained from the WHCO1, WHCO3, WHCO5, WHCO6 and SNO cell lines (Figure 2.4). This fragment was however accompanied by other fragments of varying size, which most probably arose from non-specific primer reactions (Figure 2.4). The intensity of these non-specific fragments decreased slightly upon optimisation of the amplification. The DLD 1 α- CC cell line was used as a negative control since it lacked endogeneous α-catenin expression. All of the amplifications performed utilizing the template obtained from the DLD 1 α- CC cell line yielded no fragments, whilst the control (amplification performed on the SNO cell line) yielded the correct fragments (Figure 2.4). This indicated that the amplifications utilizing RNA extracted from the DLD 1 α- cell line were successful and that this cell line was behaving in accordance to published data (Matsubara and Ozawa, 2001).

The VZA amplification, amplified the region of α-catenin spanning the last 916 nucleotides of the open reading frame. This region of α-catenin was amplified so that the vinculin, ZO-1 and actin binding domains, could be sequenced. A shorter PCR fragment

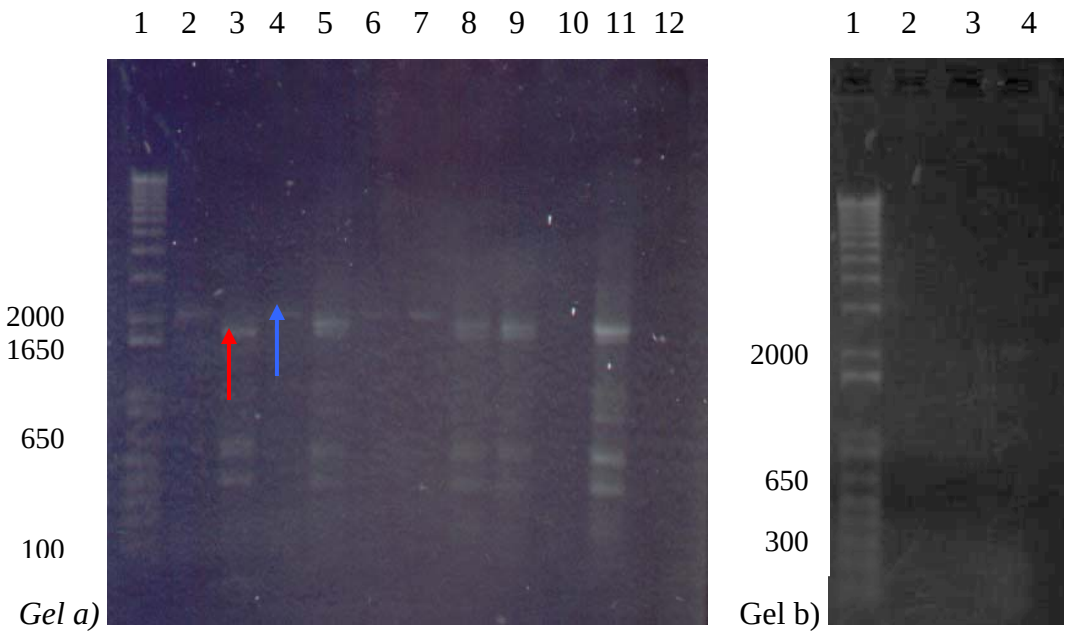


Figure 2.4: Separation of the PCR fragments produced from the 5'term and 3' term amplifications performed on the OSCC and CC cell lines. a) The 5' term amplification of α-catenin produced fragments from the following cell lines (blue arrow) (lanes indicated above the wells): WHCO1 (lane 2), WHCO3 (lane 4), WHCO5 (lane 6), WHCO 6 (lane 7) and SNO (lane 10). The 3' term amplification of α-catenin produced fragments from the following cell lines (red arrow): WHCO1 (lane 3), WHCO3 (lane 5), WHCO5 (lane 8), WHCO6 (lane 9) and SNO (lane 11). b) Neither one of the 5' and 3' term amplifications of α-catenin produced fragments when performed on material extracted from the DLD 1 α- cell line (lane 2 and 3 of gel b). A 1 kb+ molecular weight marker was loaded in lanes 1 on gel a and b respectively. A positive control (SNO 3' term amplification) was loaded in lane 4, gel b. A 2.091 kb band was produced in lanes (a) 2, 4, 6, 7 and 10 (blue arrow). A 1.773 kb band was produced in lanes (a) 3, 5, 8, 9 and 11 and (b) lane 4 (red arrow).

was amplified so as to obtain more accurate sequencing results, because the rate of errors made by *taq* polymerase increases with the length of the fragment amplified. Any errors made by the *taq* polymerase were further eliminated by using, high fidelity *taq* polymerase (Expand high Fidelity^{Plus} PCR system) (Roche) and by sequenceing the fragments bi- directionally. The VZA amplification (produced a 916 bp amplicon) was successfully amplified using template from all five of the OSCC cell lines (Figure 2.5). The amplifications produced the desired amplicon, with some smaller sized non-specific amplicons. The quantity of fragments produced depended on the origin of the template.

A portion of the electrophoretogram obtained for the WHCO6 OSCC cell line is shown in Figure 2.6. The electrophoretic peeks produced on the electrophretogram are quite large and well spaced. The results of the automated DNA sequencing (bi-directional) of the vinculin, ZO-1 and actin binding domains of α-catenin were aligned to published *bona fide* sequences of α-catenin obtained from various tissues using BLAST (www.ncbi.nih.nlm.gov/BLAST/). The BLAST results indicated that the portion of α-catenin containing the vinculin, ZO-1 and actin binding domains as amplified from the WHCO5 and SNO OSCC cell lines contained no mutations (Table 2.1). Three mutations were detected in this portion of α-catenin obtained from the WHCO1, WHCO3 and WHCO6 OSCC cell lines (Table 2.1).

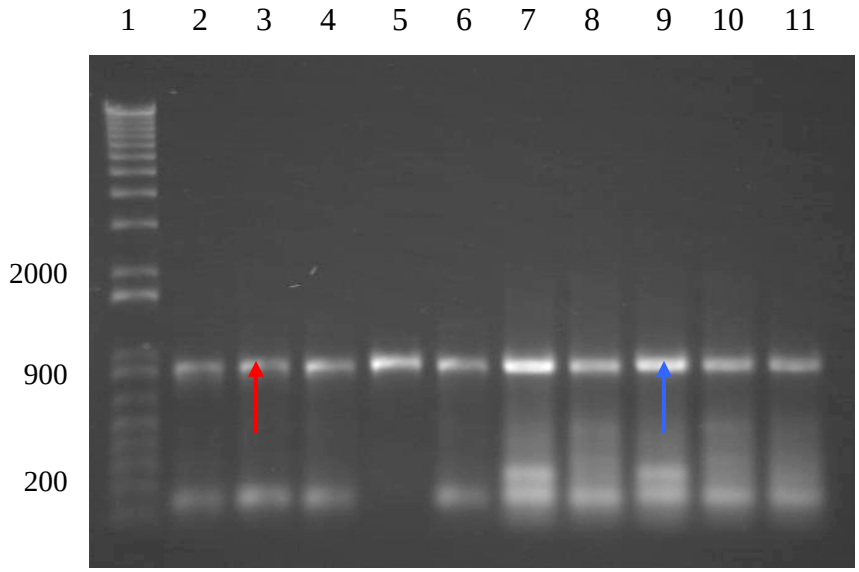


Figure 2.5: Separation of the fragments produced from the VZA amplification utilizing the OSCC cell lines as template and the separation of the same fragments post purification. The VZA amplification of α-catenin yielded fragments from the following cell lines: WHCO1, WHCO3, WHCO5, WHCO6 and SNO OSCC cell lines, were loaded in lanes 2-6 respectively (lanes indicated above the gel). The QIAquick® (Qiagen) purified PCR fragments obtained from the WHCO1, WHCO3, WHCO5, WHCO6 and SNO OSCC cell lines were loaded in lanes 7-11 respectively. A 1Kb+ molecular weight marker (Roche) was loaded in lane 1. A single band of approximately 916 bp was produced in lanes 2-6 (red arrow). A single band of approximately 916 bp was produced in lanes 7-11 (blue arrow).

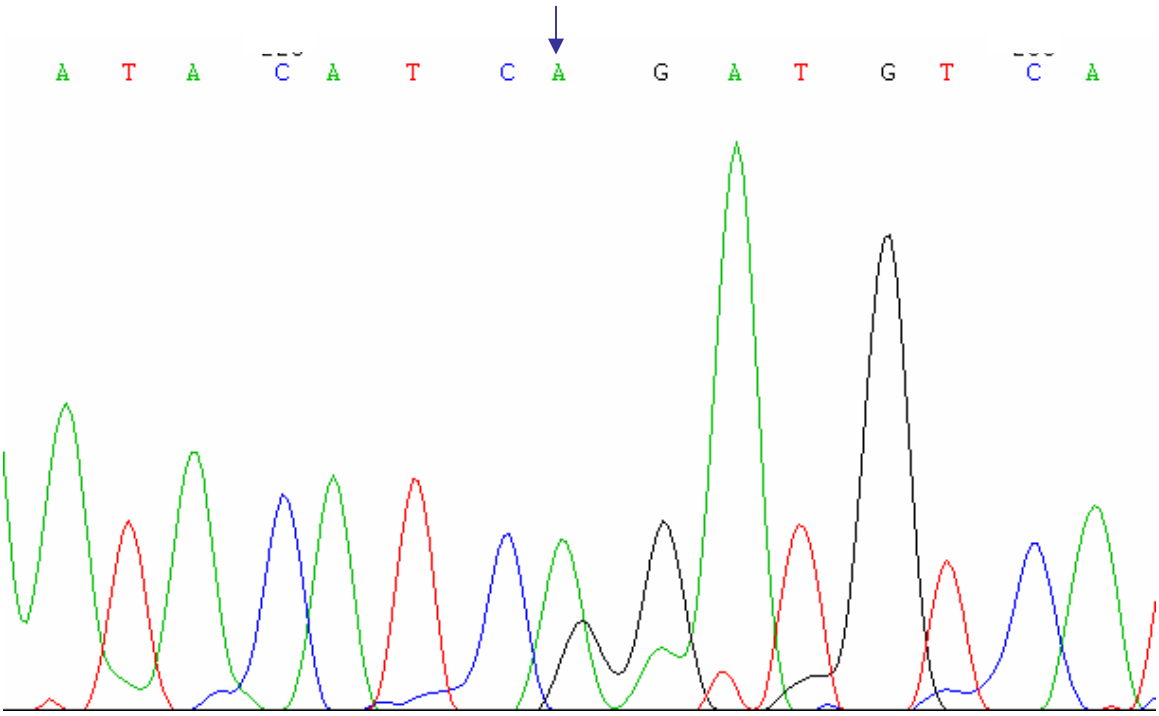


Figure 2.6: Portion of the electrophoretogram obtained from the WHCO6 OSCC cell line containing the DNA sequence depicting the single mutation at nucleotide 2220. The arrow (blue) indicates the mutation located at nucleotide 2220 of α-catenin. This mutation resulted in a G to A mutation. A = adanine, T = thymine, C = cystine and G = guanine. The height and spacing of the peaks produced, indicated that this was a high quality sequence.

Cell line	Presence of mutations	Mutation and location	Change in amino acid sequence
WHCO1	Yes	T to C (2384)	Val to Ala
WHCO3	Yes	C to A (2346)	Arg to Arg
WHCO5	No		
WHCO6	Yes	G to A (2220)	Ser to Ser
SNO	No		

Table 2.1: Mutational status of α-catenin's vinculin, ZO-1 and actin binding domains as amplified from the OSCC cell lines. α-catenin's vinculin, ZO-1 and actin binding domains as amplified from the WHCO5 and SNO cell lines contained no mutations. The above mentioned portion of α-catenin as amplified from the WHCO3 and WHCO6 cell lines each contained a single mutation, which did not alter the amino acid sequence of α-catenin. The above mentioned portion of α-catenin as amplified from the WHCO1 cell line was shown to harbour a mutation, which did alter the amino acid sequence of α-catenin. This mutation resulted in a valine being replaced with an alanine.

2.5 DISCUSSION

The RNA extraction from the five OSCC and CC cell lines was successful (Figure 2.2). This was evident by the presence of the 18 S and 28 S rRNA subunits in all of the extractions obtained from the OSCC and CC cell lines (Wilson and Walker, 1996). α-catenin has an mRNA sequence which is 2721 bp long (Rimm *et al.,* 1994). Since the size of the α-catenin cDNA sequence (open reading frame) fell well within the size range of the RNA produced, for all of the samples extracted from the OSCC and CC cell lines, it was concluded that the extracted RNA samples could serve as adequate templates for the RT-PCRs. The less intense smears, which accompanied all of the RNA extractions, indicated that a small quantity of the extracted RNA had been degraded.

Amplification of DNA through the use of PCR has been described as one of the most powerful molecular biological techniques currently in existence (Delidow *et al.,* 1989). It is possible through the use of PCR to amplify a specific region of DNA repeatedly to generate large quantities of the desired DNA region, which could then be used for further analysis (Souaze *et al.,* 1996, Delidow *et al.,* 1989). RT-PCR synthesizes cDNA, using mRNA as template, and then through the use of specific primers amplifies a target region of the cDNA. It is therefore possible through the use of RT-PCR to determine if a gene is being expressed at the mRNA level (Hayward-Lester *et al.,* 1996). The expression, at the mRNA level, of a gene may be an indication that, the gene could be producing mRNA, which may or may not be translated into protein.

The first step of the RT-PCR i.e. cDNA synthesis was successful. This could be concluded due to the presence of the smears of cDNA, which were produced for the RT's performed on the OSCC and CC cell lines (Figure 2.3). The smears were in the size range of 0.3-12 kb (Figure 2.3). The α-catenin cDNA sequence was 2721 bp long (Rimm *et al.*, Kebriaei and Morrow 1994). It was therefore possible to conclude that this cDNA produced, would serve as adequate template for the second step of the RT-PCR.

The open reading frame of α-catenin was amplified (amplifications 5' term and 3' term) in order to test for the presence of any large insertion or deletion mutations, which have been frequently observed (Oda *et al*., 1993). The 5' term amplification produced a fragment, which spanned the first 2091 bp of the open reading frame of α-catenin. The amplification of this region of α-catenin out of the OSCC cell lines indicated that this portion of the molecule was present in these cell lines. Oda and co-workers (1993) discovered two common deletions located between 106-1062 bp and 302-1062 bp. These mutations resulted in 957 bp and 760 bp deletions respectively. The size of the fragments amplified from all of the OSCC cell lines matched the predicted size of the fragments (predicted from the mRNA sequence for α-catenin) (Oda *et al*., 1993). This may indicate that the α-catenin molecule contained in these cell lines did not contain the previously observed deletion mutations. The β-catenin binding domain is located in the 5' region of the molecule (Figure 2.1). Therefore these results may indicate that this binding domain may not have been lost in the α-catenin contained in the OSCC cell lines. Based on these findings, the interaction between α-catenin and β-catenin should for the larger part be unaltered in the OSCC cell lines studied.

The 3' term amplification, amplified the last 1773 bp portion of the α-catenin open reading frame. This fragment was successfully amplified from all five of the OSCC cell lines (Figure 2.4). The size (1773 bp) of the amplified fragments matched the predicted size of the PCR fragments (Oda *et al*., 1993). This indicated that these cells contained the 3' terminus of the α-catenin mRNA fragment and that it lacked any large insertion or deletion mutations. Together with the results obtained for the first amplification, it was possible to conclude that α-catenin was present in the five OSCC cell lines and that it was being expressed at the mRNA level in these cells. The DLD 1 α- CC cell line was shown to lack endogeneous expression of α-catenin (Matsubara and Ozawa, 2001, Giannini *et al*., 2000). This was confirmed by the PCR results.

The vinculin, ZO-1 and actin binding domains of α-catenin were amplified using high fidelity *taq* polymerase (Expand high Fidelity^{Plus} PCR system) (Roche), in order to

eliminate amplification errors made by the *taq* polymerase. The fragments were sequenced bi-directionally to detect any errors made by the *taq* polymerase. The DNA sequencing and BLAST alignments, revealed that α-catenin's vinculin, ZO-1 and actin binding domains for the larger part appeared to be unaltered in the OSCC cell lines studied. However, three of the OSCC cell lines did harbour single base pair mutations in this region of α-catenin. These cell lines were; WHCO1, WHCO3 and WHCO6 (Table 2.1). The WHCO6 cell line harboured a single mutation at nucleotide 2220. This mutation resulted in a G to A mutation. This mutation however, did not alter the amino acid sequence of α-catenin in any way. Therefore this mutation resulted in a silent mutation. Other researchers have found nucleotide 2220 of α-catenin, to be frequently altered, resulting in a change in the amino acid sequence of α-catenin in breast carcinomas (Candidus *et al.*, 1996). The WHCO3 cell line contained a single base mutation at nucleotide 2346. This single mutation resulted in a C to A mutation. The amino acid sequence of α-catenin in this cell line was not altered in anyway. Therefore, this single mutation resulted in a silent mutation.

The WHCO1 cell line contained a single base mutation at nucleotide 2384. This mutation occurred in the region of α-catenin, which contained the vinculin, ZO-1 and actin binding domains (Figure 2.1). This mutation resulted in a T to C mutation. This change in the nucleotide sequence resulted in a change in the amino acid sequence. A valine was replaced with an alanine. Valine and alanine are both amino acids with non-polar side chains (Voet and Voet, 1995). Alanine and valine have short aliphatic hydrocarbon side chains (Voet and Voet, 1995). There is only a small difference in the size of the side chains of the two amino acids. Thus their may be no difference between the two amino acids with respect to there molecular crowding. Both amino acids display the same charge characteristics and polarity. Taking these facts into account, it is possible to conclude that this change in amino acid sequence may have no effect on the functionality of this portion of the α-catenin molecule, since there is no change in the chemical properties between the two amino acids. Due to these two amino acids lacking any charged groups, they may only play a minor role in the interaction between α-catenin and vinculin, ZO-1 or actin. Based on this, it could be concluded that the α-catenin molecule

contained in the WHCO1 cell line, may be functional even though it contains a single mutation.

Three out of the five OSCC cell lines harboured single mutations located in α-catenin's vinculin, ZO-1 and actin binding domains. These single mutations however, did not seem to result in any loss of function of the α-catenin protein. Therefore, it may be concluded that α-catenin's vinculin, ZO-1 and actin binding sites were still functional in all of the OSCC cell lines. These findings indicated that the α-catenin contained by the OSCC cell lines is likely to be functional. The findings also suggested that the interaction between α-catenin and the actin cytoskeleton (via vinculin, ZO-1 and actin) might have been unaffected in the OSCC cell lines, due to the lack of mutations, which affected the amino acid sequence in this region of the molecule. This would mean that α-catenin might be able, to have fulfilled its crucial role in adherens junction formation. It is therefore not possible, that an alteration in α-catenin preventing it from interacting with the actin cytoskeleton could have attributed to a reduction in cell adhesion observed in the metastatic OSCC cells.

α-catenin has been found to harbour mutations ranging from single nucleotide mutations to insertion and deletion mutations, in a wide range of cancers (Candidus *et al.*, 1996, Oda *et al*., 1993). These mutations may have affected α-catenin's interaction with the adherens junction components. This breakdown in cell adhesion may have attributed to the metastatic phenotype displayed by these cells (Candidus *et al.*, 1996, Oda *et al.*, 1993). This did not seem to be the case in the OSCC cell lines studied. The localisation and relative levels of α-catenin expression may shed more light on the functionality of α-catenin in these OSCC cell lines.

CHAPTER 3

CELL LOCALISATION AND RELATIVE LEVELS OF α-CATENIN PROTEIN EXPRESSION

3.1 INTRODUCTION

Cell adhesion has been shown to play a role in a wide range of cellular processes (Hollande *et al.,* 2002). Epithelial cells in particular are one of the cell types, which have a strong dependence on cell adhesion. One of the key cell-cell adhesions found in epithelial cells is adherens junctions (Nieset *et al.,* 1997). The adherens junctions are primarily made up of cadherins and catenins (Figure 1.1) (Pradahan *et al.,* 2001). The catenin component of the adherens junctions consists of α-catenin and β-catenin (Wijinhoven *et al.,* 1999). α-catenin is essential for the formation of the adherens junctions (Hirohashi and Kanai, 2003). α-catenin has been predicted to have a secondary structure consisting of two α-helical regions, separated by a loop at amino acid positions 113-122 (Huber *et al*., 1997). In solution, α-catenin forms a homodimer, which is disrupted upon the binding of β-catenin (Pukutta and Weiss, 2000). α-catenin and β-catenin assemble into a 1:1 hetero-dimeric complex (Aberle *et al*., 1994, Pukutta and Weiss, 2000). This interaction is mediated by a homophilic interaction mechanism and the phosphorylation of Tyr 142 of β-catenin (Huber *et al.,* 1997, Piedra *et al*., 2003).

If α-catenin is to fulfil its role in cell adhesion, it must be located at the plasma membrane. Numerous molecules in cells may have duel functions, depending on their cell locations (Seidensticker and Berherns, 2000). A prime example of this is α-catenin. At the plasma membrane α-catenin forms an essential component of the adherens junctions and so doing plays an adhesive role (Giannini *et al.,* 2000). On the other hand, when localised to the cytoplasm, α-catenin may regulate the levels of β-catenin, and in

this manner regulate the Wnt signalling pathway (chapter 1, section 1.3) (Giannini *et al.*, 2000). In this cell location, α-catenin plays a regulatory role.

Resent studies have shown that α-catenin is also able to enter the nucleus and regulate β-catenin's transcriptional activities (Giannini *et al*., 2000, Giannini *et al*., 2004). α-catenin, however, lacks a nuclear localisation signal and is unable to diffuse through the nuclear pores (Giannini *et al*., 2004). It was found that α-catenin entered the nucleus with the Tcf 4/β-catenin complex (Giannini *et al*., 2004). Once inside the nucleus, it represses the levels of Tcf 4/β-catenin transcription activation (Giannini *et al*., 2000). This could serve as another level of control for the Wnt pathway. α-catenin's cell localisation was found altered in a wide range of tumours (Ropponen *et al.*, 1999, Shiozaki *et al.*, 1994). The cells containing aberrantly localised α-catenin, demonstrated higher cytoplasmic levels of α-catenin than normal (Ropponen *et al.*, 1999, Shiozaki *et al.*, 1994). This indicates that the cytoplasmic localization of α-catenin, may play an important role in the transformation process. Elucidating the location of α-catenin inside the OSCC cell lines and the relative levels of protein expression of α-catenin may aid in the understanding of the status of α-catenin interactions in these cells.

3.2 OBJECTIVES

1. Determine the localisation of α-catenin in the OSCC and CC cell lines and estimate the relative levels of α-catenin expression in the plasma membrane and cytoplasm/nucleus of these cell lines.

3.3 METHODS

3.3.1 Tissue culture

The WHCO1, WHCO3, WHCO5, WHCO6 (Veale and Thornley, 1989) and SNO (Bey *et al.*, 1976) South African moderately differentiated human OSCC cell lines and the DLD 1 α- human CC cell line, were maintained in tissue culture as in chapter 2 (methods 2.3.1).

3.3.2 Antibodies

The primary antibody used in this study to detect α-catenin was a Rabbit anti-alpha-catenin antibody (Zymed). This antibody reacted to α-catenin contained in human, mouse, rat, chicken and Xenopus (Zymed). This antibody had been raised to react with high specificity to a unique epitope displayed by the α-catenin protein (epitope specifications not supplied by the manufacturer) (Zymed). The secondary antibodies used were anti-rabbit fluorescine isothiocyanate (FITC) conjugated antibody (Sigma) and a horseradish peroxidase-conjugated anti-rabbit secondary bond antibody (Sigma, Inc). These antibodies had been raised to bind to the heavy and light chains of the IgG antibodies (Sigma), and was used to detect the presence of the primary (anti-α-catenin) antibody.

3.3.3 Indirect immunoflurescent staining

The cell localisation of α-catenin in the OSCC and CC cell lines was visualised using indirect immunofluorescent staining. Dishes at 70 % confluence of the WHCO1, WHCO3, WHCO5, WHCO6, SNO and DLD 1 α- cell lines were washed with 1 x PBS (appendix 1.1). The cells were removed from the dish by adding 2 ml of trypsin ethylenediaminetetra-acetic acid (EDTA) (Sigma). The detached cells were then counted using a haemocytometer (appendix 2.1). 5 x 10^5 cells/ml were then seeded onto a sterile glass slide. Tissue culture medium was added and the cover slip incubated for 15 hr in a 37^0 C incubator supplemented with 5 % CO_2.

The cover slips were rinsed five times with 1 x PBS and fixed with 4 % paraformaldehyde for 30 minutes (appendix 2.2) (Jones and Veale, 2003). The cover-slips were rinsed with 1 x PBS, covered with 0.25 % Triton X 100 for 10 minutes (appendix 2.3), after which they were rinsed once more and dipped into dH_2O. The Dako® pen was used to draw two wells on each cover-slip. The one well served as the control and the second well served as the experiment. The cells were rehydrated by adding 1 x PBS for 30 minutes. 20 µl of 1:50 rabbit anti-α-catenin primary antibody was added to the experimental wells and incubated at 4^0 C for 2 hr. The same volume of 1x PBS was added to the control wells. The cells were then rinsed with 1 x PBS in order to remove any unbound primary antibody. 20 µl of anti-rabbit FITC conjugated secondary antibody (1:1000) was added to the control and experimental wells and incubated in the dark for 1 hr. Unbound secondary antibody was removed by rinsing with 1 x PBS. The cover-slips were mounted onto clean glass slides using Elvanol mounting agent (appendix 2.4). The cells were viewed using a Zeiss LEM 410 confocal Lazer scanning microscope (FITC excitation 490, emission 525).

3.3.4 Cell fractionation into plasma membrane and cytoplasm/nuclear components

α-catenin plays an integral role in the adherens junctions at the plasma membrane (Hirohashi and Kanai, 2003). Due to this structural role it is necessary to obtain an accurate measure of the plasma membrane expression of α-catenin. Therefore the following extraction was employed. The extraction, extracted all proteins associated with the plasma membrane, hence leaving the cytoplasm/nuclear proteins (sample termed cytoplasm/nuclear fraction) behind to form another sample. The WHCO1, WHCO3, WHCO5, WHCO6, SNO and DLD 1 α- cell lines were grown to 80 % confluence, and washed with 1 x PBS to remove the tissue culture medium. The cells were rinsed with 1 x phenyl-methyl-sulphonyl fluoride (PMSF)/ Trasylol solution and scraped into the remains of the solution (appendix 2.5). The cells were centrifuged at 7 500 x g for 1 minute in a MC12V Sorval bench top centrifuge (Du Pont), to separate them from the

solution and the supernatant discarded. 500 µl of hypotonic buffer (PMSF, trasylol, pepstatin and leupeptin) (appendix 2.6) containing protease inhibitors (appendix 2.7), was added in order to inhibit protein degradation due to the presence of proteases in the samples. The samples were immediately frozen at – 70^0 C so as to prevent protein degradation.

The samples were thawed on ice and dounce homogenized with 30 strokes of an eppendorf-dounce homogeniser. The homogeniser was rinsed with 50 µl of hypotonic buffer. The homogenates were transferred to polycarbonate ultra-centrifuge tubes, and the eppendorf tubes rinsed with 50 µl of hypotonic buffer in order to remove any remaining extract. The samples were centrifuged in a Beckmann L7 Ultracentrifuge using a 50 Ti Beckmann (Beckmann) rotor at 100 000 x g and 4^0 C for 30 minutes. The supernatants contained the cytoplasm/nuclear fraction of proteins. The pellet contained the plasma membrane fraction of proteins and was re-suspended in 500 µl of 0.1 % sodium dodecyl sulphate solution (SDS) (appendix 2.8), and storing at 4^0 C for 12 hr in order to re-suspend.

3.3.5 Lyophilisation of plasma membrane and cytoplasm/nuclear fractions

In order to increase the concentrations of the plasma membrane and cytoplasm/nuclear extracted protein fractions, the fractions were lyophilised. The plasma membrane and cytoplasm/nuclear samples (methods 3.3.4) were snap frozen by placing in liquid nitrogen for 5 minutes. The samples were then lyophilised by placing into an Edwards Freeze dryer for 6 hours. The lyophilised fractions were then resuspended in dH_2O (Sigma) and stored at – 20^0 C.

3.3.6 Estimation of concentration of proteins in the lyophilised samples

The concentration of the proteins found inside the lyophilised plasma membrane and cytoplasm/nuclear fractions were determined, so that equal concentrations of proteins

could be used in the Western blot analysis of the fractions (Bramhall *et al.,* 1969). This method to estimate protein concentration was used because it allowed for the estimation of protein concentration in samples, which contained high concentrations of detergents. A Whatman filter paper disc for each of the samples was placed in dH_2O for 20 minutes. The filter paper discs were then placed in 95 % ethanol for 5 minutes. This step was repeated using acetone and the discs air dried in a fume hood for 1 hour. The Whatman filter paper discs were washed in this manner to remove any loose fibers from the discs, which may affect the protein concentration estimations.

5 μl of each fraction along with the appropriate volume of standard (appendix 2.9 and 2.10) was dotted onto the dried Whatman filter paper discs. The discs were air dried in a fume hood and fixed in 7.5 % Tri-chloroacetic acid (TCA) for 40 minutes (appendix 2.11). The filter paper discs were stained with Coomasie Blue stain for 1 hour (appendix 2.12). The excess Coomasie blue was then removed by destaining using an acetic acid: methanol: H_2O solution (10:12:100) for 3 hours. The Whatman filter paper discs were then air dried and the dots of protein excised. The excised dots of proteins were placed in elution buffer (appendix 2.13) for 12 hours in the dark (Bramhall *et al.,* 1969). The absorbancies of the samples along with the standards were read at 595 nm using a Beckman DU-64 Spectrophotometer and the concentration of the extracted proteins calculated (appendix 2.14, and 2.15).

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

SDS-PAGE is the most widely used method for analysing protein mixtures qualitatively. SDS-PAGE denatures proteins into their individual subunits and separates these subunits based on their relative molecular masses (Wilson and Walker, 1996). SDS-PAGE was used to separate the plasma membrane and cytoplasm/nuclear protein fractions in order to isolate α-catenin and detect its presence via Western blot analysis.

A 10 % polyacrylamide gel was used for the separation of the plasma membrane and cytoplasm/nuclear fractions (Laemmli, 1970) (appendix 2.16). The appropriate volumes of plasma membrane and cytoplasm/nuclear fractions (Table 7.1) were mixed with equal volumes of double lysis buffer and boiled for 5 minutes to denature the proteins (appendix 2.22). The fractions were then loaded onto the gel and electrophoresed at 20 mA constant current for approximately 90 minutes (appendix 2.23). The gel was stained with Coomasie Blue stain for 1 hour after which it was distained with an acetic acid:methanol:dH_2O solution (10:12:100) for 12 hours. α-catenin is a 102 kDa protein (Matsubara and Ozawa, 2001). The position of α-catenin on the SDS-PAGE was determined, using the equation obtained from the line fitted to the standard curve of the log molecular weight of the low molecular weight marker (Pharmicia) vs. the distance migrated by the low molecular weight marker (Pharmicia) in the gel ($y = -72.478x + 161.14$ and $R^2 = 0.9957$) (Figure 7.3).

3.3.8 Western blots

The technique of Western blotting employs antibodies raised against unique epitopes of the protein under study. It is therefore possible to determine the presence of the desired protein in a mixed sample of proteins, which have been separated by PAGE. Western blots will be performed on the fractions obtained from the WHCO1, WHCO3, WHCO5, WHCO6, SNO and DLD 1 α- cell lines in order to ascertain whether the α-catenin protein is present in the cell lines.

The plasma membrane and cytoplasm/nuclear fractions obtained from the WHCO1, WHCO3, WHCO5, WHCO6, SNO and DLD 1 α- cell lines were separated using SDS-PAGE (methods 3.3.7) (Giannini *et al.*, 2000). Equal quantities of protein from each cell line were loaded so as to enable the estimation of α-catenin protein expression via Laser scanning densitometry. The area containing α-catenin (2.5 cm, measured from the stacking/separating gel interface) (as previously determined) was excised from the gel. A piece of nitocellulose membrane (Nitro Bind, Osmonics Inc.) the same dimensions as the gel was cut. The proteins were transferred to the Nitrocellulose (Nitro Bind, Osmonics

Inc.) membrane at 50 V and 400 mA for 3 hours using a Trans-blot cell (Bio Rad) (appendix 2.24) (Towbin *et al.,* 1979). Once transferred, the membrane was washed with 1 x PBS (appendix 1.1). The SDS-PAGE gel was stained for 20 minutes with Coomasie blue stain and then destained with an acetic acid:methanol:dH_2O solution (10:12:100) over night. This was done in order to determine the efficiency of the transfer.

The background proteins were prevented from reacting Non-specifically with the antibodies used to probe the nitrocellulse membrane by reacting the membrane with blocking buffer for 40 minutes (appendix 2.25). The membrane was then washed with 1 x PBS. The Western blots were then probed using anti-α-catenin antibody at a concentration of 1:1000 for 1 hour (Giannini *et al*., 2000). The unbound primary anti-α-catenin was removed by washing with 1 x PBS (5 minutes/wash). The primary anti-α-catenin antibody was then detected using a horseradish peroxidase-conjugated anti-rabbit secondary bond antibody. The secondary antibody (1:1500) was reacted for 1 hour in the dark to prevent photo bleaching of the antibody. The unbound secondary antibody was removed by washing with 1 x PBS (5 minutes/wash).

The nitrocellulose (Nitro Bind, Osmonics Inc.) blot was placed in working solution (Pierce) for 5 minutes in the dark (appendix 2.26). The blot was then sealed in polyethylene (glad wrap) and exposed to Hyperfilm^{TM} MP autoradiography film (Amersham, UK) for 3 minutes. The film was then developed for 5 minutes using D19B developer (appendix 2.27). The film was rinsed in dH_2O and fixed for 5 minutes with fixer (appendix 2.28), after which it was rinsed in dH_2O once more.

3.3.9 LASER Scanning densitometry

The intensity of the bands produced by the Western blot analysis was estimated using an LKB Bromma 2202 Ultrascan Laser densitometer (LKB Bromma). Each band was scanned individually using the same reference point and the intensity of the band was printed using a LKB Bromma 2210 Recorder (LKB Bromma). The following settings were used for the densitometer: Scan speed = 10, absorbance range = 1 (O.D), integration

factor = 1 and out put = transmission. The scanning field was adjusted for each band to start scanning 5 mm prior to the band and end 5 mm after the band. This was done so that any background signal on the blot could be more accurately detected and subtracted. The chart recorder was set on the following: chart/printer speed = 1 mm/sec and voltage = 500 mV. The area under each curve was calculated for each scan (appendix 2.29).

3.4 RESULTS

The indirect immunofluorescent staining of α-catenin indicated that α-catenin was expressed largely in the plasma membrane of the OSCC cell lines (Figure 3.1). Strong plasma membrane staining of α-catenin was observed in the WHCO3, WHCO5 and SNO OSCC cell lines (Figure 3.1, b, c and e). The WHCO6 cell line yielded plasma membrane α-catenin staining accompanied by large quantities of cytoplasmic α-catenin staining (Figure 3.1, d). No α-catenin staining was observed in the OSCC negative control (Figure 3.1, g). The DLD 1 α- CC cell line produced no α-catenin staining. The results obtained from the DLD 1 α- CC cell line paralleled those of other researchers (Giannini *et al.*, 2000).

The SDS-PAGE separation of the plasma membrane and cytoplasm/nucleus fractions obtained from the WHCO1, WHCO3, WHCO5, WHCO6, SNO and DLD 1 α- cell lines could be seen in Figures 3.2 and 3.3. The same banding pattern was produced by all of the OSCC cell lines studied (Figures 3.2 and 3.3). No smearing was present in any of the samples run (Figures 3.2 and 3.3). The smears (e.g. Figure 3.2 lane 4 and Figure 3.3 lane 5) did however differ slightly in their intensity, indicating differences in protein concentration. This was corrected for in subsequent experiments.

The presence of α-catenin in the plasma membrane and cytoplasm/nuclear fractions obtained from the OSCC and CC cell lines was analysed using Western blot (Figure 3.4). α-catenin is a 102 kD protein (Matsubara and Ozawa, 2001).

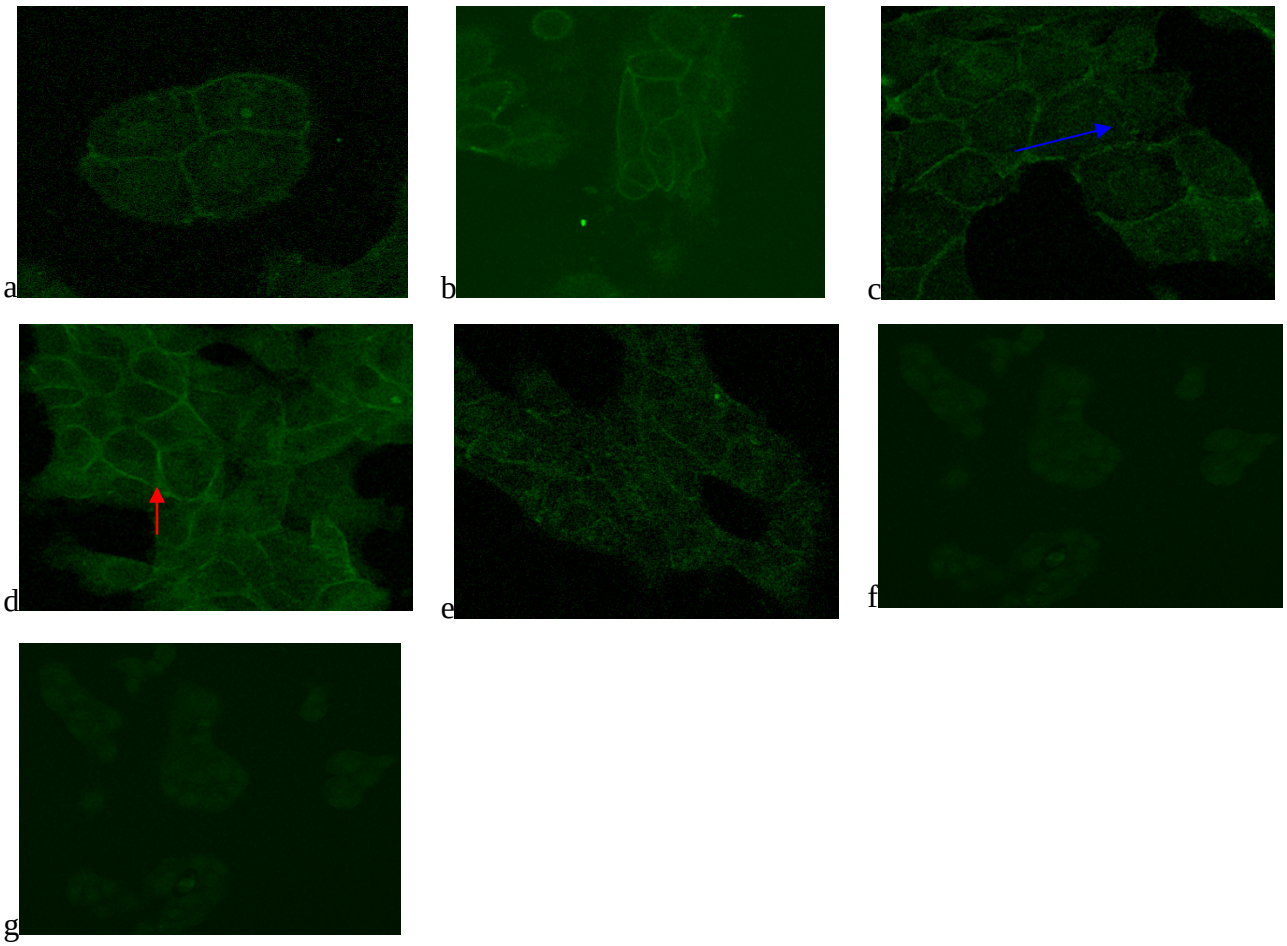


Figure 3.1: Indirect immunofluerescent staining of α-catenin in the OSCC and CC cell lines. *The localisation of α-catenin was determined in the following cell lines: WHCO1 (a) (Mag. x280), WHCO3 (b) (Mag. x190), WHCO5(c) (Mag. x260), WHCO6 (d) (Mag. x180), SNO (e) (Mag. x150) and DLD 1 α- (f) (Mag. x250). Plasma membrane α-catenin staining was observed in the WHCO1, WHCO3, WHCO5, WHCO6 and SNO cell lines (indicated by the red arrow). Slight cytoplasm/nuclear α-catenin staining was observed in all of the OSCC cell lines studied, with the WHCO6 cell lines having the highest levels (indicated by the blue arrow). No staining was detected in the OSCC negative control (g) (Mag. x). No α-catenin staining was detected in the DLD 1 α- CC cell line. The DLD 1 α- cell line served as a control, since it had been previously proven to lack α-catenin expression (Giannini et al., 2000).*

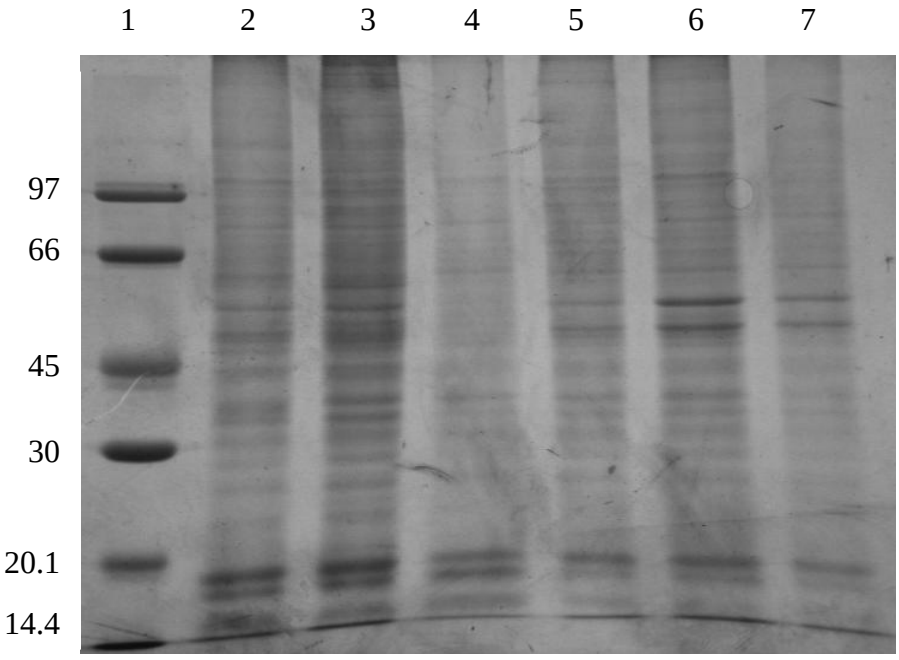


Figure 3.2: 10% SDS-PAGE separation of plasma membrane fractions obtained from the OSCC and CC cell lines (Laemmli, 1970). The following OSCC and CC cell lines were studied (lanes indicated above the gel): WHCO1 (lane 2), WHCO3 (lane 3), WHCO5 (lane 4), WHCO6 (lane 5), SNO (lane 6) and DLD 1 α- (lane 7). A low molecular weight marker (Pharmicia) was loaded in lane 1. The size of the bands produced by the low molecular weight marker (Pharmicia), was given next to the corresponding band. A clear distinct banding pattern was present for all of the samples run. The band pattern produced in lane 4 (WHCO5) was fainter than the other lanes, indicating that this sample contained a lower concentration of proteins. The concentration of this sample was corrected in the ensuing experiments.

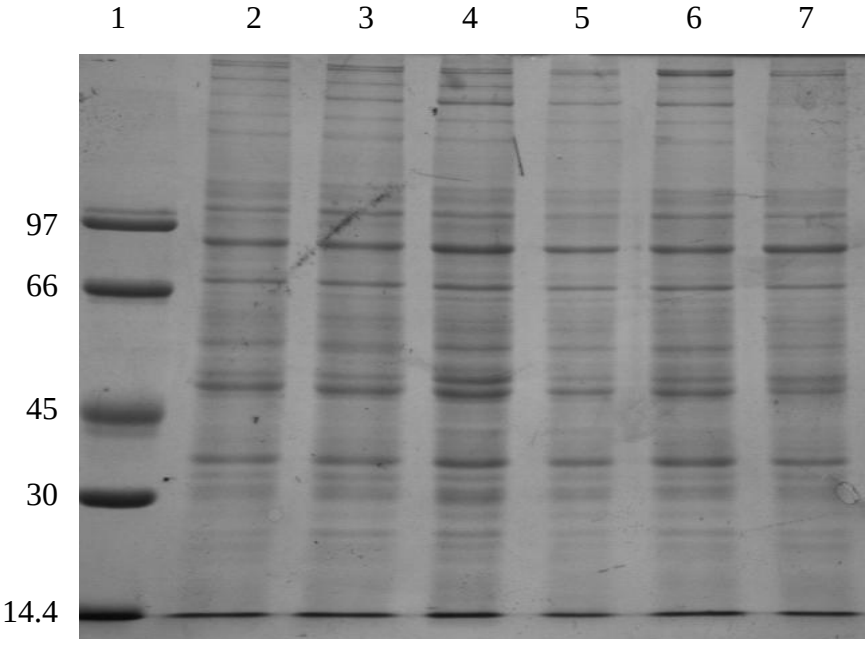


Figure 3.3: 10% SDS-PAGE separation of the cytoplasm/nuclear fractions obtained from the OSCC and CC cell lines (Laemmli, 1970). The following OSCC and CC cell lines were studied (lanes indicated above gel): WHCO1 (lane 2), WHCO3 (lane 3), WHCO5 (lane 4), WHCO6 (lane 5), SNO (lane 6) and DLD 1 α- (lane 7). A low molecular weight marker (Pharmicia) was loaded in lane 1. Clear distinct banding patterns were present for all of the samples run. The band pattern present in lane 5 (WHCO6) was fainter than the other lanes, indicating that this sample contained a lower concentration of proteins. The concentration of this sample was corrected in the ensuing experiments.

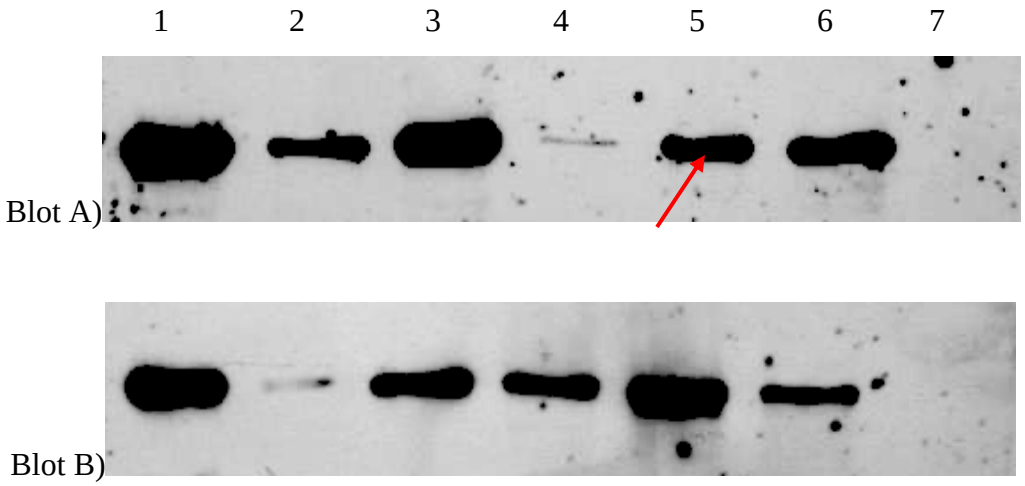


Figure 3.4: Western blots depicting α-catenin expression in the plasma membrane and cytoplasm/nuclear fractions obtained from the OSCC and CC cell lines. Blots A and B, were loaded in the following order: WHCO3 (control used to standardize Western blot results obtained from different Western blots)(lane 1), WHCO1 (lane 2), WHCO3 (lane 3), WHCO5 (lane 4), WHCO6 (lane 5), SNO (lane 6) and DLD 1 α- (lane 7). Blot A contained the plasma membrane fractionates and blot B contained the cytoplasm/nuclear fractions. All of the OSCC cell lines expressed α-catenin (red arrow). α-catenin is a 102 kD protein. The DLD 1 α- CC cell line did not express α-catenin. No other bands were present on the rest of the blots and therefore only this area of the blot was depicted.

A band at the correct molecular weight of α-catenin (α-catenin is a 102 kD protein), was present for each of the OSCC cell lines on the Western blots, indicating that all of the OSCC cell lines expressed α-catenin in the plasma membrane (Figure 3.4, blot A) (Matsubara and Ozawa, 2001). No non-specific bands were present on the blots and therefore only this portion of the blots was depicted. The Western blot analysis of the cytoplasm/nuclear fractions obtained from the OSCC cell lines indicated that α-catenin protein was expressed by all of the cell lines (Figure 3.4, blot B). The DLD 1 α- CC cell line showed no expression of α-catenin in the plasma membrane or the cytoplasm/nuclear fractions.

In order to obtain an accurate estimation of the protein expression in the fractions, and to be able to compare the levels of α-catenin expression, equal amounts of protein from each fraction was used in the respective Western blot analysis. Laser Scanning densitometry was performed on the Western blots, so as to obtain an estimate of the levels of α-catenin expression in the plasma membrane and cytoplasm/nuclear fractions obtained from the OSCC and CC cell lines. The estimated levels of α-catenin expression in the plasma membrane and cytoplasm/nuclear fractions, was calculated as percentages of the expression of α-catenin in the control (WHCO3 membrane fraction) (Figure 3.5). The WHCO3 cell line had the highest levels of expression and was therefore set as the standard at 100%. The WHCO1, WHCO3 and SNO cell lines all contained 10-40% higher plasma membrane levels of α-catenin expression, compared to the cytoplasm/nuclear levels of expression (Figure 3.5). The WHCO5 and WHCO6 cell line however, showed a 10-30% higher level of α-catenin expression in the cytoplasm/nucleus than it did in the plasma membrane (Figure 3.5).

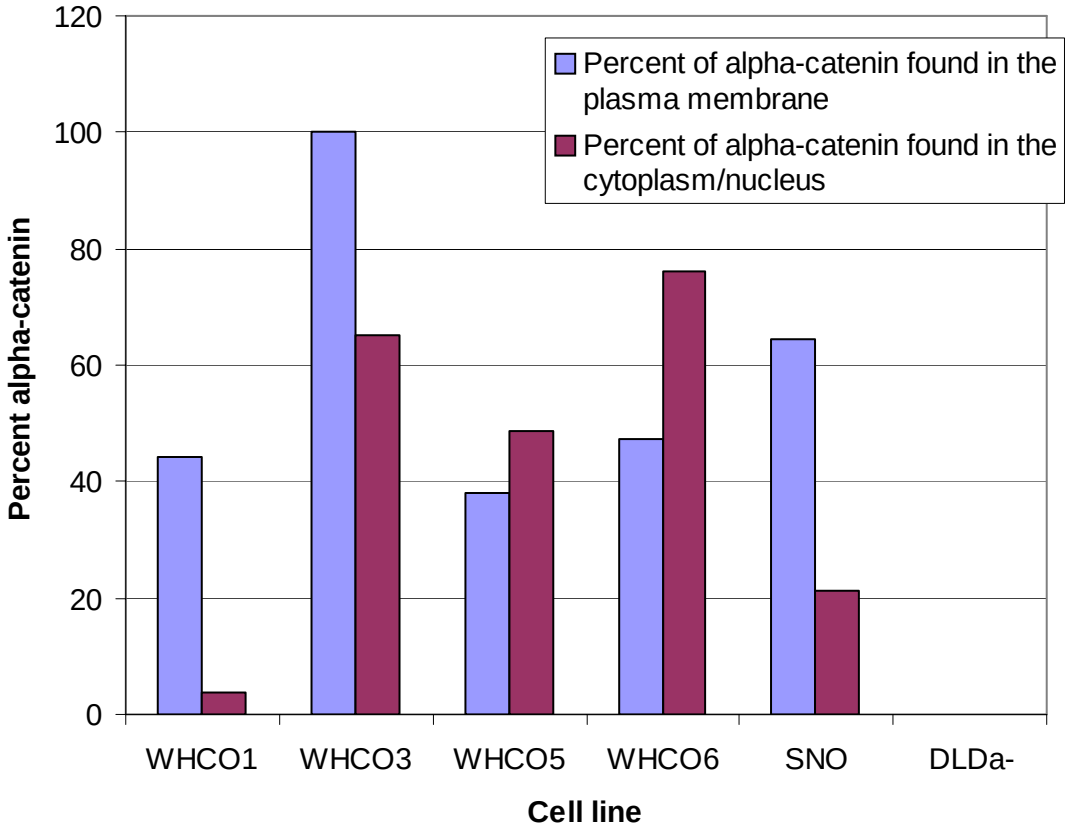


Figure 3.5: Relative levels of α-catenin expression in the plasma membrane and cytoplasm/nuclear fractions obtained from the OSCC and CC cell lines. The WHCO3 cell line contained the highest level of α-catenin expression and was therefore used as a standard for comparison with the other cell lines. The DLD 1 α- CC cell line was used as a control and contained no α-catenin expression. All of the OSCC cell lines except for the WHCO5 and WHCO6 cell line had higher levels of α-catenin plasma membrane expression then what they did in the cytoplasm/nuclear.

3.5 DISCUSSION

Since α-catenin plays a central role in the formation of adherens junctions, it was expected that the indirect immunofluorescent staining of the OSCC cell lines would largely yield plasma membrane staining of α-catenin (Nieset *et al.*, 1997). Indeed, plasma membrane staining of α-catenin was observed in all of the OSCC cell lines (Figure 3.1, a-e). No α-catenin staining was observed in the DLD 1 α- CC cell line (Figure 3.1, f). The DLD 1 α- CC cell line was expected to show no α-catenin staining, since it had been shown to lack endogeneous expression of α-catenin (Ozawa, 2001). The OSCC non-specific control (Figure 3.1, g) was probed only with secondary antibody, in order to determine if the secondary antibody bound non-specifically to any epitopes contained in the OSCC cell lines. The lack of staining indicated that the primary antibody did not bind non-specifically to any epitopes contained by the OSCC cell line. These results verify the specificity of the primary and secondary antibodies and indicated that the antibodies did not bind to other adherens junction components such as β-catenin or E-cadherin. The WHCO3, WHCO5 and SNO OSCC cell lines all exhibited strong α-catenin plasma membrane localisation. These results correlated with those obtained from colorectal carcinoma and oesophageal cancer (Shiozaki *et al*., 1995, El-Bahrawy *et al*., 2002, Ozawa, 1998).

The WHCO6 OSCC cell line yielded both plasma membrane and cytoplasm/nuclear staining of α-catenin. The WHCO1, WHCO3, WHCO5 and SNO cell lines, contained very low cytoplasm/nuclear α-catenin expression. Studies performed, have demonstrated that α-catenin may be found in two locations in cells, namely at the plasma membrane forming part of the adherens junctions or free in the cytoplasm (Giannini *et al*., 2000). Another study into the localisation of α-catenin, in intestinal mucosa also showed that the tumourogenic intestinal mucosa cells displayed a high level of cytoplasm/nuclear α-catenin staining (Ropponen *et al*., 1999). All of the OSCC cell lines studied originated from moderately differentiated OSCC (Veale and Thornley, 1989). This is a broad class of differentiation and could therefore explain the differing results obtained for the

WHCO6 cell line. The WHCO6 cell line may have been relatively more or less differentiated than the other cell lines were, which might have affected the cell expression of α-catenin in various cell compartments (e.g. plasma membrane or cytoplasm/nucleus).

The plasma membrane and cytoplasm/nuclear fractions were separated using SDS-PAGE. A distinct set of bands lacking any smearing was produced for all of the samples separated by SDS-PAGE (Figure 3.2 and 3.3). This indicated that the proteins contained in these extracts were for a large part intact, since degraded proteins would have produced smears on the gels.

One of α-catenin's key functions is the modulation of cell adhesive strength (Nieset *et al*., 1997). This is accomplished through the link which α-catenin forms with β-catenin and the actin cytoskeleton. It was for this reason, that it was expected to observe high levels of α-catenin expression in the plasma membrane samples obtained from the OSCC cell lines. The Western blot analysis confirmed the immunofluorescent results of the OSCC cell lines. α-catenin was shown to be expressed in the plasma membrane in all of the OSCC cell lines (Figure 3.5). These results support work done by Khare and co-workers (1999), who showed using Western blot analysis that α-catenin was membrane-bound. Equal amounts of protein from each fraction were used in the Western blots, so as to enable the estimation of protein concentration of α-catenin expression in each fraction via Laser scanning densitometry. The levels of protein expression of α-catenin differed from one cell line to the next (Figure 3.5). This could be due to the different cell lines having varying levels of cell-cell adhesion and therefore having varying levels of α-catenin expression.

The five OSCC cell lines showed varying levels of cytoplasm/nuclear expression of α-catenin (Figure 3.5). Holding true to the results obtained from the indirect immunofluorescent staining, the WHCO6 cell line showed a 50% higher cytoplasm/nuclear level of α-catenin expression than its corresponding plasma membrane expression. The WHCO5 cell line showed a slightly higher cytoplasmic/nuclear level of

α-catenin. As mentioned earlier, this may have been an effect of the varying levels of differentiation as the cell lines have differing growth characteristics and behaviour.

α-catenin may be present in the cytoplasm/nucleus of the OSCC cells to sequester free β-catenin and compensate for any abnormalities in other molecules (e.g. mutations in APC), which result in β-catenin not being properly degraded. It has been found that mutations in key members of the β-catenin degradation pathway (e.g. APC) may result in increased levels of β-catenin (Kawakara *et al.,* 2000). β-catenin via the wnt signalling pathway may result in an increase in cell proliferation. Previously it was mentioned that α-catenin might regulate the levels of cytoplasm/nuclear β-catenin (Giannini *et al.,* 2000). This is supported by the discovery that the reintroduction of α-catenin into α-catenin deficient cells, resulted in a decrease in proliferation and therefore the attenuation of tumour formation (Giannini *et al.,* 2000).

The heightened cytoplasm/nuclear levels of α-catenin may be, due to the above mentioned reason or due to α-catenin degradation. It has been shown that free cytoplasmic α-catenin is actively degraded within 5 hours after protein synthesis (Takahashi *et al.,* 2000). This may explain the cytoplasmic expression of α-catenin. Ropponen and co-workers (1999) observed that α-catenin accumulated in the cytoplasm of intestinal mucosal tumours, when compared to normal intestinal mucosal cells. The sustained levels of cytoplasmic α-catenin expression observed in a large range of tumours may suggest that α-catenin could be playing a signalling role in these cells.

Due to the increased cytoplasmic α-catenin staining only being observed in tumourogenic cells, one may speculate that α-catenin may play a role in the pathways activated in these cells or in the inhibition of a pathway in order to bring about some of the changes in these cells (Ropponen *et al.,* 1999, Takahashi *et al.,* 2000). Cell adhesion receptors may transduce signals (Hynes, 1999). α-catenin expression has been found to be decreased in prostatic and pituitary adenocarcinomas, colorectal carcinomas and bladder and gastric cancers (Dia *et al.,* 2001, Gofuku *et al.,* 1999, Kallakury *et al.,* 2001, Mialhe *et al.,* 1997). Tumour cells generally have decreased levels of apoptosis, which would increase their

chances of survival (McDonnell *et al.,* 1999, Sarbia *et al.,* 1999, Torzewski *et al.,* 1998). There may therefore be a link between the decrease in apoptosis and the decrease in α-catenin expression, thus linking α-catenin to activation of the apoptotic pathway. This could also be an explanation for the cytoplasm/nuclear levels of α-catenin expression in the OSCC cell lines, since the apoptotic pathway takes place in the cytoplasm (Chen *et al.,* 2001, Matsubara and Ozawa, 2001). Examining the effects on α-catenin, that EGF may exert, via the increase in EGFR, may shed further light on the distribution of α-catenin in the OSCC cell lines.

CHAPTER 4

EFFECT OF EGF SIGNALLING ON α-CATENIN EXPRESSION

4.1 INTRODUCTION

Growth factors are extracellular signalling proteins, which regulate cell processes such as cell growth, proliferation, differentiation and cell survival (Kingston *et al.,* 2003). An important growth factor for epithelial cells is epidermal growth factor (EGF). EGF is a polypeptide consisting of 53 amino acids and contains three internal disulphide bridges (Lu *et al*., 2001). The EGFR is a trans-membrane protein receptor (Andl *et al.,* 2003). The EGFR family is made up of four members, namely EGFR (or ErbB1), ErbB2, ErbB3 and ErbB4 (Kingston *et al.,* 2003). EGF binds to the EGFR with a high affinity, inducing dimerisation (Figure 1.4) (Lu *et al*., 2001). The EGF receptors form a homo - or hetero - dimer complex, with other EGFR family members (Lu *et al.,* 2001). The dimerised EGFR then, through a series of events, activates the Erk and Jnk pathways, which ultimately result in increased cell migration and decreased cell adhesion (Andl *et al.*, 2003, Solic and Davies, 1997) (Figure 1.4). Each combination of EGFR's initiates a distinct signalling pathway by recruiting different Src homolog 2 (SH2) containing effector proteins.

The increased expression of EGFR has been found to be prominent events associated with tumorigenesis and metastasis (Hazan and Norton, 1998). EGF treatment has also been shown to decrease cell adhesion (Shiozaki *et al*., 1995, Hazan and Norton, 1998). Studies have shown that the levels of E-cadherin and β-catenin were not reduced with EGF treatment (Hazan and Norton, 1998). The lack of effect, which EGF treatment had on the levels of adherens junction molecules, indicate that the reduction in cell adhesion due to EGF treatment could not be attributed to a reduction in the expression of the adherens junction components. With this in mind, it could be speculated that the

reduction in adherens junctions, due to EGF treatment, could be due to the uncoupling of the interactions responsible for the formation of the adherens junctions.

A decrease in cell adhesion upon treatment with EGF is accompanied by the tyrosine phosphorylation of β-catenin (Piedra *et al.,* 2003). This tyrosine phosphorylation of β-catenin was speculated to disrupt the interaction between α-catenin and β-catenin, resulting in the uncoupling of the adherens junctions. EGF has been shown to phosphorylate Tyr 654 of β-catenin (Piedra *et al.,* 2003). Tyrosine phosphorylation of Tyr 654 breaks the association between E-cadherin and β-catenin (Piedra *et al.,* 2003). This could result in the uncoupling of the adherens junctions. Studies performed on α-catenin indicated that it was not phosphorylated by EGF (Hoschuetzky *et al.,* 1994). Jones and Veale (2003) discovered that EGF treatment did not affect the plasma membrane location of β-catenin. These findings all lead to the suggestion that the decrease in adherens junction levels upon EGF treatment may be due to alterations in other members of the adherens junctions. In adherens junctions, α-catenin forms a direct link with the actin cytoskeleton (Figure 1.1). Upon EGF treatment and subsequent β-catenin phosphorylation, actin, α-actinin and vinculin dissociated from the adherens junctions (Hazan and Norton, 1998). These findings suggest that the breakdown in the link formed between β-catenin and the cytoskeleton (mediated via α-catenin and its associated proteins) may be the key event in the reduction in cell adhesion as a result of EGF signalling.

The dissociation of α-actinin, vinculin and actin from the adherens junctions and the disruption of the α-catenin/β-catenin interaction through the phosphorylation of β-catenin may result in unbound α-catenin. This unbound α-catenin could then freely move to the cytoplasm, were it may interact with β-catenin or participate in cell signalling pathways. The role which α-catenin may play in the decrease in cell adhesion upon EGF signalling, may therefore, be determined by treating the OSCC cell lines with EGF and studying the effects which this EGF signalling may have on the expression levels and cell localisation of α-catenin.

4.2 OBJECTIVES

1. Treat the OSCC cell lines with EGF and determine the effects, which EGF may have on the relative levels of α-catenin expression in these cell lines.

4.3 METHODS

4.3.1 Tissue culture

The WHCO1, WHCO3, WHCO5, WHCO6 (Veale and Thornley, 1989) and SNO (Bey *et al.,* 1976) OSCC cell lines were maintained in tissue culture as in chapter 2.

4.3.2 EGF treatment

The WHCO1, WHCO3, WHCO5, WHCO6 and SNO OSCC cell lines were grown to 70% confluence. The cells were then treated with EGF to obtain a final concentration of 10 ng/ml (Sigma) (Jones and Veale, 2003). The cells were treated for 1, 6 and 12 hours.

4.3.3 Cell fractionation into plasma membrane and cytoplasm/nuclear components

Upon completion of the EGF treatment of the OSCC cell lines, the plasma membrane (sample containing all of the proteins in the plasma membrane) and cytoplasm/nuclear (the remaining proteins from the plasma membrane fractionation) fractions of proteins were extracted. Therefore refered to as plasma membrane and cytoplasm/nuclear fractions. The protocol used to extract the plasma membrane and cytoplasm/nuclear fractions of proteins from the EGF treated OSCC cells was identical to that used in chapter 3 (methods 3.3.4).

4.3.4 Lyophilisation of plasma membrane and cytoplasm/nuclear fractions

The plasma membrane and cytoplasm/nuclear fractions were lyophilised in order to increase their concentrations. Refer to chapter 3 for the protocol employed (methods 3.3.5).

4.3.5 Estimation of protein concentration

In order to use equal quantities of extracted protein in the Western blots, the protein concentration in the extracted plasma membrane and cytoplasm/nuclear fractions, was estimated. The technique used to estimate the protein concentration was identical to that employed in chapter 3 (methods 3.3.6) (Figure 7.1 and 7.2 and Table 7.2) (Bramhall *et al.*, 1969).

4.3.6 Western blot

α-catenin was detected in the plasma membrane and cytoplasm/nuclear fractionates using Western blot analysis. The same quantities of proteins from each respective fraction were loaded, so as to enable the estimation of α-catenin concentration via Laser scanning densitometry. The samples were first separated on an SDS-PAGE (methods 3.3.7). Once separated α-catenin was detected using Western blot analysis (methods 3.3.8).

4.3.7 Laser scanning densitometry

The relative levels of expression of α-catenin as detected by the Western blot analysis was estimated using Laser scanning densitometry (methods 3.3.9). These relative levels of α-catenin expression were compared to the untreated samples using fold increase. The fold increase was calculated by, dividing the relative level of expression of α-catenin in the treated samples by that of the relative level of expression of α-catenin in the corresponding untreated sample.

4.4 RESULTS

Plasma membrane and cytoplasm/nuclear samples extracted from the EGF treated OSCC cell lines were tested for the presence of α-catenin, via Western blot analysis. The Western blots indicated that α-catenin was present in all of the plasma membrane and cytoplasm/nuclear fractions (Figure 4.1). The levels of α-catenin expression between each cell line, EGF treatment period and respective cell fractions differed between the OSCC cell lines, when compared to the standard (untreated) samples. The WHCO3 untreated plasma membrane fraction demonstrated the highest level of α-catenin expression. It was for this reason that this sample was used as the standard and set at 100%. The same control was used for both the plasma membrane and cytoplasm/nuclear samples, to enable the comparison between these samples.

Laser scanning densitometry was performed on all of the Western blots in an attempt to quantify the levels of α-catenin expression in the EGF treated and untreated samples (Figure 4.2). There was only a small fluctuation in the plasma membrane levels of α-catenin expression observed in the WHCO3, WHCO5, WHCO6 and SNO OSCC cell lines, which was considered to be no change, due to the small sample size not lending itself to to accurate statistical analysis (Figure 4.2, b to e). There however was a substantial increase in the cytoplasm/nuclear levels of α-catenin expression upon EGF treatment of the WHCO1 cell line (Figure 4.2, a). This increase in the cytoplasm/nuclear levels of α-catenin expression exhibited by this cell line, did appear to follow a pattern. This pattern was a steady increase in the cytoplasm/nuclear levels of α-catenin expression between 1 and 6 hr of EGF treatment, which then decreased at the 12 hr time interval. The EGF treatment of this cell line was repeated and produced the same results. No logical explanation could be obtained from the results obtained from the EGF treatment of the WHCO1 OSCC cell line. Therefore it seems that beside the WHCO1 OSCC cell line, EGF appeared to have no effect on the plasma membrane and cytoplasm/nuclear expression of α-catenin in the OSCC cell lines.

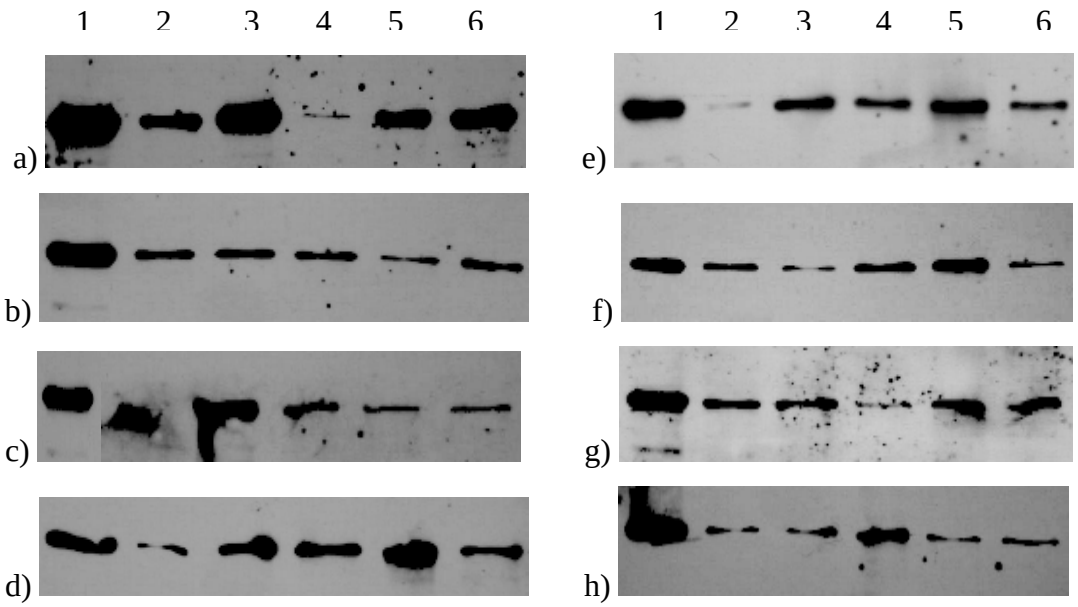
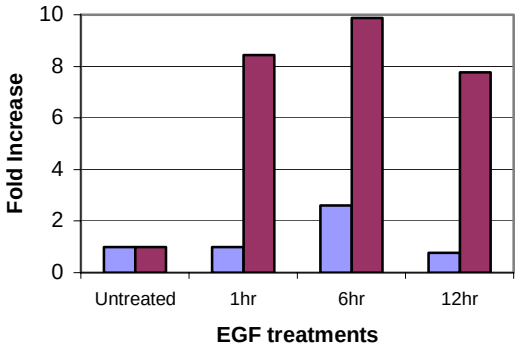
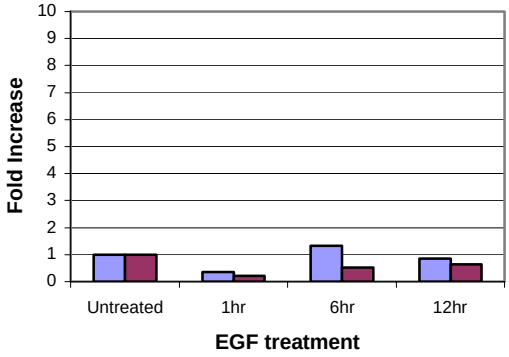


Figure 4.1: Western blots depicting the presence of α-catenin in the EGF treated and untreated plasma membrane and cytoplasm/nuclear fractions. A single band was produced by all of the fractions. All of the blots (a-h) were loaded in the following order: WHCO3 (standard) (lane 1), WHCO1 (lane 2), WHCO3 (lane 3), WHCO5 (lane 4), WHCO6 (lane 5) and SNO (lane 6)(lanes indicated above blots a and e). Blots a) and e) contained the untreated plasma membrane and cytoplasm/nuclear fractions respectively. α-catenin was detected in the plasma membrane fractions obtained from the 1 hr (b), 6 hr (c) and 12 hr (d) EGF treated OSCC cell lines. α-catenin was detected in the cytoplasm/nuclear fractions obtained from the 1 hr (f), 6 hr (g) and 12 hr (h) EGF treated OSCC cell lines.

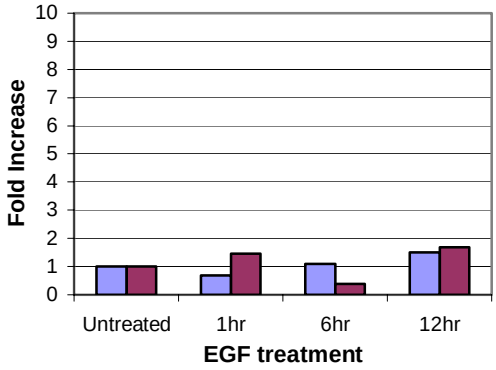
a) WHCO1



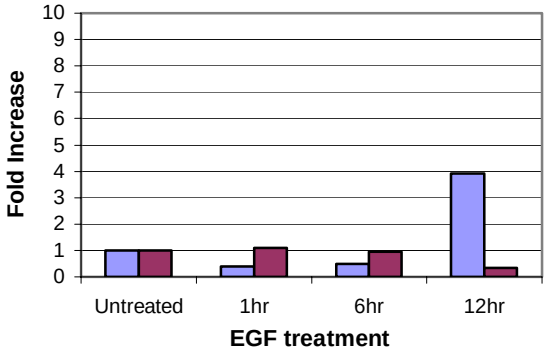
b) WHCO3



c) WHCO5



d) WHCO6



e) SNO

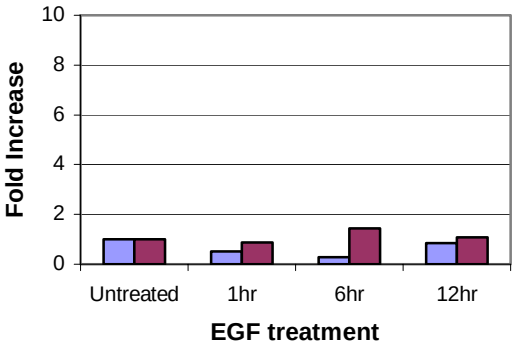


Figure 4.2: Comparison of the relative levels of α-catenin expression in the plasma membrane and cytoplasm/nuclear fractions obtained from the EGF treated and untreated OSCC cell lines. See next page for legend…

The WHCO1 (a), WHCO3 (b), WHCO5 (c), WHCO6 (d) and SNO (e) OSCC cell lines were treated with EGF for 1, 6 and 12 hr. One dish of cells was treated with EGF for each treatment for each respective OSCC cell line. The relative levels of α-catenin expression were compaired to the WHCO3 standard (Figure 3.4 and 3.5). The plasma membrane levels of α-catenin expression were indicated in blue, and the cytoplasm/nuclear levels of α-catenin expression in purple. Upon treatment with EGF a substantial increase in the cytoplasm/nuclear levels of α-catenin expression was observed in the WHCO1 OSCC cell line. EGF treatment of the WHCO3, WHCO5, WHCO6 and SNO OSCC cell lines, resulted in no notable change in the plasma membrane or cytoplasm/nuclear levels of α-catenin expression.

4.5 DISCUSSION

EGF signalling has been shown to result in a decrease in cellular adhesion (Solic, 1997). This decrease in cellular adhesion could result in an increase in the levels of free α-catenin, which in turn may enter the cytoplasm. EGF treatment of the OSCC cell lines for the larger part appeared to have no effect on the α-catenin expression levels in these cell lines. The WHCO3, WHCO5, WHCO6 and SNO OSCC cell lines, exhibited very slight fluctuations in the plasma membrane and cytoplasm/nuclear levels of α-catenin expression upon EGF treatment (Figure 4.2, b to e). Due to sample size not lending itself to accurate statistical analysis, we considered the slight fluctuations in the plasma membrane levls of α-catenin in the WHCO3, WHCO5 and SNO cell line as no change. Unlike the other OSCC cell lines, the WHCO1 OSCC cell line demonstrated marked changes in the expression levels of α-catenin upon EGF treatment.

The WHCO1 OSCC cell line produced conflicting results to the other OSCC cell lines. EGF treatment of the WHCO1 cell line, displayed a trend in the changes in α-catenin expression. The WHCO1 cell line displayed an increase in the cytoplasm/nuclear levels of α-catenin expression upon EGF treatment, which increased with the time of EGF treatment up till 6 hr after which it started to decline (Figure 4.2, a). Whilst the cytoplasm/nuclear levels of α-catenin increased, the plasma membrane levels remained unchanged. The α-catenin gene contained in the WHCO1 OSCC cell line was found to harbour a mutation. This mutation was discovered to affect the amino acid sequence of the vinculin, ZO-1 and actin binding domains of α-catenin (chapter 2). Based on reasons given in chapter 2, it was concluded that this mutation might not have affected α-catenin's binding to vinculin, ZO-1 or actin. However, the effects of EGF treatment on α-catenin expression in this cell line may indicate that this mutation in α-catenin may have an effect on its interaction with vinculin, ZO-1 and actin. This mutation may prevent α-catenin from forming a link with the actin cytoskeleton. Therefore upon the breakdown of the link with β-catenin due to EGF treatment, α-catenin in this cell line, may be fully

detached from the adherens junction and enter the cytoplasm. This cell line however, may require closer study in isolation.

Based on the results observed from the treatment of the OSCC cell lines as a whole, it was possible to conclude that EGF had no affect on the expression levels of α-catenin. The link between α-catenin and β-catenin at the adherens junctions or the link between α-catenin and the actin cytoskeleton is key for the formation of the adherens junctions. Tyr 654 of β-catenin is affected by EGF signalling (Piedra *et al.,* 2003). EGF signalling, resulted in tyrosine phosophorylation of Tyr 654 of β-catenin, causing the interaction between E-cadherin and β-catenin to be broken (Piedra *et al.,* 2003). The disruption of the α-catenin/β-catenin link may not necessarily mean that the link between α-catenin and the cytoskeleton would be compromised. In order for α-catenin to break free from the adherens junctions and enter the cytoplasm, this link to the cytoskeleton would have to be compromised. Due to there being no changes in the cytoplasm/nuclear levels of α-catenin expression in the OSCC cell lines, it would suggest that α-catenin's link to the actin cytoskeleton was not compromised. Therefore, if α-catenin has a role in the EGF signal transduction pathway, it would have to be fulfilling this role at the plasma membrane (Figure 1.4).

The increased levels of EGFR in the OSCC cells may contribute to the changes in cell characteristics observed in the OSCC cell lines. The role played by α-catenin in the EGF signalling pathway and possibly other pathways could be more clearly characterised by transfecting α-catenin into one of the OSCC cell lines. α-catenin could then be over-expressed and the subsequent effects on the adhesion mechanism followed more closely. The data obtained from the over expression of α-catenin may also aid in determining the role played by α-catenin in signal transduction pathways.

CHAPTER 5

SUB-CLONING OF α-CATENIN FOR OVER-EXPRESSION

5.1 INTRODUCTION

The technique of molecular cloning and gene over-expression revolutionised the field of molecular biology (Voet and Voet, 1995). Once cloning has identified the gene of interest, it is possible to transfect the gene into a cell. The sub-cloned gene may then be over expressed by placing the gene under the control of an inducible promoter (Grant *et al.,* 2001).

Through the use of molecular cloning, it is possible to over-express α-catenin in an OSCC cell line. This over-expression of α-catenin makes it possible to study the effects, which α-catenin may have on adhesion and cell signal transduction pathways (Grant *et al.,* 2001). The full length α-catenin cDNA fragment has been sub-cloned into several human cell lines (Giannini *et al.,* 2000, Rimm *et al.,* 1994, Matsubara and Ozawa, 2001, Oda *et al.,* 1993, Linkels *et al.,* 1997). Various vectors were used in these studies, an example of a vector used to clone α-catenin was the pBlueScript SK vector. The pBlueScript SK vector was used to clone α-catenin in order to sequence it (Linkels *et al.*, 1997, Rimm *et al.,* 1994, Oda *et al.,*, 1993).

α-catenin may regulate β-catenin levels and so doing, β-catenin signalling (Giannini *et al.,* 2000). It was suggested (discussion 4.5) that α-catenin might have a direct signal transduction role in the EGF pathway at the plasma membrane or in other signal transduction pathways. The role that α-catenin, may play in signal transduction, could be more clearly determined by sub-cloning α-catenin into an efficient expression vector and transfecting one of the OSCC cell lines and inducing its over expression. The effects which this over expression of α-catenin might have on signal transduction and cell adhesion could then be quantified.

5.2 OBJECTIVES

1. Over express α-catenin in the WHCO5 OSCC cell line in order to determine the effects, which over expression of α-catenin may have on signalling pathways and cell adhesion.

5.3 METHODS

5.3.1 Complete Control® inducible mammalian expression system (Stratagene)

Stratagene's Complete Control® inducible mammalian expression system (Stratagene) is a gene transfer system that allows for the precise control of gene expression in a wide variety of mammalian cell types (Stratagene). In the Complete Control® System, the transcription of the inserted gene is stimulated by the insect hormone ecdysone or its analog ponasterone A (pon A) (Stratagene). Pon A has no known measurable effect on mammalian physiology, has a short half-life and its lipophilic nature allows it to efficiently penetrate mammalian tissues (Stratagene). The OSCC cell lines are sensitive to dexamethasome, which is commonly used by other expression vectors to stimulate the expression of the inserted gene (laboratory data not published). Therefore, because Staratagene's Complete Control® inducible mammalian expression system does not utilize dexamethasome, it was decided to use this system.

The Complete Control® inducible mammalian expression system (Stratagene) is made up of two vectors, namely the pERV 3 receptor vector and pEGSH expression vector (Figures 5.1 and 5.2) (Stratagene). The pERV 3 receptor vector contains the ecdysone receptor (VgEcR) and retinoid-x-receptors, which are the receptors for Pon A (Figure 5.1) (Stratagene).

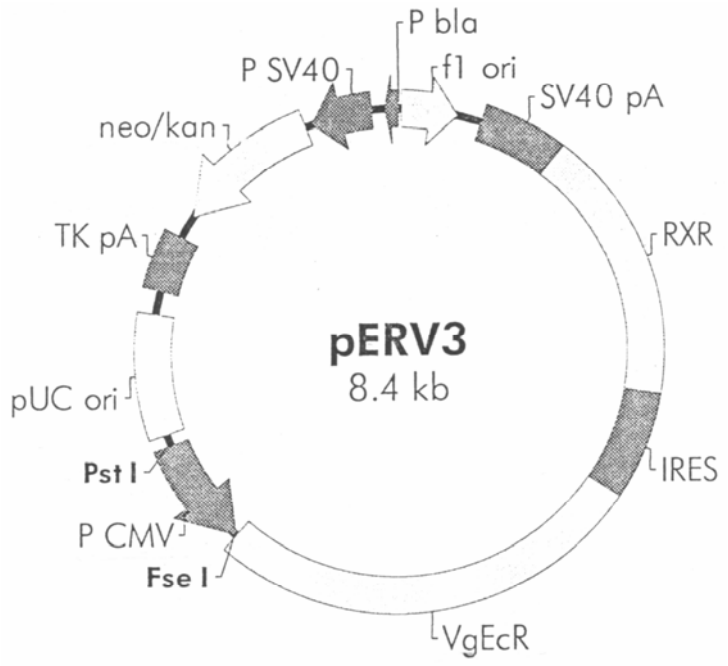


Figure 5.1: Map of the pERV 3 vector (Stratagene). This vector contains the ecdysone-receptor element (VgEcR) and the retinoid-x-receptor (RXR) (Stratagene). The plasmid also contains the neomycin-resistance gene and the β-lactamase promoter, to allow for selection of mammalian cell transfectants (with G418) and E.coli transfectants (with kanamycin) (Stratagene). Key: f1 ori = f1 origin of ss-DNA replication, SV40 pA = SV40 poly a signal, IRES = internal ribosome entry site, p CMV = CMV promoter, P UC ori = pUC origin of replication, TkpA = HSV-thymidine kinase (TK) poly A signal, neo/kan = neomycin/kanamycin resistance ORF, P SV40 = SV40 promoter, P bla = bla promoter (Stratagene).

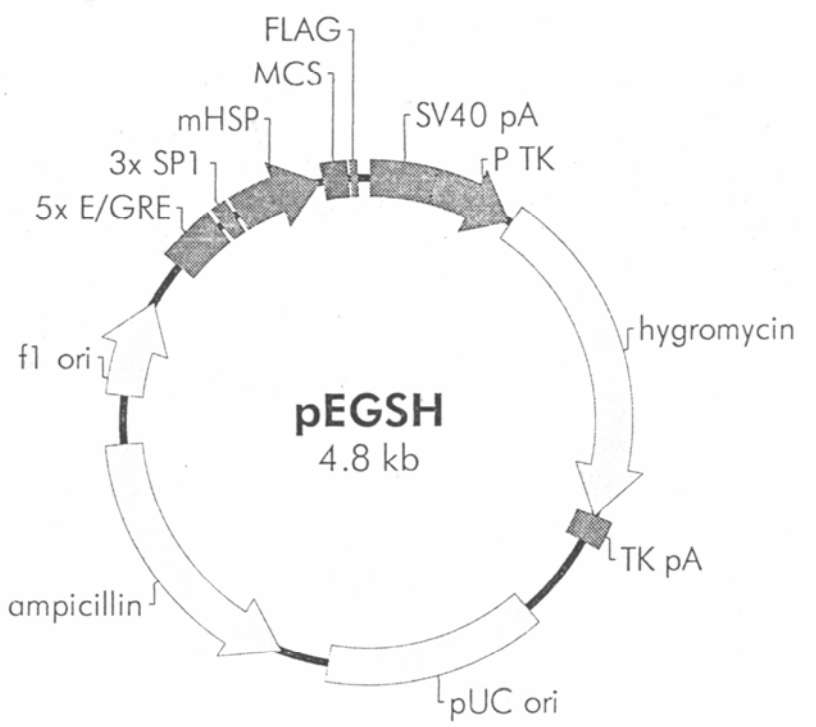


Figure 5.2: Map of the pEGSH vector (Stratagene). This vector contains a pon A-inducible expression cassette and a multiple cloning site (MCS) containing 11 unique restriction sites (Stratagene). The T3 promoter is positioned for convenient sequencing of the 3' junction of the inserted DNA (Stratagene). Key: P TK = HSV-thymidine kinase promoter, hygromycin = hygromycin resistance ORF, ampicillin = ampicillin resistance ORF, 5x E/GRE = 5 ecdysone/glococorticoid responsive elements, 3x SP1 = 3x Sp1 binding sites, m HSP = minimal heat shock promoter, FLAG = FLAG tag (Stratagene).

The pEGSH expression vector contains the Pon A inducible expression cassette and the MCS (Figure 5.2) (Stratagene). The MCS contains an array of restriction sites where in the DNA fragment of interest may be inserted. Through the utilization of this system it is possible to transfect mammalian cells and to stimulate the expression of a desired gene, without triggering any changes in the cell brought about by the molecule used to stimulate the expression of the gene.

5.3.2 Endonuclease restriction digestions

Restriction endonucleases are enzymes, which recognised specific DNA sequence, and cut the DNA with in these sequence (Wilson and Walker, 1996). Restriction endonucleases only cleave double stranded DNA at sites located inside the molecule. The complete mRNA fragment of α-catenin was kindly supplied by Dr. Yai Kanai (National Cancer Centre Research Institute, Pathology Division, Tsukiji, Chuo-Ku, Tokyo, Japan). As a control, EGFP was kindly supplied by Prof. Greg Blatch (Chaperone Research Lab, Department of Biochemistry and Microbiology, Rhodes University).

The α-catenin fragment was excised from the pBlueScript SK vector using the *Kpn I* and *Spe I* restriction endonucleases (Amersham Biosciences). The pEGSH vector was restricted using the same combination of restriction endonucleases. The restriction sites for these enzymes were located outside of the ORF of α-catenin and inside the MCS of the pEGSH vector. This combination of restriction endonucleases were used to enable the directional and in frame sub-cloning of the α-catenin fragment.

The α-catenin fragment and the pEGSH vector were restricted first using the *Kpn I* restriction endonuclease (Amersham Bioscience) (appendix 3.1). Two separate restriction digestions were performed due to the salt concentration of the two buffers differing. The restricted DNA was extracted from the reaction mixture using phenol-chloroform (appendix 1.2). The restricted α-catenin fragment and pEGSH vector were then restricted using the *Spe I* restriction endonuclease (Amersham Bioscience) (appendix 3.3). All

restriction digestions were performed at 37^{o} C for 3 hr. The restricted fragments were gel purified (methods 5.3.3).

Ligation of the α-catenin fragment to the pEGSH vector proved to be problematic. It was attempted to solve this by making use of the following combinations of restriction endonucleases: *Sal I* and *Not I* (Amersham Biosciences) and the pEGSH vector was blunt end restricted using the blunt end restriction endonuclease *EcoRV* (Boehringer Mannheim). The same protocol as used in earlier restriction digestions were employed.

The EGFP fragment was excised from the pCiNeoEGFP plasmid using the *Not I* and *Xho I* (Amersham Bioscience) restriction endonucleases. Due to the compatibility of the salt concentrations of the two buffers, a double digestion of the EGFP fragment and pEGSH vector was set up (appendix 3.5). The restricted fragment was gel purified (methods 5.3.3). This combination of restriction endonucleases were used to enable the directional and in frame sub-cloning of the EGFP fragment. All restriction digestions were performed at 37^{o} C for 3 hr.

5.3.3 Gel purification

The restricted α-catenin and EGFP fragments and pEGSH vector were gel purified to reduce non-specific competition for ligation. The WIZARD® SV gel and PCR Clean-up system (Promega) was used to purify the restricted samples. This gel purification system was used for its high yield (95%) (Promega).

The restricted samples (EGFP, α-catenin and pEGSH) were separated on a 1% agarose gel (methods 2.3.3). The area of the gel containing the desired DNA fragment was excised and weighed. Membrane binding solution was added at a ratio of 10 μl of solution per 10 mg of agarose gel, and the resulting mixture incubated at 55^{0} C for 10 minutes in order to melt the agarose gel (appendix 3.7). The mixture was transferred to a SV Minicolumn (Promega) and incubated at room temperature for 1 minute. The column was centrifuged for 1 minute, any remaining agarose gel was removed by washing the

column with 700 µl of membrane wash solution (appendix 3.8). The column was centrifuged as above. The wash step was repeated using 500 µl of membrane wash solution. The elute was discarded and the SV Mini column centrifuged for 1 minute. 25 µl of Nuclease-Free H_2O (Promega) was added to the column and incubated for 1 minute at room temperature. The DNA was eluted by centrifuging for 1 min. All of the centrifugation steps were carried out at 10 000 x g at room temperature using a Sorvall® MC12V bench top centrifuge (Du Pont). The success of the extraction was determined by separating an aliquot on a 1% agarose gel (methods 2.3.3).

5.3.4 Dephosphorylation of the linearised pEGSH vector

Dephosphorylation of linearised plasmid DNA results in the removal of 5'-phosphate groups (Sambrook *et al.,* 1989). By removing the 5'-phosphate group of the linearised pEGSH vector, bacteriophage T4 DNA ligase is unable to re-ligate the 5' and 3' end of the linearised vector to each other.

The Calf intestine alkaline phosphatase (CIP) dephosophorylation reaction was incubated at 37^0 C for 1 hour (appendix 3.9) (Boehringer Mannheim). The CIP was inactivated by adding 1 µl of 0.5 M EDTA and 5 µl of 10% SDS and heating to 75^0 C for 10 minutes (appendix 3.11 and 3.12). The dephosphorylated vector was extracted using phenol-chloroform (methods 2.2.4.1).

5.3.5 End filling

T4 DNA polymerase fills 3' recessive DNA and excises 5' overhanging DNA fragments from "sticky ended" DNA (Amersham Biosciences). In this manner T4 DNA polymerase produces "blunt ended" DNA from the "sticky ended" DNA fragments.

The end filling reaction was incubated at 70^0 C for 5 minutes and placed at 37^0 C (appendix 3.13). 2.5% T4 DNA polymerase was added and incubated at 37^0 C for 1 hour. The end filled α-catenin fragment was extracted using phenol-chloroform (appendix 1.2).

5.3.6 Spectrophotometic analysis of extracted samples

The concentration and purity of the WIZARD® SV gel and PCR Clean-up system extracted DNA (Promega), was determined spectrophotometrically. Samples were diluted 1:150 and the absorbancies read spectrophotometrically at 260 nm and 280 nm using a Beckman DU 64 Spectrophotometer (Beckman). The DNA concentration and 260 nm:280 nm ratio was calculated (appendix 3.15 and 3.16).

5.3.7 Ligation

Bacteriophage T4 ligase is capable of covalently joining single stranded DNA molecules containing 5'-phosphate termini and 3'-hydroxyl termini (Sambrook *et al.*, 1989). The efficiency of the ligation reaction may be affected by the ratio (e.g. 3:1) of the molar concentration of available ends of DNA of the DNA to be inserted into the vector (e.g. α-catenin) compared to that of the vector (e.g. pEGSH). In attempt to ligate the α-catenin and EGFP fragments into the pEGSH vector, the following ligation strategies were employed.

5.3.7.1 Manual ligation

The following ratios were employed in ligating the α-catenin and EGFP fragments to the pEGSH vector: a) 1:1, 2:1, 3:1 and 4:1 and b) 2:1, 3:1 and 6:1 (appendix 3.17 and 3.18). Due to difficulties encountered with the ligation reaction, the DNA was heat denatured by heating to 70^{o} C for 5 minutes prior to ligation. The reaction was incubated at 4^{0} C or 16^{0} C for 4 to 16 hours. The products from the ligation were then used to transform *E.coli* (methods 5.3.8).

5.3.7.2 FAST-LINK™ DNA ligation and Screening kit (Epicentre Technologies)

The Fast-Link™ DNA ligation and Screening kit (Epicentre technologies), ligates DNA rapidly (5 minutes). The following ratios were employed to ligate the α-catenin and EGFP fragments to the pEGSH vector: a) 1:1, 2:1, 3:1, 4:1, 6:1 and 7:1 and b) 2:1, 4:1, 5:1 and 6:1. The Fast-Link™ reaction (appendix 3.21) was incubated at room temperature for 5 minutes. Heating to 70^{o} C for 15 minutes inactivated the T4 ligase. The ligation products were then used to transform *E.coli* (methods 5.3.8).

5.3.7.3 Rapid DNA ligation kit (ROCHE)

The rapid DNA ligation kit enables the rapid ligation of DNA (5 minutes) (Roche). The following ratios were employed to ligate the α-catenin and EGFP fragments to the pEGSH vector: a) 3:1, 4:1, 5:1 and 6:1. and b) 3:1, 5:1, 10:1 and 20:1. 10 μl of T4 DNA ligation buffer was added to the reaction mixture, and mixed (appendix 3.23 and 3.25). 1 μl of T4 DNA ligase (Roche) was added, mixed and incubated at 20^{0} C for 5 minutes. The ligation products were used to transform *E.coli* (methods 5.3.8).

5.3.8 High-voltage electroporation

The application of a brief high-voltage electric pulse leads to the formation of pores in bacterial cells, thus enabling these cells to absorb DNA (Hengen, 1996). The transformation of bacterial cells via electroporation can be extremely efficient and produced 10^{4}-10^{6} transformants/μg of DNA (Hengen, 1996). Transformation of *E.coli* enables large quantities of the transforming factor (ligation products) to be obtained for testing and further use.

Electroporation competent XL1 Blue *E.coli* cells were thawed on ice (appendix 3.26), and one tenth of the ligation products added (methods 5.2.7). The cells were transferred to a BIO-RAD Gene Pulser®/E.coli Pulser™ Cuvette (BIO-RAD), and pulsed using a

BIO-RAD Gene Pulser™ (BIO-RAD), set on 1700 V, 200 Ω and 25 μF. The cells were immediately re-suspended in 37^0 C SOC medium (appendix 3.29), transferred to a 15 ml 2059 Falcon polypropylene tube (Corning) and incubated at 37^0 C with vigorous shaking (140 r.p.m) for 1 hr in a Labcon incubator (Labcon). An aliquot of the cells were spread plated on LB agar plates containing ampicillin (Roche) (LA) (appendix 3.33) and incubated at 37^0 C for 20 hr in a Labcon incubator (Labcon).

5.3.9 PCR analysis of transfected cells

The following two primers were supplied with the Complete Control® Inducible Mammalian Expression system (Stratagene), for the PCR analysis of the transforming factors contained in the transformed *E.coli*:

- Primer T3: 5' ATTAACCCTCACTAAAG 3' (Stratagene)
- Primer pEGSH: 5' CTCTGAATACTTTCAAAAGTTAC 3' (Qiagen)

Colonies produced on the LA plates were replica plated using sterile tooth picks, and the tooth picks placed in 50 μl of cracking buffer (appendix 3.35). The samples were incubated at 95^0 C for 10 minutes and centrifuged at room temperature and 12 000 x g for 5 minutes in a Sorvall® MC12V (Du Pont) centrifuge. The following amendments were made to the protocol used in methods 2.3.4.2; T3 and pEGSH primers used and the supernatant from each respective cracked colony used as template. The amplified fragments were separated on a 1% agarose gel (methods 2.2.3).

5.3.10 DNA sequencing

Colonies, indicated by PCR analysis, to contain the desired EGFP construct (methods 5.2.7) were sequenced, to verify results. The colony was sent to Inqaba Biotech for sequencing in both directions. The sequence obtained was aligned to that of EGFP using BLAST (www.ncbi.nlm.nih.gov/BLAST/) (appendix 3.36).

5.3.11 Transfection of the WHCO5 OSCC cell line

The FuGENE™ 6 Transfection Reagent (Roche) was used to transfect the WHCO5 OSCC cell line due to its high efficiency (Roche). The FuGENE™ 6 Transfection Reagent (Roche) is a liposome based transfection system (Roche).

Four 5 cm tissue culture dishes were seeded with sufficient WHCO5 cells to reach a density of 60% within 24 hr. Four dishes were seeded so as to obtain two experiments and two controls. The FuGENE™6/pERV 3 mixtures were incubated at room temperature for 15 minutes (table 7.3). The FuGENE™ 6/pERV 3 mixtures were added drop wise to the cells, and incubated at 37^0 C in the presence of 5% CO_2 for 24 hr. The TC medium and FuGENE™ 6/pERV 3 mixtures were replaced with fresh TC medium. The plates were re-incubated as above for 24 hr. 150 μl of neomycin (G418) (20 mg/ml) (Promega) was added to the cells and incubated as above for 3 weeks (appendix 3.36). Colonies, surviving the 3 week incubation period, were maintained in tissue culture (methods 2.2.1). The same procedure was employed for the transfection of the WHCO5 OSCC cell line with the pEGSH-EGFP construct.

5.4 RESULTS

5.4.1 Sub-cloning of the EGFP control

The endonuclease restriction of the EGFP fragment produced two bands respectively (Figure 5.3, lane 8). The larger of these bands represented the pCiNeo vector (Figure 5.3, lane 8). This band was not present in the gel purified samples (Figure 5.3, lane 9). The same result was obtained for the gel purified pEGSH vector (Figure 5.5 lane 7). The gel purified EGFP and pEGSH samples had a 260:280 nm ratio of 1.7 and both contained 0.2 ng/μl of DNA (appendix 3.15 and 3.16).

Several different ligation strategies (methods 5.3.7) and restriction endonuclease digestions (methods 5.3.2) had been employed, in attempt to ligate the EGFP fragment to

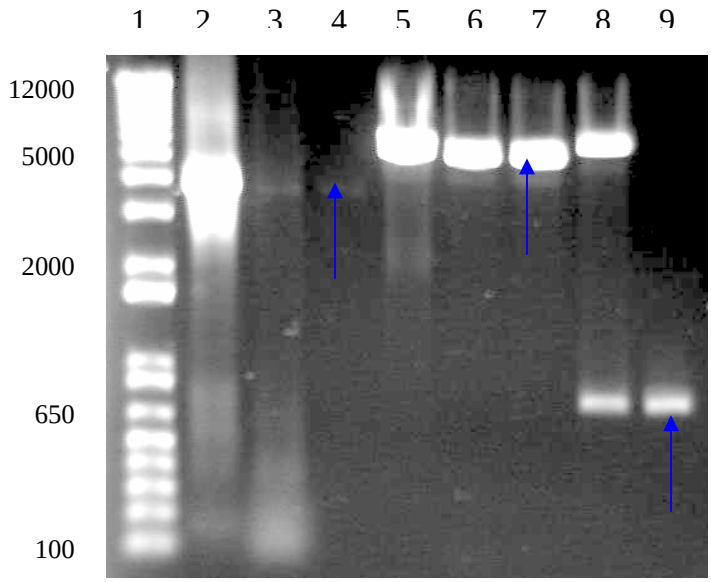


Figure 5.3: Endonuclease restricted α-catenin, EGFP fragments and pEGSH vector and their respective gel purified samples. The gel was loaded in the following order (lanes indicated above the gel): Kpn I restricted α-catenin (lane 2), Kpn I and Spe I restricted α-catenin (lane 3), gel purified restricted α-catenin (lane 4), Kpn I restricted pEGSH (lane 5), Kpn I and Spe I restricted pEGSH (lane 6), gel purified pEGSH (lane 7), Not I and Xho I restricted EGFP (lane 8), gel purified restricted EGFP (lane 9). A 1 kb + molecular weight marker (Roche) was loaded in lane 1 (molecular size of bands indicated next to gel). The gel purified samples (lanes 4, 7 and 9) (indicated by blue arrows) contained the endonuclease restricted samples only. Two bands were present in lanes 2, 3 and 8. The α-catenin restricted fragment (lane 3 and 4) was 3.5 kb in size. The restricted pEGSH vector (lanes 6 and 7) was 4.8 kb in size. The restricted EGFP fragment (lanes 8 and 9) was 0.74 kb in size. A very faint (almost not visible) band was produced in lane 9.

the pEGSH vector. The success of the ligation of the EGFP fragment to the pEGSH vector was determined by transforming competent *E.coli*, with the constructs produced from the ligation reactions. To determine the effectiveness of the transformation, a control transformation, was included, where the cells were transformed with the pEGSH vector. The control plate produced a large number of colonies. A single colony was produced on the plate containing the pEGSH-EGFP construct, produced from the manual ligation reaction. This colony exhibited the same morphological characteristics as that of *E.coli*. An 900 bp fragment was amplified from the colony produced by the bacteria transformed with the EGFP ligation product (Figure 5.4, lane 5). This result was verified by DNA sequencing (Figure 5.5). The fragments were sequenced in both directions. DNA sequencing products were aligned to the EGFP sequence using BLAST (www.ncbi.nlm.nih.gov/BLAST/) (BLAST I D:1098177056-27224-41496202813.BLASTQ4). The sequence produced aligned to that of EGFP, and the BLAST alignment indicated that the sequence was in the correct orientation in the vector for expression.

The four dishes of WHCO5 OSCC cells transfected with the pERV 3 vector using the FuGENE™ 6 reagent (Roche) survived the initial 24 hr incubation with TC medium. Both negative controls [FuGENE™ 6 reagent (Roche) only and only TC medium] did not survive the three week incubation with G418. Only selected cells transfected with the pERV 3 vector and pEGSH-EGFP construct grew after the three week G418 incubation period.

5.4.2 Sub-cloning of α-catenin

Endonuclease restriction of the α-catenin fragment yielded two bands (Figure 5.3, lane 2). The larger band represented the pBlueScript SK vector, from which the α-catenin fragment had been excised. The gel purified α-catenin fragment contained only the endonuclease digested fragment (Figure 5.3, lane 4). The 260:280 nm ratio for α-catenin was 1.6. The gel purified α-catenin sample, was half the concentration (0.1 ng/μl) of that of the EGFP and pEGSH samples.

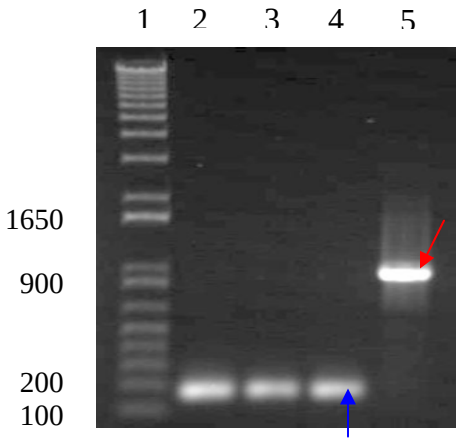


Figure 5.4: PCR analysis of the transforming factors contained in the transformed E.coli cells. The 0.8% agarose gel was loaded in the following order (lanes indicate above the gel): PCR fragments amplified from DNA extracted from E.coli transformed with α-catenin (lanes 2-4) and EGFP (lane 5) ligation products. A 1 kb+ molecular weight marker (Roche) was loaded in lane 1. A 183 bp band was produced in lanes 2-4 (indicated by blue arrow). A 900 bp band was produced in lane 5 (indicated by red arrow).

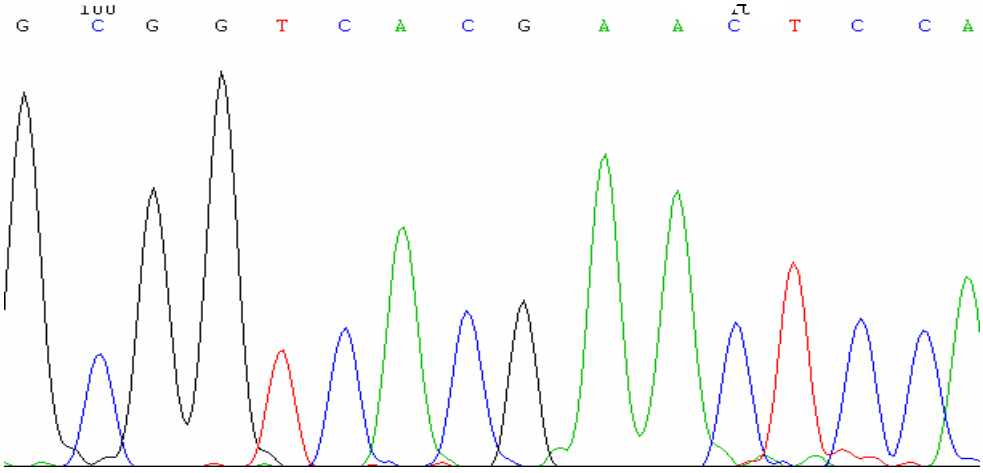


Figure 5.5: Sequence of the pEGSH-EGFP construct. The peaks produced on the electrophoretogram did not overlap and had a high frequency. This portion of the electrophoretograph was taken 100 bp into the sequence, so as to illustrate the EGFP sequence (this sequence spanned nucleotides 72-86 of EGFP) (BLAST ID: 1098344902-11987-169033755335.BLASTQ2).

Varying numbers of colonies were produced on the different LB ampicillin plates containing the various pEGSH-α-catenin constructs produced by the different ligation techniques. The colonies that grew exhibited the same morphological characteristics as that of *E.coli*. A 180 bp fragment was amplified out of all of the colonies transformed with the α-catenin ligation products (Figure 5.4, lanes 2-4). A fragment of this size would be amplified if the pEGSH vector relegated. Despite all the attempts, the α-catenin fragment was not successfully ligated to the pEGSH vector.

5.5 DISCUSSION

The presence of the two bands produced by the endonuclease restriction digestions of the α-catenin and EGFP fragments and the pEGSH vector, indicated that the endonuclease restriction digestions were successful (Figure 5.3). The larger of the bands in the case of the α-catenin and EGFP endonuclease restriction digestions, represented the pBlueScript SK and pCiNeo vectors respectively. The smaller fragments were calculated to be 3.5 kb and 0.74 kb in size respectively, representing the α-catenin and EGFP fragments (Figure 5.3) (Rimm *et al.*, 1994). In the case of the pEGSH vector, the larger band represented undigested vector and the smaller band represented the fully digested linearised vector (Figure 5.3). The absence of these bands (pBlueScript KS, pCiNeo and undigested pEGSH) in the WIZZARD® SV gel and PCR Clean up system (Promega) purified samples, indicated that the gel purification was successful (Figure 5.3). These results were verified by those obtained from the spectrophotometric analysis of the samples.

The 260 nm:280 nm ratios produced by the gel purified α-catenin, EGFP and dephosphorylated pEGSH samples were all within 0.2 of 1.8. This indicated that the samples contained very low levels of proteins, and high levels of DNA. It also indicated that the gel extractions of the endonuclease restricted fragments via the WIZZARD® SV gel and PCR Clean up system (Promega) were successful, in removing almost all traces of agarose and proteins (restriction endonucleases). It was essential that pure samples be

used in the ligations, so as to eliminate any interference of the reaction, caused by contaminating proteins or remaining fragments (e.g. MCS) from the restriction digestion.

Several different restriction endonucleases and ligation strategies were employed in attempt to ligate the EGFP fragment to the pEGSH vector. The EGFP fragment (control) was successfully ligated to the pEGSH vector. A 0.80 kb fragment was amplified from the colony containing the ligation product. The size of the amplified fragment correlated with that of the EGFP insert. DNA sequencing results confirmed the PCR analysis. The fragment was sequenced in both directions. The sequence produced, was aligned to that of EGFP using BLAST. The BLAST results indicated that the sequence produced aligned perfectly to that of EGFP and it was in the correct orientation in the vector for expression. It was therefore concluded that the ligation had been a success, and that the EGFP fragment had been inserted into the pEGSH vector. This indicated that it was possible to ligate foreign DNA to the pEGSH vector. The necessity to denature the vector prior to the ligation reaction indicated that the linearised vector might have formed secondary structures, which could have interfered with the ligation reaction.

All four dishes of WHCO5 OSCC cells tranfected with the pERV 3 vector using the FuGENE™ 6 reagent (Roche) grew within the initial 24 hr incubation. This indicated that the WHCO5 OSCC cells were not negatively affected by the transfection. The ability of the WHCO5 OSCC cells which had been transfected with the FuGENE™ 6 reagent alone to grow in the TC medium was an indication that the FuGENE™ 6 reagent was not toxic to the cells. Both controls failed to grow in the presence of G418. The pERV 3 vector contained the neomycin-resistance gene (G418) (Stratagene). During the selection process, only cells, which have integrated the construct, were able to grow (Domann, 1994). The inability of the WHCO5 OSCC cells to grow in the presence of G418 indicated that this cell line contained no innate resistance to neomycin. This meant that the WHCO5 OSCC cells which where capable of growing in the presence of G418 should have contained the pERV 3 vector. Based on these results, it was possible to conclude that the WHCO5 OSCC cell line had been successfully transfected with the pERV 3 vector and that the vector was being expressed in order to produce the antibiotic

resistance trait being displayed by the transfected cells. The transfection of the WHCO5 OSCC cells with the pEGSH-EGFP construct yielded the same results, indicating that the WHCO5 OSCC cell line had been successfully transfected with the pEGSH-EGFP construct. The pEGSH-EGFP construct was transfected into the WHCO5 OSCC cell line as a control to determine if the pEGSH vector and insert had any affect on the cell characteristics of the WHCO5 OSCC cell line. The cell characteristics of the pEGSH-EGFP transfected WHCO5 OSCC cell line were unaltered when compared to the untransfected WHCO5 cells. It was therefore, concluded that the transfection of the WHCO5 OSCC cell line with the pEGSH-α-catenin construct should have no effect on the cell characteristics of the this cell line. This would mean that any changes in these characteristics upon α-catenin over expression, could be attributed solely to α-catenin signalling.

The same ligation strategies used to ligate the control EGFP fragment, were employed in ligating the α-catenin fragment to the pEGSH vector. All of the control transformations utilised during the transformation of the *E.coli* with the pEGSH-α-catenin constructs were successful. This indicated that if the experimental transformation did not yield any growth, it could be attributed to an unsuccessful ligation reaction and not the failure of the transformation. A 180 bp fragment was amplified from all of the colonies produced by the transformation of *E.coli* using the α-catenin ligation products (Figure 5.4). This indicated that the transformed colonies contained re-ligated pEGSH vectors. It was for this reason that the 5' phosphate group of the pEGSH vector was removed. Without this group the pEGSH vector would be unable to re-ligate (Sambrook *et al.*, 1989). This however, did not improve the success of the ligations. This lead to the thought that there may have been an anomaly occurring at one of the restriction sites resulting in the restriction endonucleases not cleaving the DNA. Different combinations of restriction endonucleases were therefore used to restrict the α-catenin fragment and pEGSH vector in attempt to rectify this problem. This however did not aid in increasing the success rate of the ligations. It was then thought that α-catenin's base composition (long stretches of GC and AT rich areas) might have facilitated a high degree of secondary structure formation. The secondary structures may have masked the cleaved restriction sites and

prevented these sites from coming into close enough proximity to be joined by the T4 bacteriophage ligase.

In an attempt to solve this problem, the α-catenin fragment was heat denatured prior to commencing with the ligation reaction. The success of the ligations was however, not improved. It was therefore concluded that some undetermined factor was hindering the ligation of the α-catenin fragment to the pEGSH vector. The α-catenin gene had been successfully cloned or sub-cloned into other expression vectors (Giannini *et al*., 2000, Rimm *et al.,* 1994, Matsubara and Ozawa, 2001, Oda *et al.,* 1993, Linkels *et al.,* 1997). Unfortunately the details of the strategies used by Matsubara and co-workers (2001) and Giannini and co-workers (2000) to sub-clone the α-catenin fragment, are not available. Oda and co-workers (1993), Rimm and co-workers (1994) and Linkels and co-workers (1997), all sub-cloned the α-catenin gene into the pBlueScript SK vector from cDNA libraries, in order to sequence the α-catenin gene. In order to sub-clone α-catenin, Oda and co-workers (1993) attached synthetic linkers to the α-catenin gene. Contained in the synthetic linkers was an EcoRI restriction site, which was then utilised to insert the α-catenin gene into the pBlueScript SK vector (Oda *et al.,* 1993). However, it was not possible to utilise the same restriction sites as used by Oda and co-workers (1993) to insert the α-catenin gene into the pEGSH vector, because the pEGSH vector did not contain an EcoRI restriction site in the MCS. The pBlueScript SK vector, however is not an expression vector. Therefore the studies using this vector had no bearing on our investigation. The OSCC cell lines are sensitive to dexamethasone (lab data not published). Therefore, by using a different expression vector, the advantages of the pon A regulation of expression which the pEGSH expression vector offers, would be lost. Three ligation strategies and fourteen different insert to vector ratios had been attempted. This lead to the conclusion that it was not possible at present to ligate the α-catenin fragment into the pEGSH vector. Unfortunately due to time constraints the ligation of the α-catenin fragment to the pEGSH vector had to be abandoned.

The ligation of the EGFP fragment to the pEGSH vector had been used as a control to monitor the success of the insertion of foreign DNA to the pEGSH vector and to ascertain the effects, which the pEGSH-EGFP construct would have on the WHCO5 OSCC cell line. The successfulness of the control ligation, indicated that the problem did not lie with the ligation techniques used, but rather the pEGSH vector. Had it worked, it would have allowed us to ascertain the affects that an increase in the cytoplasmic pool of α-catenin would have had on the adhesion complex and the effects, which the increased pool may have had on gene expression with respect to β-catenin/Tcf/Lef.

CHAPTER 6

OVERALL DISCUSSION

6.1 OUTCOMES OF THE INVESTIGATION

The adherens junctions are key adhesions found in epithelial cells (Nieset *et al.,* 1997). α-catenin forms a vital component of the adherens junctions. In the adherens junctions, α-catenin links the E-cadherin/β-catenin complex to the actin cytoskeleton (chapter 1, section 1.2.2). This link is essential for the formation and maintenance of the adherens junctions. Some of the cell adhesion molecules, such as α-catenin and β-catenin have duel functions, as adhesion and signal transduction molecules (Hynes, 1999). α-catenin has been shown to regulate the cytoplasmic and nuclear levels of β-catenin (Giannini *et al.,* 2000). This prevents β-catenin from fulfilling its signalling role in the Wnt pathway (chapter 1 section 1.3). Changes in cell adhesion, may be brought about by multiple pathways (Nicholson *et al.,* 1991).

EGF signalling results in an increase in cellular proliferation, migration and a decrease in cellular adhesion (Andle *et al.,* 2003, Solic and Davies, 1997) Heightened levels of expression of EGFR have been observed in OSCC cell lines (Andl *et al.,* 2003, Veale and Thornley, 1989). This suggests that some of the changes in the cell adhesive properties of the OSCC cell lines may be attributed to the effects of EGF signalling. The effects, which the EGF and Wnt signal transduction pathways have, on cell adhesion highlights the important role which α-catenin may play in these pathways and their regulation. The expression levels of α-catenin are decreased in a large number of tumours (Giannini *et al.,* 2000, Roe *et al.,* 1998, Vermeulen *et al.,* 1999). α-catenin has also been found to harbour mutations in a large number of malignancies (Giannini *et al.,* 2000, Roe *et al.,* 1998, Vermeulen *et al.,* 1999). It is therefore crucial to study the status of α-catenin in the OSCC cells and attempt to elucidate the role which it may play in bringing about changes in cell adhesion and signal transduction.

6.2 INVESTIGATION OF THE α-CATENIN GENE IN OSCC

All of the OSCC cell lines contained the α-catenin gene. α-catenin was being expressed at the mRNA level in all of the OSCC cell lines. For the larger part, the α-catenin gene contained in the OSCC cell lines was intact. α-catenin forms the link between the β-catenin/E-cadherin complex and the actin cytoskeleton. It is essential for the formation of the adherens junctions that this link is formed (Janssens *et al.*, 2000). This was the first study into the α-catenin gene contained by OSCC cell lines.

Three of the five OSCC cell lines harboured mutations in the vinculin, ZO-1 and actin binding domains of α-catenin. All of these mutations were single nucleotide mutations. However only the mutation contained in the WHCO1 OSCC cell line altered the amino acid sequence of α-catenin. This mutation did not appear to alter the interaction between α-catenin and vinculin, ZO-1 or actin. There have only been two other studies examining the specific location of mutations in the α-catenin gene (Oda *et al.*, 1993, Candidus *et al.*, 1996). These studies were carried out in lung, breast and gastric cancers (Oda *et al.*, 1993, Candidus *et al.*, 1996). Two mutations had been found by Oda and co-workers (1993) to alter the amino acid sequence of α-catenin in the above mentioned cells. The effect, which these mutations had on the ability of α-catenin to interact with the members of the adherens junctions was, however, not determined. The mutation that was discovered in this investigation in the WHCO1 OSCC cell line was the first to be reported at this location in α-catenin. This mutation did not appear to have affected the ability of α-catenin to interact with the actin cytoskeleton. Further investigation however, is required to verify that the link between α-catenin and the actin cytoskeleton is unaltered by this mutation. Due to three out of the five OSCC cell lines studied harbouring mutations, valuable information may be gained by increasing the sample size of the study to determine the role, which mutations in the α-catenin gene may play in OSCC.

6.3 CELL LOCALISATION AND RELATIVE LEVELS OF α-CATENIN PROTEIN EXPRESSION

It is necessary that α-catenin be expressed at the protein level in order for it to form the link between the E-cadherin/β-catenin complex and the actin cytoskeleton. α-catenin was found to be expressed in both the plasma membrane and cytoplasm/nucleus of the OSCC cell lines, at varying levels. It was generally found that α-catenin was mainly localized to the plasma membrane of the OSCC cell lines. This was expected due to α-catenin's pivotal role in adherens junction formation and the fact that epithelial cells generally contain large numbers of adherens junctions (Raven and Johnson, 1996).

The cytoplasmic levels of α-catenin protein expression observed in the OSCC cell lines, were lower than the plasma membrane levels, but were still higher than expected. This however, correlated with the findings from other studies, which showed that abnormal α-catenin expression appeared to be a hallmark of cancerous cells (Shiozaki *et al.,* 1994). Upon closer examination of the studies performed, it was discovered that the cancers which were shown to exhibit abnormal α-catenin expression were all of gastrointestinal (e.g. pancreatic, gastric, stomach colorectal and liver) or epithelial origin (e.g. oral squamous carcinoma, basal cell carcinoma, Barrett's oesophagous and oesophageal adencarcinoma) (Karayiannakis *et al.,* 2001, Kooy *et al.,* 1999, Xiangming *et al.,* 1999, Ropponen *et al.,* 1999, Gofuku *et al.,* 1999). All of these studies found that α-catenin expression was either shifted (to the cytoplasm) or reduced in the tumourogeneic cells (Karayiannakis *et al.,* 2001, Kooy *et al.,* 1999, Xiangming *et al.,* 1999, Ropponen *et al.,* 1999, Gofuku *et al.,* 1999). These findings were not too surprising, when taking into account the cell adhesive characteristics of the non cancerous cells, which were the progenitors for the above mentioned cancers. Therefore it would stand to reason that changes in the expression and localization of cell adhesion molecules would affect the cell adhesive characteristics of these cells. These findings highlight the important part played by α-catenin in the formation and maintenance of cell adhesion and the critical role which cell adhesion plays in cellular processes.

Changes in the expression of α-catenin, appear to be a common occurrence in tumours, which traditionally would be strongly dependent on cell adhesion (e.g. tumours which have an epithelial origin). This suggests that the increase in cytoplasm/nuclear levels of α-catenin could be attributed to more than just a decrease in cell adhesion. There may be numerous explanations for the abnormal levels of α-catenin protein expression observed in the cytoplasm/nucleus of the OSCC cell lines (see chapter 3, discussion). It was noted that cell adhesion molecules also have signal transduction roles in cells (Hynes, 1999). Based on the evidence provided (see chapter 3, discussion), α-catenin may play a role in the activation of the apoptotic pathway. However, in order to shed more light on this in OSCC, it was deemed necessary to examine the other molecules (e.g. EGFR), which are altered in the OSCC cell lines to determine the effect, which they may have on α-catenin.

6.4 EFFECTS OF EGF SIGNALLING ON α-CATENIN EXPRESSION

The adherens junctions, who form cell-cell contacts in epithelial cells, have an important function in maintaining tissue organization (Piedra *et al.*, 2003, Solic and Davies, 1997). A loss of cell adhesion may be brought about by various factors. An example of one of the factors responsible for a reduction in cell adhesion is EGF signalling (Solic and Davies, 1997). The EGFR has been found to be localized near the adherens junctions (Watabe *et al.*, 1994). New information has lead to the speculation in the manner in which EGF signalling reduces cell adhesion (Figure 6.1).

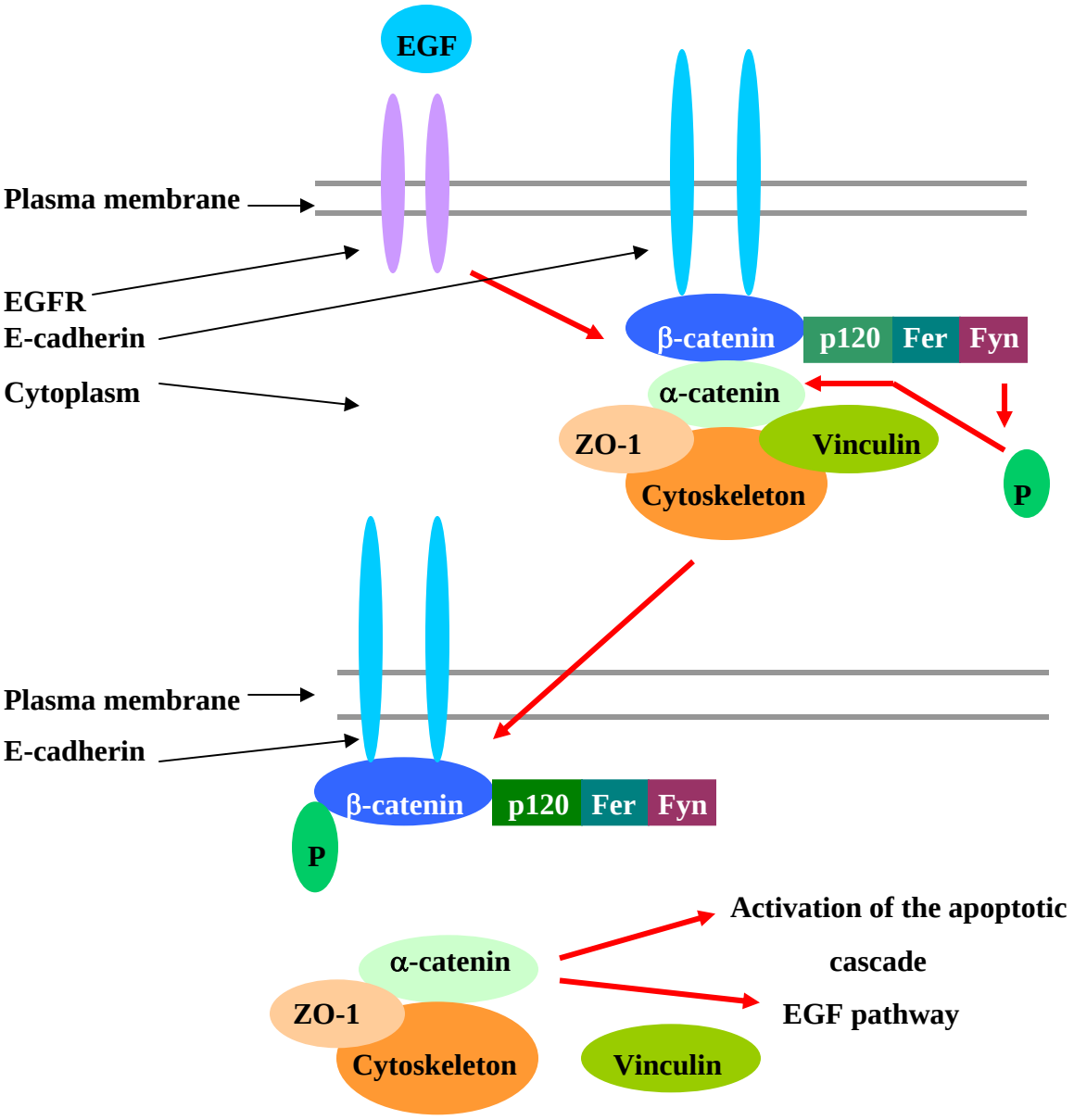


Figure 6.1 Hypothesized mechanism for EGF's breakdown of the adherens junction (Piedra et al., 2003, Watabe et al., 1994). Upon EGF signalling, Fyn and Fer tyrosine kynases phosphorylate p120, strengthening its interaction with the β-catenin/E-cadherin complex. Fyn or Fer tyrosine kinases, then phosphorylates Tyr 142 of β-catenin resulting in the disruption of the α-catenin/β-catenin link. Vinculin detaching from the adherens junction (Hazan and Norton 1998). The link between α-catenin and the actin cytoskeleton however, is still unaltered by EGF signalling. The cytskeleton bound α-catenin may then form part of the EGF signalling pathway or the apoptotic cascade.

The link between α-catenin and β-catenin is essential for the sequestration of β-catenin to the plasma membrane. Therefore, once the α-catenin/β-catenin link is broken by EGF signalling, it would be expected to see an increase in the levels of cytoplasm/nuclear β-catenin. EGF signalling, however, was shown to have no effect on the cytoplasm/nuclear levels of β-catenin (Jones and Veale, 2003). Taking this into account, along with the fact that α-catenin's interaction with the cytoskeleton does not seem to be altered by EGF signalling, it could be speculated that the cytoplasm/nuclear levels of α-catenin should remain unaltered upon EGF treatment, which is what was observed in the OSCC cell lines.

This study was the first to examine the effects of EGF signalling on α-catenin expression. It was observed that EGF treatment of the OSCC cell lines, as a whole had no effect on the expression levels of α-catenin. EGF treatment of the WHCO1 OSCC cell line however, resulted in an increase in the cytoplasm/nuclear levels of α-catenin expression. It was concluded that the mutation detected in α-catenin's vinculin, ZO-1 and actin binding domains of α-catenin in this cell line, could have affected its interaction with these molecules. Therefore upon EGF treatment the link between β-catenin would be compromised resulting in free cytoplasm/nuclear α-catenin. This could not be confirmed by results from other studies, since other studies into α-catenin to determine the presence of mutations, did not determine the effect that these mutations had on the interaction of α-catenin with vinculin, ZO-1 or actin (Candidus *et al.,* 1996, Oda *et al.,* 1993).

The lack of effect which EGF treatment had on the expression levels of α-catenin in the OSCC cell lines as a whole indicated that EGF did not affect the expression levels of α-catenin. A similar study performed into the effects of EGF on β-catenin in the same cell lines indicated that EGF had no effect on the expression levels of β-catenin (Jones and Veale, 2003). It was however mentioned that EGF phosphorylates β-catenin, resulting in the disruption of the α-catenin/β-catenin interaction (Piedra *et al.,* 2003). Due to there being no increase in the cytoplasm/nuclear levels of α-catenin expression observed in the OSCC cell lines, it may indicate that the α-catenin/cytoskeleton link is

unaltered upon EGF treatment. Free α-catenin has been shown to be degraded within 5 hrs (Takahashi *et al.,* 2000). Therefore, due to there being no increase in the cytoplasm/nuclear levels of α-catenin it would suggest that α-catenin remains at the plasma membrane upon EGF treatment.

6.5 SUB-CLONING OF α-CATENIN FOR OVER-EXPRESSION

The EGFP control was successfully sub-cloned into the pEGSH vector. This indicated that it was possible to ligate DNA to the pEGSH vector. The pEGSH-EGFP construct was successfully transfected into the WHCO5 OSCC cell line. The α-catenin fragment was unfortunately not successfully ligated to the pEGSH vector. It was therefore, not possible to determine the signal transduction role which, α-catenin may play in the apoptotic and EGF pathways. α-catenin has been successfully sub-cloned into an array of cells (Giannini *et al.,* 2000, Rimm, 1994, Matsubara and Ozawa 2001, Oda *et al.,* 1993, Linkels *et al.,* 1997, Watabe *et al.,* 1994, Breen *et al.,* 1993). Most of these studies sub-cloned α-catenin using the pBlueScript SK vector in order to sequence it (Oda *et al.,* 1993, Rimm, 1994, Linkels *et al.,* 1997). Unfortunately the expression vectors used by the remaining researchers to sub-clone the α-catenin gene were not supplied (Watabe *et al.,* 1994, Breen *et al.,* 1993, Matsubara and Ozawa 2001, Giannini *et al.,* 2000).

Transfecting of α-catenin into α-catenin deficient lung and colon carcinoma cells, resulted in the cells recovering their cell-cell adhesiveness (Watabe *et al.,* 1994, Breen *et al.,* 1993). In the case of the colon carcinoma cell lines, this observation led to the conclusion that α-catenin fulfils an indispensable role in adherens junction formation (Breen *et al.,* 1993). Transfecting of α-catenin into α-catenin deficient PC9 lung carcinoma cell lines resulted in adherens junction formation, without affecting the levels of expression of the adherens junction components (e.g. E-cadherin, ZO-1, β-catenin etc) (Watabe *et al.,* 1994). This further illustrated α-catenin's pivotal role in adherens junction formation and regulation of cell adhesion. These studies however, did not attempt to ascertain the signal transduction role which α-catenin may play. Our study was the first

attempt to over express α-catenin in an OSCC cell line. If this had been successful, indispensable knowledge could have been gained into the part played by α-catenin in OSCC.

6.6 CONCLUDING STATEMENTS

Alterations in expression levels, cell localization and the gene structure of α-catenin have been found to be frequent events in a large range of cancers (Giannini *et al.,* 2000, Roe *et al.,* 1998, Vermeulen *et al.,* 1999). The α-catenin gene, in particular the region containing the vinculin, ZO-1 and actin binding domains were shown to be unaltered in South African OSCC. Therefore the interaction between α-catenin and the actin cytoskeleton should be formed in the OSCC cell lines.

In the OSCC cell lines studied, α-catenin appears to be largely expressed at the plasma membrane, which is crucial for the adherens junction formation. EGF signalling was shown to have no effect on the expression levels of α-catenin in the OSCC cell lines. α-catenin expression in the WHCO1 OSCC cell line was altered. It was speculated that this alteration in expression upon EGF treatment in this cell line was due to a mutation contained in the vinculin, ZO-1 and actin binding domain of α-catenin which may have affected its interaction with these molecules. If the interaction between α-catenin and vinculin, ZO-1 and actin does not occur correctly, then α-catenin may detach from the adherens junction and translocate to the cytoplasm/nucleus.

EGFR was found to be over expressed in OSCC and the apoptotic cascade has been found to be decreased in tumours (Veale and Thornley, 1989, McDonnell *et al.,* 1999, Sarbia *et al.,* 1999). Therefore if the over expression of α-catenin had been successful it would have provided invaluable insights into α-catenin's signalling role in these pathways and the part which it may play in the multi-faceted disease of OSCC. This unfortunately did not prove to be possible in this study and would certainly be appropriate for perusing in the future. All of the results obtained in this study indicated

that α-catenin plays an indispensable role in the adherens junction formation and may be an important intermediate in pathways stimulated by EGF.

CHAPTER 7

APPENDIX:

1.1 Composition of 1 x PBS: 1.37 mM of $NaCl_2$, 0.023 mM of KCl, 0.08 mM of $Na_2HPO_4.12H_2O$ and 0.015 mM of KH_2PO_4 at pH 7.2-7.3.

1.2 Chloroform removal of DNA and proteins: 0.2 ml of chloroform was added to the cells and incubated at room temperature for 3 minutes. The tube was then centrifuged at 12 000 x g for 15 min at 4^0 C in a MC 12V Sorvall Bench top centrifuge and the aqueous phase kept.

1.3 1% agarose gel (White Sci): 1% of agarose was added to 30 ml of 1 x TAE (appendix 1.4) and melted. 1.5 µl of EtBr was added and the gel poured and set.

1.4 Composition of 1 x TAE buffer: 40 mM Tris base, 20 mM of $CH_3COONa.\ 3H_2O$ and 1 mM of EDTA at pH 7.2 (adjusted using acetic acid). Volume made up to 1 L using dH_2O.

1.5 Agarose gel loading buffer: 50% glycerol, 50% 2 x TAE and 1 g of bromophenol blue.

1.6 Reaction mixture for first step of the RT-PCR: 5µl of 5 x buffer (Roche), 0.6 µl of RNAsin, 2µl of dNTP mix, 1µl of MMLV-RT (Roche) and 12.4 µl of dH_2O.

1.7 3M sodium acetate solution: A 3 M sodium acetate solution was made. The pH adjusted to 5.2 using glacial acetic acid and the volume made up using dH_2O and filter sterelized using a 0.45 µm filter (Micron Separations Inc.).

1.8 PCR reaction mixture (the mixture was set up under standard conditions):

- 1 µl of each primer.
- 1 µl of cDNA (synthesized in 2.3.4.1).
- 5 µl 10 x PCR buffer (Roche) (appendix 1.9).
- 3 µl of 1.5 mM $MgCl_2$ (Roche).
- 1µl of dNTP mix and 33 µl dH_2O (Sigma).
- 1 µl Expand high Fidelity^{plus} PCR System *taq* polymerase (Roche)

1.9 10 x PCR buffer: 50 mM KCl and 10 mM Tris.Cl pH 8.3. The volume was made up using dH_2O.

1.10 Buffer PB (Qiagen): Composition of buffer not supplied by manufacturer.

1.11 Buffer PE (Qiagen): Composition of buffer not supplied by manufacturer.

1.12 Sequence of α-catenin's vinculin, ZO-1 and actin binding domains as apmplified from the WHCO1 OSCC cell line (Mutated base in red):

```
5′ 1 TCGCGGGACGAGATCGAGTGGCTTAGTAGTTCCCTTGTAAGCAAAAAGCG
  51 AAGATTGCGGAACAGGTGGCCAGCTTCCAGGAAGAAAAGAGCAAGCTGG
 100 ATGCTGAAGTGTCCAAATGGGACGACAGTGGCAATGACATCATTGTGCT
 149 GGCCAAGCAGATGTGCATGATTATGATGGAGATGACAGACTTTACCCGA
 198 GGTAAAGGACCACTCAAAAATACATCAGATGTCATCAGTGCTGCCAAGA
 247 AAATTGCTGAGGCAGGATCCAGGATGGACAAGCTTGGCCGCACCATTGC
 296 AGACCATTGCCCCGACTCGGCTTGCAAGCAGGACCTGCTGGCCTACCTG
 345 CAACGCATCGCCCTCTACTGCCACCAGCTGAACATCTGCAGCAAGGTCA
 394 AGGCCGAGGTGCAGAATCTCGGCGGGGAGCTTGTTGTCTCTGGGGTGGA
 443 CAGCGCCATGTCCCTGATCCAGGCAGCCAAGAACTTGATGAATGCTGTG
 492 GTGCAGACAGTGAAGGCATCCTACGTCGCCTCTACCAAATACCAAAAGT
 541 CACAGGGTATGGCTTCCCTCAACCTTCCTGCTGTGTCATGGAAGATGAA
 590 GGCACCAGAGAAAAAGCCATTGGTGAAGAGAGAGAAACAGGATGAGACA
 639 CAGACCAAGATTAAACGGGCATCTCAGAAGAAGCACGTGAACCCGGTGC
 688 AGGCCCTCAGCGAGT 3′
```

1.13 Sequence of α-catenin's vinculin, ZO-1 and actin binding domains as amplified from the WHCO3 OSCC cell line (Mutated base in red):

```
5′ 1 TAACATGAAGTCTATGCGCCGAGACTGTGAGTGGTGGCGTAGGCGCGCA
  51 AGTTGGAGGATCGCGAGCCCCAAAGCGTAGGACAGGGTGGAGTCCGTAT
 100 GAAAGAAGAAGGCACAGGTGTCTTCAGTCGGGCTAGATTAAACCGACAG
 149 AGGCAATGAACAGTGGTGTGTAGTGCCACCCGATAAGCGAAAGTTGGGA
 198 AGTAAATGATTAACTCGGTCCTGGTGGTAAAGTCAGCCGATAAGGTTCT
 247 CCGGATGTCACTTGTGCTGCCAAGAAATTTGCTGATGCAGGTGCCAAGA
 296 TGAAACAAGGTTGGCCGCACCATTGCGACCATTGCCCCGACTCGGCTTG
 345 ACAAGCCGGACCCGCTGGCCTACCTGCAACGAATCGCCCTCTACTGCCA
 396 CCAGCTGAACATCTGCAGCAAGGTCAAGGCCGAGGTGCACAATCTCGGC
```

443 GGGAAGCTTGTTGTCTCTGGGGATGGACAGCGCCATGTCCCTGATCCAG
492 GCAGCCAAAAACTTGATGAATGCTGTGGTGCAGACAGTGAAGGCATCCT
541 ACGTCGCCTCTACCAAATACCAAAAGTCACAGGGTATGGCTTCCCTCAA
590 CCTTCCTGCTGTGTCATGGAAAATGAAGGCACCAGAGAAAAAGCCATTG
639 GTGAAGAGAGAGAAACAGGATGAGACCCATACCAAGTATTAAA 3′

1.14 Sequence of α-catenin's vinculin, ZO-1 and actin binding domains as amplified from the WHCO5 OSCC cell line:

5′ 1 GAGGACATGGACGGAATGCCCGAGAATGTATGCGTGGTTTATATGTCCA
51 ATTGTAGGATGGAGGCTCAATACTTTAGGACAGGAGTCGGACGACTACA
100 GAAAGAAACCATCACAGTTGTCTTCAGAAGTGGCTAGGTTAAATAGAAA
149 AGGCAAAGAATACTGCCATCGTAGGGGGGCTCTAGTAACGGAGTAGCTG
198 GGAGAAATAAAGAGTGAACCAGTCCAGGTGTTAAATTTAATTCAAAAAG
247 GCCGCGGATGTCATCAGTGCTGCCAAGAAAATTGCTGAGGCAGGATCCA
296 GGATGGACAAGCTTGGCCGCACCATTGCAGACCATTGCCCCGACTCGGC
345 TTGCAAGCAGGACCTGCTGGCCTACCTGCAACGCATCGCCCTCTACTGC
394 CACCAGCTGAACATCTGCAGCAAGGTCAAGGCCGAGGTGCAGAATCTCG
443 GCGGGGAGCTTGTTGTCTCTGGGGTGGACAGCGCCATGTCCCTGATCCA
492 GGCAGCCAAGAACTTGATGAATGCTGTGGTGCAGACAGTGAAGGCATCC
541 TACGTCGCCTCTACCAAATACCAAAAGTCACGGGGTATGGCTTCCCTCA
590 ACCTTCCTGCTGTGTCATGGAAGATGAAGGCACCAGAGAAAAAGCCATT
639 GGTGAAGAGAGAGAAACAGGATGAGACACAGACCAAGATTAAACGGGCA
688 TCTCCAAAAAAGCACGTGAACCCGGTGCA 3′

1.15 Sequence of α-catenin's vinculin, ZO-1 and actin binding domains as amplified from the WHCO6 OSCC cell line (Mutated basein red):

5′ 1 TCGCGGGACGAGATCGAGTGGCTTAGTAGTTCCCTTGTAAGCAAAAAGC
51 GAAGATTGCGGAACAGGTGGCCAGCTTCCAGGAAGAAAAGAGCAAGCTG
100 GATGCTGAAGTGTCCAAATGGGACGACAGTGGCAATGACATCATTGTGC
149 TGGCCAAGCAGATGTGCATGATTATGATGGAGATGACAGACTTTACCCG
198 AGGTAAAGGACCACTCAAAAATACATC**A**GATGTCATCAGTGCTGCCAAG
247 AAAATTGCTGAGGCAGGATCCAGGATGGACAAGCTTGGCCGCACCATTG
296 CAGACCATTGCCCCGACTCGGCTTGCAAGCAGGACCTGCTGGCCTACCT

345 GCAACGCATCGCCCTCTACTGCCACCAGCTGAACATCTGCAGCAAGGTC
394 AAGGCCGAGGTGCAGAATCTCGGCGGGGAGCTTGTTGTCTCTGGGGTGG
443 ACAGCGCCATGTCCCTGATCCAGGCAGCCAAGAACTTGATGAATGCTGT
492 GGTGCAGACAGTGAAGGCATCCTACGTCGCCTCTACCAAATACCAAAAG
541 TCACAGGGTATGGCTTCCCTCAACCTTCCTGCTGTGTCATGGAAGATGA
590 AGGCACCAGAGAAAAAGCCATTGGTGAAGAGAGAGAAACAGGATGAGAC
639 ACAGACCAAGATTAAACGGGCATCTCAGAAGAAGCACGTGAACCCGGTG
688 CAGGCCCTCAGCGAGTTCAAAGCTATGGACAGCATCTAA 3′

1.16 Sequence of α-catenin's vinculin, ZO-1 and actin binding domains as amplified from the SNO OSCC cell line:

5′ 1 CGAGCGCACATGAACGGCATGCGCCGATGAATGTAGTTGTGGTTTAAGT
51 GTCGCAGTATGTAGGTTCGGAGGCCCAAAAGACTAGAACGGCAGTTGGG
100 GGATAAAGAAAGAGAGACAGCACAGTTGTCTTCAGTACGGGCTGGATTT
149 AACAGACAAAGGCAATGAACACTGCTGATGTTAGTGCGGCTCTATAGAC
198 GGAGAGCTGGGAAGAGAAATGGTGAACCTGTCCAGGTGGTGAATGTATA
247 GCTCAAAAAGATTGCGTATGTCATTTGTGCTGCCAAGAAATTTGCTGAG
296 GCAGGAGCCAAGATGTGAACAAGCTTGGCCGCACCATTGCAGACCATTG
345 CCCCGACTCGGCTTGCAAGCGGGACCTGCTGGCCTACCTGCAACGAATC
394 GCCCTCTACTGCCACCAGCTGAACATCTGCAGCAAGGTCAAGGCCGAGG
443 TGCAGAATCTCGGCGGGGAGCTTGTTGTCTCTGGGGATGGACAGCGCCA
492 TGTCCCTGATCCAGGCAGCCAAGAACTTGATGAATGCTGTGGTGCAAAC
541 AGTGAAGGCATCCTACGTCGCCTCTACCAAATACCAAAAGTCACAGGGT
590 ATGGCTTCCCTCAACCTTCCTGCTGTGTCATGGAAGATGAAGGCACCAG
639 AGAAAAACCCATTGGTGAAGAGAGAGAAACAGGATGAGACACAGACCAA
688 GATTAAACGGGCATCTTCAAAAGAACGCA 3′

2.1 Counting of detached cells: 8 ml of tissue culture medium (methods 2.3.1) was added to the detached cell/TE mixture. 20 μl of cell suspension was mixed with an equal volume of 4% trypan blue, and placed onto a heamocytometer. The average number of cells in ten squares of the heamocytometer were counted, corrected for the dilution factor and multiplied by the volume of the square (10^4) to calculate the number of cells/ml.

2.2 4% paraformaldehyde (Sigma): 4% paraformaldehyde was dissolved in solution 1 [83% of a 0.14 M Na_2HPO_4 (anhydrous) and 17% of a 0.63 M NaOH]. This was heated to 80^0 C and stirred until the solution went clear, were after it was left to cool and the pH adjusted to 7.2-7.4. This solution was then filtered and stored at 2^0 C.

2.3 0.25% Triton X 100: 0.25% of Triton X 100 was added to 10 ml of 1 x PBS.

2.4 Elvanol mounting agent: 40% of glycerol was dissolved in dH_2O. 10% of polyvinyl alcohol (grade 51-05) was added. 7.5 ml of dH_2O was added and stirred for 24 hours at room temp. 15 ml of 0.1 M Tris.HCl (pH 8.5) was added and the solution stirred in a 50^0 C H_2O bath for 48 hours. The tube was then centrifuged at 1 500 x g for 30 minutes at room temp in an Universal bench top centrifuge (Hettich). The supernatant was stored at 4^0 C. Prior to use, a granual of p-Phenylenediamine (anti bleaching agent) was added.

2.5 1 x PMSF/Trazylol solution: A 20 mM PMSF solution was made in methanol.

2.6 Hypotonic buffer: 20 mM Tris.HCl (pH7.4-7.5), 25 mM sodium fluoride and 1 mM EDTA, which was made up to 100 ml using dH_2O.

2.7 Protease inhibitors added to the hypotonic buffer:

- 0.5% PMSF stock solution (appendix 2.5).
- 1% Trazylol.
- 0.1% Pepstatin (Sigma USA).
- 0.05% Leupeptin (Sigma USA).

2.8 0.1 % SDS solution: 0.1% SDS was made up in a 20 mM Tris.HCl pH 7.4 solution.

2.9 Standards used in the estimation of protein concentration of the plasma membrane fractions: The following concentrations of bovine serum albumin (BSA) suspended in SDS-Tris buffer (appendix 2.8) was used: 2 μg/μl, 6 μg/μl, 12 μg/μl, 18 μg/μl, 24 μg/μl and 30 μg/μl.

2.10 Standards used in the estimation of protein concentration of the cytoplasm/nuclear fractions: The following concentrations of BSA suspended in hypotonic buffer (appendix 2.6) were used: 2 μg/μl, 6 μg/μl, 12 μg/μl, 18 μg/μl, 24 μg/μl and 30 μg/μl.

2.11 7.5% TCA: 7.5% TCA was made up to 100 ml using dH_2O.

2.12 Coomasie blue stain (Merck): 0.26% of Coomasie blue was added to 50% methanol and 10% acetic acid. The volume was made up to 500 ml using dH_2O.

2.13 Elution solution: 66% methanol, 33% dH_2O and 1% ammonia solution.

2.14 Calculation of the concentration of proteins in the plasma membrane fractions: The equation for the line fitted to the standard curve (Figure 7.1) was y = 0.0318 x – 0.0183. The absorbance value for each sample was fitted into the equation and the concentration of the proteins in that sample calculated. E.g. WHCO1 membrane fraction:

- A = 0.2880, equation: y = 0.0318 x – 0.0183
- 0.2880 = 0.0318 x – 0.0183
- x = 9.632 μg/5 μl →
- Divide by 5 to get μg/μl
- 9.632 μg/5 μl / 5 = 1.927 μg/μl of protein →

The concentration of the other cell lines could be found in Table 7.1.

2.15 Calculation of the concentration of proteins in the cytoplasm/nuclear fractions: The equation for the line fitted to the standard curve was: y = 0.0276 x + 0.0077 (Figure 7.2). The method used to calculate the protein concentrations in the cytoplasmic/nuclear fractions was the same as that used in appendix 2.14. The concentration of the other cell lines could be found in table 7.1.

2.16 10% polyacrylamide gel:

- 10% acrylamide.
- 25% separating buffer (appendix 2.17).
- 4% of 0.17 M SDS-solution (appendix 2.18).
- 4% of NN-methylene Bisacrylamide (bis) (appendix 2.19).
- 1.8% of ammonium persulfate (appendix 2.20).
- 0.25% of NNNN-tetramethylene-diamine (TEMED)

The volume was made up to 10 ml using dH_2O.

A 5% stacking gel was then poured onto of the separating gel.

- 5% acrylamide.
- 25% of stacking buffer (appendix 2.21)

- 4% of 0.17 M SDS-solution.
- 4% μl of bis.
- 1.8% of ammonium persulfate.
- 0.2% of TEMED.

The volume was made up to 10 ml using dH_2O.

2.17 Separating buffer: 0.5 M Tris was dissolved in dH_2O. The pH was adjusted to 8.8 using 1 N HCl and the volume made up to 25 ml using dH_2O.

2.18 0.17 M SDS-solution: 5% of SDS was made up to 25 ml using dH_2O.

2.19 Bis: 2.5% of bisacrylamide was made up to 10 ml using dH2O.

2.20 0.05 M ammonium persulfate solution: 0.05M ammonium persulphate was made up to 20 ml using dH_2O.

2.21 Stacking buffer: 0.1 M Tris was dissolved in dH_2O. The pH was adjusted to 6.8 using 1 N HCl and the volume made up to 25 ml using dH_2O.

2.22 Double lysis buffer:

- 3% Tris.HCl (pH 6.8).
- 4% SDS.
- 20% glycerol.
- 10% β-mercaptoethanol.

The volume was made up using dH_2O.

2.23 SDS-PAGE running buffer:

- 0.1% of SDS.
- 1.45% of glycine.
- 0.3% of Tris.

The pH was adjusted to 8.3 using 5 N HCl and the volume made up to 0.8 L using dH_2O.

2.24 Western blot transfer buffer (Towbin *et al.,* 1979): The following was mixed with 20% methanol:

- 0.3% glycine.
- 0.4 M Tris.

The pH was adjusted to 8.3 using 5 N HCl and the volume made up to 3 L using dH_2O.

2.25 Blocking buffer:

- 0.05 M Tris.Cl (pH 7.8).
- 2 mM calcium chloride (dehydrate).
- 5% non-fat milk powder (Elite).

2.26 Working solution: Equal volumes of the following were mixed:

- Luminol (Super Signal® West Pico Chemiluminescent kit, Pierce, USA).
- Peroxidase (Super Signal® West Pico Chemiluminescent kit, Pierce, UAS).

The total volume of solution made was dependent on the size of the nitro cellulose blot.

2.27 D19B developer:

- 6.4 M Metol
- 0.6 M Sodium sulphite (anhydrous).
- 80 mM Hydroquinone.
- 0.45 M Sodium carbonate (anhydrous).
- 34 mM Potassium bromide.

The volume was made up using dH_2O.

2.28 Fixer:

- 0.8 M Sodium thiosulfate.
- 0.2 M Potassium metabisulphite.

The volume was made up to 1 L using dH_2O.

2.29 Estimation of the concentration of α-catenin expression: The expression of α-catenin from the Western blots was calculated as follows:

- The ratio of the original standards concentration (WHCO3 membrane fraction) was compared to that of the concentration of the standard (WHCO3 membrane fraction) used in each Western blot.
- The ratio of the intensity of the concentration of the experiment was compared to the ratio calculated in step 1.
- The percent of α-catenin was calculated using the standard (WHCO3 membrane fraction) as 100 percent.

3.1 *Kpn I* restriction digestion reaction mixture:

- x μl of α-catenin or pEGSH.

- 10% *Kpn I*.
- 10% of 10 x buffer low salt concentration (appendix 3.2)

The volume was made up to 20 µl using dH_2O.

3.2 10 x low salt concentration buffer:

- 100 mM Tris.Cl (pH 7.5).
- 100 mM $MgCl_2$.
- 10 mM dithiothreitol.

3.3 *Spe I* restriction digestion reaction mixture:

- 50% of Kpn I restricted α-catenin or pEGSH.
- 7.5% *Spe I* (Amersham Bioscience).
- 10% of 10 x medium salt concentration buffer (appendix 3.4).

The volume was made up to 20 µl using dH_2O.

3.4 10 x medium salt concentration buffer (Ameraham Bioscience):

- 100 mM Tris.Cl (pH 7.5).
- 100 mM $MgCl_2$.
- 10 mM dithiothreitol.
- 500 mM NaCl.

3.5 *Not I* and *Xho I* restriction digestion reaction mixture:

- 50% of EGFP or pEGSH.
- 10% of *Not I* (Amersham Bioscience).
- 10% of *Xho I* (Amersham Bioscience).
- 10% of 10 x high salt concentration buffer (appendix 3.6).
- The volume was made up to 20 µl using dH_2O.

3.6 10 x high salt concentration buffer (Amersham Bioscience):

- 500 mM Tris-HCl (pH 7.5).
- 100 mM $MgCl_2$.
- 10 mM dithiothreitol.
- 1000 mM NaCl.

3.7 Membrane binding solution (Promega):

- 4.5 M guanidine isothiocyanate.
- 0.5 M potassium acetate (pH 5.0).

3.8 Membrane wash solution (Promega):

- 10 mM potassium acetate (pH 5.0).
- 80% ethanol.
- 16.7 μM EDTA.

3.9 The CIP dephosphorylation reaction mixture:

- 10% of 10 x CIP dephosphorylation buffer (Boehringer Mannheim) (appendix 3.10).
- 4% of CIP (Boehringer Mannheim).
- Linearised gel purified pEGSH vector.

The volume was made up to 100 μl using dH_2O (Sigma).

3.10 10 x CIP dephosphorylation buffer (Boehringer Mannheim):

- 10 mM $ZnCl_2$.
- 10 mM $MgCl_2$.
- 100 mM Tris.Cl (pH 8.3).

3.11 0.5 M EDTA: A 0.5 M EDTA solution was made and the pH adjusted to 8.1.

3.12 10% SDS solution: A 10% SDS was added to 100 ml using dH_2O.

3.13 T4 DNA polymerase end filling reaction (Amersham Bioscience):

- x μl of restricted α-catenin fragment.
- 10% of 10 x T4 DNA polymerase buffer (Amersham Biosciences) (appendix 3.14).
- 2.5% dNTP mix.

The volume was made up to 40 μl using dH_2O (Sigma).

3.14 10 x T4 DNA polymerase buffer (Amersham Biosciences): 330 mM Tris-HCl, pH 8.8, 660 mM potassium acetate, 100 mM magnesium acetate and 5 mM DTT.

3.15 Equation used to calculate the concentration of DNA:

- Absorbance at 260 nm x 50* x dilution factor = μg/ml.
- Example: The purified α-catenin fragment:
  - 0.43 x 50 x 150 = 322.5 μg/ml →
  - The same method was used to calculate the concentration of the other samples.

*This value was due to the fact that an optical density of 1 of double-stranded DNA corresponds to approximately 50 μg/ml.

3.16 Calculation of the 260 nm:280 nm ratios:

Example, α-catenin:

- $\frac{\text{Absorbance 260 nm}}{\text{Absorbance 280 nm}} = \frac{0.043}{0.020} = 2.15 \rightarrow$

The same method was used to calculate the 260 nm:280 nm ratios of the other samples.

3.17 Calculation of the molar concentration of available ends of DNA: The following equation was used to calculate the molar concentration of available ends of DNA:

- pico mole ends/micro gram of DNA = $\frac{2 \times 10^6 \quad \text{(Stratagene)}}{\text{number of base pairs} \times 660}$
- For example: The α-catenin fragment:
- Pico mole ends/mico gram of DNA $= \frac{2 \times 10^6}{3500 \times 660}$

$= 0.866 \rightarrow$

The molar concentration of available ends of DNA of the pEGSH and EGFP fragment and pEGSH vector were calculated in the same manner.

3.18 Manual ligation reaction mixture:

- 10% of 10 x T4 DNA ligase buffer (Amersham Bioscience) (appendix 3.19).
- x μl of restricted pEGSH vector.
- x μl of restricted α-catenin or EGFP fragment.
- 10 mM ATP (appendix 3.20).
- 2.5% of T4 DNA ligase (Amersham Bioscience).

The volume was made up to 20 μl using dH_2O (Sigma).

3.19 10 x T4 DNA ligase buffer (Amersham Bioscience):

- 200 mM Tris.Cl (pH 7.6).
- 50 mM $MgCl_2$.
- 50 mM dithiothreitol.
- 500 μg/ml of BSA

The volume was made up using dH_2O.

3.20 10 mM ATP (Sigma): A 10 mM ATP solution was made up using dH_2O.

3.21 Fast-Link™ reaction mixture (Epicentre technologies):

- 7.5% of 10 x ligation buffer (appendix 3.22).
- 3.75% of 10 mM ATP.
- x µl of restricted α-catenin.
- x µl of restricted pEGSH vector.
- 5% of T4 DNA ligase.

The volume was made up to 20 µl using dH_2O (Sigma).

3.22 10 x ligation buffer (Epicentre technologies): Composition of buffer not supplied.

3.23 Rapid DNA ligation reaction mixture (Roche):

- x µl of restricted α-catenin or EGFP.
- x µl of restricted pEGSH.

The volume was made up to 10 µl using 1 x concentration DNA dilution buffer (appendix 3.24).

3.24 1 x concentration DNA dilution buffer (Roche): Composition of buffer not supplied.

3.25 T4 DNA ligation buffer (Roche): Composition of buffer not supplied.

3.26 Protocol used to produce competent XL1 Blue *E.coli* cells (Henyen., 1996): 0.5 L of Luria burtani broth (LB) (appendix 3.27) was grown to an optical density of 0.6 at 600 nm. The cells were then chilled to harvest and centrifuged. The pellet was re-suspended in 0.5 L of cold dH_2O and re-centrifuged as previous. The pellet was re-suspended in 250 ml of cold dH_2O and re-centrifuged. The pellet was re-suspended in 10 ml of 10 % glycerol and re-centrifuged as previous (appendix 3.28). The pellet was re-suspended in 2 ml of cold 10 % glycerol, so as to obtain a cell concentration of 1-3 x 10^{10} cells/ml. The cells were then snap freezed and stored at -70^0 C. All of the centrifugation steps were carried out at 4^0 C and at 4 000 x g using a JA20 rotar in a Beckman J2-21 centrifuge (Beckman) for 15 minutes.

3.27 LB broth: The following was added to 300 ml of dH_2O: 1 % NaCl, 1 % tryptone (OXOID) and 0.5 % yeast extract (OXOID). The pH was adjusted to 7 using 5 N

NaOH and the volume made up to 500 ml using dH_2O. The broth was autoclaved and stored at 4^0 C.

3.28 10 % glycerol solution: A 10 % glycerol solution was made up and filter sterilised using a 0.45 μm filter (Micron Separations Inc.).

3.29 SOC medium: The following was added to 890 μl of SOB medium (appendix 3.30): 10 % 0f 1 M $MgCl_2$ and 1 M $MgSO_4$ solution (appendix 3.31) and 1 % of 2 M glucose solution (appendix 3.32).

3.30 SOB medium: The following was added to 40 ml of dH_2O: 2 % tryptone, 0.5 % yeast extract and 0.05 % NaCl. The pH was adjusted to 7 using 5 N NaOH and the volume made up to 50 ml using dH_2O. The broth was autoclaved and stored at 4^0 C.

3.31 1 M $MgCl_2$ and 1 M $MgSO_4$ solution: 1 M $MgCl_2$ and 1 M $MgSO_4$ was made up to 50 ml using dH_2O, and filter sterilized using a 0.45 μm filter (Micron Separations Inc.).

3.32 2 M glucose solution: A 2 M glucose solution was made up to 10 ml and filter sterilized using a 0.45 μm filter (Micron Separations Inc.).

3.33 LA agar plates: The following was added to 100 ml of dH_2O: 1 % NaCl, 1 % tryptone, 0.5 % yeast extract and 2 % agar. The pH was adjusted to 7 using 5 N NaOH. The broth was autoclaved, 200 μl of ampicillin (50mg/ml) (appendix 3.34) added and poured into petri-dishes.

3.34 Ampicillin solution (Roche): A 50 mg/ml ampicillin solution was made and filter sterilized using a 0.45 μm filter (Micron Separations Inc.).

3.35 Cracking buffer: 20 mM Tris (pH 8.0), 2 mM EDTA and 1 % Triton X-100. The volume was made up to 50 ml using dH_2O.

3.36 Seqeunce of the pEGSH-EGFP construct:

```
5′ 1 CACAGCAGAAACAGTAATTGGTACCGGATCCGATATCGATGCGGCCGCC
  51 ATCGAATTCTTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCG
 100 GCGGTCACGAACTCCAGCAGGACCATGTGATCGCGCTTCTCGTTGGGGT
 149 CTTTGCTCAGGGCGGACTGGGTGCTCAGGTAGTGGTTGTCGGGCAGCAG
 198 CACGGGGCCGTCGCCGATGGGGGTGTTCTGCTGGTAGTGGTCGGCGAGC
 247 TGCACGCTGCCGTCCTCGATGTTGTGGCGGATCTTGAAGTTCACCTTGA
```

296 TGCCGTTCTTCTGCTTGTCGGCCATGATATAGACGTTGTGGCTGTTGTA

345 GTTGTACTCCAGCTTGTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCG

394 ATGCCCTTCAGCTCGATGCGGTTCACCAGGGTGTCGCCCTCGAACTTCA

443 CCTCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTTGAAGAAGATGGTGCG

492 CTCCTGGACGTAGCCTTCGGGCATGGCGGACTTGAAGAAGTCGTGCTGC

541 TTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTAGGTCAGGG

590 TGGTCACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGAT

639 GAACTTCAGGGTCAGCTTGCCGTAGGTGGCATCGCCCTCGCCCTCGCCG

688 GACACGCTGAACTTGTGGCCGTTTACGTCGCCGTCCAGCTCGACCAGGA

736 TGGGCACCACCCCGGTGAACAGCTCCTC 3′

3.37 G418 stock solution (Promega): A 20 mg/ml stock solution of G418 was made and filter sterilized using a 0.45 μm filter (Micron Separations Inc.).

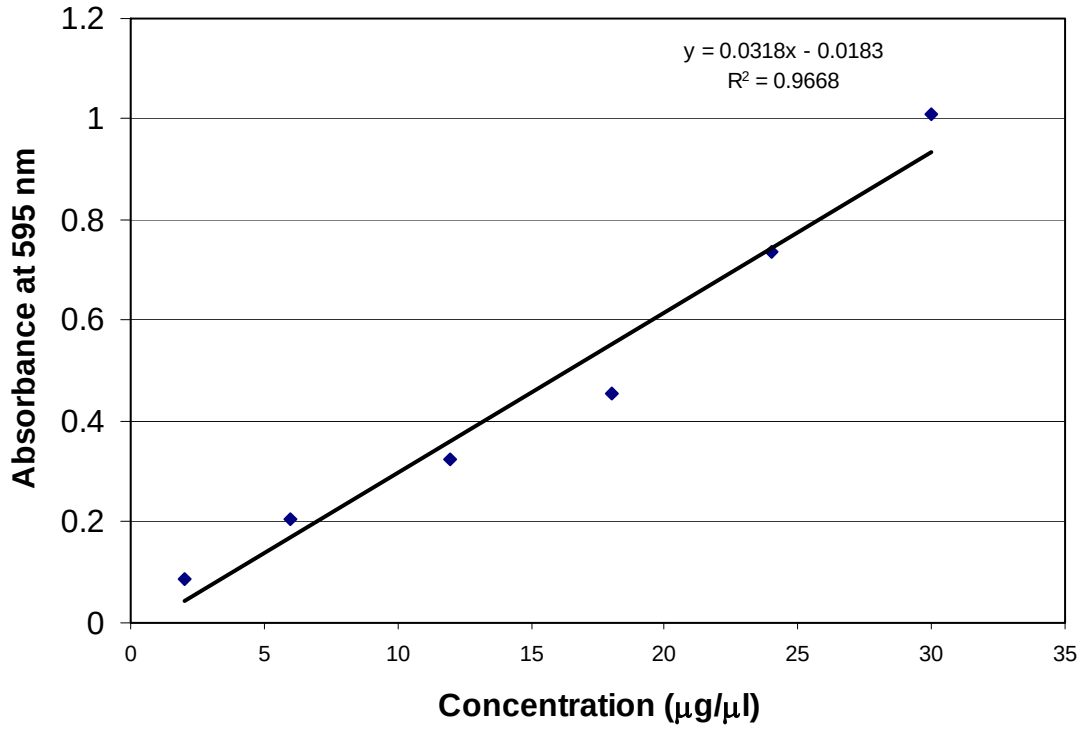


Figure 7.1: Standard curve of BSA concentration used to estimate the concentration of proteins contained in the plasma membrane fractions obtained from the OSCC and CC cell lines. The absorbance values of the six BSA standard (the standards were resuspended in SDS-Tris buffer) solutions were used to establish this standard curve. The standard curve was then used to calculate the concentration of the proteins in the plasma membrane fractions. The equation of the line fitted to the datum points was: y = 0.0318x – 0.0183. The degree of fit was R^2 = 0.9668.

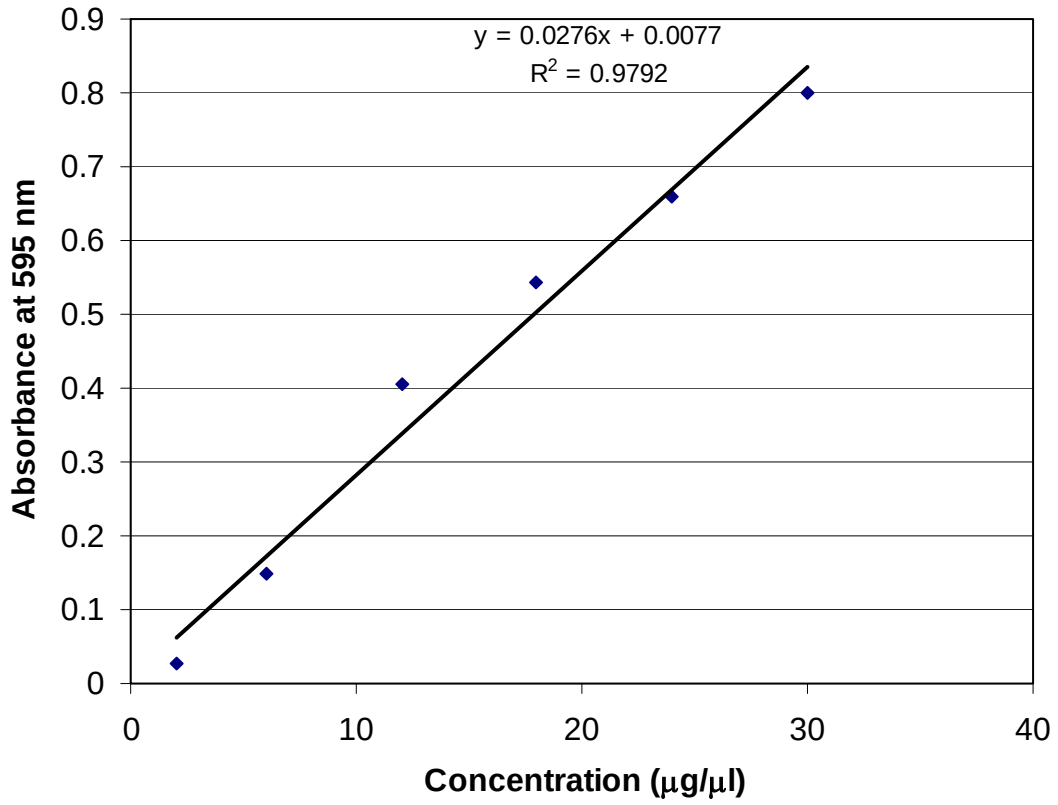


Figure 7.2: Standard curve of BSA concentration used to estimate the concentration of proteins in the cytoplasmic/nuclear fractions obtained from the OSCC and CC cell lines. The absorbance values obtained from the six BSA standard solutions (standards resuspended in hypotonic buffer) were used to set-up this standard curve. This standard curve was then used to calculate the concentration of the cytoplasmic/nuclear fractions. The equation of the line fitted to the datum points was: $y = 0.0276x – 0.0077$. The degree of fit was $R^2 = 0.9792$.

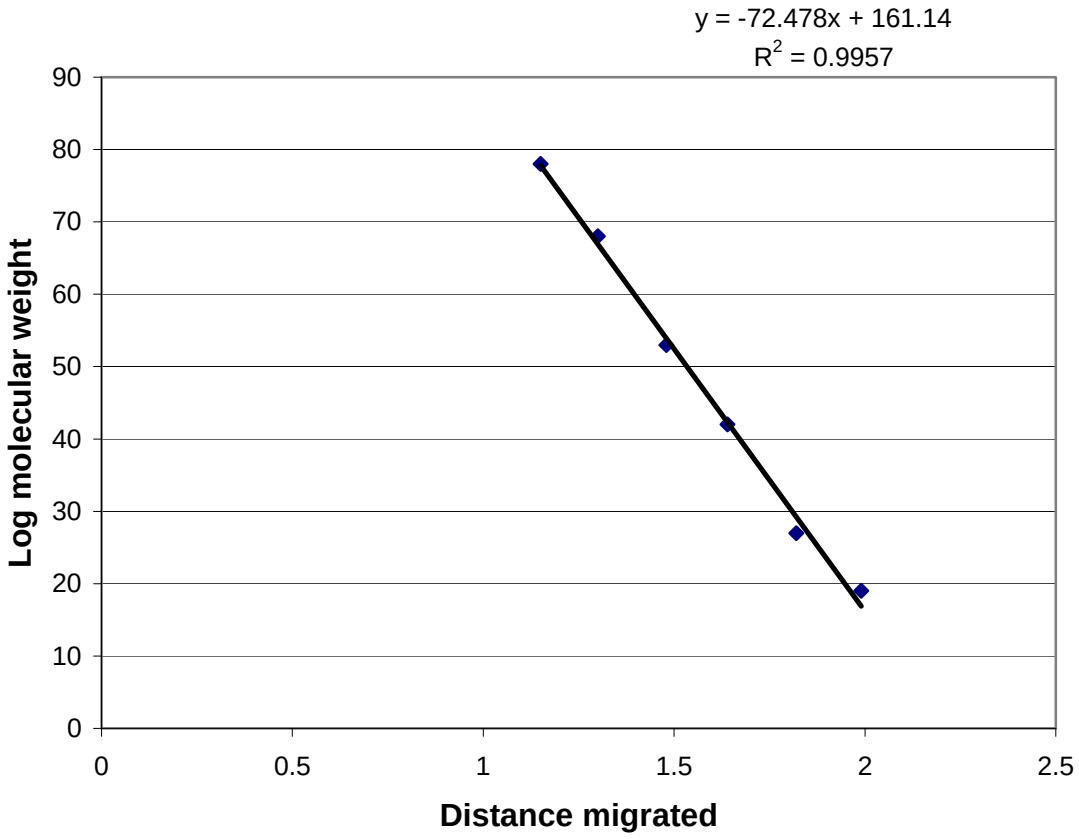


Figure 7.3: Standard curve of log molecular weight of the low molecular weight marker (Pharamicia) verse the distance migrated by the marker. The equation of the line fitted was y = -72.478x + 161.14 and the degree of fit was R^2 = 0.9957 .

F	**C L**	**O V**	**V L**	**C F**	**A**	**C E**	**C S**	**V R**
Membrane	CO1	300	100	3	0.288	9.62	1.93	2.60
Membrane	CO3	300	100	3	0.29	9.68	1.94	2.58
Membrane	CO5	300	300	1	0.112	4.10	0.82	6.09
Membrane	CO6	300	200	1.5	0.265	8.91	1.77	2.81
Membrane	SNO	300	100	3	0.181	6.27	1.24	3.99
Membrane	DLD 1 α-	300	200	1.5	0.136	4.84	0.96	5.14
Cyto/Nuc	CO1	300	50	6	0.307	10.83	2.17	2.31
Cyto/Nuc	CO3	300	50	6	0.278	9.78	1.96	2.54
Cyto/Nuc	CO5	300	50	6	0.882	31.68	6.34	0.79
Cyto/Nuc	CO6	300	50	6	0.521	18.60	3.72	1.33
Cyto/Nuc	SNO	300	50	6	0.422	15.00	3.00	1.67
Cyto/Nuc	DLD 1 α-	300	50	6	0.574	20.52	4.09	1.22

Table 7.1: *Estimated protein concentrations of the plasma membrane and cytoplasm/nuclear fractions obtained from the OSCC and CC cell lines and the volumes loaded onto the SDS-PAGEs: Cyto/nuc: Cytoplasmic/nuclear* ***F****: Fractionate,* ***C L****: Cell line,* ***O V****: Original sample volume (μl),* ***V L****: Volume lyophilised (μl),* ***C F****: Concentration factor,* ***A****: Absorbance,* ***C E****: concentration of estimated sample in μg/μl,* ***C S****: Concentration of sample in μg/μl and* ***V R****: Volume required to obtain 5μg of protein.*

Treatment	**C L**	**O V**	**V L**	**C F**	**A**	**C E**	**C S**	**V R**
EGF 1hr	CO1	300	100	3	0.332	11.016	2.202	2.27
EGF 1hr	CO3	300	200	1.5	0.114	4.160	0.831	6.00
EGF 1hr	CO5	300	100	3	0.205	7.021	1.403	3.55
EGF 1hr	CO6	300	200	1.5	0.13	4.664	0.933	5.35
EGF 1hr	SNO	300	100	3	0.166	5.796	1.158	4.30
EGF 6hr	CO1	300	100	3	0.273	9.160	1.831	2.73
EGF 6hr	CO3	300	100	3	0.271	9.096	1.818	2.75
EGF 6hr	CO5	300	100	3	0.176	6.100	1.221	4.08
EGF 6hr	CO6	300	100	3	0.708	22.840	4.568	1.10
EGF 6hr	SNO	300	100	3	0.37	12.211	2.441	2.05
EGF 12hr	CO1	300	100	3	0.264	8.876	1.774	2.82
EGF 12hr	CO3	300	100	3	0.205	7.021	1.403	3.55
EGF 12hr	CO5	300	100	3	0.16	5.607	1.120	4.46
EGF 12hr	CO6	300	300	1	0.202	6.928	1.386	3.61
EGF 12hr	SNO	300	100	3	0.201	6.895	1.378	3.61

Table 7.2: Estimated concentration of plasma membrane extracted fractions from the EGF treated OSCC cell lines: Treatment: *Length of time which cells were treated with EGF*. ***C L***: *Cell line treated.* ***O V***: *Original sample volume prior to lyophilysation (μl). V L: Volume lyophalised (μl).* ***C F***: *Concentration factor.* ***A***: *Absorbance of sample at 595 nm.* ***C E***: *Concentration of sample used to estimate the concentration of proteins in the lyophalised sample in 5μg/μl.* ***C S***: *Concentration of lyophalised sample in μg/μl.* ***V R***: *Volume (μl) loaded onto SDS-PAGE so as to load 5μg of protein.*

Treatment	**C L**	**O V**	**V L**	**C F**	**A**	**C E**	**C S**	**V R**
EGF 1hr	CO1	300	50	6	0.273	9.611	1.921	2.60
EGF 1hr	CO3	300	50	6	0.125	4.25	0.85	5.88
EGF 1hr	CO5	300	50	6	0.168	5.808	1.162	4.30
EGF 1hr	CO6	300	50	6	0.681	24.395	4.879	1.03
EGF 1hr	SNO	300	50	6	0.249	8.743	1.749	2.85
EGF 6hr	CO1	300	50	6	0.244	8.562	1.711	2.91
EGF 6hr	CO3	300	50	6	0.175	6.062	1.211	4.11
EGF 6hr	CO5	300	50	6	0.359	12.727	2.546	1.95
EGF 6hr	CO6	300	50	6	0.816	29.285	5.856	0.84
EGF 6hr	SNO	300	50	6	0.303	10.698	2.140	2.34
EGF 12hr	CO1	300	50	6	0.194	6.75	1.35	3.70
EGF 12hr	CO3	300	50	6	0.185	6.424	1.285	3.88
EGF 12hr	CO5	300	50	6	0.329	11.640	2.327	2.15
EGF 12hr	CO6	300	50	6	0.548	19.575	3.914	1.28
EGF 12hr	SNO	300	50	6	0.605	21.640	4.327	1.14

Table 7.3: Estimated concentration of cytoplasm/nuclear extracted fractions from the EGF treated OSCC cell lines: ***Treatment:*** *Length of time which cells were treated with EGF.* ***C L:*** *Cell line treated.* ***O V:*** *Original sample volume prior to lyophilysation (μl).* ***V L:*** *Volume lyophilised (μl).* ***C F:*** *Concentration factor.* ***A:*** *Absorbance of sample at 595 nm.* ***C E:*** *Concentration of sample used to estimate the concentration of proteins in the lyophalised sample in 5μg/μl.* ***C S:*** *Concentration of lyophalised sample in μg/μl.* ***V R:*** *Volume (μl) loaded onto SDS-PAGE so as to load 5μg of protein.*

Sample	Serum free TC medium (µl)	FuGENE™ 6 reagent (µl)	pERV 3 (µl)
A	94	6	0
B	94	6	5
C	96.5	6	2.5

Table 7.4: Composition of the FuGENE™ 6/pERV 3 mixtures: The pERV 3 vector used had a concentration of 0.99 µg/µl. Methods 2.2.1 contained the composition of the serum free tissue culture medium. TC stands for tissue culture.

CHAPTER 8

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