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THE ROLE OF E-CADHERIN IN MODULATING THE BEHAVIOURAL CHARACTERISTICS OF ORAL SQUAMOUS CARCINOMA CELLS

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This Thesis is dedicated to my family; Dad, Mom, Brother and Sister.

ABSTRACT

The incipient invasion of epithelial tumour cells, characterised by disruption of normal cell-cell adhesion, marks the point of transition to malignancy. Cadherins are a family of calcium-dependent, transmembrane, cell-cell adhesion molecules whose function requires association with the actin cytoskeleton of the cell through cytoplasmic proteins called catenins. Oral keratinocytes express E-cadherin, which is considered a regulator of the epithelial phenotype. Loss of E-cadherin commonly is seen in oral squamous cell carcinoma (OSCC), particularly in poorly differentiated tumours, allowing cells to detach from the tumour mass, invade and metastasise.

An E-cadherin negative OSCC cell line was identified where E-cadherin expression and function was restored through cDNA transfection resulting in functional cell surface expression of the protein and the formation of cell-cell attachments, with significant alteration in levels and distribution of catenins. Alteration of cell morphology, from a spindle to an epitheloid form, was observed. Additionally, restoration of E-cadherin showed a marked inhibition of the invasive potential of this OSCC cell line. These effects were not observed in the control cell line transfected with the expression vector containing antisense E-cadherin cDNA.

Most findings in cell and molecular biology, presented in the literature, are based, primarily, on *in vitro* studies obtained from the culture of cells growing in 2-dimensional (2-D) tissue culture dishes. In this study I have investigated if E-cadherin plays a role in modulating tumour cell growth in a 3-D, as opposed to a 2-D, environment. Results showed that the epitheloid-like morphology brought about by E-cadherin expression under 2-D conditions allows differences in density dependent inhibition to occur accompanied by enhancement in the apoptotic capacity of the cells. However, where constraints of 3-D culture are imposed, these variations between E-cadherin expressing and non-expressing lines disappear. To explore the basis of these changes I have examined Western blot analysis of proteins known to play a role in mediating cell proliferation or regulation of apoptosis, such as Bcl-2, Akt-1, ILK and N-cadherin. I showed that changes in levels of expression of these proteins under 2-D conditions is markedly affected when cells were implanted in 3-D collagen I cultures.

I also investigated the possible molecular cross talk between E-cadherin and the β4-integrin subunit. Results obtained from β4-knock-down experiments showed that β4-integrin plays a role in the maturation of E-cadherin-dependent cell-cell contacts. I investigated the possible mechanisms of this apparent cadherin-integrin cross-talk and preliminary results showed that the function of β4-integrin was, potentially, executed through the Rac1 small Rho GTPase.

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ABBREVIATION LIST

Ajs	adherens junctions
Akt-1	protein kinase B alpha
α-MEM	Alpha modification of Eagle's medium
APC	adenomatous polyposis coli
bcl-2	B cell leukaemia/lymphoma-2
Bp	base pair
BSA	bovine serum albumin
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
CAMs	Cell Adhesion Molecules
CDH1	E-cadherin gene
cDNA	complementary DNA
CE	cornified envelop
CMV	Cytomegalovirus
c-myc	cellular homologue of myelocytomatosis virus
CO_2	carbon dioxide
CRIB	Cdc42/Rac Interactive Binding
DAPI	4^1, 6-αdiamidino-2-phenylindole dihydrochloride
DEPC	diethyl pyrocarbonate
ddH_2O	distilled deionised water
DMSO	Dimethylsulphoxide
DNA	deoxyribonucleic acid
DNTPs	Deoxyribonucleotides
doc-1	deleted in oral cancer gene
Dsc	Desmocollins
Dsg	Desmogleins
DTT	Dithiothreitol
EC	European Community
ECM	extracellular matrix
EDTA	Ethyldiaminotetraacetic acid
EGF(R)	Epidermal Growth Factor (receptor)
EMT	Epithelial-mesenchymal transition
ERK	extracellular signal-related kinase
FACS	fluorescence activated cell sorting
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
G	Gram
G	acceleration due to gravity
GDP	guanosine diphosphate
GST	glutathione *S*-transferase
GTP	guanosine triphosphate
HandE	haematoxylin and eosin (stain)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	hepatocyte growth factor/scatter factor
HRP	horse radish peroxidase

ILK	integrin-linked kinase
IRES	internal ribosome entry site
kb	kilo base
KCl	potassium chloride
kDa	Kilo Dalton
KGM	keratinocyte growth medium
K	Keratin (eg. K14 is Keratin-14)
LEPs	late envelope proteins
MAPK	mitogen activated protein kinase
MCS	multiple cloning site
MDCK	Madin-Darby canine kidney
MEM	modified Eagle's medium
$MgCl_2$	magnesium chloride
MMP	matrix metalloprotease
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	messenger RNA
MT-MMP	matrix-MMP
NaCl	sodium chloride
NaOH	sodium hydroxide
LAP	Latency-associated peptide
OD	optical density
OPCS	Office of Population Censuses and Surveys
OSCC	oral squamous cell carcinoma
PAGE	polyacrylamide gel electrophoresis
PAK	p21 activated kinase 1
PBD	p21 Binding Domain
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PET	polyethylene tetraphthallate
pH	potential of Hydrogen
PI3-kinase	Phosphatidylinositol 3-kinase
PKB	protein kinase B
PKC	protein kinase C
PMSF	phenylmethanesulphonyl fluoride
PRAD-1	parathyroid adenomatosis-1
PTP	protein tyrosine phosphatase
PVDF	polyvinylidene difluoride
Ras	rat sarcoma oncogene
Rb	Retinoblastoma
RNA	ribonucleic acid
RNAi	RNA interference
Rpm	rotations per minute
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulphate
siRNA	small inhibitory RNA
SPR	small proline rich
SSC	squamous cell carcinoma
TAE	Tris-acetate-EDTA buffer

TBS	Tris-buffered Saline
TEMED	N,N,N', N'-tetramethylethylenediamine (1,2 di(dimethyamino)ethane)
TJs	tight junctions
Thr	Threonine
TE	Buffer
Tris	tris(hydroxymethyl)aminomethane
TRITC	tetramethylrhodamine isothiocyanate
VASP	vasodialator-stimulated phosphoprotein
WB	Western blotting
WHO	World Health Organisation
Wnt	Wingless
ZO-1	zonula occludens
2-D	2-dimensional
2DE	H376 E-cadherin cell line growing in 2-D collagen I culture
3-D	3-dimensional
3DE	H376 E-cadherin cell line growing in 3-D collagen I culture

CHAPTER 1

Introduction

1.1 Overview

Cancer is the second leading cause of death in the Western world. Growth and proliferation of normal cells at all levels (biochemical, genetic and cellular) usually are under strict control. Loss of control over these processes leads to the development of malignancy; characterised by abnormal, non-stop cell divisions. Cancer usually starts as a primary tumour that is capable of invading the surrounding tissues, and eventually metastasises to distant sites where it settles as a secondary lesion. Metastasis entails a series of events involving adhesion and de-adhesion where the transformed cells gain the ability to alter their cell-cell and cell-extracellular matrix interactions. For example, loss of intercellular adhesion is a prerequisite for the cell to break away from the primary tumour mass.

Tumours arising from epithelial origin account for the majority of human cancers, while E-cadherin is one of the major cell-cell adhesion molecules in epithelial cells (and also is responsible for conveying signals in and out of the cell), and is the molecule that I have chosen to study. Understanding the biological basis of E-cadherin's importance and the underlying signalling cues it triggers in tumours will, hopefully, provide the basis of improved interventional therapeutic strategies.

1.2 Normal Oral Mucosa

The oral mucosa is divided into two components; an outer epithelial lining (equivalent to the epidermis of the skin), and an underlying connective tissue support (equivalent to the dermis of the skin). The two components are divided by a highly specialised basement membrane.

1.2.1 Epithelium

The oral epithelium is of a stratified squamous type, it forms a protective layer that shields the oral mucosa from the outer environment thus providing a barrier against environmental stresses, chemical damage, microorganisms and other noxious substances, as well as preventing excessive transepithelial loss of body fluids. To maintain this critical barrier the oral epithelium undergoes programmed terminal differentiation, as well as continual desquamation, which establishes the basis of epithelial homeostasis. Epithelial homeostasis implies that cell proliferation at the deeper layers is balanced by the loss of cells from the surface.

Oral epithelium shows regionally distinctive patterns of maturation. Depending on the presence of keratinisation, the oral epithelium is divided into three main types: the keratinised, or cornified, epithelium, parakeratinised epithelium and non-keratinised epithelium.

The keratinised epithelium, like that of the epidermis of the skin, covers the masticatory mucosa of the hard palate, the gingiva and some areas of specialised mucosa on the dorsum of the tongue. The parakeratinised epithelium covers the remaining part of the gingiva where superficial epithelial cells are nuclear, compared to the superficial anuclear cells of the keratinised oral epithelium which contain flattened remnants of nuclei (Scully *et al.*, 2005). The non-keratinised epithelium forms the lining of the distensible oral mucosa. It is generally thicker, but more permeable, than keratinised mucosa, covering areas that are subjected to the least mechanical stresses such as the floor of the mouth, the ventral surface of the tongue, the vestibule, the alveolar mucosa, the cheeks, and the inner lining of the lips.

In general, the stratified squamous epithelium consists of multiple layers of epithelial cells (keratinocytes). The proliferative basal layer (stratum basale), which lies adjacent to the basement membrane, consists of a single layer of keratinocytes, cuboidal or columnar in

shape, that exhibit a mitotic potential. Basal keratinocytes are of two types: the pluripotent stem cells, which maintain high capacity for self-renewal throughout adult life, and the transit amplifying cells, which a have lower capacity for self-renewal and possess the capability for undergoing terminal differentiation after few rounds of cell division (Potten, 1981; Hall and Watt, 1989; Watt, 2001). These cells generate a post-mitotic cell population that irreversibly withdraws from the cell cycle; the cells increase in size and lose their proliferative capacity (Barrandon and Green, 1985). Cells committed to terminal differentiation become less adherent to the basement membrane as well as to one another (Watt, 1984), detach from the basement membrane, as a consequence of inactivation and loss of β1-integrin (Hotchin *et al.*, 1993), and express proteins associated with differentiation, such as envoplakin, periplakin, involucrin and new cytokeratins (Watt and Green, 1981; Watt and Green, 1982; Barrandon and Green, 1985; Fuchs, 1990, Steinert, 2000). Basal cells also are characterised by their cytokeratin profile, containing an intracellular cytoskeleton composed of a relatively dispersed, but extensive, network of the keratin pair; keratin-5/keratin-14 (K5/K14) (Presland and Jurevic, 2002). In contrast stem cells were claimed to express K15/K19 (Squier and Kremer, 2001) though this is not proven unequivocally.

The spinous, or prickle-cell, layer (stratum spinosum) is composed of 3-5 layers of cells that show a gradient in shape, size and orientation with their protein synthesising machinery devoted to the manufacture of new pairs of keratins; K1/K10 in epidermal and gingival tissue and K4/K13 in non-keratinised epithelium such as the buccal and soft palate mucosa (Presland and Jurevic, 2002). At histological levels it can be seen that the spines (which give this layer its name) correspond to the insertion of the keratin tonofilament bundles into neighbouring cells. As the cells move towards the surface they gradually become larger, flatter and contain more kertain bundles.

The granular cell layer (stratum granulosum) lies superficial to the spinous cell layer and is characterised by irregularly shaped cells, condensed with 0.5-5μm keratohyaline granules, which become progressively larger as the keratinocytes move into the outermost granular layer. In this layer cells stop generating keratin and envelope proteins, that become uniformly deposited at the inner surface of the cell membrane to serve as a scaffold or template for subsequent maturation and assembly of the cornified envelope (CE) (stratum corneum), and start producing a new set of reinforcement proteins such as loricrin, filaggrin and the small proline rich (SPR) family of proteins (Fleckman *et al.*, 1985; Mehrel *et al.*, 1990; Steinert, 2000; Kalinin *et al.*, 2001). In oral epithelium, reinforcement proteins are composed of ~70% SPRs and ~10% loricrin (Steinert, 2000). These proteins mediate the cross-linking of the structural proteins that together form the cornified envelope of the stratum corneum. Also present in these cells are the lipid-rich cytoplasmic lamellar granules, which release their contents into the intercellular space to form lamellae between the granular and first cornified layer. The lamellar granule-derived sheet contributes to the protective barrier properties of the epithelium (Elias and Menon, 1991).

The stratum corneum is a 15nm multilayered zone of terminally differentiated keratinocytes, in keratinised epithelium. It is composed of flattened cells with the proteinaceous envelope cross-linked to the keratin cytoskeleton inside the cells and to lamellar granule-derived lipids on the surface (Presland and Jurevic, 2002). Keratinising epithelial cells are anuclear and are devoid of organelles. Proteins in the CE are of two types. They are either proteins that are produced exclusively in epithelia to be incorporated into the CE, such as loricrin, involucrin, the small proline-rich proteins (SPRs) and a new family of proteins, called the late envelope proteins (LEPs) (Nemes and Steinert, 1999; Kalinin *et al.*, 2001; Marshall *et al.*, 2001), or they are proteins that are known to regulate other functions in epithelial cells, such as

desmosomal proteins (for example, envoplakin, plakoglobin, desmogleins) or proteins associated with differentiation and/or cytoskeletal functions (for example, filaggrin) (Nemes and Steinert, 1999; Presland and Dale, 2000).

In non-keratinised stratified epithelium, the most superficial cells become flatter and larger but retain their nuclei and a variety of organelles and do not form a CE (Presland and Jurevic, 2002).

Beside keratinocytes the stratified squamous epithelium harbours a variety of other cells, such as Langerhans cells, lymphocytes, melanocytes and Mercle cells but such cells comprise only a small proportion of the total cellular content.

1.2.2 Basement membrane

Basement membranes (basal laminae) are complex, delicate, lace-like structures, often characterised by undulated appearance over connective tissue papillae, and composed of a mixture of collagenous and non-collagenous glycoproteins and proteoglycans, which separate cells from underlying connective tissue. They form cell-associated, sheet-like extracellular matrices under epithelial cells and around endothelial cells, and surrounding individual muscle, fat and peripheral nerve cells. Bone osteoid and cartilage are among the few tissues that lack basement membranes completely. Basement membranes act as a selective barrier between tissue compartments and provide a mechanical scaffold to the overlying cellular component, as well as being critically involved in regulation of cellular biological activities such as differentiation, morphogenesis, survival and migration (Adams and Watt, 1990; Adams and Watt, 1993; Erickson and Couchman, 2000; Miner and Yurchenco, 2004). The basic constituents of basement membranes are type IV collagen, laminin, the glycoprotein nidogen/entactin and proteoglycans of the perlecan type (Timpl and Brown, 1996; Erickson and Couchman, 2000). Recently new components have been described such as agrin and type

XVIII collagen which have been classified as heparan sulphate proteoglycans (Erickson and Couchman, 2000). The laminin family recently has been expanded to include new isoforms with new functions elucidated (Miner and Yurchenco, 2004), with laminin-1 being the most abundant non-collagenous protein in the basement membrane (Sasaki *et al.*, 2004). A small amount of fibronectin also is present within the basement membrane (Fleischmajer and Timpl, 1984) along with other proteoglycans and these molecules are capable of binding to growth factors and presenting them at the cell surface (Aviezer *et al.*, 1994). Basement membrane proteins are synthesised by both keratinocytes and fibroblasts (Marinkovich *et al.*, 1993).

Under conventional electron microscopy, the basement membrane comprises the lamina lucida, lamina densa and the reticular layer (Briggaman and Wheeler, 1975).

The lamina lucida is a 10-50nm thick electron-lucent layer that lies adjacent to the epithelium and which is traversed by bundles of anchoring filaments, running from the basal keratinocytes to the lamina densa. Laminin-5 (also known as kalinin, epiligrin and nicein) constructs the anchoring filaments that run from the hemidesmosomes into the basement membrane and which play an important role in maintaining the epithelial-ECM junction (Verrando *et al.*, 1991). The extracellular domain of the $\alpha 6\beta 4$ integrin interacts with components of the basement membrane such as laminin-1 and -5 (Niessen *et al.*, 1994).

The lamina densa is a 55nm thick electron-dense collagen IV-rich layer, which lies between the lamina lucida and the reticular layer.

The reticular layer is an electron-lucent layer traversed by collagen IV connecting the dermis to the hemidesmosomes.

1.2.3 Connective tissue

The connective tissue component, the corium or the lamina propria, of the oral mucosa consists of a dense network of collagen and elastin embedded in a viscoelastic ground substance of proteoglycans and glycoproteins. It forms a papillary layer that is made of connective tissue papillae and a dense fibrous region comprising the bulk of the lamina propria (Moss-Salentijn, 1980; Gage, 1989). In adult oral mucosa, type I collagen constitutes the bulk of the connective tissue proper and represents 80-90% of the total collagen content, type III collagen represents 10-15% and type IV and V less than 5%. Type I collagen is found throughout the lamina properia, in contrast, type III is localised mainly to the papillae and adjacent to blood vessels. In addition, other components, like elastin, tenascin and fibronectin, and vitronectin, are found associated with the dermal elastic fibres (Dahlback *et al.*, 1989; Yamada, 1989; Chiquet-Ehrismann, 1991).

Connective tissue also contains sparse cell populations of fibroblasts, macrophages, Mast cells, and migrating cells such as polymorphonuclear leukocytes, lymphocytes and plasma cells (Moss-Salentijn, 1980; Gage, 1989).

Connective tissue of the non-keratinised epithelium is more elastic and flexible than that of the keratinised epithelium.

1.3 Malignant oral mucosa

1.3.1 Oral cancer

Oral cancer refers to malignancies that include cancer of the lip ((9th International Classification) ICD9 140), cancer of the tongue (ICD9 141) and cancer of the mouth (ICD9 143-145) (Parkin, 1993). Oral cancer is the sixth most common solid cancer accounting for about 5% of all malignancies (Parkin, 1993), with squamous cell carcinoma (SCC) of the

covering epithelium being the most common type of oral cancer and accounting for more than 90% of cases (Thomas and Speight, 2001).

World-wide, the incidence of oral cancer shows remarkable geographical variations. Generally the highest rates are found in the developing countries, such as India and Sri Lanka, where oral cancer accounts for as many as 50% of all malignancies. In Saudi Arabia oral cancer happens to be relatively common accounting for about 5% of all malignancies (WHO report, 1997), particularly in the Jizan area (Allard *et al.*, 1999), where oral cancer was found to be the most common cancer accounting for about one-fifth of all cancers, probably due to the widespread use of oral tobacco preparations (Tandon, 1995).

Despite the rapid advances in treatment modalities of oral sqamous cell carcinoma (OSCC) as well as improvements in the early detection of oral cancer, the overall 5-year survival of patients remains poor, with it being estimated as approximately 50%; a figure which has not improved over the past 25 years (Greenlee *et al.*, 2001). Mortality from oral and pharyngeal cancer has shown increased rates over the past three decades in the European Community (EC) countries (Johnson and Warnakulasuriya, 1993), with 50% of the cases expected to die within a period of 5 years (OPCS, 1994). In the United State an estimated 29,370 new cases of oral cancer are expected to be diagnosed in 2005 and approximately 7,320 people will die of the disease (ASC, 2005). The poor prognosis is thought to be attributable to the fact that over 60% of patients presenting with oral cancer have tumours greater than 2cm in diameter, and prognosis is known to be worse for larger lesions (Platz, 1986). Moreover, prognosis is affected adversely by the occurrence of distant metastatic lesions; a situation which appears to be increasing (Carr and Langdon, 1989).

The ventral and lateral surfaces of the tongue are the most common sites of oral cancer (Scully, 2005). The floor of the mouth, the retromolar area and the posterior lower alveolus

come next in order of frequency of involvement followed by the buccal mucosa. Involvement of the other areas of the oral-pharyngeal region showed a lesser incidence.

1.3.2 Characteristics of oral cancer

1.3.2.1 Development and proliferation

Cancer involves disruption of the normal developmental programmes of the cell, both in terms of proliferation and in terms of differentiation, hence the term "development disorder" (Dean, 1998). The transformed cell escapes these developmental restraints and gives rise to a malignant cell population that is, in a sense, immortal and exhibits many characteristics which appear to be quite foreign to the mature differentiated cell. However, it should be noted that the morphological identification of many tumours is based on their expressing a repertoire of differentiated functions.

Oral carcinogenesis is a complex multi-step process in which the basic cellular functions are being disturbed by multiple genetic alterations with progressively increasing capacity for proliferation, survival, invasion and metastasis. The first step in the process is "tumour initiation" which includes activation of oncogenes and inactivation of tumour suppressor genes. An accumulated body of evidence suggests that there are at least 6 to 10 genetic events required for the development of full-blown oral carcinogenesis (Williams, 2000). Oncogenes encode proteins which normally activate and drive cell proliferation and several oncogenes have been implicated in oral carcinogenesis, for example overexpression of the proto-oncogene epidermal growth factor receptor (EGFR) has been detected frequently in oral cancers (Partridge *et al.*, 1988), and was found to be due to gene amplification in almost 30% of oral cancers (Scully, 1993). Members of the ras oncogene family, together with other genes like c-myc, int-2, hst-1, PRAD-1 and bcl-1, are also found to be implicated in the

development of oral cancer (For review see: Williams, 2000). Oncogenes however only act as initiators for carcinogenesis and their activation alone is not sufficient for the development of a tumour. In general, for any malignancy to develop activation of oncogenes must be coupled with the inactivation of tumour suppressor genes. Tumour suppressor genes encode proteins which normally suppress cell proliferation. The p53 gene, the most widely studied tumour suppressor gene, has been shown to be functionally inactivated in oral cancer (Schantz, 1993; Partridge *et al.*, 1999). Other tumour suppressor genes, such as doc-1, the retinoblastoma (Rb) gene, and the adenomatous polyposis coli (APC) gene, have been mutated frequently in oral cancers (For review see: Williams, 2000). In general, oncogenes and tumour suppressor genes feed into common cellular regulatory systems, implicated in proliferation and/or differentiation, which change the structure and behaviour of transformed cells. Continuous efforts have been made to identify new genes that are expressed differentially in oral cancers (Arora *et al.*, 2005). However, further studies are required to determine the biological importance of these newly discovered genes to assess their validity as future biomarkers and as targets for new therapeutic strategies.

The second step in cancer evolution is "tumour progression" where additional mutations continue to occur within the transformed cells giving rise to more rapid-growing and increasingly malignant cell populations.

Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity, and it is considered that here the cancer cells are less stringently regulated by cell-cell and cell-matrix interactions. Contact-inhibition is a characteristic of normal epithelial cells, where normal cells proliferate until they reach a finite cell density and become quiescent. Contact inhibition is lost in oral squamous cell carcinoma, where transformed cells are no longer subjected to the density-dependent inhibition of cell proliferation. These changes are attributed to the downregulation or loss of the E-cadherin-dependent cell-cell contacts in

oral carcinoma (Li *et al.*, 1998), where re-expression of E-cadherin conferred density-dependent growth inhibition. However, on the other hand, E-cadherin was found to confer survival advantage, in other OSCC cell lines, for detached cell growing in suspension (Kantak and Kramer, 1998).

1.3.2.2 Growth and survival

The growth of a malignant tumour is dependent upon the balance between cell growth and cell death (Carson and Ribeiro, 1993). Programmed cell death, or apoptosis, is an integral part of normal tissue homeostasis. Failure of neoplastic cells to undergo apoptosis contributes substantially to tumour growth and development. Among other regulating effectors, the B cell leukaemia/lymphoma-2 (Bcl-2) family of proteins is implicated in regulation of apoptosis (Adams and Cory, 2001). In oral cancers over-expression of Bcl-2 protein was reported in poorly differentiated tumours (Jordan *et al.*, 1996; Badaracco *et al.*, 2000; Chen *et al.*, 2000; Teni *et al.*, 2002; Loro *et al.*, 2003). In normal epithelial cells, apoptosis is initiated by loss of cell anchorage, i.e. cell-cell and cell-matrix interactions (Frisch and Francis, 1994; Hermiston and Gordon, 1995). However, transformed cells are resistant to the loss of cell anchorage and exhibit anchorage-independent survival capabilities, a phenomenon which, it is thought, enables malignant cells to survive and metastasise to distant sites (Grossmann, 2002). E-cadherin has been implicated in regulation of anchorage-independent growth and in survival of tumour cells in oral squamous cell carcinoma (Kantak and Kramer, 1998).

1.3.2.3 Differentiation

There generally has been an inverse relation between tumour growth and differentiation (Harris, 1987). Dedifferentiation (or loss of the differentiated phenotype) is an important

step in carcinogenesis and often correlates with poor prognosis. Dedifferentiation involves morphological alterations to the cell-cell structures and to the cytoskeleton of the transformed cell, as well as changes to the cell-matrix organisation and gene expression profile (Thiery, 2003); such a change, manifested by alterations in morphology, often is referred to as Epithelial-Mesenchymal Transition (EMT) where the carcinoma cells are thought to lose their epithelial characteristics at the invasive front and to express a new set of mesenchymal-specific genes (Boyer *et al.*, 2000). In sqamous cell carcinoma, E-cadherin, an epithelial marker which mediates cell-cell interactions, frequently is lost in oral squamous cell carcinoma and its loss is implicated in EMT (Behrens *et al.*, 1993; Bagutti *et al.*, 1998; Williams *et al.*, 1998; Lo Muzio *et al.*, 1999).

1.3.2.4 Invasion and metastasis

OSCC is characterised by its ability to spread locally and regionally, where malignant keratinocytes break through the underlying basement membrane and move through the connective tissue stroma. Alteration in the expression and/or function of cell adhesion molecules results eventually in tumour invasion and metastasis. In OSCC, loss or aberrant function of the E-cadherin (invasion-suppressor) cell adhesion molecule has been correlated with invasion and metastasis of tumour cells (Behrens *et al.*, 1993; Mattijssen *et al.*, 1993; Bagutti *et al.*, 1998; Shinohara *et al.*, 1998; Hoteiya *et al.*, 1999; Kudo *et al.*, 2004). Also, thinning and discontinuity of the basal lamina were observed in OSCC and such changes are thought to promote invasion (Kannan *et al.*, 1994; Cheng and Hudson, 2002). The ability of the malignant cells to degrade ECM components, mediated by matrix metalloprotease (MMP) enzymes, is an important determinant of the invasive potential of any tumour. The Gelatinases (MMP-2 and -9), Stromelysins (MMP-3, -10, -11), Collagenases (MMP-1 and -13) and membrane-bound MMP (MT1-MMP) are all expressed in oral cancer where they may

contribute to tumour progression (Polette *et al.*, 1991; Polette *et al.*, 1993; Gray *et al.*, 1992; Pyke *et al.*, 1992; Kusukawa *et al.*, 1992; Kusukawa *et al.*, 1993; Kusukawa *et al.*, 1995; Muller *et al.*, 1993; Okada *et al.*, 1995; Yoshizaki *et al.*, 1997; Charous *et al.*, 1997; Airola et al., 1997; Johansson *et al.*, 1997; Thomas *et al.*, 1999).

1.4 Cell adhesion Molecules

Eukaryotic cells communicate with their extracellular environment, both cellular and acellular, through a variety of cell surface located cell adhesion molecules (CAMs). At the present time there are six major subfamilies of CAMs: integrins, syndecans, selectins, immunoglobulin superfamily members, cadherin superfamily members, and those which fall into a separate group, such as the hyaluronan receptor CD44, with little resemblance to the other five specific categories (Skubitz, 2002).

In general, broad terms it may be viewed that interactions with the extracellular matrix (ECM) are carried out by integrins and syndecans while interactions with neighbouring cells, via heterophilic or homophilic binding, are attributable to the members of the cadherin, selectin and immunoglobulin (Ig-CAM) families (Aplin *et al.*, 1999).

The CAMs do not serve simply as "inert" molecules binding a cell to the recognised ligand. Rather they function in a dynamic fashion to control a number of cellular events, such as proliferation, survival, differentiation and migration, by participating in cellular signal transduction pathways (Juliano, 2002). The ultimate outcome of the generation of these signal transduction pathways are changes in gene expression and, consequently, changes in cell behaviour (Juliano, 2002).

1.4.1 Epithelial cell-cell and cell-matrix adhesion

Formation of a polarised epithelium is dependent upon a high-order organisation involving formation of cell-cell and cell-matrix adhesive structures.

Epithelial cell-cell adhesion is mediated by a variety of membrane proteins that accumulate to form different adhesive structures; tight junctions (TJs), adherens junctions (AJs) and desmosomes. Collectively they form the intercellular junctional complexes (for review see: Kowalczyk *et al.*, 1999; Tsukita *et al.*, 2001; Perez-Moreno *et al.*, 2003). Tight junctions and adherens junctions are both connected to the actin cytoskeleton of the cell, desmosomes, in contrast, are linked to the intermediate filament network. Tight junctions are formed mainly by glycoproteins, such as claudin/occluding and nectin. However, adherens junctions and desmosomes are cadherin-dependent adhesive structures.

The AJs play a key role in synchronising cellular organisation and movements within epithelia. Moreover, they are directly involved in conveying information from the surrounding to the interior of the cell (Perez-Moreno *et al.*, 2003).

Epithelial cell-matrix adhesion is mediated by integrins which cluster to form organised adhesive structures, such as focal adhesions and hemidesmosomes. Focal adhesive complexes connect components of the extracellular matrix (ECM) to the actin cytoskeleton and are made mainly by the β1-containing members of the integrin family (Clark and Brugge, 1995). In contrast, hemidesmosomes are multi-protein complexes that are dependent upon α6β4 integrin and link the intermediate filament (keratin) network of the cell with the ECM (Borradori and Sonnenberg, 1996; Green *et al.*, 2005)

The work described in this report has focused primarily on E-cadherin, a classical member of the cadherin superfamily, and thus a brief review of the characteristics of these molecules is presented.

1.4.2 Cadherins

The cadherin superfamily represents a family of adhesion molecules that are structurally related. Sequence similarities among members of the cadherin superfamily provide the basis for their assignment into four main groups: classical cadherins (type I, II, and III), desmosomal cadherins (desmocollins and desmogleins), protocadherins, and other cadherin related proteins (Angst *et al.*, 2001; Truong and Ikura, 2002). The cadherin superfamily, and its known members, is presented in Table 1.1 (also see Figure 1.1)

Table 1.1: Cadherin Superfamily

Superfamily	Members
Classical cadherins	E-, N-, P-cadherin VE-cadherin
Desmosomal cadherins	Desmocollin Desmoglein
Proto-cadherins	μ-Protocadherin CNR-cadherin
Seven transmembrane (7TM)	Flamingo
T-cadherin	T-cadherin
FAT family	Dachsous Fat

Taken from (Angst *et al.*, 2001)

Cadherins are calcium-dependent (hence the name calcium-dependent-adherins) intercellular adhesion molecules described originally by Takeichi (1977). These molecules generally bind in an homophilic (i.e. like-binding-like) fashion though an heterophilic interaction between the E-cadherin molecule and integrin αEβ7 has been demonstrated (Higgins *et al.*, 1998). Also, it has been reported that E-cadherin can interact directly with α2β1 (Whittard *et al.*, 2002). These generally transmembrane molecules (T-cadherin is unique in being linked to the

plasma membrane via a glycosyl-phosphatidylinositol "GPI" anchor) link through to elements of the cytoskeleton (Figure 1.1). Indeed it is the nature of the filaments to which the cytoplasmic tail binds, eventually, which places some of the cadherins into their appropriate sub-families. Thus the desmosomal cadherins bind to intermediate filaments whereas the classical cadherins bind to actin filaments (Figure 1.1). VE-cadherin, a classical cadherin expressed in endothelial cells, is unusual in being able to bind both actin filaments and intermediate filaments (Angst *et al.*, 2001). Much that is known about the functions of the cadherins comes from studies on the classical cadherins (Wheelock and Johnson, 2003b; Wheelock and Johnson, 2003a).

In general, the cadherins play an important role in histogenesis and maintenance of the normal tissue architecture, where they contribute to the formation of intercellular organised adhesive structures. Classical cadherins constitute the adherens junctions (AJs) whereas desmosomal cadherins; the desmocollins (Dsc) and desmogleins (Dsg), form the spot-like desmosomes.

1.4.2.1 Classical Cadherins

These may be regarded as the prototype of all cadherins. Named for the tissue from which they first were isolated (epithelial, E-cadherin Yoshida-Noro *et al.*, 1984; placental, P-cadherin Nose *et al.*, 1987; neuronal, N-cadherin Hatta *et al.*, 1988; vascular endothelial, VE-cadherin Lampugnani *et al.*, 1995).

E- and P-cadherins as well as desmosomal cadherins are the primary cadherins expressed by the stratified squamous epithelium (Thomas and Speight, 2001; Wheelock and Johnson, 2003b). E-cadherin is the molecule we are interested in.

1.4.2.2 Epithelial-cadherin (E-cadherin)

E-cadherin, the predominant intercellular adhesion molecule of epithelial cells, is a 5758 amino acid, transmembrane protein with a molecular mass of 120 kDa; formed by the enzymatic cleavage of a 135 kDa precursor in a step which is thought to be crucial for a mature, functional adhesive molecule (Figure 1.2). It is also known as uvomorulin, L-CAM, cell-CAM 120/80 or Arc-1 (Bremnes *et al.*, 2002). E-cadherin is, arguably, the prototypic member of the classical cadherins.

The gene encoding human E-Cadherin (CDH1) recently has been cloned and characterised and found to be situated on chromosome 16q22.1, within a large conserved linkage group that includes loci for haptoglobin, chymotrypsinogen B, metallothionine-1, metallothionine-2 and tyrosine aminotransferase (Bremnes *et al.*, 2002).

Like the other members of the type I classical cadherins, E-cadherin consists of three main domains: extracellular domain, transmembrane domain and an intracellular highly conserved domain.

The extracellular region of these molecules consists of five repeats (EC1-EC5; amino to carboxyl terminus) of negatively-charged unique calcium-binding motifs. Calcium is essential for cadherin-mediated adhesion and also serves to protect the cadherins from degradation by trypsin (Hyafil *et al.*, 1981). It is thought that the HAV (histidine-alanine-valine) amino acid triplet displayed on the EC1 sub-domain at the N-terminus acts as a recognition sequence essential for the homophilic binding of the cadherin molecules (Nöe *et al.*, 1999a; Nöe *et al.*, 1999b). In order for cadherins to bind they must dimerise; first a parallel dimer is formed between two adjacent cadherin molecules on the same cell and then an antiparallel dimer is formed between cadherin dimers of adjacent cells; the so-called "adhesion zipper" (Shapiro *et al.*, 1995; Yap *et al.*, 1997; Vasioukhin *et al.*, 2000).

1.4.2.2.1 E-cadherin in normal oral mucosa

E-cadherin is expressed during embryonic life as early as the two-cell stage (Larue *et al.*, 1994; Riethmacher *et al.*, 1995), where E-cadherin was shown to play an important role in development, regulation of morphogenesis and tissue formation. Epithelial differentiation and polarisation occurs early during the morula stage, during which E-cadherin plays an important role (Fleming *et al.*, 1992). In normal adult epithelial tissue, E-cadherin's function lies primarily in the formation of adherens junctions. E-cadherin is also involved in maintenance of epithelial tissue homeostasis, by being part of the signal transduction pathways which not only regulate adhesion but regulate other cellular process such as cellular differentiation, migration, proliferation and apoptosis (Barth *et al.*, 1997; Pećina-Šlaus, 2003; Wheelock and Johnson, 2003a). E-cadherin is implicated in the regulation of growth of normal ginigival epithelium in a density-dependent manner (Kandikonda *et al.*, 1996).

1.4.2.2.2 Adherens junctions (AJs)

In epithelial tissue, AJs are E-cadherin-dependent adhesive structures that are knotted to the actin cytoskeleton of the cell. In order for functional adhesive activity to occur it also is essential that the cytoplasmic domain of E-cadherin molecule interacts with the actin cytoskeleton. Linkage to this structure is via a series of cytoplasmic plaque proteins, known as catenins; α- (102 kDa, chromosome 5q21-22), β- (88 kDa, chromosome 3q21), γ-catenin (82 kDa, chromosome 11q11), and the catenin $p120^{ctn}$ (120 kDa, chromosome 12q13). The β-catenin is homologous to the Drosophila segment polarity gene armadillo (Gumbiner, 1995) and binds directly to the E-cadherin molecule at the C-terminal end of the cytoplasmic domain (Ozawa *et al.*, 1990). The β-catenin leads a dual life in that, through binding to E-cadherin, it plays a role in AJ formation (Gumbiner, 1996), and also, it acts as a transcriptional factor when stimulated by the Wnt signal transduction pathway (Ben-Ze'ev

and Geiger, 1998). The α-catenin shares sequence homology with vinculin (Herrenknecht *et al.*, 1991) and binds the E-cadherin/β-catenin complex either directly to the actin cytoskeleton (Rimm *et al.*, 1995) or via α-actinin (Knudsen *et al.*, 1995). Clustering of E-cadherin/catenin complex and formation of proper AJ are highly dependent on α-catenin. Transformed cells lacking α-catenin showed disturbed cell-cell adhesion (Shimoyama *et al.*, 1992) and restoration of α-catenin restored the normal cell-cell contacts (Hirano *et al.*, 1992). α-catenin complexes with β-catenin (or γ-catenin) and other cytoskeletal proteins, including vinculin, to organise the cadherin/catenin complex at the AJ of the polarised cell (Perez-Moreno *et al.*, 2001). α-catenin can also bind to proteins involved in actin polymerisation such as VASP (vasodilator-stimulated phosphoprotein) and Mena (Vasioukhin *et al.*, 2000); an association thought to be necessary for maturation of E-cadherin-dependent AJs (Vasioukhin and Fuchs, 2001). α-catenin also possesses a binding region for ZO-1 (Imamura *et al.*, 1999). The γ-catenin bears homology to β-catenin and is identical to the desmosomal plakoglobin. The β- and γ-catenins appear to form mutually exclusive complexes with the E-cadherin cytoplasmic domain, where the two catenins can substitute for one another when one is missing. Like β-catenin, $p120^{ctn}$ binds directly to defined, and distinct, portions of the E-cadherin cytoplasmic domain (Yap *et al.*, 1998).

An intact cadherin-catenin complex is crucial for the development of a functional adherens junction.

1.4.2.2.3 Regulation of adherens junction formation

Theoretically the life of a cadherin junction can be divided into three stages: formation, maintenance and disassembly. Junction formation is further subdivided into three phases involving the formation of initial contacts, which then grow and mature and, eventually, form clusters of cadherins and associated molecules that can be recognised ultrastructurally

as AJs. Sometimes, cadherin is recruited from intracellular stores following initiation of cell-cell contact formation (Mary *et al.*, 2002). AJs are dynamic in nature; the turnover of molecules in and out of the adhesive contacts is marked while internalisation and recycling of cadherins can affect junction formation and maintenance substantially (Le *et al.*, 2002). Disassembly could result from the extensive breaking of contacts, for example by proteolysis while different cell types may not share identical mechanisms of how junctions are initiated and maintained; thus formation of AJs appears to be cell-type-specific as well as cadherin-specific (Wheelock and Johnson, 2003b).

The process of AJ formation (assembly and disassembly) is vital for certain biological processes such as growth, wound healing and differentiation, as well as during pathological conditions such as tumourigenesis. Several mechanisms are known to be implicated in the regulation of AJs, including: tyrosine phosphorylation/dephosphorylation of components of the AJs, ubiquitination (internalisation and degradation of plasma membrane proteins) and endocytosis of E-cadherin, and regulation of AJs by members of the Rho family GTPases.

In epithelial cells, tyrosine kinases induce tyrosine phosphorylation of components of the AJs (Takata and Singer, 1988). Activation of various tyrosine kinases, such as the src kinase family (Tsukita *et al.*, 1991), the protein tyrosine phosphatase PTPμ family (Brady-Kalnay *et al.*, 1995)), the epidermal growth factor (EGF) receptor (Hazan and Norton, 1998), the hepatocyte growth factor (HGF) receptor "c-met" (Hiscox and Jiang, 1999) and the oncogene product c-erbB-2 (Shibata *et al.*, 1996b), all induce tyrosine phosphorylation of adhesion molecules such as E-cadherin and β-catenin, with subsequent scattering of epithelial cells and the adoption of a fibroblast-like morphology (Weidner *et al.*, 1990; Fukuyama and Shimizu, 1991; Behrens *et al.*, 1993; Hoschuetzky *et al.*, 1994; Shibata *et al.*, 1996b). Moreover, inhibition of tyrosine phosphatases, using sodium pervanadate for

example, resulted in increased tyrosine phosphorylation of E-cadherin, β- and γ-catenins, with the occurrence of incomplete junctions and scattering of the treated keratinocytes (Soler *et al.*, 1998). These findings suggest that tyrosine phosphorylation and dephosphorylation play an important role in regulation of the cadherin-dependent AJs.

E-cadherin trafficking (dynamic recycling and endocytosis) play an important role in control of AJs disassembly (Le *et al.*, 2002). It has been reported that E-cadherin molecules were internalised following Ca^{2+} depletion (Kartenbeck *et al.*, 1991). Several mechanisms have been proposed to explain this phenomenon. However, this aspect of cadherin biology has not yet been thoroughly investigated. Le *et al.* (2002) showed that recycling of the E-cadherin molecule was dependent upon activation of the protein kinase C (PKC) family (Le *et al.*, 2002). Hakai, a novel E-cadherin binding protein, is found to mediate E-cadherin endocytosis through enhancement of cell-cell adhesion disassembly (Fujita *et al.*, 2002; Pece and Gutkind, 2002).

In addition to the potential role of kinases in regulation of adherens junctions, the Rho family of GTPases (Rho, Rac, and Cdc42) also have been implicated in remodelling actin for regulation of cell-cell contacts (for review see: Hall, 1998; Van Aelst and D'Souza-Schorey, 1997; Bishop and Hall, 2000; Braga, 2000). Blocking the activity of Rho or Rac1 resulted in the removal of cadherin adhesion complexes and prevention of cell-cell contact assembly in epithelial cells (Braga *et al.*, 1997). Conversely the introduction of constitutively active Rac1 accumulates cadherins, catenins, and actin at junctions; resulting in the formation of E-cadherin-mediated cell-cell adhesion contacts (Ridley, 1995; Braga *et al.*, 1999). In contrast, the expression of dominant negative Rac1 showed disruption in the E-cadherin-dependent cell-cell contacts (Lambert *et al.*, 2002).

1.4.2.2.4 E-cadherin signalling

E-cadherin is thought to play an important role in the in-out cellular signalling. There are several models that have been used to study signals downstream of cadherin engagement (Wheelock and Johnson, 2003a). The best studied signalling pathway involving cadherin is the β-catenin/Wnt (or wingless) pathway. Activation of this signalling pathway, by the Wnt ECM-associated growth factor, triggers the synthesis of proteins involved in cell growth, such as myc and cyclin D1, with unbound β-catenin being a co-activator (Conacci-Sorrell *et al.*, 2002). Over-expression of E-cadherin decreases the total amounts of unbound β-catenin available for signalling, and thus provided strong evidence that E-cadherin is involved in modulating the Wnt signal transduction pathway.

E-cadherin also has been linked recently to the Rho family GTPases signalling pathways (Braga, 2002; Yap and Kovacs, 2003) with Rac1 being reported to be activated following E-cadherin engagement. Moreover, $p120^{ctn}$, one of the catenin proteins that interact directly with E-cadherin's cytoplasmic domain, is involved in Rho GTPase signalling pathway; a pathway which is implicated in the regulation of cell morphology, migration and gene transcription (Noren *et al.*, 2000, Anastasiadis and Reynolds, 2001; Perez-Moreno *et al.*, 2003). E-cadherin is thought to play a major role in controlling cell fate and survival through its interaction with the Epidermal Growth Factor Receptor (EGFR) and the subsequent activation of the MAP kinase pathway. E-cadherin was found to induce a ligand-independent activation of the EGF receptor (Pece and Gutkind, 2000).

1.4.2.2.5 Loss of E-cadherin function during carcinogenesis

It has been well accepted that the E-cadherin molecule functions as an invasion-suppressor (for review see: Behrens, 1999; Guilford, 1999; Wijnhoven *et al.*, 2000; Wheelock *et al.*, 2001; Thiery, 2002; Wheelock and Johnson, 2003a). Loss of E-cadherin expression leads to

epithelial tumourigenesis (Perl *et al.*, 1998) and disruption of the cadherin-catenin complex. Such decreased E-cadherin expression often is observed in many advanced, poorly differentiated carcinomas (Berx and Van Roy, 2001; Hajra and Fearon, 2002).

Multiple mechanisms of inactivation of the E-cadherin/catenin complex in epithelial tumours have been proposed; including transcription and genomic regulation of E-cadherin expression where either transcription of E-cadherin is reduced through binding of transcription factors, like Snail, to the E-boxes sites of the E-cadherin promoter (Batlle *et al.*, 2000; Cano *et al.*, 2000; Comijn *et al.*, 2001; Perez-Moreno *et al.*, 2001; Yokoyama *et al.*, 2003), or through DNA hypermethylation of the E-cadherin promoter, thereby making the promoter inaccessible for transcription (Graff *et al.*, 2000; Nass *et al.*, 2000; Nojima *et al.*, 2001).

Switched expression of different types of cadherins, the so-called "Cadherin switch" where N-cadherin is expressed instead of E-cadherin, has been reported as one of the main causes for E-cadherin suppression in human squamous carcinoma cells (Tomita *et al.*, 2000; Hazan *et al.*, 2004). Mutation or deletions of cadherin or catenin genes is another mechanism where E-cadherin expression and function are compromised in epithelial tumours (reviewed by: Berx *et al.*, 1998; Nollet *et al.*, 1999).

Also inhibition of E-cadherin function where both cadherin and catenins molecules are expressed and intact has been attributed either to the shedding of its ectodomain (Vallorosi *et al.*, 2000), as a result of cleavage by metalloproteinases, like matrilysin, stromelysin-1 (Steinhusen *et al.*, 2001; Nöe *et al.*, 2001) and plasmin (Ryniers *et al.*, 2002), or through the cleavage of its cytoplasmic domain by caspases (Steinhusen *et al.*, 2001). The cleaved soluble fragments of the E-cadherin molecule (sE-cadherin) have been found in patients diagnosed

with cancer (Katayama *et al.*, 1994). These sE-cadherin fragments were reported to downregulate E-cadherin function in a paracrine manner (Nöe *et al.*, 2001).

Alteration of adhesive functions by intracellular signalling pathways, such as growth factor receptors, oncogenes and small GTPases, may change cadherin adhesive properties (Lozano *et al.*, 2003).

Mutation of the E-cadherin gene (CDH1) has been reported in familial gastric carcinoma (Guilford *et al.*, 1998), however the majority of carcinomas appear to lack mutations in this gene. Nevertheless, immunohistochemical studies show that loss of E-cadherin is common in many tumours (Behrens, 1999) and it is thought that tumour cells must downregulate E-cadherin in order to invade surrounding tissues (Hajra and Fearon, 2002). In many cases, E-cadherin frequently is lost or rendered dysfunctional by mechanisms that do not involve irreversible, genetic alterations. Hypermethylation of 5' CpG islands, which is another mechanism by which the E-cadherin gene is silenced in normal cells as well as during the process of carcinogenesis, has been demonstrated in many human cancers (Graff *et al.*, 2000; Nass *et al.*, 2000; Kanazawa *et al.*, 2002; Ribeiro-Filho *et al.*, 2002) including oral cancer (Saito *et al.*, 1998; Nakayama *et al.*, 2001b; Chang *et al.*, 2002; Chen *et al.*, 2004).

Frequently in tumours, it has been shown that loss of E-cadherin is transient and shows a heterogeneous and unstable dynamic pattern (Mareel *et al.*, 1995); where, in some tumours, E-cadherin was absent in the primary tumour but re-expressed in the metastatic lesions of the same patient (Schipper *et al.*, 1991). It is proposed that the microenvironment at the invasive front transiently downregulates E-cadherin and it is thought that this facilitation might contribute to the dynamic heterogeneity that drives metastatic progression (Berx and Van Roy, 2001). Similar mechanisms also might be responsible for the occurrence of the cadherin switch (Chen *et al.*, 2004). Regardless of the precise mechanism, it appears that loss of E-cadherin, indeed is required for the tumour cells to detach from the primary tumour,

invade and metastasise and that there might well be re-expression of E-cadherin, enhancing tumour-host adhesion, at the metastatic site (Guilford, 1999).

Disruption of the cadherin/catenin complex, as a result of any of the proposed mechanisms, has been demonstrated in a wide range of carcinomas and was found to be correlated with various pathological and clinical features, such as de-differentiation, infiltrative growth, lymph node metastasis and poorer patient prognosis (Berx and Van Roy, 2001).

1.4.2.2.6 E-cadherin and Oral Squamous Cell Carcinoma (OSCC)

Up to date there have been a limited number of studies which have correlated the expression of E-cadherin with the behaviour of head and neck tumours. In oral squamous cell carcinoma (OSCC) loss of cadherin expression also has been shown to be associated with tumourigenicity and tumour progression (Thomas and Speight, 2001). In different tumour cell lines, restoration of E-cadherin expression resulted in reduced invasiveness (Miyaki, 1995), growth suppression (St Croix *et al.*, 1998), and terminal differentiation (Zhu and Watt, 1996).

In an attempt to correlate the expression of E-cadherin and β-catenin with the clinical progression and prognosis of cancer, Bánkfalvi *et al.* (2002) found that there is a perturbed expression of E-cadherin and β-catenin during the stepwise course of OSCC, dysplastic lesions showed general transient increase in the immunoreactivity of all adhesion molecules and only some showed restricted loss of E-cadherin and β-catenin; indicative of at least two possible mechanisms that could be involved in the malignant transformation of oral epithelial cells: expansion of the germinative basal cell layer, which normally express high levels of E-cadherin and β-catenin, and the *de novo* inactivation of the respective genes by mutation (Bánkfalvi *et al.*, 2002). As tumours acquire an invasive phenotype, there was a marked reduction of both E-cadherin and β-catenin especially in poorly differentiated

tumours which was consistent with the findings of others (Sakaki *et al.*, 1994; Sorscher *et al.*, 1995; Yamada *et al.*, 1997; Williams *et al.*, 1998; Lo Muzio *et al.*, 1999). Loss of E-cadherin in poorly differentiated OSCC lesions usually is accompanied by loss of P-cadherin, α- and γ-catenins and β1-integrin (Bagutti *et al.*, 1998; Williams *et al.*, 1998). In the majority of OSCC lesions, E-cadherin was lost at the advancing front of the tumour; localisations which play a role in determining the aggressiveness of oral tumours (Downer and Speight, 1993; Bánkfalvi and Piffko, 2000; Bánkfalvi *et al.*, 2002). Alternatively it could be that this is suggestive of the possible molecular cross-talk between the tumour cell and the surrounding micro-enviroment of the host (Bryne *et al.*, 1998; Bánkfalvi and Piffko, 2000; Bánkfalvi *et al.*, 2002). However, in general E-cadherin has shown an heterogeneous distribution patterns. Thus Downer and Speight (1993) found varied expression with no correlation between the pattern of staining and the histological grade of the tumour. However some infiltrative islands and tumour cells were found to be negative suggesting, perhaps, that E-cadherin could be implicated in the infiltrative process (Downer and Speight, 1993). Similarly, Kudo *et al.* (2004) had also observed an heterogeneous downregulation of E-cadherin within metastatic areas and at the invasive front of OSCC resulting from hypermethylation of the E-cadherin promoter (Kudo *et al.*, 2004). Indeed methylation of the E-cadherin promoter is accountable for about 67% of nodal metastasis in OSCC (Chang *et al.*, 2002).

Reduction or loss of E-cadherin expression has been associated with poor differentiation and higher metastatic potential of OSCC (Mattijssen *et al.*, 1993; Shinohara *et al.*, 1998; Hoteiya *et al.*, 1999), as well as the development of the malignant phenotype, characterised by loss of the epithelial morphology and the acquisition of mesenchymal characteristics (Yokoyama *et al.*, 2003).

Regional and lymph node metastasis has been the most important diagnostic factor in predicting OSCC outcome (Jones *et al.*, 1993). Schipper *et al.* (1991) found that metastatic

lymph node deposits, whether they were derived from well or poorly differentiated tumours, were generally E-cadherin negative (Schipper *et al.*, 1991). In contrast to these findings, Bowie *et al.* (1993) and Sorscher *et al.* (1995) had found that metastatic tumour deposits in lymph nodes showed the same pattern of staining as the primary tumour (Bowie *et al.*, 1993; Sorscher *et al.*, 1995). E-cadherin expression also has been used in attempts to predict patient survival. In OSCC loss of E-cadherin has been correlated with clinical stage and the increased incidence of occurrence of metastatic lesions (Yamada *et al.*, 1997; Tanaka *et al.*, 2003) which in itself is associated with poor survival. Mattijssen *et al.* (1993) found significant correlation between strong E-cadherin expression and improved patient survival whereas others found no such correlation (Mattijssen *et al.*, 1993).

Epithelial-mesenchymal transition has not been well studied in OSCC, however, it has been suggested the activation of the Wnt family, in OSCC, might be a contributory factor to such changes (Lo Muzio, 2001; Uraguchi *et al.*, 2004).

In OSCC there is a strong correlation between reduced E-cadherin expression, or loss, and increased invasiveness, where the E-cadherin molecule is considered to act as a tumour-suppressor (Mattijssen *et al.*, 1993; Birchmeier and Behrens, 1994; Andrews *et al.*, 1997; Hoteiya *et al.*, 1999; Kudo *et al.*, 2004). However, Kinsella *et al.* (1994) found no correlation between E-cadherin expression and invasiveness when commencing invasion assays on cell lines derived from tongue SCC (Kinsella *et al.*, 1994).

E-cadherin however often is considered to function as a tumour suppressor gene. The interference of E-cadherin-mediated cell-cell contact formation by using anti-E-cadherin antibodies (St Croix *et al.*, 1998), incubation of cells in a low-calcium culture medium (Kantak and Kramer, 1998), or by modulation of cell density (Takahashi and Suzuki, 1996) has abrogated the growth-inhibitory effects mediated by E-cadherin.

Inappropriate expression of the non-epithelial/mesenchymal cadherin (N-cadherin) was shown in OSCC (Islam *et al.*, 1996; Chen *et al.*, 2004). These cell lines exhibited a fibroblastic phenotype and showed reduced expression of E-cadherin. The actual mechanism by which this cadherin-switch takes place is still not known. However, Chen *et al.* (2004) showed that hypermethylation of the E-cadherin promoter is a possible mechanism (Chen *et al.*, 2004).

1.4.3 Integrins

Integrins are a large family of heterodimeric, cation-dependent, transmembrane cell-surface proteins. Each integrin consists of an α and β glycoprotein subunit non-covalently associated (Hynes, 1992; Sonnenberg, 1993). There are about 19 α and 8 β subunits which have been identified. Many integrin α and β subunits have the ability to associate with more than one partner, resulting in redundancy of integrin heterodimerisation. For example, the β1 subunit can dimerise with about 12 α subunits and the α6 subunit can dimerise with β1 and β4 subunits. About 24 integrin receptors have been recognised and each exhibits a different ligand profile (for review see: Hynes, 2002). Integrins also express a high degree of redundancy in ligand binding. Most integrin heterodimers recognise several ECM proteins, whereas the α5β1 integrin binds only to fibronectin (Wehrle-Haller and Imhof, 2003). Figure 1.3 illustrates the known αβ integrin combinations with their ligand interactions.

In the epidermis of the skin, integrin expression mostly is confined to the basal layer. The most abundant integrins expressed by normal keratinocytes are α2β1, α3β1 and α6β4 ((Bagutti *et al.*, 1998)). Basal cells have also been reported to express other integrins such as α8β1 and α9β1 (Bossy *et al.*, 1991; Ojakian and Schwimmer, 1994). Reports on α5β1 integrin expression in the epidermis have been contradictory. Klein *et al.* (1990) found that α5β1 was not expressed, however, on the other hand Hertle *et al.* (1991) found weak pericellular expression in the basal keratinocytes (Klein *et al.*, 1990; Hertle *et al.*, 1991). There is some

evidence suggesting that the α5β1, if present, may not be fully functional (Guo *et al.*, 1991). Moreover, Hertle *et al.* (1991) also demonstrated weak pericellular expression of αvβ5 in basal keratinocytes (Hertle *et al.*, 1991).

α2β1 and α3β1 are components of focal contacts in keratinocytes (Carter *et al.*, 1990) and they both have a pericellular distribution (Zambruno *et al.*, 1991; Watt and Jones, 1993). High levels of α2β1 have been demonstrated in proliferating epithelium; indicative of a possible role for this integrin in promoting cell proliferation (Zutter and Santoro, 1990).

α6β4 integrin is a component of the hemidesmosomes and is expressed at the basal aspect of the basal cells with lower expression at the lateral aspects of the cell (Hertle *et al.*, 1991).

In oral epithelium, integrin expression showed a similar pattern. Oral keratinocytes showed strong expression of α2β1, α3β1 and α6β4 with weaker and more variable expression of α5β1 and αvβ5 (Jones *et al.*, 1993). α6β4 is relatively confined to the basal aspects of the basal cells whereas the other integrins have a uniform pericellular distribution in the basal cells. In gingival epithelium α6β4 showed a strong linear staining along the basement membrane zone (Hormia *et al.*, 1990). Larjava (1991) reported strong pericellular expression of α2, α3 and β1 in the basal layer with low expression of α5β1 (Larjava, 1991).

In contrast to the epidermis of the skin where integrin expression largely is confined to the basal cells, oral epithelium showed an extensive suprabasal staining, particularly in the floor of the mouth and lateral borders of the tongue (Jones *et al.*, 1993, Jones *et al.*, 1995). In these areas integrin expression is seen to be as high as in the prickle cell layer, possibly indicative of the increased turnover rate of the oral epithelium.

In tissue culture, keratinocytes were shown to express α2β1, α3β1, α5β1, α6β4, αvβ5 and αvβ6 (Adams and Watt, 1991; Haapasalmi *et al.*, 1996).

Table 1.2: Epithelial integrins

Integrin	Ligand	Reference
α2β1	Collagen type I	(Wayner and Carter, 1987)
	Collagen type IV	(Carter *et al.*, 1990)
	Laminin 1	(Languino *et al.*, 1989)
α3β1	Laminin 1	(Carter *et al.*, 1990)
	Laminin 5	(Carter *et al.*, 1991)
α5β1	Fibronectin	(Adams and Watt, 1991)
α6β4	Laminin 1	(Niessen *et al.*, 1994)
	Laminin 5	(Niessen *et al.*, 1994)
α9β1	Tenascin	(Yokosaki *et al.*, 1994)
	Vitronectin	(Weinacker *et al.*, 1995)
αvβ5	Vitronectin	(Adams and Watt, 1991)
αvβ6	Fibronectin	(Busk *et al.*, 1992)
	Vitronectin	(Thomas *et al.*, 2002)
	Tenascin	(Thomas *et al.*, 2002)
	Latency-associated peptide (LAP)	(Thomas *et al.*, 2002)

All integrins, except for α6β4, mediate adhesion via the actin cytoskeleton of the cell.

1.4.3.1 Integrin signalling

In addition to their role as cellular mechanical anchors, integrins also mediate bi-directional intracellular signalling (for review see: Hynes, 2002; Lee and Juliano, 2004).

Engagement of cells with the ECM proteins via integrins is crucial for transduction of a variety of signal events that serve to modulate many biological processes, including cell adhesion, spreading, cell shape, polarity, proliferation, differentiation, migration, survival/apopotosis and gene induction, contributing to maintenance of tissue integrity,

embryogenesis, wound healing and the metastasis of tumour cells (Hynes, 2002; Juliano, 2002).

Interaction of integrins with ECM proteins initiates clustering of integrins and recruitment of intracellular signalling molecules and actin filaments (Hynes, 2002). Integrins interact with different intracellular sigalling molecules, such as focal adhesion kinase (FAK), Erk and c-Jun kinase (JNKs), MAP kinases, and Rho GTPase family members (RhoA, Rac1 and Cdc42) (Juliano, 2002; Lee and Juliano, 2004).

One of the major functions of an integrin is to link ECM proteins to the actin cytoskeleton of the cell by virtue of interactions of the integrin cytoplasmic tail with actin binding proteins, such as talin, filamin, integrin-linked kinase (ILK), and α-actinin (Pozzi and Zent, 2003). Through these interactions, integrins mediate actin filament reorganisation (Coppolino and Dedhar, 2000), as well as mediating signals critical for cell growth, survival, migration and cell polarity. Also, integrin-mediated cell adhesion regulates formation, turnover, and linkage of the actin cytoskeleton via stimulation of the Rho small GTPase family members including RhoA, Rac1 and Cdc42 (Lee and Juliano, 2004). During early stages of adhesion, integrins mediate the protrusion of the cell membrane through activation of Rac1 and Cdc42, followed by the adhesion/spreading phase which involves RhoA activation and increased contractility. On the other hand, assembly of integrins and associated proteins in focal adhesion contacts is mediated by members of the Rho small GTPases (Hotchin and Hall, 1995; Hotchin and Hall, 1996). Mechanisms of integrin-dependent activation of the Rho GTPases are being actively studied.

Another major function of integrins is promoting cell survival in an anchorage-dependent manner, whereby engagement of the integrin with ECM proteins mediates survival signals in the cell (Frisch and Screaton, 2001). Cells that lose integrin-mediated attachment with the ECM undergo apoptosis. Adhesion of integrin receptors with ECM generates survival signals

and blocks apoptosis via PI3-kinase and Akt (King *et al.*, 1997) and stimulates cell cycle progression via ERK and cyclin D1 (Hynes, 2002). In tumours, anchorage-*in*dependent survival is a hallmark of neoplastic transformation and is critical for tumour progression. In transformed cells survival signals generally are mediated by oncogenes or by loss of tumour suppressor genes (Lukashev and Werb, 1998).

1.4.3.2 Integrins in tumours

There is a large body of literature providing evidence that integrins dictate the malignant behaviour of tumour lesions. In general, integrins are implicated in different biological processes of great significance to tumourigenesis, such as migration, proliferation, protease production, apoptosis and angiogenesis (Marshall, 2000). Different tumours show different integrin expression profiles, probably reflecting the biological activity of each tumour. Variable loss of α2β1, α3β1, αvβ5 and α6β6 has been reported in many carcinomas (Pignatelli and Bodmer, 1990; Koretz *et al.*, 1991). Loss of polarised integrin expression has been observed constantly in all tumours, for example α6β1 and α6β4, normally expressed at the basolateral domain of epithelial cells, showed a uniform distribution and loss of polarity in transformed malignant cells (Keely *et al.*, 1998). Increased expression of αvβ3 and αvβ6 has been reported in several carcinomas and the presence of these heterodimers has been thought to contribute to the development of a more malignant phenotype (Albelda *et al.*, 1990; Felding-Habermann *et al.*, 1992; Seftor *et al.*, 1993; Agrez *et al.*, 1994; 1996; Thomas *et al.*, 2001). The α5β1 integrin plays a complex role in tumour progression. Restoration of α5β1 integrin expression was shown to inhibit anchorage-independent growth of malignant cells (Giancotti and Ruoslahti, 1990). However, it also was reported that α5β1 integrin plays an important role in cell survival and inhibition of apoptosis (Zhang *et al.*, 1995).

So as a general rule, tumours tend to retain or express integrins that support the malignant phenotype, for example those involved in increased cell migration, and lose expression of integrins known to maintain tissue organisation and stable adhesion.

1.4.3.3 Integrins in OSCC

In OSCC, integrins show variable expression between tumours and within different areas of the same tumour (Jones *et al.*, 1993; Jones *et al.*, 1997; Cortesina *et al.*, 1995; Thomas *et al.*, 1997). As with other carcinomas, focal and extensive loss of α2β1, α3β1, αvβ5 and α6β6, has been associated with the poorly differentiated OSCC lesions and loss of the α6β4 correlated with the loss of basement membrane integrity (Downer *et al.*, 1993). However, others reported retained expression of the α6β4 integrin in highly malignant head and neck carcinoma (Cortesina *et al.*, 1995), probably indicative of a failure of the transformed cells to differentiate rather than a true upregulation of the α6β4 (Thomas and Speight, 2001). Moreover, *in vitro* studies showed high expression of α6β4 integrin consistent with epithelioid colonies in head and neck carcinoma (Tomson *et al.*, 1996). Alterations in localisation and expression of α6-integrin were also observed, with a relative increase in α6-integrin expression compared to the β4-integrin subunit, in both premalignant and malignant oral mucosa (Garzino-Demo *et al.*, 1998). Also, strong expression of α2-, α3-, α4-, α5- and α6-integrin subunits have been oberved in metastatic OSCC cell lines as compared to non-metastatic cell lines (Shinohara *et al.*, 1999).

Alteration of αv-integrin has also been observed in OSCC (Jones *et al.*, 1997). Thus the αvβ6 integrin, which normally is not expressed by normal epithelium, was expressed in OSCC and was correlated with increased motility and invasiveness (Breuss *et al.*, 1995; Jones *et al.*, 1997), whereas αvβ5 integrin showed a reduced expression in OSCC as compared to the normal epithelial cells (Jones *et al.*, 1993; Jones *et al.*, 1997). The αv-integrin might be of significant

importance in OSCC as it has been reported that re-expression of this particular integrin has reversed the malignant phenotype (Jones *et al.*, 1996b; Thomas *et al.*, 1997; 2001).

1.5 Synergy and cross-talk between E-cadherin and integrins

Molecular cross-talk is a term used to refer to the concept of two different classes of adhesion molecules signalling to each other. There is growing indication of cross-talk between different CAM families. In a multicellular environment, the collaborative actions of multiple CAMs are essential to maintain tissue balance and homeostasis.

In cultured keratinocytes, inhibition of E-cadherin-mediated cell-cell contacts resulted in decreased integrin expression in proliferating cells and *de novo* expression by differentiating cells (Hodivala and Watt, 1994). Moreover, downregulation of E-cadherin levels in keratinocytes showed a concomitant decrease in the expression of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins and enhancement of cell motility (Zhu and Watt, 1996). Recently, Hintermann *et al.* (2005) provided evidence of a potential cross talk between E-cadherin and the $\alpha 6\beta 4$ integrin, where activation of the $\alpha 6\beta 4$ integrin resulted in an augmentation of the E-cadherin-dependent cell-cell contacts and a concomitant decrease in cell motility (Hintermann *et al.*, 2005).

Evidence of cross-talk between adhesion receptors has a particular relevance to malignant epithelial cells that show decreased levels of adhesion molecules complemented with increased motility and invasiveness potential. In malignant lesions there is a strong suggestion that loss of E-cadherin and integrins are closely related. Bagutti *et al.* (1998) found that E-cadherin, but not P-cadherin, strongly associated with integrin expression and tumour differentiation; indicative that downregulated expression levels of E-cadherin and integrins are likely to confer selective growth advantage in squamous cell carcinoma and possibly are correlated with poor prognosis (Bagutti *et al.*, 1998).

One possible mechanism pertaining to the cross-talk between cadherins and integrins involves ILK. ILK interacts with the cytoplasmic domain of β1 and β3 integrins (Dedhar *et al.*, 1999). It has been reported that epithelial cells overexpressing ILK showed additional activation of the Wnt signalling pathway, and dramatic reduction in E-cadherin expression accompanied by loss of cell-cell contacts and increased invasion (Novak *et al.*, 1998; Huttenlocher *et al.*, 1998; Wu *et al.*, 1998). Thus, such findings provide an interesting potential connection between cadherins and integrins.

Decoding the underlying mechanisms by which this adhesion molecules cross-talk may provide a better understanding of mechanisms relevant to tumour differentiation, invasion and metastasis.

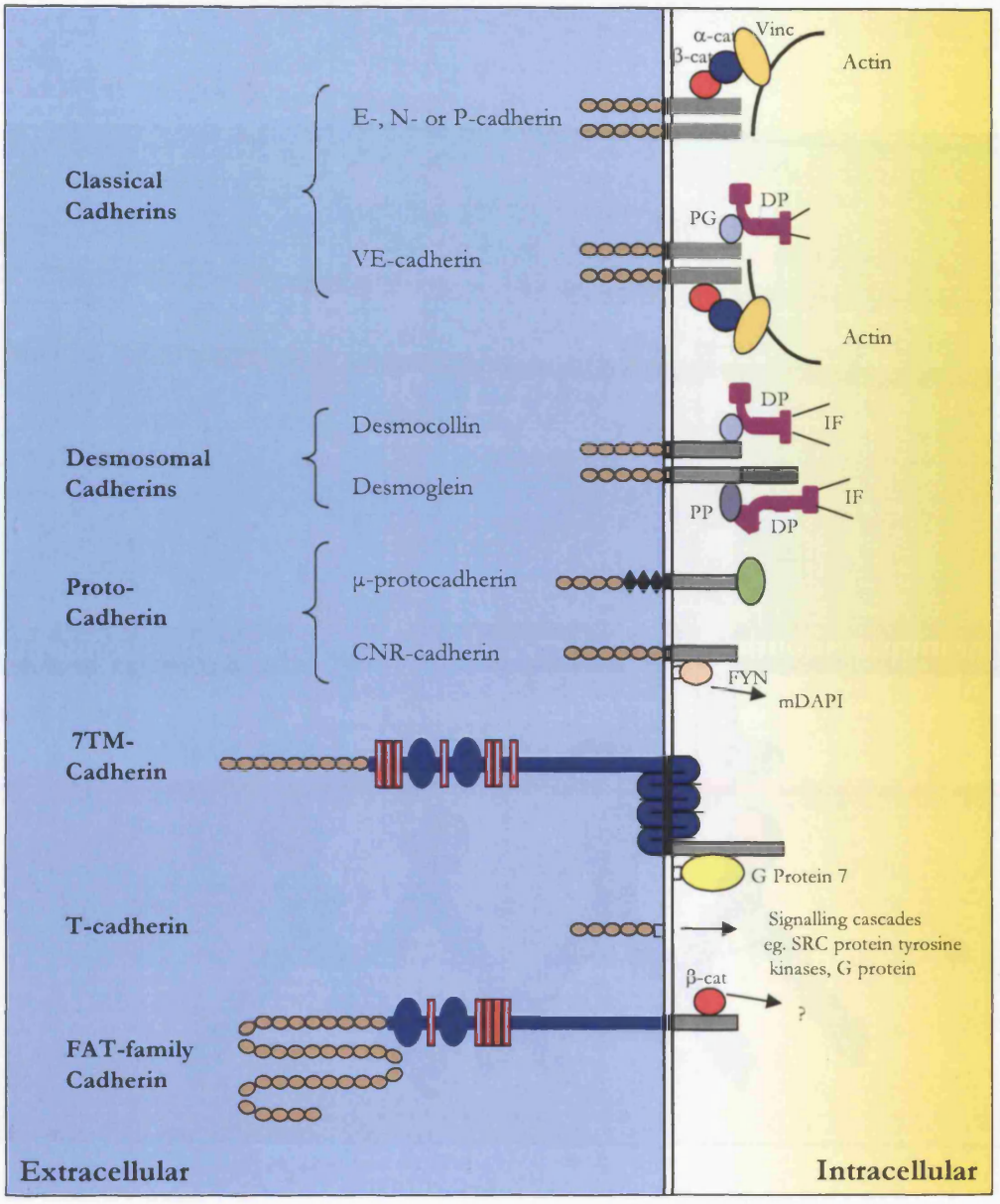


Figure 1.1
Schematic representation of the Cadherin Superfamily
α-cat: α-catenin; β-cat: β-catenin; PG: plakoglobin; PP: plakophilin; Vinc: vinculin; IF: intermediate filaments; DP: desmoplakin. Desmosomal cadherins (Desmocollins and Desmogleins) can bind DP via PG or PP. Classical and desmosomal cadherins are represented as dimers, while other cadherins are represented as monomers because of lack of evidence as to how they bind. VE-cadherin is known to bind to both the actin cytoskeleton and to the intermediate filament network; for simplicity only one interaction is illustrated (Angst *et al.*, 2001).

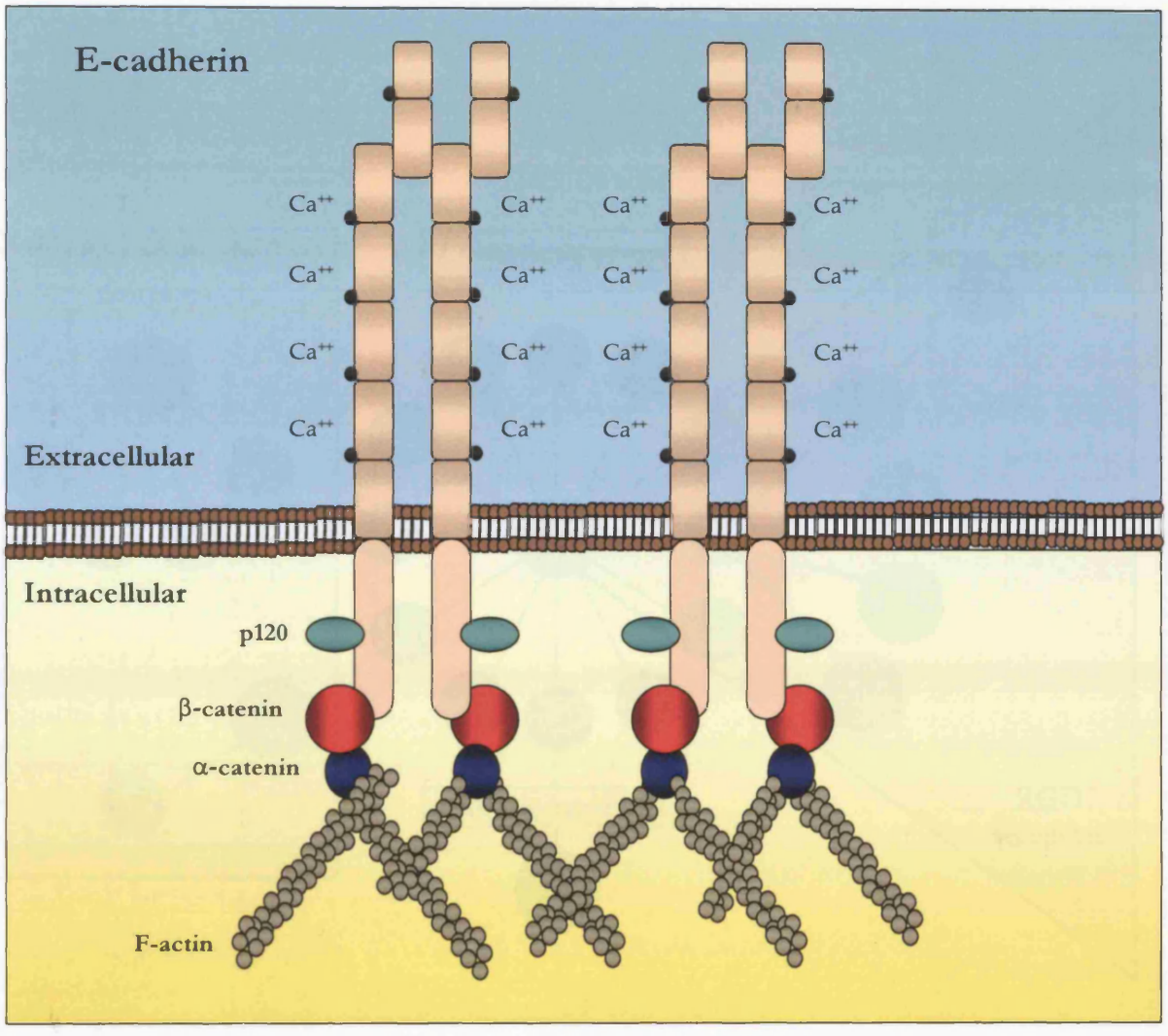


Figure 1.2
Schematic representation of the mature E-Cadherin molecule
E-cadherin molecules in one cell appear as dimers interacting with E-cadherin molecules on adjacent cells forming the "Adhesion Zipper". Intracellularly, E-cadherin interacts with the actin cytoskeleton of the cell via cytoplasmic plaque proteins named the catenins (α-, β-, γ-catenin and p120ctn).

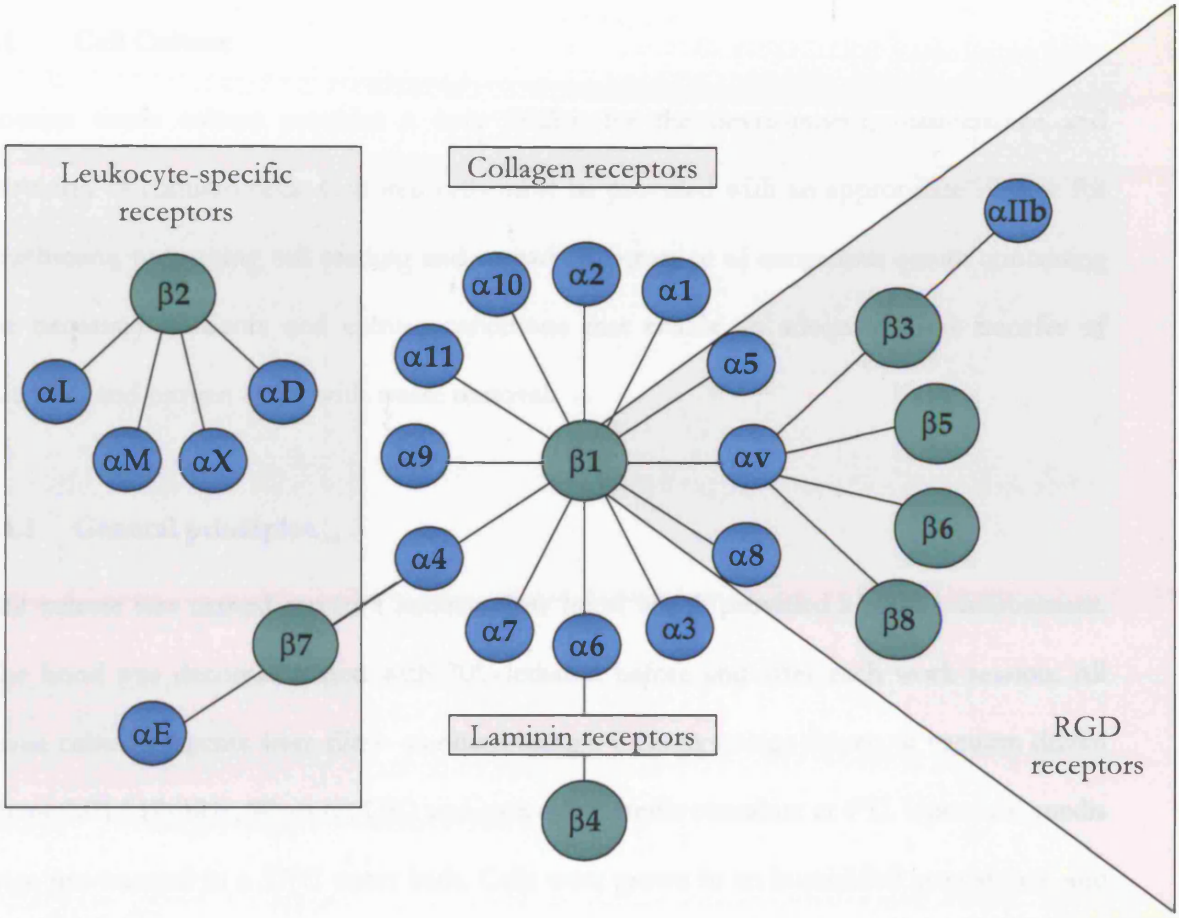


Figure 1.3
Schematic representation of the integrin family and ligands
The figure illustrates the mammalian integrin subunits and their αβ associations, which are subdivided further into groups according to their ligand-specificity (adapted from Hynes, 2002).

CHAPTER 2

Materials and Methods

2.1 Cell Culture

Routine tissue culture provides a core facility for the development, maintenance and provision of cultured cells. Cultured cells must be provided with an appropriate surface for attachment, promoting cell seeding and spreading, presence of exogenous serum containing the necessary nutrients and culture conditions that enable an adequate mass transfer of nutrients and oxygen along with waste removal.

2.1.1 General principles

Cell culture was carried out in a laminar flow hood which provided a sterile environment. The hood was decontaminated with 70% ethanol before and after each work session. All tissue culture reagents were filter- sterilised using a 0.22μm syringe-driven or vacuum driven filters (MILLIPORE, Watford, UK) and stored in a sterile container at 4°C. Upon use, media were pre-warmed in a 37°C water bath. Cells were grown in an humidified atmosphere and 8% CO_2 at 37°C in incubators. Closed-front lab coats and gloves were worn routinely.

2.1.2 Cell lines and growth requirements

All cell lines used were derived from oral squamous cell carcinomas. H357 is an OSCC line derived from the lateral border of the tongue and H376 is an OSCC line derived from the floor of the mouth. Both lines were provided by Professor S. Prime (Prime *et al.*, 1990). H376β4 is an H376 cell line previously transfected with the β4-integrin subunit (Jones *et al.*, 1996a).

Cells were grown in either full- or antibiotic-supplemented keratinocyte growth medium (KGM) (Appendix I). The cell lines and their growth requirements are shown in Table 2.1.

Table 2.1: Cell Lines: Origin and growth requirements

Cell lines	Origin	Medium	Supplements
H357	Oral keratinocytes derived from a squamous cell carcinoma of the tongue (Prime *et al.*, 1990)	KGM	Nil
H376	Oral keratinocytes derived from a squamous cell carcinoma of the tongue (Prime *et al.*, 1990)	KGM	Nil
H376β4	H376 transfected with β4 integrin subunit (Jones *et al.*, 1996a)	KGM	G418 1 mg/ml
IA2	H376 transfected with empty vector	KGM	G418 1 mg/ml

2.1.3 Cell Culture

2.1.3.1 Cells growing on tissue culture plastic

Cells were grown as adherent monolayers in sterile, serum-coated tissue culture flasks (Nunc/Falcon) in an humidified atmosphere and 8% CO_2 at 37°C in incubators. Cells were subcultured, when they were 80-90% confluent, 1-3 times a week, at a ratio 1:2 to 1:10 depending on the growth rate. After removal of the spun medium, cells were washed once with PBS washing buffer (Appendix I), then 2ml of trypsin/EDTA (0.05% trypsin (w/v) / 5mM EDTA) were added to the cells for 3-5 minutes at 37°C. Once the cells had been detached from the plastic, the trypsin was inactivated by serum-containing medium. Cells were spun at 1,200 rpm for 3 minutes, supernatant was discarded, and then cells were resuspended in fresh medium and transferred to new flasks.

2.1.3.2 Cells growing in 2-D/3-D collagen I cultures

2-dimensional (2-D) and 3-dimensional (3-D) Type I collagen cultures were established as illustrated in Figure 2.1. Collagen I, rat tail (Upstate Cell Signalling Solutions, Milton Keynes, UK) mix was prepared at a final concentration of 2.9 mg/ml. Kept on ice, the collagen I was mixed with 10x MEM (ICRF, Clare Hall Labs., South Mimms, UK), 0.34N NaOH and Foetal calf serum (FCS) in an 8:1:1:1 ratio.

For formation of the 2-D substrate, 25µl of the collagen I mix was spread evenly, with the aid of a pipette tip to ensure an even lining, on the bottom of each well in a 24-well plate. The plates were incubated for 45 minutes at 37°C; to allow for the collagen I mix to set. Then $2x10^4$ cells were seeded on top of the set matrix and fed with 2ml selection medium.

For 3-D cultures, $2x10^4$ cells were embedded in 500µl of the collagen I mix prior to setting. The plates were incubated for 45 minutes at 37°C before adding 2ml selection medium. Cells in both 2-D and 3-D collagen I cultures were allowed to grow until harvested using collagenase 1 enzyme (Worthington Biochemical Corporation, Lakewood, NJ, USA) for counting or for obtaining total cell lysates.

2.1.4 Storage and recovery of liquid nitrogen stocks

2.1.4.1 Cryopreservation of cell culture

Cultures can be preserved effectively in the presence of a cryoprotectant, such as dimethylsulphoxide (DMSO), which reduces the damage from ice crystals (Brown, 2000). Cells to be stored were harvested in log phase growth. Following trypsinisation, cells were washed in complete medium and resuspended in medium containing 10% DMSO (Sigma-Aldrich, Dorset, UK) at $1\text{-}2x10^6$ cells per ml. One ml of cell suspension was placed in a Nunc

cryotube in an insulated container and placed at –80°C for 8 hours, before transferring vials to –196°C liquid nitrogen for long-term storage.

2.1.4.2 Thawing cell stocks

Cryopreserved cells are fragile and require quick thawing and immediate retrieval into full medium. Vials to be recovered from liquid nitrogen were placed in a water bath at 37°C to thaw rapidly. As soon as the vial contents were thawed they were transferred to 15ml tubes (Falcon) containing 10ml of pre-warmed KGM and centrifuged at 1,200 rpm for 3 minutes. The supernatant was removed to get rid of cryopreservative and cells were resuspended in 1ml of KGM and transferred to a 75cm^2 flask containing fresh medium. If cells required drug selection pressure, selection medium was used once growth in culture was re-established.

2.1.5 Cell counting

As a part of standardising a proper experiment, cell counting is the commonly used method to quantify cells in a cell culture. There are two methods used to count cells: the haemocytometer and the automated cell counter.

2.1.5.1 Cell counting using an haemocytometer

Cell count was obtained by placing harvested cell suspension under a girdded, optically flat chamber viewed under the microscope. Different defined fields of cells, within the confines of the grid, were counted. The cell concentration was obtained from the count. Along with this method "Trypan blue exclusion" was used to determine cell viability. Viable cells are impermeable to trypan blue dye, while non-viable cells, characterised by breakdown of the cell membrane, uptake the dye and appear blue under the microscope. 0.4% (w/v) Trypan

blue dye (Sigma-Aldrich) was added 2 minutes prior to counting a time just enough to allow for staining of non-viable, but not viable, cells.

2.1.5.2 Cell counting using an automated cell counter

Harvested cells were counted on a CASY automated cell counter (Shärfe System, Reutlingen, Germany). Basically, cells suspended in isotonic buffer (Coulter Euro Diagnostics GMBH, Buckinghamshire, UK) were passed through a fine orifice changing a current flow which produces a series of pulses that are sorted and counted.

2.2 Analysis of the whole cell

2.2.1 Flow cytometry (FACS analysis)

Flow cytometry is a commonly used method for characterising and separating individual cells. It provides a technology that can deliver rapid, quantitative, multi-parameter analyses on a single cell based on measurement of fluorescent light emission intensity, produced by fluorescent-labelled antibody bound to a specific cell-associated molecule, using a flow cytometer.

2.2.1.1 FACS analysis for E-Cadherin

Cells to be labelled with E-Cadherin monoclonal antibody "HECD-1" (M. Takeichi, Tokyo, Japan) were allowed to reach confluence, washed twice in PBSA containing 1mM calcium chloride, 1mM magnesium chloride (PBSABC) (Appendix I) and incubated in EDTA free trypsin (0.1% (w/v) (Sigma-Aldrich) supplemented with 1mM calcium chloride for 25-30 minutes (adapted from Takeichi, 1997), conditions which minimise the proteolysis of the extracellular domain of E-Cadherin. Antibodies used were diluted in PBSABC containing

10%FCS, which also was used as a washing buffer. Dissociated cells were incubated with primary antibody for 60 minutes at 4°C and washed twice with 10%FCS-PBSABC washing buffer. Alexa 488-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes, Paisley, UK) was applied to the cells for 45-60 minutes at 4°C. The cells were then washed three times with 10%FCS -PBSABC washing buffer and resuspended in 0.5ml PBS containing 10% FCS. Control samples were labelled with Mouse IgG1 negative control (DakoCytomation Ltd., Cambridgeshire, UK). Labelled cells were scanned on a FACScaliber Cytometer (BD Bioscience, Oxford, UK) connected to an Apple Macintosh computer fitted with Cellquest software, acquiring $1x10^4$ events. With each cell line, the same settings were used for each experiment.

2.2.1.2 FACS analysis for Integrins

Subconfluent cells were washed twice with PBSA and harvested by trypsin/EDTA (0.25% w/v, 5mM). The cells were washed once in PBSA 0.1% heat-inactivated BSA and 0.1% Azide (0.1/0.1 blocking/washing buffer), incubated with primary antibody for 60 minutes at 4°C and washed twice with 0.1/0.1 washing buffer. Alexa 488-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes) or FITC-conjugated Rabbit anti-Rat Immunoglobulin secondary antibody (DakoCytomation Ltd.) were applied to the cells for 45 minutes at 4°C (a complete list of antibodies used in this experiment is given in Appendix II, Table 2). The cells were then washed three times with 0.1/0.1 blocking buffer and resuspended in 0.5ml PBSA containing 10% FCS. Control samples were labelled with Mouse or Rat IgG1 negative control (DakoCytomation Ltd.). The procedure was as described previously in Section 2.2.1.1.

2.2.2 Indirect immunocytochemical staining

A suspension of $2x10^4$ cells was plated on uncoated 13mm glass coverslips in 24-well plates and cells were allowed to grow for 48 hours in complete medium. Cells were rinsed twice in PBSABC, fixed for 15 minutes in 4% formaldehyde (BDH Laboratories, Poole, UK), rinsed once with PBSA. Cells were then permeabilised using 0.1% Triton X-100 (Sigma-Aldrich) for 3-4 minutes at room temperature, rinsed once with PBSA and blocked with PBSA containing 0.1% heat-inactivated BSA and 0.1% Azide (0.1/0.1 blocking/washing buffer) for 30 minutes at room temperature. Antibodies used (Appendix II, Table 2) were diluted in 0.1/0.1 blocking buffer. 200µl of the diluted primary antibody was used to cover one coverslip in 24-well plate; otherwise, the coverslip was inverted on a 20µl drop placed on parafilm (a cost-effective method). The coverslips were incubated with the primary antibody for 60 minutes at 4°C. Then, the cells were washed three times with 0.1/0.1 blocking buffer. The coverslips must not be allowed to dry at any time. Secondary antibody, also diluted in 0.1/0.1 blocking buffer, was added for 60 minutes at 4°C. F-actin was visualised using phalloidin-tetramethylrhodamine isothiocyanate "phalloidin-TRITC" 50 ng/ml (Sigma-Aldrich: 5ng/ml), or using Alexa Fluor ® 647 phalloidin (Molecular probes: Invitrogen Ltd., Paisley, UK) while nuclei were detected with 4^1, 6-α diamidino-2-phenylindole dihydrochloride "DAPI" (Polyscience Inc., Warrington, PA, USA). The coverslips were incubated with the counterstains for 10 minutes at room temperature. The coverslips were washed three times, for 5 minutes each, in washing buffer and mounted with MOWIOL 4-88 (0.1g/ml dissolved in 25% (v/v) Citifluor AFI (Citifluor Ltd, London, UK)/ 100 mM Tris-HCL pH 8.5) and viewed with a confocal inverted laser scanning microscope (Zeiss Axiovert 200M: Welwyn Garden City, UK).

2.3 Analysis of cellular proteins by Western blotting (WB)

Western blot analysis is used to determine some important characteristics of a protein antigen, including: the presence, the quantity and the molecular weight. Polyacrylamide gel electrophoresis separates individual proteins based on their size, shape and charge. Transferring separated proteins allows for detection of specific proteins using specific antibodies.

2.3.1 Preparation of whole cell lysates of cells growing on plastic

Confluent and subconfluent cells were washed twice in PBSABC. A 1ml solution of RIPA buffer containing Protease inhibitor (Appendix I), was added to each $75cm^2$ flask and the cells were scraped into a microcentrifuge tube, and incubated on ice for 1 hour. Lysates were cleared by centrifugation for 10 minutes at 13,000 rpm. Samples were stored at –70°C until use.

2.3.2 Preparation of whole cell lysates of cells growing in 2-D/3-D collagen I cultures

Cells were plated as described in Section 2.1.3.2 but with the cultures instead being set in 6-well plates. For the 2-D cultures 60µl of the collagen I mix was spread on the bottom of each well in a 6-well plate and a starting number of $6x10^4$ cells were seeded on top of the set matrix and fed with 6ml selection medium. For 3-D cultures the same number of cells was added to 1.5ml of the collagen I mix prior to setting and 6ml selection medium were added after setting. Cells were allowed to grow for 5 days, until cells growing in 2-D collagen I cultures were about confluent and cells growing in 3-D collagen I cultures were sufficient to provide appropriate levels of protein lysate as determined in preliminary experiments. Cells

were retrieved from the collagen I culture prior to lysing, as follows: After washing the cultures, twice, with PBSABC, 500µl of collagenase 1 (Worthington Biochemical Corporation) were added to the 2-D cultures and 2ml to the 3-D cultures and incubated for about 1-2 hours at 37°C before cells were collected in 15ml Falcon tubes and spun down and the supernatant was discarded. To get rid of excess collagenase, the cell pellet was washed twice with PBSA. After the second wash the supernatant was discarded carefully and excess was removed using a 200µl pipette. RIPA buffer, containing Protease inhibitor, was added to the cell pellet and mixed. The lysates were transferred into a microcentrifuge tube, and incubated on ice for 1 hour. Lysates were cleared by centrifugation for 10 minutes at 4,000 rpm. Samples were stored at –70°C until use.

2.3.3 Estimation of protein concentration

Protein concentration was estimated using the Bio-Rad DC protein assay (Bio-Rad, Hercules, Hertfordshire, UK), a colorimetric assay based on the Lowry assay, according to the manufacturer's instruction. The assay depends on the reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein. A blue colour develops which is read at absorbance 650nm.

BSA protein standard solutions were set up at protein concentrations of 0, 125, 250, 500, 750, 1000, 1500 and 2000 µg/ml. 1ml reagent B was added, first, into 2.5ml SM Cuvettes (Kartell Plastics UK Ltd., Cambridge, UK), followed by 25µl of standard solutions and lysate samples. 125µl of reagent A' were added last. The tubes were incubated at room temperature for 15 min and read at OD 650nm on a spectrophotometer (SLT Labinstruments GmbH, Salzburg, Austria) and the unknown protein concentration determined against the standard curve using UV WinLab software (PerkinElmer™ Molecular SPECTROPSCOPY, Boston, USA)

2.3.4 Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Cellular proteins were separated using a polyacrylamide gel mounted on a vertical gel electrophoresis apparatus system (Xcell SureLock™ Novex Mini-Cell system: Ivitrogen Ltd.) and the 1mm thick gels assembled according to the manufacturer's instructions.

The desired percentage of acrylamide resolving gel (Appendix I) was poured into the gel cassette and covered with propan-2-ol to eliminate the air interface (which inhibits acrylamide polymerisation). The gel was allowed to polymerise for about 45 minutes, the propan-2-ol was poured off and the space on top of the set gel was rinsed with 0.5M Tris (pH 6.8). 4% stacking gel (Appendix I) was poured, and allowed to polymerise for about 30 minutes before placing the gel into the electrophoresis tank. The chamber was filled with SDS-running buffer (Appendix I). Samples were diluted in loading buffer (Appendix I), boiled for 10 minutes; the contents were collected by centrifugation and loaded using capillary disposable pipette tips. The gel was run for 10 minutes at 120 volts, then 140 volts for about 1 hour, until sufficient protein migration had occurred, as judged by the migration of the loaded Molecular weight markers (Amersham Life Science, Buckinghamshire, UK) and the dye in the loading buffer.

After running the gel the assembly was dismantled and the gel carefully removed and equilibrated in transfer buffer (Appendix I).

2.3.5 Immunoblotting

After separation of the protein sample on the polyacrylamide gel, the proteins were transferred to either a supported nitrocellulose membrane or a polyvinylidene difluoride (PVDF) membrane (Hybond™-C extra or Hybond™-P: Amersham Life Science), in a pattern reproducing the separation seen on the gel. The resolved gel was laid on the blot membrane and sandwiched between two 3MM Whatman Chromatography papers

(Whatman Int., Maidstone, UK). Using a transfer cassette unit, the sandwiched gel was compressed in the cassette and assembled according to the manufacturer's instructions (XCell II™ Blot Module: Ivitrogen Ltd.). The cassette was immersed in transfer buffer. Proteins were transferred at 26 Volts for 2 hours.

Once the transfer was complete, depending on the primary antibody used, each blot was processed differently to generate an optimum signal. In general each blot was incubated in blocking solution, to minimise non-specific binding, for 1-2 hours with agitation. The blot was then incubated with the primary antibody diluted in a buffer with agitation for a variable length of time. Then the blot was washed several times in washing buffer before incubation, with the appropriate HRP-conjugated secondary antibody diluted in a buffer, and then the blot was again washed for several times (a list of primary antibodies used in this experiment is given in Appendix II, Table 3).

Specifically bound antibody was detected using enhanced chemiluminescence (ECL™, ECL plus™ or ECL™ advance: Amersham Life Science) on Kodak X-Omat film (Sigma-Aldrich). Images were scanned, and densitometry was performed by comparing treatment and control band densities using the image analysis program NIH Image (NIH, USA).

2.3.5.1 General blocking protocol

To generate optimal signal the following blocking protocol was used with all antibodies listed in Table 2.4 (Appendix II), except when blocking for phospho-Akt (Thr 308), Rac1 and Cdc42. Protein was transferred to nitrocellulose membrane. After the transfer was complete, the membrane was rinsed once with ddH_2O, then was incubated in 5% milk in PBSA blocking buffer (blocking buffer 1: Appendix I) for 2 hours at room temperature. Then, the blot was incubated with the primary antibody in blocking buffer over night, with agitation, at 4°C. Then, the membrane was washed for four times in 0.05% PBSA-Tween (washing buffer

1: Appendix I), 5 minutes for the first two washes and 10 minutes for the second two washes. The blot was then incubated with 1:1000 goat-anti-mouse HRP secondary antibody (BioSource United Kingdom, Nivelles, Belgium) diluted in 5% milk-PBSA blocking buffer for one hour at room temperature. Finally, the blot is washed for five times in 0.05% PBSA-Tween, 5 minutes each, then developed using either ECL™ or ECL plus™ (Amersham Life Science).

2.3.5.2 Blocking for phospho-Akt (Thr 308)

50μg reduced sample protein were loaded in 12.5% polyacrylamide gel. Proteins were then transferred into nitrocellulose membrane which was washed in 0.05% TBS-Tween (washing buffer 2: Appendix I) for three times, 5 minutes each. The blot was incubated, with 1:1000 primary antibody diluted in 1% BSA in 0.05% TBS-Tween blocking buffer (blocking buffer 2: Appendix I), overnight at 4°C. Subsequently, the blot was washed for three times in 0.05% TBS-Tween, 5 minutes each, and then incubated with 1:1000 the goat-anti-Rabbit secondary antibody (BioSource United Kingdon) diluted in 5% milk-0.05% TBS-Tween blocking buffer (blocking buffer 3: Appendix I). To wash off excess secondary layer the blot was washed for three times in 0.05% TBS-Tween, 5 minutes each, then developed using ECL plus™ (Amersham Life Science).

2.3.5.3 Blocking for Rac1

Sample were run in 15% polyacrylamide gel, the proteins were then transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was rinsed twice with ddH_2O, then was incubated in 3% milk in PBSA blocking buffer (blocking buffer 4: Appendix I) for 2 hours at room temperature. Subsequently, the blot was incubated with the primary antibody (anti-Rac1 "23A8" monoclonal antibody: Upstate Cell Signaling Solutions), diluted

1:1000 in 3% milk-PBSA, with agitation, over night at 4°C, followed by two washes with ddH_2O, 5 minutes each, and a final wash with PBSA for 10 minutes. The blot was then incubated with the 2° antibody diluted in 3% milk-PBSA, with agitation, for 1.5 hour at room temperature. To minimise the high back ground, the blot was washed extensively. The blot was first rinsed, once, with ddH_2O, followed by three washes in ddH_2O, 5 minutes each. Additionally, it was washed twice using 0.05% TBS-Tween for 10 and 5 minutes, respectively. Prior to development, using ECL plus, the blot was quickly rinsed using ddH_2O.

2.3.5.4 Blocking for Cdc42

Sample were run in 15% polyacrylamide gel, the proteins were then transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was rinsed twice with ddH_2O, and then was incubated in 5% milk in 0.05% TBS-Tween blocking buffer for 1 hour at room temperature. Subsequently, the blot was incubated with the primary antibody (anti-Cdc42 "3707" monoclonal antibody: diluted 1:250 in 0.01% TBS-Tween- no blocking reagent was added), with agitation, over night at 4°C. The blot was rinsed twice in 0.01% TBS-Tween and then washed using the same buffer for three times, 5 minutes each. The blot was then incubated with the 2° antibody diluted in 3% milk-PBSA, with agitation, for 1.5 hour at room temperature. Using 0.05% TBS-Tween, the blot was first rinsed twice, and then washed three times, 10 minutes each. Prior to development, using ECL plus the blot was quickly rinsed using ddH_2O.

2.3.6 Western blot recycling

To re-probe the same blot with different antibody (or to correct errors in incubation with the wrong antibody) the bound antibodies can be removed effectively from the membrane without affecting the immobilised proteins using stripping buffer (Re-Blot™ Western blot

recycling kit: CHEMICON® international, Hampshire, UK). The blots were incubated with stripping buffer (diluted in distilled water at 1:10 ratio) at room temperature for 7 minutes with agitation. Following stripping the blots was blocked for 1 hour before incubating with primary antibody.

2.4 Molecular biology

Several molecular biological techniques were used in an attempt to isolate and clone E-cadherin cDNA to create a cell line, originally E-cadherin negative, that expresses functional E-cadherin molecules and further study the effect of E-cadherin expression on the function of this particular cell line.

2.4.1 RNA isolation and RT-PCR

2.4.1.1 RNA isolation

Total cellular RNA was extracted from the E-Cadherin positive H357 cell line (Sugiyama *et al.*, 1993), using RNA STAT-60™ total RNA/mRNA isolation reagent (Tel-Test "B" Inc., Friendswood, Texas, USA). Briefly, confluent cells in a 75cm^2 flask were washed once with whole medium (KGM) and trypsinised using 3ml of trypsin/EDTA. The trypsin was inactivated by 7ml fresh medium and cells were centrifuged at 1,200 rpm for 3 minutes. Supernatant was removed completely and the pellet kept on ice for immediate use. 2ml of RNA STAT-60 (phenol and guanidinium thiocyanate in mono phase solution) were added and mixed to homogenise the cells, and the homogenate was stored at room temperature for 5 minutes. 0.2ml of chloroform per 1ml of RNA STAT-60, was added, and the tube was shaken vigorously for 15 seconds, and then was incubated at room temperature for 3 minutes. The mixture was centrifuged at 13,000 rpm for 15 minutes at 4°C, after which the

homogenate was separated into two phases: colourless upper aqueous phase, containing total RNA, and a red lower organic phase, containing DNA and proteins. The aqueous phase was carefully transferred into a sterile microcentrifuge tube. 0.5ml isopropanol per 1ml RNA STAT-60 was added and the sample incubated at room temperature for 5-10 minutes, then centrifuged at 13,000 rpm for 10 minutes at 4°C, to allow the RNA precipitate to form as a white pellet at the bottom of the tube. The supernatant was removed completely, and the RNA pellet was washed by adding 1ml of 75% ethanol, vortexed and centrifuged at 13,000 rpm for 5 minutes at 4°C. The washing buffer was removed completely and the pellet was left to dry for 5-10 minutes. Then the RNA pellet was resuspended in 20µl ddH_2O, incubated on ice for 30 minutes and vortexed every 5 minutes to dissolve RNA completely.

The concentration and purity of RNA was determined by absorbance at 260 and 280nm. A solution with an optical density OD_{260} of 1.0 contains 40µg RNA/ml, and the ratio OD_{260} /OD_{280} of ~ 1.8 specifies a clean preparation. Contamination with protein or salts leads to a lower ratio.

5µl of sample were added to 995µl ddH_2O, and the concentration of RNA was calculated using the following equation:

Final concentration of RNA = Spectrophotometer reading at 260nm x dilution factor x conversion factor

Where: Dilution factor = 1000/5 = 200

Conversion factor = 40 µg/ml

2.4.1.2 Reverse transcription of RNA to make complementary DNA (cDNA)

DNA complementary to the total RNA was synthesised using the SUPERSCRIPT First-Strand Synthesis System (GIBCO BRL®: Invitrogen Ltd.) that has been engineered to eliminate the RNAse activity that degrades mRNA during the first-strand reaction.

1μl total RNA (2.34 μg/μl), 9μl DEPC-treated distilled water, 1μl (0.5μg) of oligo$(dt)_{12\text{-}18}$ and 1μl of 10mM dNTP mix were added to a nuclease-free 0.5ml tube. The mix was incubated at 65°C for 5 minutes, and then was chilled on ice for 2 minutes. The contents were collected by brief centrifugation. 4μl of (5x) first-strand buffer, 1μl RNAseOUT RNAse inhibitor and 2μl of 0.1 M DTT were added to the mix, mixed gently and the contents were collected by centrifugation and incubated at 42°C for 1 minute. 1μl of SUPERSCRIPT II RT was added and the contents were mixed, and further incubated at 42°C for 1 hour. The reaction was terminated at 70°C for 15 minutes, followed by 2 minutes incubation on ice. The contents were collected by brief centrifugation. To increase the sensitivity of PCR, the RNA template was removed by adding 1μl of RNAse H before proceeding to PCR, , and the mix was incubated at 37°C for 20 minutes.

2.4.2 Purification of cDNA product

cDNA was purified with a Microcon-100 column (MILLIPORE). The column was placed in a tube and 400μl ddH_2O with 2μg oil-free RT-product product was added. The column was spun at 2,200 rpm in a microfuge for 15 minutes, and two washes were performed. The column was then inverted in a new sterile Microcon tube, and the product was eluted by centrifugation at 2,200 rpm for 2 minutes, and ddH_2O was added to make up to 10μl solution.

2.4.3 Amplification of cDNA using the polymerase chain reaction (PCR)

For cDNA amplification, 5µl of purified RT product was incubated in the presence of 100 ng/µl of two specific primers (see below), 10mM of each of the four dNTPs (Sigma-Aldrich) and 1µl of (6x) pfuTurbo®DNA polymerase (Stratagene, Cambridgeshire, UK) with 5µl reaction buffer (GeneSys Ltd., Surrey, UK). The mixture was made up to 50µl with ddH_2O. The reaction mixture was thermal cycled in the DNA Thermal Cycler (Perkin Elmer, Wellesley, MA, USA) using the following setting parameters:

Segments	Cycles	Temperature	Time
1	1	95°C	5 minutes
2	30	95°C	1 minutes
		63°C	1 minutes
		72°C	4 minutes
3	1	72°C	10 minutes
4	1	4°C	Hold

The PCR product was purified with a Microcon-100 column prior to DNA restriction digestion.

Following amplification, 10µl of PCR products were added to 2µl DNA loading buffer (Appendix I) and electrophoresed as described below in Section 2.4.5.

The primers for determination of the E-Cadherin cDNA were obtained from the ICRF Oligonucleotide Synthesis Laboratory at Clare Hall (South Mimms, UK). The primer sequences were as follows

GCGCCCGGGCTCGAGTCGGCGTCCCCGGCCAGCCATGG E1 (forward primer)

GCGCCCGGGCTCGAGGCATTTCCCAGCACATGGGTCTGG E2 (reverse primer)

2.4.4 Preparation of the plasmid

The pIRES-puro plasmid was digested with Xho I restriction endonuclease (New England Biolabs, Hitchin, Hertfordshire) at the Multiple Cloning Site (MCS) (Figure 2.2) and was prevented from religation by dephosphorylation with alkaline phosphatase (New England Biolabs) as follows

Plasmid DNA was digested using 1 unit of Xho I restriction endonuclease enzyme per 1μg of DNA at 37°C overnight (New England Biolabs) and used with the appropriate buffers supplied by the manufacturers. Xho I restriction enzyme is supplied in an inactive form and, to activate it, it has to be diluted to 1:10 dilution, and the volume of each reaction was adjusted accordingly as follows:

	Plasmid DNA	pIRES-puro Vector (0.75 μg/μl)
DNA	10μl	7μl
Xho I	6μl	8μl
Buffer # 2	6μl	8μl
ddH_2O	38μl	57μl
Total	60μl	80μl

Religation was prevented by dephosphorylation using alkaline phosphatase enzyme (New England Biolabs). 2μl alkaline phosphatase enzyme was added to the 80μl sample of the digested vector, incubated at 37°C for 30 minutes, then, another 2μl alkaline phosphatase enzyme was added, and the mix was incubated for another 30 minutes at 37°C, chilled on ice. The reaction was terminated by the addition of loading buffer.

2.4.5 Agarose gel electrophoresis

Samples were run on 1% agarose gels prepared by adding 1g of agarose (Amresco: NBS Biologicals, Huntingdon, UK) to 100ml of 1xTAE buffer (National Diagnostics, Hassle, Hull, UK) (Appendix I) and boiled in a microwave oven until the agarose was dissolved. On cooling for a few minutes 1μg ethidium bromide (Sigma-Aldrich) was added and the solution poured into a flat gel former with a well-comb in place. Once the gel had set it was transferred to an electrophoresis tank and immersed in TAE buffer. Loading buffer (Appendix I) was added to the DNA solutions which were then loaded into the wells and electrophoresed at 100 V for 30 minutes. 5μl of HinDIII digested λ phage DNA ladder (New England Biolabs) were loaded in the first lane to enable DNA fragment size to be determined. The gel was transilluminated with ultraviolet (UV) light and the DNA bands were visualised and photographed using an EagleEye II camera, monitor and printer (Stratagene).

2.4.6 DNA recovery from agarose gel

The amplified gene of interest (Section 2.4.3) and the linearised pIRES-puro plasmid (Section 2.4.4) were recovered from the agarose gel and purified using the QIAEX II kit (QIAGEN Ltd, Crawley, UK), according to the manufacturer's instructions. DNA, contained in either aqueous solution or in an agarose gel in TAE buffer, was solubilised in a high salt buffer containing a chaotropic salt, which dissociates DNA from DNA binding proteins and solubilises agarose. DNA was adsorbed onto silica particles, any agarose was washed off with the original buffer, and salts were washed off in two washes with an ethanol-containing buffer. The silica particles were allowed to air-dry thoroughly, and the DNA was eluted with ddH_2O.

2.4.7 Ligation of DNA fragment into vector

The purified cDNA and pIRES-puro plasmid were ligated using T4 DNA ligase enzyme (New England Biolabs). Ligation reaction comprised 1 unit T4 DNA ligase enzyme, 1μl (10x) T4 ligase buffer, a mixture of insert and linearised vector DNA, made up to 10μl with dH_2O. Reaction was carried out overnight at 16°C.

2.4.8 Transformation of bacteria

Plasmid DNA was chemically transformed into One Shot® Top10 Chemically Competent *E. coli* (Invitrogen Ltd.), 2μl of each ligation reaction were added directly into the competent cells and mixed by tapping gently, and incubated on ice for 30 minutes. The vials were then incubated in the pre-heated water bath at 42°C and for exactly 30 seconds, and then chilled on ice for 2 minutes. 450μl of pre-warmed SOC medium (rich L-broth medium) were added to each vial. The vials were incubated at 37°C for exactly 1 hour at 225 rpm in a shaking incubator. 100-200μl of each transformation from the vials were spread onto 9cm Petri dishes containing LB agar (L-Broth with 1.5% (w/v) agar) with ampicillin at a final concentration of 100 μg/ml. The plates were inverted and allowed to dry, then incubated at 37°C overnight.

2.4.9 Small-scale preparation of DNA ("minipreps")

For preliminary analysis, plasmid DNA was prepared from bacteria, using QIAprep® Miniprep plasmid DNA purification system (QIAGEN Ltd.), the microcentrifuge spin procedure, according to the manufacturer's instructions. In this protocol, plasmid DNA is extracted from bacterial cells using alkaline lysis, and is then selectively adsorbed onto silica-based resin in the presence of high salts.

Single colonies of transformed bacteria were picked from plates using sterile yellow Gilson pipette tips, inoculated into 5ml of L-Broth containing ampicillin 100 μg/ml, and incubated overnight in a shaking incubator at 37°C. On the next day, 1.5ml of each culture were centrifuged at 13,000 rpm for 2 minutes, and the bacterial pellets resuspended in 250μl of P1 buffer and transferred to a microcentrifuge tube. 250μl of buffer P2 were added and the tube was gently inverted 4-6 times to mix the contents. 350μl of buffer N3 were added and the tube was immediately, but gently, inverted to avoid localised precipitation. This inversion was repeated for 4-6 times. The tube was centrifuged at 13,000 rpm for 10 minutes, and the cleared supernatant was transferred to a QIAprep column, that had been centrifuged at 13,000 rpm for 30-60 seconds, before the flow-through was discarded. The QIAprep spin column was then washed with 0.5ml buffer PB, to remove trace nuclease activity, centrifuged for 30-60 seconds and the flow-through was discarded. The QIAprep spin column was further washed by adding 0.75ml buffer, centrifuged for 30-60 seconds and the flow-through was discarded. The QIAprep spin column was centrifuged for another 1 minute to remove the residual wash buffer, and placed in a clean 1.5ml microcentrifuge tube. The DNA was eluted by the addition of 50μl buffer EB (10 mM Tris-Cl, pH 8.5) to the centre of the QIAprep spin column. After 1 minute the column was centrifuged for 1 minute and the flow-through, containing the DNA, was collected and analysed by restriction enzyme digestion and agarose gel electrophoresis as described above.

Xho I restriction digestion (New England Biolabs) was used to determine the presence of the plasmid in the selected colony, while EcoR I restriction digestion (New England Biolabs) was used to verify the direction of insertion of the PCR-generated cDNA within the plasmid.

2.4.10 Medium-scale preparation of DNA ("maxipreps")

The identified sense and antisense constructs were built up for large scale DNA preparation using the QIAGEN® Plasmid Maxi Kit (QIAGEN Ltd.). A modified alkaline lysis procedure was performed, followed by binding of plasmid DNA to an anion exchange resin under appropriate low salt and pH conditions. Impurities (RNA, proteins etc.) were washed away by a medium salt wash. Plasmid DNA was eluted by a high salt buffer and concentrated and desalted by isopropanol precipitation.

5μl of bacterial culture, shown to have the correct plasmid insert, were added to 100ml L-Broth containing ampicillin 100 μg/ml and incubated overnight at 37°C in a shaking incubator. The bacterial cells were harvested by centrifugation at 3,840 *g* for 15 minutes at 4°C (J2-HS centrifuge, Beckman, Palo Alto, CA, USA), and the bacterial pellet resuspended in 10ml of chilled buffer P1 (50mM Tris-HCL, pH 8.0; 10mM EDTA; 100 μg/ml RNAse A). The bacteria were lysed by the addition of 10ml buffer P2 (200mM NaOH; 1% SDS) and, as soon as lysis was complete, the lysate was neutralised by the addition of 10ml of buffer P3 (3.0 M potassium acetate, pH 5.5). The mixture was filtered through a QIAfilter cartridge, incubated at room temperature for 10 minutes, and the flow-through was poured into a QIAGEN-tip containing the anion exchange resin which had been equilibrated with 10ml buffer QBT (750mM NaCl; 50mM MOPS, pH 7.0; 15% ethanol; 0.15% Triton X-100). The cell lysate was allowed to flow through under gravity, and the resin was then washed twice with 30ml buffer QC (1.0 M NaCl; 50mM MOPS, pH 7.0; 15% ethanol). Plasmid DNA was eluted from the column by the addition of 15ml buffer QF (1.25 M NaCl; 50mM Tris-HCL, pH 8.5; 15% ethanol). The plasmid DNA was precipitated and desalted by adding 10.5ml isopropanol and recovered by centrifugation at 15,000 *g* for 30 minutes. The pellet was washed twice in 5ml 70% ethanol, air dried for 20 minutes, and re-dissolved in 200μl TE buffer, incubated at room temperature for 10 minutes and the DNA solution was transferred

into a clean microcentrifuge tube. To ensure that the DNA was completely dissolved, the sample was further incubated at 4°C before measuring the yield.

DNA concentration was measured using a Spectrophotometer. The concentration of DNA was determined by absorbance at 260 and 280nm.

A solution with 4µl of the sample was added to 396µl ddH_2O, and the concentration of DNA was calculated using the following equation:

Final concentration of DNA = Spectrophotometer reading at 260nm x dilution factor x conversion factor

Where: Dilution factor = 400/4 = 100
Conversion factor = 50 µg/ml

2.4.11 Sequencing of DNA

Primers were obtained from the ICRF Oligonucleotide Synthesis Laboratory at Clare Hall (Appendix III), and diluted to 3.2 pmol/µl.

For the sequencing reactions, the following 20µl reaction mixture were made using ABI PRISM® BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystem, Foster City, CA, USA):

	Test reaction	Control reaction
Purified PCR product	200-500ng	2µl
Primer (3.2 pmol/µl)	1µl	4µl
Stock mix (Big dye*)	8µl	8µl
ddH_2O	Make up to 20µl	6µl

The Terminator Ready Reaction mix contains: A, C, T and G terminators labelled with high-sensitivity dyes; deoxynucleoside triphosphatase; $MgCl_2$; and Tris-HCL buffer, pH 9.0.

The mixture was overlaid with 20µl mineral oil and thermal cycled in the DNA Thermal Cycler (Perkin Elmer) using the following setting parameters:

Segments	Cycles	Temperature	Time
1	1	94°C	4 minutes
2	17	94°C	30 seconds
		60°C	30 seconds
		72°C	5 minutes
3	1	72°C	5 minutes
4	1	15°C	Hold

The results were analysed using ABI Prism sequencer software (Perkin Elmer).

2.5 Gene transfer into eukaryotic cells

2.5.1 Introduction of E-cadherin cDNA, Rac^{V12} and Rac^{N17} into OSCC cell line using Fugene 6 transfection reagent

FuGENE 6 Transfection reagent (Roche Diagnostic Corporation, East Sussex, UK) is a proprietary blend of lipids and other components supplied in 80% ethanol. Transfection was done using FuGENE 6 reagent: μg DNA at a 3:1 ratio.

On the day prior to transfection, 5 x 10^5 cells were seeded into 10cm petri-dishes, and allowed to grow in KGM to 50-60% confluency, which is optimal for transient transfection. On the day of transfection, the transfection complex was prepared in a small sterile tube, where 500μl Opti-MEM was added, and directly into the medium 30μl of FuGENE 6 reagent was added (undiluted FuGENE 6 reagent should not be allowed to come in contact with plastic surfaces other than the pipette tip). For E-cadherin cDNA, 10μg of DNA were added to the mix, and the tube was gently tapped. However, for constitutively active Rac1 variant (Rac^{V12}-GFP) and the dominant negative Rac1 variant (Rac^{N17}-GFP), 1μg was added to mix. The transfection complex was incubated at room temperature for 15-20 minutes.

Meanwhile, KGM was removed and the cells were washed once with Opti-MEM®I "serum-free medium" (GIBCO™: Invitrogen Ltd.). 10ml Opti-MEM were added to the adherent cells. The transfection complex was added dropwise to the cells and then they were incubated in 8% CO_2 at 37°C in an humidified incubator for 5-6 hours. Transfection complex was removed and the cells were washed once with KGM, and 12ml KGM were added, and the cells were allowed to grow to confluency.

For transient transfection, the transfection was terminated after 48 hours at which time the transfected cell lines (Table 2.2, Figure 2.3) were analysed using FACS analysis, western blot and indirect immunostaining.

For stable transfection, the transfected cell lines were allowed to grow in KGM for 1-2 weeks before being drug-selected or magnetic bead-sorted.

Table 2.2: Transfected Cell Lines

Origin (Parental cell lines)	cDNA plasmid Orientation	Transfected Cell line
H376	**E-Cadherin Sense**	**H376 E-cadherin**
	E-Cadherin Anti- Sense	H376 AS
H376β4	**E-Cadherin Sense**	**H376β4 E-cadherin**
	E-Cadherin Anti- Sense	H376β4 AS
IA2	**E-Cadherin Sense**	**IA2 E-cadherin**
	E-Cadherin Anti- Sense	IA2 AS

2.5.2 Introduction of siRNA duplexes into OSCC cell line using Oligofectamine™ transfection reagent

Oligofectamine™ reagent (Invitrogen Ltd.) interacts efficiently with oligonucleotides to form transfection complexes. A 6-well plate format was used according to the manufacturer's instructions.

The β4 integrin siRNA oligonucleotides were supplied as a pool of four siGENOME duplexes (siGENOME SMARTpool: Dharmacon RNA technologies, Chicago, IL, USA) provided in a desalted form at 50nM concentration (see Appendix III for oligonucleotide sequences). The SMARTpool reagent was resuspended in (1x) siRNA buffer (100mM KCl, 30mM HEPES-pH 7.5, 1.0mM $MgCl^2$) to a 40μM stock solution then stored at -20°C until use.

24 hours before transfection, 2 x 10^4 cells were plated in each well of a 6-well plate, and allowed to grow in an antibiotic-free KGM medium up to 40-50% confluency, which is optimal for transfection. On the day of transfection, for each well in a 6-well plate the following oligo solutions were prepared. 2.5μl of SMARTpool stock solution was diluted in 182.5μl Opti-MEM®I "serum-free medium" (GIBCO™: Invitrogen Ltd.) to form 185μl duplex solution (solution A). 3μl Oligofectamine™ reagent was diluted in 12μl Opti-MEM®I, forming 15μl transfection solution (solution B). Both solution A and B were incubated for 15 minutes at room temperature. Then both diluted solutions were added and mixed gently to form a 200μl mix (solution C), that was incubated at room temperature for 20 minutes. Meanwhile, cells were washed once with Opti-MEM®I and, for each well containing cells, 800μl of Opti-MEM®I was added. 200μl of solution C was then added dropwise onto each well. Cells were incubated at 37°C in an 8% CO_2 humidified incubator for 4 hours. Transfection was terminated by adding 500μl (3x) antibiotic-free KGM into each well. Gene expression was assayed at 48-72 hours post-transfection.

2.5.3 Selection

Puromycin acts as an analogue of aminoacyl-tRNA thus causing premature chain termination; it therefore inhibits protein synthesis in eukaryotic cells. *Streptomyces alboniger* contains the puromycin-N-acetyl transferase gene which can be expressed in mammalian

cells and can be used as a selectable marker for puromycin resistance. The puromycin (Sigma-Aldrich) concentration required to kill each parental cell line was determined by a "Kill Curve", and the minimum concentration of puromycin required to kill all parental cells after 1 week was used to select transfected cells.

2.5.4 Magnetic bead sorting of cells

E-Cadherin expressing cells were selected by two rounds of magnetic bead sorting (Dynabeads®, Dynal Ltd, Norway) using Anti-E-Cadherin Antibody HECD-1 (M. Takeichi). Cells were washed twice in PBSABC and incubated in EDTA free trypsin/ calcium (0.1% (w/v) trypsin (Sigma-Aldrich)/ 1mM $CaCl_2$) for 25-30 minutes. Detached cells were spun down and resuspended in 1ml cold PBSABC containing 10%FCS. Cells were incubated with primary antibody HECD-1 (1:200 dilution) on ice for one hour. Then, cells were washed three times in PBSABC containing 10%FCS, and incubated on ice with prewashed beads coated with goat anti-mouse antibody (10^7 beads/ml) for 30 minutes with frequent resuspension. To isolate labelled cells, cells and the coated beads were placed in a microcentrifuge tubes in a Dynal magnet, and left for 2 minutes. The unlabelled cells were removed by gentle pipetting. The tubes were removed from the magnet, and cells were gently resuspended in wash buffer. This was repeated for 6-7 times, until no further enrichment was possible. Selected cells were incubated in KGM in 6-well plates and, once they were adherent, KGM was replaced by selection medium. Selected cells naturally shed the magnetic beads over a period of seven days.

Stable expression of E-Cadherin was achieved after 2 sorts. Cells were grown continually in KGM supplemented with puromycin (Sigma-Aldrich) (0.5 μg/ml).

2.6 Functional Assays

2.6.1 Cell Growth Assays

2.6.1.1 Cell Growth Assay of cells growing on plastic

$2x10^4$ cells were seeded into 6-well plates with a well diameter of 35mm and grown as described in Section 2.1.3.2. Cells were fed every 3 days. Every 24 hours cells were removed from triplicate wells by trypsinisation with 1ml of trypsin/EDTA. Once the cells had detached, the trypsinisation was stopped with 1ml of KGM and the cells were pipetted vigorously to disrupt any cellular aggregates. The wells were examined to ensure that no cells remained. Between 50-500µl of cell suspension, depending on cell confluency, was added to Isoton solution (Coulter Euro Diagnostics GMBH) to make a total volume of 10mls. The cells were counted on a Casy I counter (Shärfe System) and the total number of cells per well was calculated. Readings were recorded every day for 5 days, and the experiment was repeated 3 times for each cell line.

2.6.1.2 Cell Growth Assay of cells growing in 2-D/3-D collagen I cultures

Cells were grown as described in Section 2.1.3.2 and allowed to grow for 10 days. Cells were harvested for the growth assay, at two day intervals, as follows. After washing the cultures, twice, with PBSABC, 200µl of collagenase 1 (Worthington Biochemical Corporation) were added to the 2-D cultures and 1ml to the 3-D cultures and incubated for about 1-2 hours at 37°C before cells were collected and spun down and the supernatant was discarded. A measured amount of Trypsin was added to the cell pellet and incubated at 37°C for about 15 minutes to break the cell clumps. Prior to cell counting, the cell aggregates were further broken down by gentle pipetting of the supernatant. Cell number was determined by haemocytometer counting using Trypan blue exclusion to monitor cell viability.

2.6.2 Invasion Assay

Cell invasion was measured using Transwells divided into upper and lower chambers by a Matrigel-coated polyethylene tetraphthallate (PET) membrane with 8μm pores (BD Bioscience). 1 x 10^5 cell/well were plated in the upper chamber, incubated for 48 hours and allowed to invade through the membrane and into the lower chamber towards full keratinocyte growth medium which was used as a chemoattractant.

Inserts were coated with 70μl of growth factor reduced-Matrigel (BD Bioscience) (diluted 1:2 in α-MEM) and allowed to set for 60 minutes at 37°C. Cells approaching confluency were washed twice with PBSABC and harvested using the E-cadherin preserving technique as described in Section 2.2.1.1. Detached cells were collected in PBSABC, spun down at 1,200 rpm for 3 minutes and resuspended in α-MEM. Cells were plated in quadruplicate with 1x10^5 cells seeded in the upper chamber of each Transwell. 0.5ml full KGM was used as a chemoattractant in the lower chamber.

After incubating for 48 hours at 37°C the KGM in the lower chamber was removed, and the chamber was washed with PBSA. Cells at the bottom of the lower chamber and at the undersurface of the Transwell insert were harvested by the addition of 0.5ml trypsin/EDTA into the lower chamber and, with the Transwell insert in place, incubated at 37°C for 45-60 minutes. The undersurface of the Transwell was washed twice carefully using trypsin in the lower chamber. The whole amount of trypsin (0.5ml) with the detached cells was pipetted up and added to 9.5ml Isotone solution, and the tubes were incubated on ice, in preparation for counting. Cells were counted using the CASY counter.

2.6.3 TUNEL Assay for biochemical detection of apoptotic DNA fragmentation

DNA fragmentation is one of the defining characteristics of apoptosis (Bursch *et al.*, 1990). The TUNEL assay, one of the methods used to detect apoptosis, is based on a procedure in which the ApopTag® Fluorescein Apoptosis Detection kit (CHEMICON® international) specifically detects DNA cleavage using indirect immunostaining.

Transfected cell lines (H376 E-cadherin and H376 antisense) were plated, in triplicate, at an initial density of $2x10^4$ cell/well, either atop 2-D, or within 3-D, collagen I cultures, and allowed to grow for 5 days.

At day 5, cells growing on 2-D collagen I- coated cover slips were washed once using PBSA. Residual PBS was then removed and cells were fixed using 1% paraformaldehyde (in PBSA) for 10 minutes at room temperature. Excess liquid was drained off and the cells were washed twice in PBSA (5 minutes each). Cells were permeabilised using Ethanol:Acetic acid (at a ratio of 2:1) for 5 minutes at –20°C. Excess liquid was drained off and cells were washed twice in PBSA (5 minutes each). Working strength Terminal deoxynucleotidyl Transferase (TdT) enzyme was added to the pre-equilibrated cells (using the supplied Equilibration Buffer) and incubated for 1 h at 37°C. Stop/Wash Buffer was added to the slide and agitated for 15 sec followed by 10 minutes incubation at room temperature. After washing three times with PBS for 1 minute each, Anti-Digoxigenin Fluorescein (1:100 dilution: Roche Diagnostic Corporation) conjugate was added to the slides and incubated for 30 min. After cells were washed for four times with PBSA, cover slips were mounted in MOWIOL 4-88, containing 1 μg/ml of Propidium Iodide, for counterstaining.

In contrast cells, growing in 3-D collagen I were fixed in Formol Saline and were embedded in paraffin and sectioned in the Histopathology Lab (Cancer Research UK Central Services). Sections were deparaffinised by washing in three changes of Xylene (5 minutes each). The

sections were washed twice in 2 changes of Absolute Ethanol (5 minutes each), followed by one wash in 95% Ethanol and a final wash in 70% Ethanol (3 minutes). Lastly, the section was washed once in PBSA for 5 minutes. Sections were pretreated with a Proteinase-K digesting enzyme (20 μg/ml) for 15 minutes and then washed twice with PBSA (2 minutes each). Then slides were treated, as described for cells growing in 2-D, from the equilibration step onwards.

Detached cell populations were evaluated by the Trypan blue dye exclusion test using a Vi-CELL™ XR Viability Analyser (BECKMAN COULTER, Buckinghamshire, UK).

2.6.4 Rac1 and Cdc42 Pull-down Assay

The Rho switch functions by alternating between an active, GTP-bound state and an inactive, GDP-bound state (discussed in Chapter 6). This assay is based on the fact that p21 activated kinase 1 (PAK), a downstream effector of both Rac1 and Cdc42 members of the Rho family GTPAses, specifically recognises the GTP bound form of the protein, thus monitoring the activity of Rac1 and Cdc42 proteins (Lim *et al.*, 1996). The assay uses the Cdc42/Rac Interactive Binding (CRIB) region (also called the p21 Binding Domain, PBD) of the Cdc42 / Rac effector protein, p21 activated kinase 1 (PAK). The CRIB/PBD protein motif has been shown to bind specifically to the GTP-bound form of Rac and/or Cdc42 proteins. The PAK-PBD is also in the form of a glutathione *S*-transferase (GST) fusion protein which allows one to "pull-down" the PAK-PBD/GTP-Rac (or GTP-Cdc42) complex with glutathione affinity beads (Lim *et al.*, 1996; Sells *et al.*, 1997).

Rac1 and Cdc42 activity assay is called (GST)-PAK-CRIB pull-down assay. Active Rac1 and Cdc42 were pulled down with the GST-PAK-CRIB fusion protein.

$5x10^5$ cells were plated in 10cm Petri dishes in full KGM medium and allowed to grow for 48 hours, where it was about 80-90% confluent. As for the β4-integrin knock-down experiment, an RNAi experiement was carried out 24 hours later and cell lysates for all cell lines were obtained altogether. Cells were washed in ice-cold PBSABC saline and lysed on ice in 800 μl of lysis buffer (Appendix I). Total lysates were cleared by centrifugation at 13,000 rpm for 2 minutes at 4°C. Each sample of cleared lysate was incubated for 40-60 minutes, in a rotor, at 4 °C with 5μl GST-PAK-CRIB domain (PAK-CD) pre-coupled to glutathione-Sepharose beads (prepared in the Lab by D. McCulloch). Subsequently, the beads were spun down by centrifugation at 13,000 rpm for 1 minute at 4°C, and supernatant, containing inactive Rac1, was aspirated into a fresh Eppendorf. Precipitated complexes were washed three times in an ice-cold washing buffer (Appendix I). After the final wash, the supernatant was discarded, leaving about 15μl on top of the precipitated complexes, and 5μl of 4x Laemmli sample buffer (Appendix I) was added to the beads. Samples were boiled for 5 minutes, spun down briefly at 4°C to sediment the beads, and the entire volume was loaded into a 15% polyacrylamide gel. Amounts of active versus inactive proteins were assessed with Western blot analysis using human monoclonal antibodies against Rac1 and Cdc42 (as described in Section 2.3.4.3 and 2.3.4.4).

2.7 Statistical Analysis

Data are expressed as the standard error of mean (sem) of a given number of observations. Where appropriate, samples of equal variance of paired groups were analysed using Student's t-Test. Comparison was set at 5% significance. A p value of ≤ 0.05 was considered to be significant.

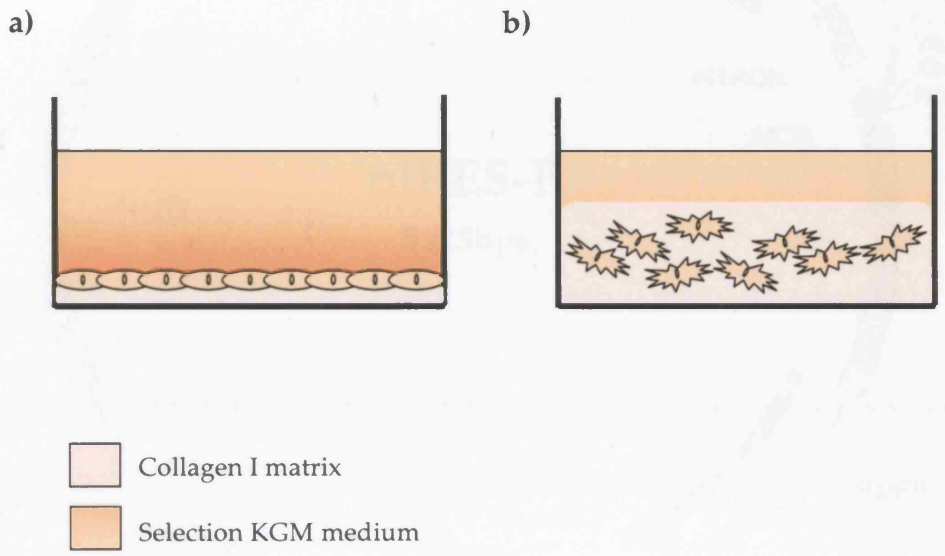


Figure 2.1
2-D/3-D collagen I cultures

a) cells growing atop of a thin layer of collagen I matrix.
b) cells growing within a thick layer of collagen I matrix.

Cells were fed with complete selection medium.

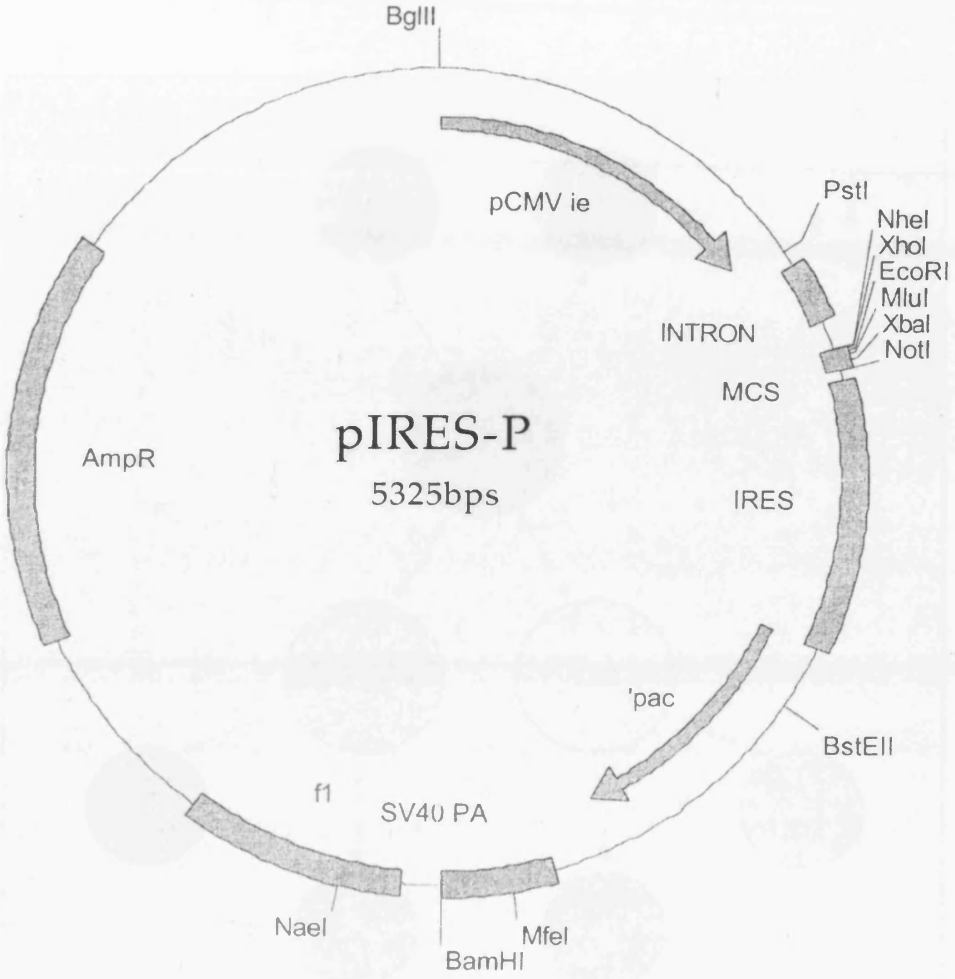


Figure 2.2
Structure of the pIRES-puro bicistronic vector
Features indicated are as follows: pCMV – cytomegalovirus immediate early promoter; INTRON – chimaeric intron; MCS – multiple cloning site, unique restriction sites indicated; IRES- internal ribosome entry site; 'pac – modified pac gene encoding puromycin N-acetyl transferase; SV40 PA – SV40 terminator/poly (A) signal; f1 – phage f1 origin; AmpR – bla ampicillin resistance gene. (Hobbs *et al.*, 1998)

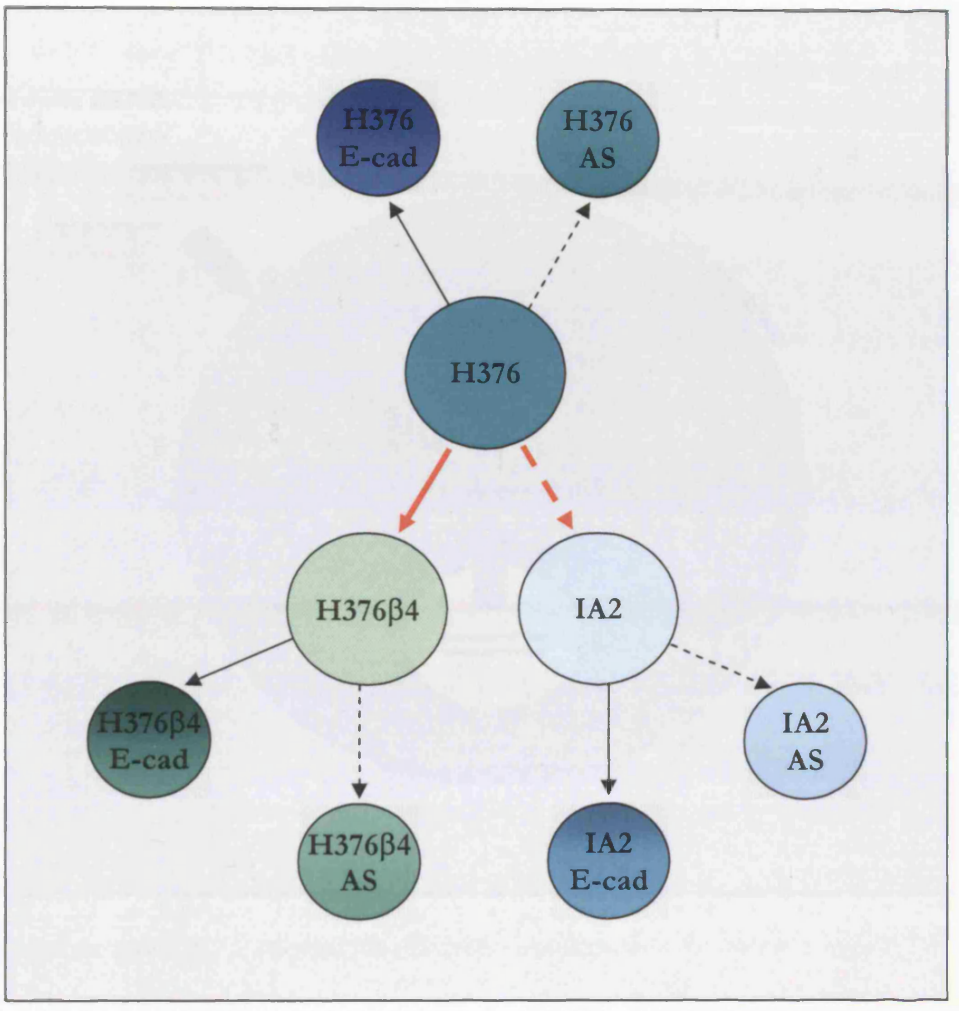


Figure 2.3
Schematic representation of the transfected cell lines

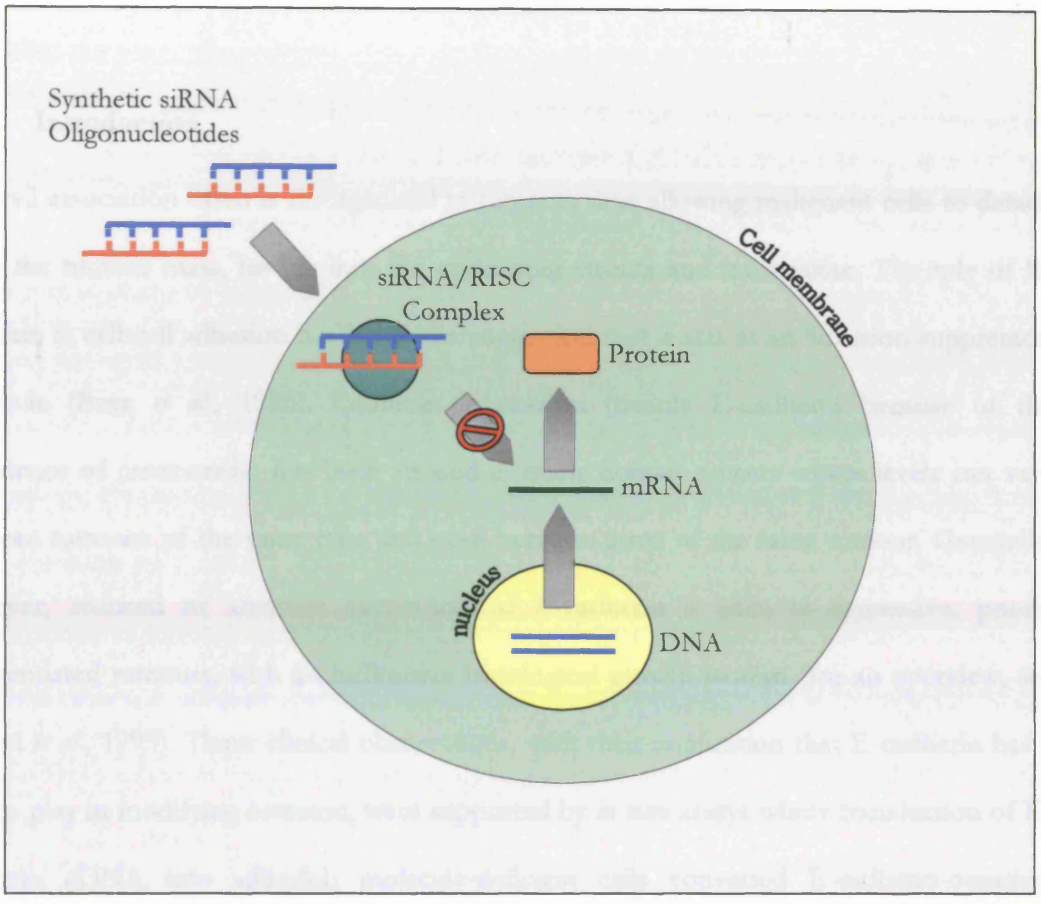


Figure 2.4
Schematic representation of RNAi technology
Synthetic small interfering (si) RNA nucleotides can be introduced into Eukaryotic cells. siRNA nucleotides interact with RNA-induced silencing complex (RISC) which uses the attached siRNA sequence as a template to seek out and destroy the single stranded mRNA with a matching sequence, thus inhibiting the production of the protein of interest.

CHAPTER 3

Introduction of Full-Length E-cadherin cDNA Into Oral Squamous Cell Carcinoma Cells and Their Characterisation

3.1 Introduction

Cell-cell association often is disorganised in tumours thus allowing malignant cells to detach from the tumour mass, invade into the underlying stroma and metastasise. The role of E-cadherin in cell-cell adhesion has led to the suggestion that it acts as an 'invasion suppressor' molecule (Berx *et al.*, 1995). Cadherin expression (mainly E-cadherin because of the prevalence of carcinomas) has been studied in many human cancers where levels can vary between tumours of the same type and even between areas of the same tumour. Generally, however, reduced or aberrant expression of E-cadherin is seen in aggressive, poorly differentiated tumours, with an infiltrative histological growth pattern (for an overview, see Mareel *et al.*, 1995). These clinical observations, with their implication that E-cadherin has a role to play in modifying invasion, were supported by *in vitro* assays where transfection of E-cadherin cDNA into adhesion molecule-deficient cells converted E-cadherin-negative, fibroblastic, invasive cell lines to an epithelial and non-invasive phenotype (Frixen *et al.*, 1991). Conversely, blocking E-cadherin function in epithelial cells was shown to induce an invasive phenotype (Behrens *et al.*, 1989; Takeichi, 1993). However, it is not only normal levels of E-cadherin but also correct localisation and proper cadherin-catenin interactions, which are necessary for the adherens junctions to be functional (Navarro *et al.*, 1993; Bullions *et al.*, 1997).

While these general comments appear applicable to many carcinomas, their relevance to head and neck cancer seems much more equivocal. Attempts to correlate E-cadherin expression

with tumour behaviour in head and neck SCC largely have been unsuccessful. Most carcinomas show variable degrees of loss of expression although this often is most prominent in poorly differentiated tumours or at the advancing front of lesions (Williams *et al.*, 1998).

My goal was to develop an oral squamous cell carcinoma (OSCC) model to study the effect of the E-cadherin molecule on the behaviour of epithelial cells in OSCC. This was achieved by the introduction of full length E-cadherin cDNA into the E-cadherin-negative OSCC line using a bicistronic vector "pIRES-puro" (Chapter 2, Figure 2.5). Such a vector has allowed both the recombinant cDNA and the puromycin resistance gene to be transcribed as a single message. In this system, therefore, clones that are resistant to puromycin express high levels of the protein encoded by the cloned cDNA. The internal ribosome entry site (IRES) ensures efficient translation of the second gene (Hobbs *et al.*, 1998).

In this Chapter, I describe the production and characterisation of this E-cadherin model system derived from OSCC.

3.2 Results

3.2.1 Characterisation of the primary OSCC lines

Two starting lines, derived from oral squamous cell carcinomas, were used. H357 is an OSCC line derived from the lateral border of the tongue and H376 is an OSCC line derived from the floor of the mouth. Both lines were kindly provided by Professor S. Prime of the University of Bristol (Prime *et al.*, 1990).

H357 OSCC cell line as an E-cadherin positive control:

As reported previously (Sugiyama *et al.*, 1993) the H357 line proved positive for E-cadherin expression. E-cadherin expression was determined by FACS analysis and Western blotting. Cells used for assessment of E-cadherin expression were allowed to grow to confluency. As described in Chapter 2 Section 2.2.1.1, cells were harvested using a procedure adapted from Takeichi (1997), which minimises the proteolysis of the extracellular domain of the E-Cadherin molecule (Takeichi, 1977). Using anti-E-cadherin (HECD-1) monoclonal antibody (M. Takeichi), FACS analysis and Western blotting results confirmed that the H357 cell line was E-cadherin positive (Figure 3.1). Accordingly this line was used both as the source of total RNA, from which the full length E-cadherin cDNA was purified, and as a constitutively positive control for E-cadherin expression throughout the rest of the study.

Characterisation of H376 OSCC cell line for E-cadherin expression:

As described above, the H376 cell line was examined for the presence and expression of the E-cadherin molecule. Figure 3.1 shows the lack of E-cadherin expression, as assessed by FACS analysis and Western blotting, in cell populations from this line.

Accordingly, it was determined that the H376 line would be a suitable recipient for transfection with E-cadherin cDNA.

Characterisation of H376 OSCC cell line for catenin expression:

Prior to introducing E-cadherin cDNA into recipient cells (H376 OSCC cell line) it was thought necessary to check that they expressed the appropriate catenins, to ensure that a functional cadherin/catenin complex could be formed. Using Western blot analysis, the H376 cell line was checked for the expression of α-, β- and γ-catenins. Results showed that the H376 line appears to express the 102 kDa α-catenin, the 88 kDa β-catenin and the 82 kDa γ-catenin (Figure 3.2: a). Several attempts were made to blot for $p120^{ctn}$, another catenin member, all of which failed. Had I had the time to recheck for its presence, I would have done so since I believed this result most likely was attributable to technical problems rather than to absence of this specific catenin. Differences in apparent levels of expression of these proteins may be a consequence of variations in protein loading, as no consistent patterns of dramatic alterations emerged from the replicate experiments.

Characterisation of H376 OSCC cell line for other cadherins:

The H376 line was also checked for the expression of other cadherins. Western blot analysis, using anti-P-cadherin (clone 56) monoclonal antibody (BD Transduction Laboratories), showed that the H376 cell line was negative for P-cadherin as compared to the H357 control cell line (Figure 3.2: b). As for the expression of N-cadherin, using Western blot analysis and the anti-N-cadherin (clone 32) monoclonal antibody (BD Transduction Laboratories), when loading the same amount of protein, the H376 parental cell line was strongly positive for N-cadherin as compared to the H357 control cell line (Figure 3.2: b).

The starting cell lines, H376 and H357, also were checked for the expression of Desmoglein, one of the desmosomal cadherins, using Western blot analysis and the monoclonal antibody against desmoglein (BD Transduction Laboratories), both cell lines were found to be almost equally positive for this molecule (Figure 3.2: b).

3.2.2 Introduction of full length E-cadherin cDNA into recipient starting cell lines

As mentioned above, the H357 cell line was used as the source of total RNA and this was extracted using RNA STAT-60™ isolation reagent (Tel-Test "B" Inc.) (Figure 3.3). The concentration and purity of RNA was determined by measuring absorbance at 260 and 280nm. A solution with an optical density OD_{260} of 1.0 contains 40μg RNA/ml, and the ratio OD_{260}/OD_{280} of ~ 1.8 specifies a clean preparation. Contamination with protein or salts leads to a lower ratio and resulted in the preparation being discarded.

DNA complementary to the total RNA was synthesised using the SUPERSCRIPT First-Strand Synthesis System (GIBCO BRL®).

Using two E-cadherin specific primers (Chapter 2, Section 2.4.3), the purified cDNA was amplified using polymerase chain reaction (PCR). The PCR product (Figure 3.4) was purified with a Microcon-100 column (MILLIPORE) prior to DNA restriction digestion.

The isolated E-cadherin gene was sequenced using PRISM® BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystem) and specific primers obtained from the ICRF Oligonucleotide Synthesis Laboratory at Clare Hall, South Mimms (Appendix III). The results were analysed using ABI Prism sequencer software (Perkin Elmer). Using sequence matching software, Lalign, supplied by the GeneStream network server where the nucleic acid sequence of the isolated E-cadherin gene was matched with the E-cadherin gene sequence template obtained from EMBL/Genbank Data Libraries under the accession number Z13009. Point mutations, resulting from substitution of Cytosine (C) with Thymidine (T), were detected at two different locations (Appendix IV). However both point mutations were found to be 'silent mutations' because the resultant codons encoded for the same amino acid. (AAC and AAT codons both encode for Tyrosine).

The pIRES-puro plasmid was digested with Xho I restriction endonuclease (New England Biolabs), prevented from religation by dephosphorylation with alkaline phosphatase (New England Biolabs) and the full length E-cadherin cDNA was ligated into the Xho I site of the pIRES-puro plasmid.

The plasmid DNA was digested with Xho I and EcoR I restriction endonucleases (New England Biolabs) to verify the direction of insertion of the PCR-generated E-cadherin cDNA (Figure 3.5). The identified sense and anti-sense constructs were expanded for large scale DNA preparation using the QIAGEN® Plasmid Maxi kit (QIAGEN Ltd.).

The E-cadherin negative cell line (H376) either was transiently or stably transfected, using FuGENE™ 6 Transfection reagent (Roche). Figure 2.6 (Chapter 2) shows a schematic illustration of the transfection plan and outcome.

3.2.3 *De novo* expression of E-cadherin in H376 Oral Squamous Cell Carcinoma Cell line

In order to verify the ability of the various constructs to cause E-cadherin expression in recipient cells originally I used transient transfection of both murine NIH 3T3 cells and H376 cells.

That expression of the transfected E-cadherin cDNA resulted in expression of the molecule on the cell surface was confirmed by flow cytometry using anti-E-cadherin (HECD-1) monoclonal antibody. In Figure 3.6, Dot plots showed that both transiently transfected cell lines, H376 and NIH 3T3, contained populations of cells positive for E-cadherin expressed at the cell membrane. The efficiency of expression was almost similar in both cell types with about 25.56% of the transfected NIH 3T3 cells being positive for surface E-cadherin (represented by cells in the upper right quadrant) and about 29.96% of the H376 E-cadherin

transfected cells being positive for surface E-cadherin. Control cell lines, transfected with the antisense construct, were negative for E-cadherin.

Expression of the E-cadherin molecule was further confirmed by Western blot analysis. Whole cell RIPA lysates from the transiently transfected cell lines and 28.6μg protein/lane were probed for E-cadherin using the anti-E-cadherin (HECD-1) monoclonal antibody. As can be seen from Figure 3.6, a strong E-cadherin band at 120kDa was apparent in both these lines after transfection with the E-cadherin sense, but not the antisense, construct. H357 was used as a positive control, while NIH 3T3 cells and pEGFP transfected-3T3 cells were used as negative controls.

Since the transient transfection experiments indicated that the constructs were functioning correctly and producing protein of the correct size, I then established stable lines.

Transfection studies with the E-cadherin sense and antisense plasmids were repeated on the H376 cell line. The E-cadherin expressing cells were selected by two rounds of magnetic bead sorting and the resulting cell populations were grown in puromycin-supplemented KGM "Selection Medium", with a concentration of 0.5μg/ml; a value previously determined with preliminary "kill curve" experiments. Isolated positive colonies, capable of growing in this selection medium, were pooled as a means of avoiding clonal heterogeneity.

Over the next 24 months of continuous culture, expression was checked on a routine basis using both FACS and Western blot analyses, so that confirmatory results were obtained at different times over this period. Representative results are illustrated in Figure 3.7.

3.2.4 Immunocytochemical localisation of E-cadherin in transfected lines

For indirect immunofluorescence experiments, cells were grown on coverslips in the presence of 10% FCS for 48 hours in order to allow for the formation of mature adherens

junctions. Cells were fixed with 4% formaldehyde, and stained for E-cadherin using anti-E-cadherin (HECD-1) monoclonal antibody. Triton X-100 was used to permeabilise cell membranes and permit access of antibodies to the cytoplasm. It was also used as an indirect assessment of the strength of association of the transfected E-cadherin molecule with the actin cytoskeleton, as molecules not associated with the actin cytoskeleton were removed by Triton X-100 (Ozawa *et al.*, 1989). Antibodies were visualised with fluorochromes linked to second layer antibodies. Actin cytoskeleton and nuclei were counterstained in some experiments using Phalloidin and DAPI respectively.

Images from H376 transfected cell lines were taken using confocal laser scanning microscopy. In Figure 3.8 (Panels A-C), it can be seen that the antisense transfected lines, as expected, demonstrated no detectable definite positive staining for E-cadherin. In contrast the cells into which the sense E-cadherin cDNA had been introduced (Panels D-F) exhibited strong cell surface positivity, mostly evident at sites of cell-cell contact. Actin filaments evident in the antisense transfected line (Panel B) appeared to be re-distributed into bundles in the E-cadherin positive cells (Panel E), and these co-localised with the cell surface E-cadherin molecule (Panel F). Moreover it was apparent that the E-cadherin positive lines had a more epithelial morphotype, compared to the antisense controls, with the cells coming together in close cell-cell apposition (Figure 3.8). However the actin belt, which is characteristic of epithelial cells, is missing as compared to the control cell line, H357, which has perfect epithelial morphology with a well-formed, E-cadherin-dependent, cell-cell junction (Figure 3.9).

From subjective morphological assessment alone the formed E-cadherin cell-cell junction appeared as a stitched zipper-like junction rather than as a straight line (marked by open arrows in Figure 3.8); what I referred to as 'immature' junctions with the formation being

stuck somewhere at the adhesion zipper phase, a phase described by Vasioukhin *et al.* (2000) as an intermediate step in Adherens junction formation (Vasioukhin *et al.*, 2000).

Under the phase-contrast microscope the E-cadherin transfected cell lines did not attain a typical epithelial morphotype although they certainly looked more epithelial than their antisense-transfected counterparts (Figure 3.10).

3.2.5 Effect of E-cadherin re-expression on catenin levels

E-cadherin is linked, via β- catenin or γ-catenin, to α-catenin which mediates the anchorage of the E-cadherin/catenin complex to the actin cytoskeleton (Kemler, 1993). Having established that there is a significant amount of E-cadherin expressed in the transfected cell lines, it was necessary to examine if there was any alteration in catenin expression and distribution in such lines, after *de novo* expression of E-cadherin, using Western blot analysis and indirect immunostaining. Whole cell lysates from the transfected cell lines and their anti-sense controls were used to blot for the various catenins. Figure 3.11 shows a representative Western blot analysis of α-, β-, and γ-catenin in transfected cell lines. Densitometric analysis of three independent experiments showed that E-cadherin re-expression resulted in about a 2-fold increase in total α-catenin levels in the H376 E-cadherin cell line as compared to the antisense control (p value $\leq$ 0.01). β-catenin levels increased by about 1.8-fold in H376 E-cadherin cell line as compared to the antisense control (p value $\leq$ 0.02). However, total γ-catenin was downregulated in the E-cadherin positive cell lines by about 1.4-fold, apparently trivial but statistically significant (p value $\leq$ 0.02).

Cellular catenins (β- and γ-catenin, but not α-catenin) also were examined with confocal laser scanning microscopy. Figure 3.12 shows confocal images of the H376 transfected cell lines stained for β- and γ-catenin. Figures demonstrated positive staining for β-catenin in both E-cadherin positive transfected cell lines (Panel A) and in its antisense-transfected counterpart

(Panels B). However there appeared to be stronger positivity in the E-cadherin positive line for this molecule. In the H376 E-cadherin cell line more of the β-catenin was redirected to the cell-cell junction.

In Figure 3.12, the Panel D showed that the stained γ-catenin appeared to be cytoplasmic in the H376 antisense-transfected cell line and was recruited to the cell-cell junction in the H376 E-cadherin cell line (Panel C) only. Inconsistent with the Western blot results, which showed downregulation of the total γ-catenin in the E-cadherin positive cell line, was the confocal image of the H376 E-cadherin cell line which showed stronger positivity for E-cadherin and for the γ-catenin.

3.2.6 Effect of E-cadherin re-expression on levels of other cadherins

Western blot analysis was used to assess changes in levels of previously identified cadherins (N-cadherin and Desmoglein) as a consequence of *de novo* E-cadherin expression. Using anti-N-cadherin (clone 32) monoclonal antibody showed that there is a marked reduction of total N-cadherin expressed in the E-cadherin transfected cell line (H376 E-cadherin) as compared to the antisense-transfected control (Figure 3.13) (Levels were re-assessed in Chapter 4).

Although the H376 parental cell line was marked positive for Desmoglein (Section 3.3.1, see Figure 3.2), I failed to blot for this molecule in both descendant cell lines (H376 E-cadherin and H376 antisense counterpart). I believe that this was attributed to technical problems and since investigation in this area was not my primary interest, I decided not to pursue it further.

3.2.7 Effect of E-cadherin re-expression on the differentiation potential of the transfected OSCC cell lines

K14 and involucrin are epithelial cell markers. H376 cells are known to express very low levels of involucrin (Sugiyama *et al.*, 1993), suggestive of a low level of differentiation. To

determine whether re-expression of E-cadherin had any effect on the ability of the cells to differentiate, immunoblotting with both anti-involucrin (Abcam Ltd.) and anti-K14 antibodies (Abcam Ltd.) was carried out on whole cell RIPA lysates. Protein extracts from HaCaT (normal keratinocyte cell line) were used as a positive control. Neither involucrin nor K14 was detected in either the parental cell line or the transfected descendant cell lines. The restoration of E-cadherin expression had no apparent effect on the capacity of differentiation of the H376 cell line as monitored by measuring involucrin (data not shown).

I next conducted functional assays to monitor the invasive and growth capacity of these cell lines in order to determine what effect, if there was any, the re-expression of E-cadherin had on these parameters.

3.2.8 Effect of E-cadherin re-expression on the invasive potential of the transfected OSCC cell lines

Cell invasion was measured using Transwells divided into upper and lower chambers by a Matrigel-coated polyethylene tetraphthallate (PET) membrane with 8μm pores (Becton-Dickinson). 1 x 10^5 cell/well were plated, in quadruplicates, in the upper chamber, incubated for 48 hours and allowed to invade through the membrane and into the lower chamber towards full keratinocyte growth medium which was used as a chemoattractant. Results showed that the E-cadherin positive line (H376 E-cadherin) was 60-80% less invasive than its antisense counterpart (Figure 3.14).

In an attempt to further assess the effect of E-cadherin re-expression on the invasive potential of the cells I tested my cells for the expression of MMP-2 and MMP-9 using zymographic analysis. Both cell lines were shown to be negative for both MMP's (data not shown).

3.2.9 Effect of E-cadherin re-expression on the proliferative potential of the transfected OSCC cell lines

Similar experiments on the proliferative activities of these cells were conducted where the growth rates of the transfected cell lines were compared on plastic.

2 x 10^4 cell/well were plated onto 24-well plates and cells were harvested at at least five time points over a five day period, where triplicate wells were counted for each reading. Experiment was repeated three times and results showed that there was no apparent difference between the growth rates of the E-cadherin expressing cells and the antisense control cell line (Figure 3.15).

3.2.10 Integrin profile of transfected H376 cell lines

Because of the increasing evidence of co-ordinated regulation or, 'cross-talk', between different families of adhesion molecules, such that changes in the functional capacity of one class of receptor has an impact on the activity of another class of receptor, I decided to monitor integrin expression by my various cell lines. Weaver *et al.* (1997) observed that tumourigenic variants of a breast carcinoma cell line could be induced to re-express functional E-cadherin by using antibodies to block β1-integrin (Weaver *et al.*, 1997). Moreover, integrins and E-cadherin cooperate with insulin-like growth factor to induce migration in colon tumour cells (Andre *et al.*, 1999). Therefore, I thought it would be relevant to investigate the integrin profile of my transfected cell lines. Flow cytometry can provide a relative measure of cell surface integrin levels. In order to compare expression in different cell lines, the mean fluorescence intensity of each integrin subunit in each cell line was determined by subtracting the fluorescence value of cells labelled with control antibody. The integrin flow cytometry results are summarised in Figure 3.16 grouped by cell line. In general, FACS analysis of the surface expression of α1, α2, α3, α4, α5, α6, α9, αv, β1, αvβ3, αvβ5 and

αvβ6, showed that there was no significant difference in expression at the cell surface of the integrins tested. The two exceptions to this observation were that there was a hint that E-cadherin re-expression might have resulted in alteration of β1- and β4-integrin expression (Figure 3.17). Thus in the Figure shown there is a change in the levels of β1- and β4-integrin expressed after transfection with E-cadherin. Upon E-cadherin restoration in the H376 transfected cell line there was about 1.8-fold, statistically significant, increase in the level of the β1-integrin subunit (p value $\leq$ 0.01) (Figure 3.17). Among the different α integrin subunits that were being tested, only α2-integrin subunit showed a concomitant 1.7-fold increase in the H376 E-cadherin cell line as compared to the antisense counterpart, which was consistent in three independent experiments (p value $\leq$ 0.04) (Figure 3.17). This particular cell line (H376) was previously characterised as expressing a very low basal level of β4-integrin (Sugiyama *et al.*, 1993). E-cadherin re-expression resulted in about a 2.5-fold increase in β4-integrin levels. The increase was, although apparently relatively trivial, statistically significant (p value $\leq$ 0.01) (Figure 3.17). H376 transfectants were also found to be negative for αvβ6 (data not shown).

These early results later were expanded to form the basis of Chapter 5 of my thesis.

3.3 Discussion

Loss of cell-cell adhesion is considered to be a main determinant of the invasive properties and metastasising capacities of tumour cells (Hajra and Fearon, 2002).

In the present Chapter I found that E-cadherin essentially was lacking in H376 OSCC cells. The membrane localisation of β-catenin in the E-cadherin-negative cell line (H376), as it appeared in preliminary immunocytochemical staining experiments (data not shown), presumably indicates the presence of a catenin-binding protein, probably N-cadherin, as suggested by other groups (Husmark *et al.*, 1999; Islam *et al.*, 1996). Accordingly, I tested for the expression of N-cadherin, and found that, in the H376 cell line, cell-cell adhesion, instead, was mediated by N-cadherin. There is a large body of data suggesting that the inappropriate expression of non-epithelial cadherins, such as N-cadherin and cadherin-11, either in association with or even instead of E-cadherin loss, may play a significant role in invasion and metastasis of cancer cells (Shibata *et al.*, 1996a; Tomita *et al.*, 2000). Concomitant upregulation of N-cadherin and downregulation of E-cadherin has been termed "cadherin switching" and this phenomenon has been demonstrated in primary tumours (Hajra and Fearon, 2002).

Since N-cadherin was not detected in normal epithelium (Christofori, 2003), E-cadherin may be replaced by N-cadherin in poorly differentiated OSCC. In support of this possibility is the finding of Islam *et al.* (1996) that N-cadherin is expressed in SCC but not in the surrounding normal tissue. The presence of N-cadherin appears to correlate with the loss of epithelial differentiation in breast carcinoma cell lines (Hazan *et al.*, 1997). In addition, N-cadherin transfected into squamous epithelial cells resulted in tumour cells which manifested a scattered phenotype (Islam *et al.*, 1996). N-cadherin therefore may be one of several aberrantly expressed molecules determining the malignant properties of epithelial tumours. One proposed mechanism (Hazan *et al.*, 1997) is that N-cadherin, present at the surface of

tumour cells, mediates adhesion to stromal cells, thereby facilitating the local and metastatic spread of the tumour. Re-expression of E-cadherin in my cell lines resulted in the down-regulation of N-cadherin and this might account, at least partially, for why invasion was markedly inhibited in the E-cadherin-transfected cell line.

Under the microscope, cells of the H376 line exhibited a fibroblastic morphotype. Navarro *et al.* (1991) reported that the complete absence of P-cadherin was only observed in fibroblast-like cells or among different epidermal keratinocyte cell lines representative of different stages of carcinogenesis. Moreover these authors also suggested that loss of both E-cadherin and P-cadherin could be associated with the final stage of carcinogenesis; that is the development of spindle cell carcinomas (Navarro *et al.*, 1991). Therefore I checked for the expression of P-cadherin in my primary H376 cell line. Figure 3.2, shows that the H376 cell line was negative for P-cadherin as compared to the H357 control.

Apart from the fact that the starting H376 cell line was found to be positive for Desmoglein, a desmosomal cadherin considered as an epithelial marker (Figure 3.2), the characterisation of these lines by others for other epithelial markers provided evidence that these cells are not fibroblasts (as might be thought from their absence of E-cadherin and their fibroblastic morphology) but rather are unusual epithelial cells.

Thus, for example, the H376 cell line has been characterised as expressing Epidermal Growth Factor Receptor (EGFR), a cell surface receptor even though the cells showed poor patchy staining for the EGF-receptor with the majority of cells being negative or weakly positive (Prime *et al.*, 1994). Sugiyama *et al.* (1993) used Western blot analysis to detect the total amount of involucrin, a marker for terminal differentiation, in 8 different OSCC cell lines among which both the H376 and H357 cell lines expressed very low total involucrin levels compared to other cell types and the results were consistent with immunohistological sections which were stained for involucrin (Sugiyama *et al.*, 1993).

Thus it appears that the H376 cell line does express markers of epithelial origin, albeit at a low level, suggesting it represents a poorly-differentiated example of this histiotype.

As a consequence of *de novo* E-cadherin expression, the H376 cells attained a more epithelial morphotype; although not typically epithelial in appearance they certainly were more epithelial than the antisense counterpart cell line, with the cells coming together in close cell-cell apposition (Figure 3.8 and Figure 3.10). These changes in cellular morphology are very similar to those reported by others who have transfected E-cadherin cDNA into various cell lines (e.g. Navarro *et al.*, 1991).

However, solely by morphological criteria, the formed E-cadherin cell-cell junctions looked immature. From subjective visual assessment alone the formation of adherens junctions appeared to be stuck somewhere at the adhesion zipper phase, a phase described by Vasioukhin *et al.* (2000) and believed to be an intermediate step in adherens junction formation. This phase manifested itself as a stage between the formation of the E-cadherin-dependent punctata at sites of cell-cell contact prior to their merging fully into a single line (Vasioukhin *et al.*, 2000). In this study several actin-binding proteins, and other proteins that reside at the cell-cell border, were studied for effect of cadherin function. The authors suggested that VASP/Mena-dependent reorganisation and the calcium-dependent directed polymerisation of actin were implicated as providing the necessary physical force to push the stabilised two rows of punctata together to further seal the cell-cell membrane (Vasioukhin *et al.*, 2000). Had I had more time available I would have explored the possibility of incomplete adherens junction formation in my transfected cell lines by using this elegant study as a guide for further experiments I might have conducted. This is one line of thought. Based on my results in this Chapter I could suggest other molecules that might be involved in the maturation of the E-cadherin-dependent cell-cell junctions in my model system, such as $p120^{ctn}$ and Keratin-14 (K14).

In vitro studies demonstrated that p120^{ctn} acts as a regulatory molecule of the adhesive function of E-cadherin, binding to the same region of this molecule as the rest of the catenins (Shibamoto *et al.*, 1995). Moreover, some studies have suggested that p120^{ctn} positively regulates cadherin clustering in the cell membrane (Yap *et al.*, 1998) and cell-cell junction formation (Yap *et al.*, 1998; Thoreson *et al.*, 2000; Grosheva *et al.*, 2001). These pieces of evidence made me think that p120^{ctn} might be a key role player in the formation and maturation of E-cadherin-dependent adherens junctions. The fact that, despite my continuous efforts, I failed to blot for p120^{ctn} in my cell lines, made the role of p120^{ctn} in the maturation of the E-cadherin-dependent cell-cell junction in my model system obscured, or non-discernible, in the results presented in this thesis.

K14 and involucrin are epithelial cell markers. Thus K14 is a keratin of the basal cells of all stratified squamous epithelia (Moll *et al.*, 1982; Quinlan *et al.*, 1985; Galvin *et al.*, 1989), while involucrin is a protein precursor of the cross-linked envelope that forms late in the terminal differentiation of keratinocytes (Rice and Green, 1979). In contrast to K14, involucrin is made only in suprabasal cells (Banks-Schlegel and Green, 1981; Watt and Green, 1982). I tested my primary and transfected cell lines for the expression of these two epithelial/differentiation markers. Whole cell RIPA lysates from all cell lines, were probed for K14 using anti-K14 monoclonal antibody (Abcam Ltd.) and a strong K14 band at 50 kDa was apparent only in the control lane (HaCaT, normal keratinocyte cell line), while H376 primary and transfected cell line lanes were negative for K14. The same negative result was obtained when blotting for involucrin. From these rather preliminary results I have considered it is possible that the lack of some of the differentiation markers in my cells might have impaired maturation of the cell-cell junction. I would suggest that in the future this idea should be considered and experiments designed to address this question. Again, had I had more time, this is an area I would have explored.

Concomitantly re-expression of the E-cadherin molecule in the H376 OSCC cell line resulted in the upregulation of both α- and β-catenin. On the other hand, γ-catenin was downregulated as a result of the *de novo* E-cadherin expression in this particular cell line (Figure 3.11).

It has been reported that E-cadherin is required for the efficient translation of the α-catenin mRNA (Takahashi *et al.*, 2000). Jamora and Fuchs (2002) argued if this was true, then in cases where E-cadherin is downregulated, like wound healing and tumour progression, there should be a concomitant decrease in levels of α-catenin (Jamora and Fuchs, 2002). Therefore, it is only logical to think that α-catenin levels were upregulated simultaneously with E-cadherin re-expression in my OSCC model system.

It is known that two distinct complexes can be found in the same cell, either E-cadherin complexing with β-catenin or E-cadherin complexing with γ-catenin but not with both occurring at the same time (Butz and Kemler, 1994). Moreover, from analysis of the interaction of purified recombinant cadherin and catenins in combinatorial protein-protein interactions, it is known that E-cadherin interacts with a higher affinity with β-catenin as compared to γ-catenin (Aberle *et al.*, 1994). This might explain why, upon E-cadherin re-expression, γ-catenin levels dropped down while levels of β-catenin increased, as determined by Western blot analysis.

Husmark *et al.* (1999) tested the role of E-cadherin and the catenins in the progression of human anaplastic thyroid-carcinoma cell lines and showed that there was a correlation with the amount of cytokeratin present in the same cell line. They suggested that, since cytokeratin is an epithelial marker, this finding supports the idea that γ-catenin is closely associated with the epithelial phenotype (Husmark *et al.*, 1999). Using the MNF 116 antibody (Dakopatts, Denmark) which recognises cytokeratins 5,6,8,17 and 19, the H376 primary cell line showed a very weak staining for cytokeratin (Thompson M., MSc thesis, University of London,

1993). In repeated experiments with my transfected cell lines and using a cocktail of two pan cytokeratin antibodies (clone AE1/AE3) to detect at least 19 of the major human cytokeratins (DakoCytomation), they consistently appeared negative for cytokeratin in paraffin sections (data not shown). In Western blot analysis, using the monoclonal antibody against Keratin-14 (Abcam Ltd.), my transfected cell lines appeared to be negative for that particular molecule (data not shown). In light of this, such observations might explain why, upon transfection with E-cadherin, the E-cadherin expressing cells in my studies did not restore the typical epithelial morphotype completely and still showed a downregulation of γ-catenin.

The invasive potentials of the transfected cell lines were measured and it showed that upon E-cadherin transfection invasion was greatly inhibited in the H376 E-cadherin cell line as compared to the H376 antisense control cell line (Figure 3.14). This finding is consistent with those of others (e.g. Behrens *et al.*, 1989). In an attempt to further assess the effect of E-cadherin re-expression on the invasive potential of the cells I tested my cells for the expression of MMP-2 and MMP-9 using zymographic analysis. Both cell lines were shown to be negative for both MMP's (data not shown). However, Barbletz *et al.* (2000) showed a marked activation of MMP-1 and MMP-7 by β-catenin in colorectal carcinoma. And with further time, I would have tested my transfected cell lines for the expression of other MMP's, especially including these.

It has been acknowledged that normal cells growing as a monolayer in tissue culture become quiescent when they reach confluency by a process known as "contact inhibition" (Aoki *et al.*, 1991; Kandikonda *et al.*, 1996; Takahashi and Suzuki, 1996). Loss of contact inhibition is considered one of the hallmarks of cancer, yet, many tumour cells were shown to maintain a certain degree of contact inhibition that has been referred to as "contact-dependent growth

inhibition/suppression" (St Croix *et al.*, 1998). E-cadherin was shown to contribute to contact-dependent growth inhibition (Aoki *et al.*, 1991; Kandikonda *et al.*, 1996; Takahashi and Suzuki, 1996). E-cadherin expression was shown to reduce tumour cell growth rate (Navarro *et al.*, 1991; Miyaki *et al.*, 1995; Hsu *et al.*, 2000; Fricke *et al.*, 2004). However, it has been suggested that contact-dependent growth suppression in tumour cells, growing as a monolyer, was incomplete as compared to normal cells which might explain the reduced sensitivity of tumour cells to undergo contact-dependent growth suppression (St Croix *et al.*, 1998). However, cells growing in three-dimensional tissue culture showed a significant, although still incomplete, reduction in cell growth (St Croix *et al.*, 1998). In my transfected cell lines (the H376 E-cadherin and the H376 antisense control), E-cadherin seems not to affect the growth rates (Figure 3.15).

This unremarkable finding did however cause me to re-think the direction of my project and stimulated me to embark on what will constitute a large section of the remainder of my studies. That is that one assay (the invasive assay) monitors the behaviour of cells in a 3-D situation, i.e. passaging through a Matrigel layer, whereas, generally, most investigators have confined their assessment of growth to that which occurs under conditions of 2-D constraint, as I have done here. Accordingly I determined to examine the proliferative and survival characteristics of the matched lines under 3-D conditions. These results will be presented in the subsequent Chapter.

There is a growing body of data suggesting that there is a kind of synergistic 'cross-talk' interaction between different CAM families (Aplin *et al.*, 1999). In some instances the expression levels of cadherins and integrins are linked. For example, in primary myoblasts, over-expression of either α5- or β1-integrin subunits leads to the increased expression of N-cadherin (Huttenlocher *et al.*, 1998). One possible mechanism in connection with the 'cross-

talk' between cadherins and integrins involves the serine/threonine kinase; integrin-linked kinase (ILK) (Delcommenne *et al.*, 1998; Novak *et al.*, 1998; Wu *et al.*, 1998; Dedhar *et al.*, 1999).

The β1-integrin subunit dimerises with various α integrin subunits to assemble into about 12 integrins (Chapter 1, Figure 1.3) (Hynes, 2002). Upon E-cadherin re-expression in my H376 transfected cell line, there was about a 1.8-fold (statistically significant) increase in the level of β1-integrin subunit (p value $\leq$ 0.01) (Figure 3.17). Among the different α integrin subunits that were being tested, only α2-integrin subunit showed a concomitant 1.7-fold increase, in the H376 E-cadherin cell line as compared to the antisense counter part, which was consistent in three independent experiments and statistically significant (p value $\leq$ 0.04) (Figure 3.17). In keratinocytes, downregulation of E-cadherin was shown to decrease the expression of α2β1 and α3β1 integrins (Zhu and Watt, 1996). The MCF-7 breast carcinoma cell line was transformed, under the effect of melatonin, into a less invasive phenotype through upregulation of E-cadherin and β1-integrin subunit, which further promoted cell differentiation (Cos *et al.*, 1998), suggesting the both E-cadherin and β1-integrin subunit contribute to invasion and differentiation in a similar manner. Furthermore, in breast carcinoma, poorly differentiated cancer cells were able to differentiate into gland-like structures when α2β1 integrin expression was restored (Zutter *et al.*, 1995). This might explain why *de novo* E-cadherin expression in this particular OSCC cell line resulted in upregulation of β1-integrin subunit and perhaps, more specifically, α2β1 integrin.

α6β4 integrin is expressed in stratified epithelia and cultured keratinocytes adhering to basement membrane substrates (De Luca *et al.*, 1990; Marchisio *et al.*, 1991; Jones *et al.*, 1993). In poorly differentiated oral squamous cell carcinomas loss of α6 and β4 was detected at the advancing edges of the tumour lesion (Jones *et al.*, 1993). The β4-integrin subunit previously has been reported to be expressed 10-fold less in the H376 primary cell line

compared to normal keratinocytes (Sugiyama *et al.*, 1993). *De novo* E-cadherin expression, in this particular cell line, resulted in about a 2.5-fold increase in β4-integrin levels (Figure 3.17), suggesting that there might be a 'cross-talk' between these two epithelial adhesion molecules which will be investigated further in Chapter 5.

Based on the results that I achieved so far, in the work described in this Chapter, I dedicated the next two years work to investigate two major areas. Firstly, I chose to examine the possible cross-talk between E-cadherin and the β4-integrin subunit. For this reason, I utilised the H376β4 cell line, an H376 cell line previously transfected with β4-integrin subunit by J.Jones (Jones *et al.*, 1996a), and its control counterpart cell line, IA2, which was transfected with the empty vector. The plan was to transfect these cell lines with my E-cadherin construct and then to study the possible cross talk between E-cadherin and the β4-integrin subunit.

Secondly the E-cadherin molecule is one of the most extensively studied adhesion molecules and my initial functional assay results showed not much difference than similarly published observations. However, in my opinion, most of these tests have a major limitation. That is they were conducted on cells under 2-D conditions in tissue culture; a non-physiological state for mammalian cells. In an effort to give my studies greater *in vivo* relevance I chose to expand the basis of my investigations by adapting some of these assays to 3-D culture conditions using my E-cadherin OSCC model cell lines. These findings constitute the basis of the next Chapter.

Figure 3.1

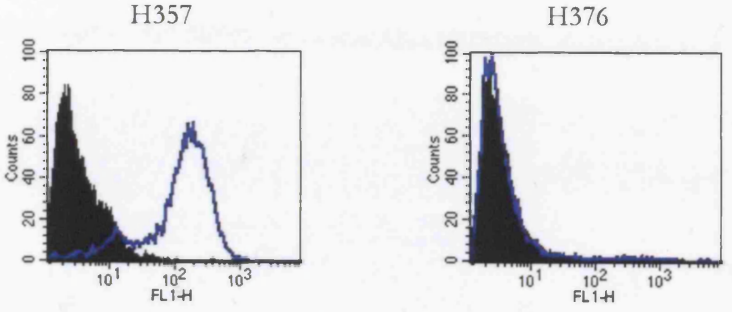
E-cadherin profile of the H376 parental cell lines using FACS analysis and Western blotting

a) ***FACS analysis:*** Cells were allowed to grow to confluency. Harvested using E-cadherin preservation method (Chapter 2, Section 2.2.1.1). FACS results showed E-cadherin surface expression of the parental cell lines, the black histogram represents the negative control labelled with Mouse IgG1 negative control (DAKO), while the blue histogram represents the positive cell population expressing E-cadherin labelled with anti-E-cadherin antibody "HECD-1" (M. Takeichi). H357 cell line was used as a positive control and shown to express high levels of E-cadherin, while H376 is shown to be an E-cadherin negative line.

b) ***Western blot analysis:*** Equal cell numbers of each cell line were plated for 48 hours in KGM prior to lysing. Whole cell RIPA lysates were obtained, and equal protein was determined by the BCA protein assay. Proteins (25 μg/lane) were resolved by SDS-PAGE and blotted onto nitrocellulose membrane. The blot was probed for E-cadherin using HECD-1 antibody and shows a 120 kDa band in relation to H357 line "positive control", whereas H376 cell line was negative for E-cadherin. The bottom blot was probed for β-actin used as a loading control.

Figure 3.1

a)


H357
H376
Counts
FL1-H

b)

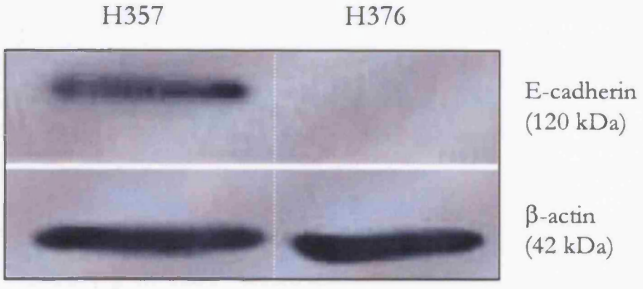

H357
H376
E-cadherin
(120 kDa)
β-actin
(42 kDa)

Figure 3.2

Catenins and other cadherins profile of the H376 parental cell lines using Western blot analysis

Equal cell numbers of each cell line were plated for 48 hours in KGM prior to lysing. Whole cell RIPA lysates were obtained, and equal protein was determined by the BCA protein assay. Proteins (25 μg/lane) were resolved by SDS-PAGE and blotted onto nitrocellulose membrane.

a) *Catenin Profile:* The top blot was probed with monoclonal antibody to α-catenin (BD Transduction Laboratories) and shows a 102 kDa α-catenin band in relation to both cell lines, H357 and H376.

The second blot was probed with monoclonal antibody to β-catenin (BD Transduction Laboratories) and shows a 92 kDa β-catenin band in relation to both cell lines, H357 and H376.

The third blot was probed with monoclonal antibody to γ-catenin (BD Transduction Laboratories) and shows a 82 kDa γ-catenin band in relation to both cell lines, H357 and H376.

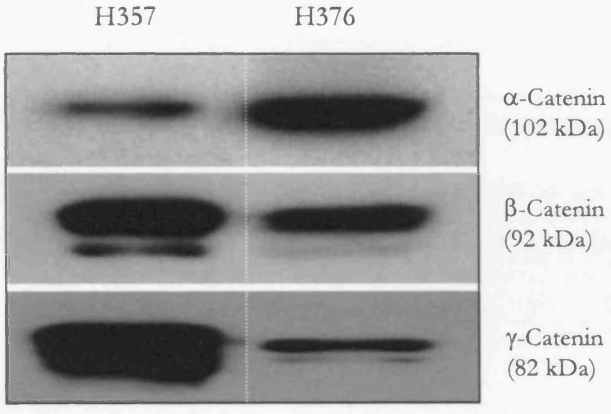
b) *Cadherin profile:* The top blot was probed using monoclonal antibody to Desmoglein (BD Transduction Laboratories) and shows a 165 kDa band in relation to both H357 and H376 lines.

The second blot was probed with monoclonal antibody to N-cadherin (BD Transduction Laboratories) and shows a 130 kDa N-cadherin band in relation to both cell lines, H357 and H376, with strong expression in the H376 lane.

The third blot was probed with monoclonal antibody to P-cadherin (BD Transduction Laboratories) and shows a 120 kDa P-cadherin band in relation to the H357 cell line, whereas the H376 cell line was negative for P-cadherin.

Figure 3.2

a)


H357
H376
α-Catenin
(102 kDa)
β-Catenin
(92 kDa)
γ-Catenin
(82 kDa)

b)

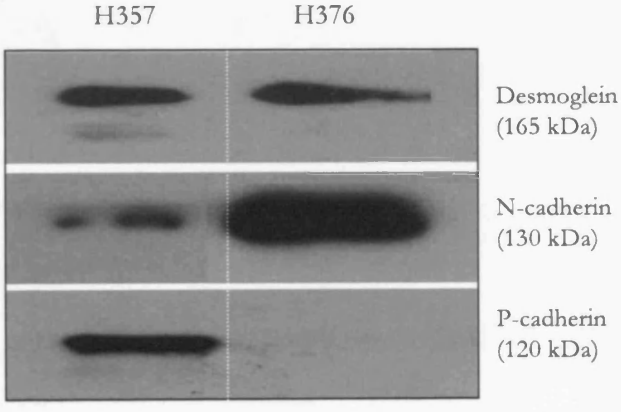

H357
H376
Desmoglein
(165 kDa)
N-cadherin
(130 kDa)
P-cadherin
(120 kDa)

Figure 3.3

Total RNA purification from the H357 cell line

Total RNA of $OD_{260/280}$ 1.8 ratio was isolated from the E-cadherin- expressing H357 cells using the RNA STAT-60™ total RNA/mRNA isolation reagent (Tel-Test "B" Inc.). The bands were resolved using 1% agarose gel electrophoresis. The image shows the ribosomal RNA bands.

Figure 3.4

Amplification of the E-cadherin gene

E-cadherin cDNA was amplified using pfuTurbo®DNA polymerase (Stratagene) and specific primers. Electrophoresis image shows a band of 2700 bp.

Figure 3.5

Miniprep restriction digestions

Colonies were selected for small scale minipreparation

The template was identified using XhoI restriction digestion and fragments of 5284 bp and ~2700 bp were expected to appear in the 1% agarose gel electrophoresis image.

The orientation of the insert was identified using EcoRI restriction digestion, and the sense orientation was expected to give fragments of ~6234 bp, 1350 bp and 500 bp, while the antisense orientation was expected to give fragments of ~5934 bp, 1350 bp and 800 bp.

Figure 3.3

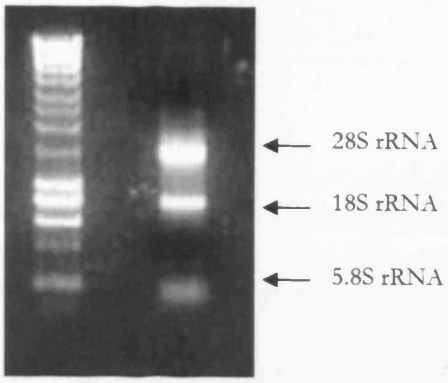


Figure 3.4

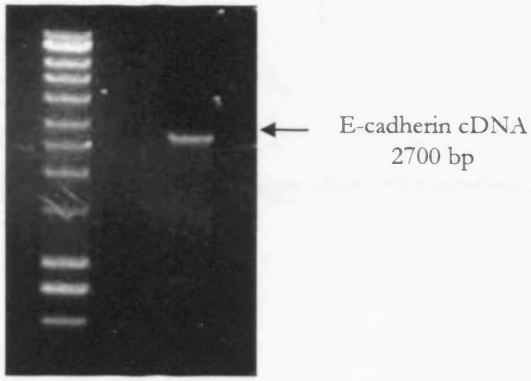


Figure 3.5

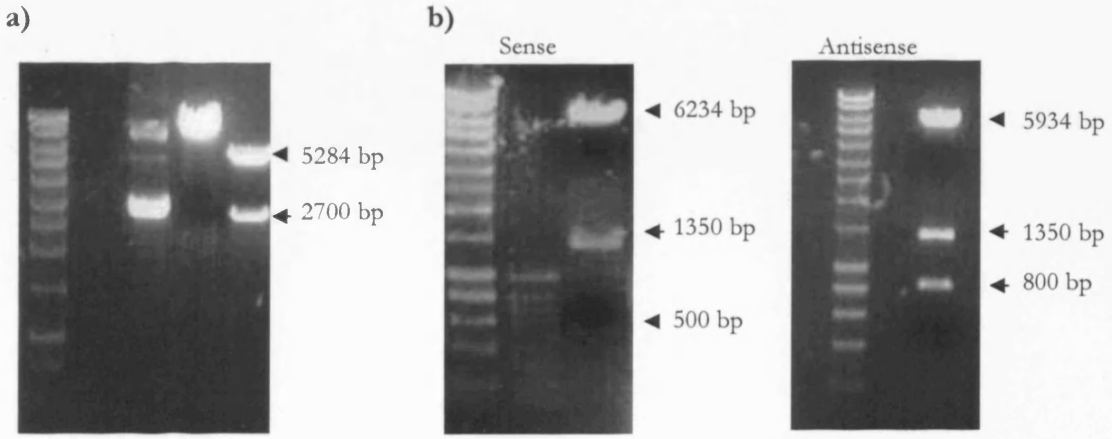


Figure 3.6

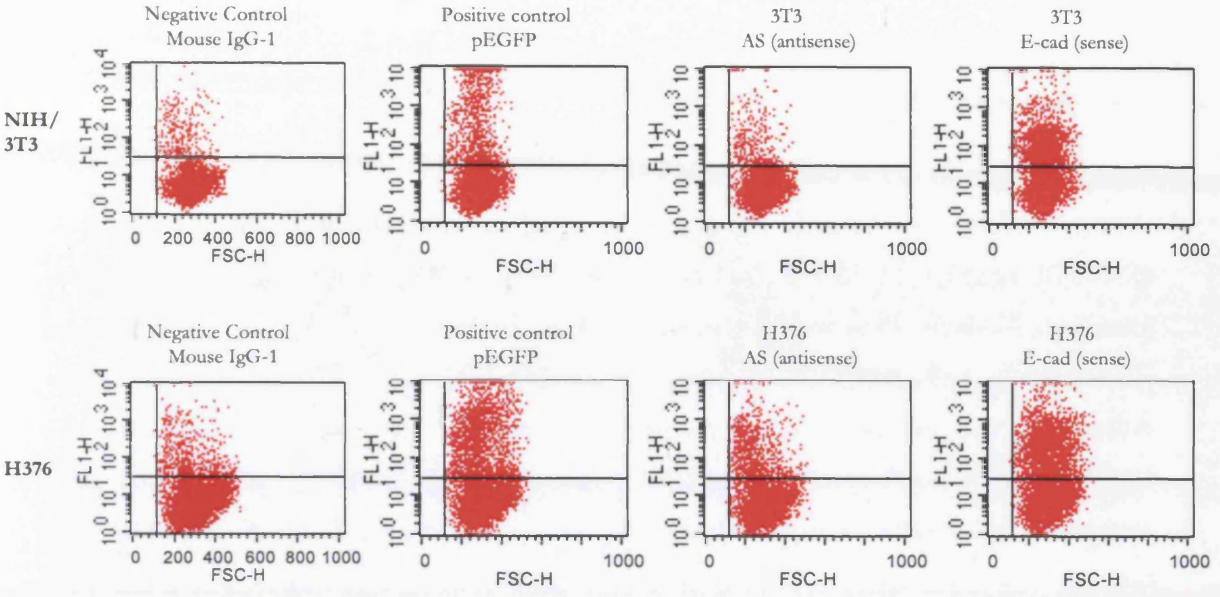
E-cadherin expression in transiently transfected cell lines using FACS analysis and Western blotting

a) ***FACS analysis:*** NIH/3T3 and H376 cell lines were transiently transfected with E-cadherin sense and anti-sense cDNA, using FuGENE 6 Transfection reagent. The transfected cells were labelled for E-cadherin using HECD-1 antibody. The negative control was labelled with Mouse IgG1 negative control (DAKO), and the positive control was labelled for pEGFP. Dot blots showed total surface E-cadherin expression of 25.56% of cells in the NIH/3T3 transfected cell line and 29.96% of cells in H376 transfected cell line.

b) ***Western blot analysis:*** Whole cell RIPA lysates (28.6 μg/lane) were resolved by SDS-PAGE and blotted onto nitrocellulose membranes. The blot was probed for E-cadherin using HECD-1 antibody and shows an E-cadherin band of 120kDa detected in NIH/3T3 and H376 cell lines transfected with the E-cadherin sense construct. The H375 cell line was used as a positive control

Figure 3.6

a)


Negative Control
Mouse IgG-1
Positive control
pEGFP
3T3
AS (antisense)
3T3
E-cad (sense)
NIH/
3T3
H376
Negative Control
Mouse IgG-1
Positive control
pEGFP
H376
AS (antisense)
H376
E-cad (sense)
FL1-H
FSC-H
0
200
400
600
800
1000

b)

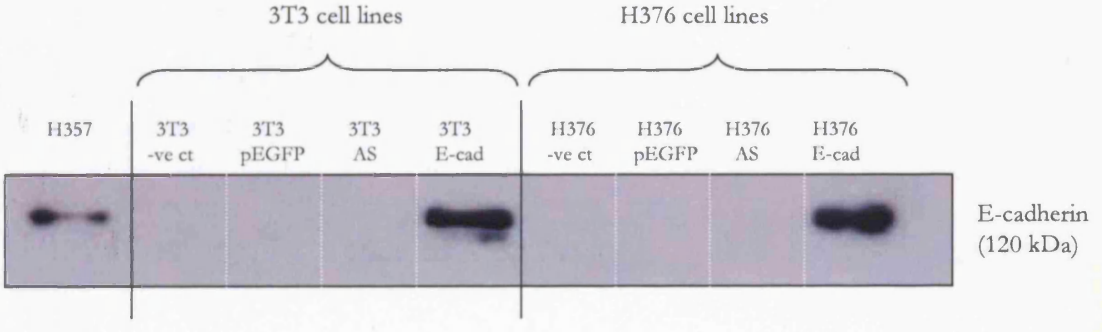

3T3 cell lines
H376 cell lines
H357
3T3
-ve ct
3T3
pEGFP
3T3
AS
3T3
E-cad
H376
-ve ct
H376
pEGFP
H376
AS
H376
E-cad
E-cadherin
(120 kDa)

Figure 3.7

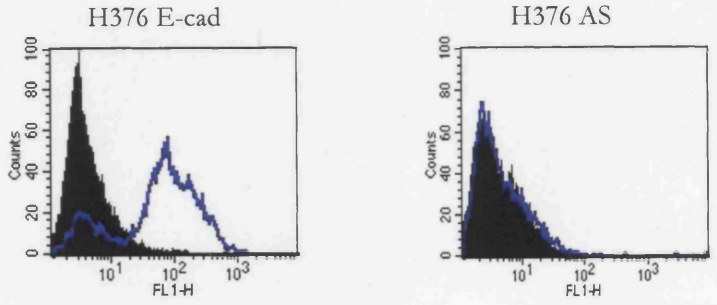
Determination of E-cadherin expression in H376 stably transfected cell lines

a) ***FACS analysis:*** H376 cell line was transfected with E-cadherin sense and anti-sense constructs. Positive cell populations expressing E-cadherin were sorted using magnetic beads (Dynabeads®), and were selected using 0.5 μg/ml puromycin. FACS results showed E-cadherin surface expression of the transfected cell lines, the black peak represents the negative control labelled with Mouse IgG1 negative control (DAKO), while the blue peak represents the positive cell population expressing E-cadherin labelled with HECD-1 antibody. H376 antisense cell line showed no expression of E-cadherin while the H376 E-cadherin cell line expressed high levels of E-cadherin.

b) ***Western blot analysis:*** Whole cell RIPA lysates (38 μg/lane) were resolved by SDS-PAGE and blotted onto nitrocellulose membranes. The top blot was probed for E-cadherin using HECD-1 antibody and shows a 120 kDa band in relation to the "positive control" H357 line and the H376 cell line transfected with the E-cadherin sense construct (H376 E-cadherin). In contrast H376 cell line transfected with the anti-sense construct (H376 antisense) was negative for E-cadherin. The bottom blot was probed for β-actin which was used as a loading control.

Figure 3.7

a)


H376 E-cad
H376 AS
Counts
0
20
40
60
80
100
10^1
10^2
10^3
FL1-H

b)

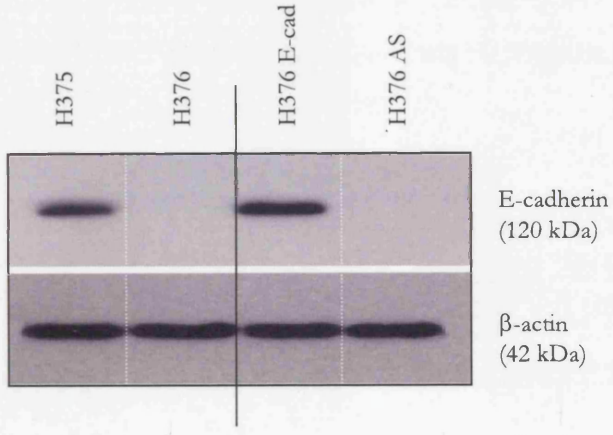

H375
H376
H376 E-cad
H376 AS
E-cadherin
(120 kDa)
β-actin
(42 kDa)

Figure 3.8

Determination of E-cadherin expression in H376 stably transfected cell lines using indirect immunostaining

2 x 10^4 cells were plated onto 13mm coverslips in a 24-well plate and allowed to grow for 48 hours with selection medium. Cells were examined on a confocal laser scanning microscope, and images collected digitally.

Panels A-C are confocal images of the H376 cells transfected with the anti-sense construct (H376 antisense).

Panels D-F are confocal images of the H376 cells transfected with the sense construct (H376 E-cadherin).

In Panels A and D, E-Cadherin, "green", was detected by HECD-1 antibody. H376 antisense cell line was negative for E-cadherin (Panel A). H376 E-cadherin cell line was positive for E-cadherin (Panel D) which appeared on the cell surface at cell-cell junctions but the junctions appeared to be morphologically immature (as denoted with open arrows).

In Panels B and E, actin cytoskeleton, "red", was detected with phalloidin-TRITC, and showed that the actin filaments co-localised with E-cadherin and redistributed into bundles in the H376 E-cadherin cell line (Panel E).

Panels C and F are composite confocal images showing E-cadherin, "green", actin cytoskeleton, "red", and nuclei, "blue", detected by DAPI.

Figure 3.8

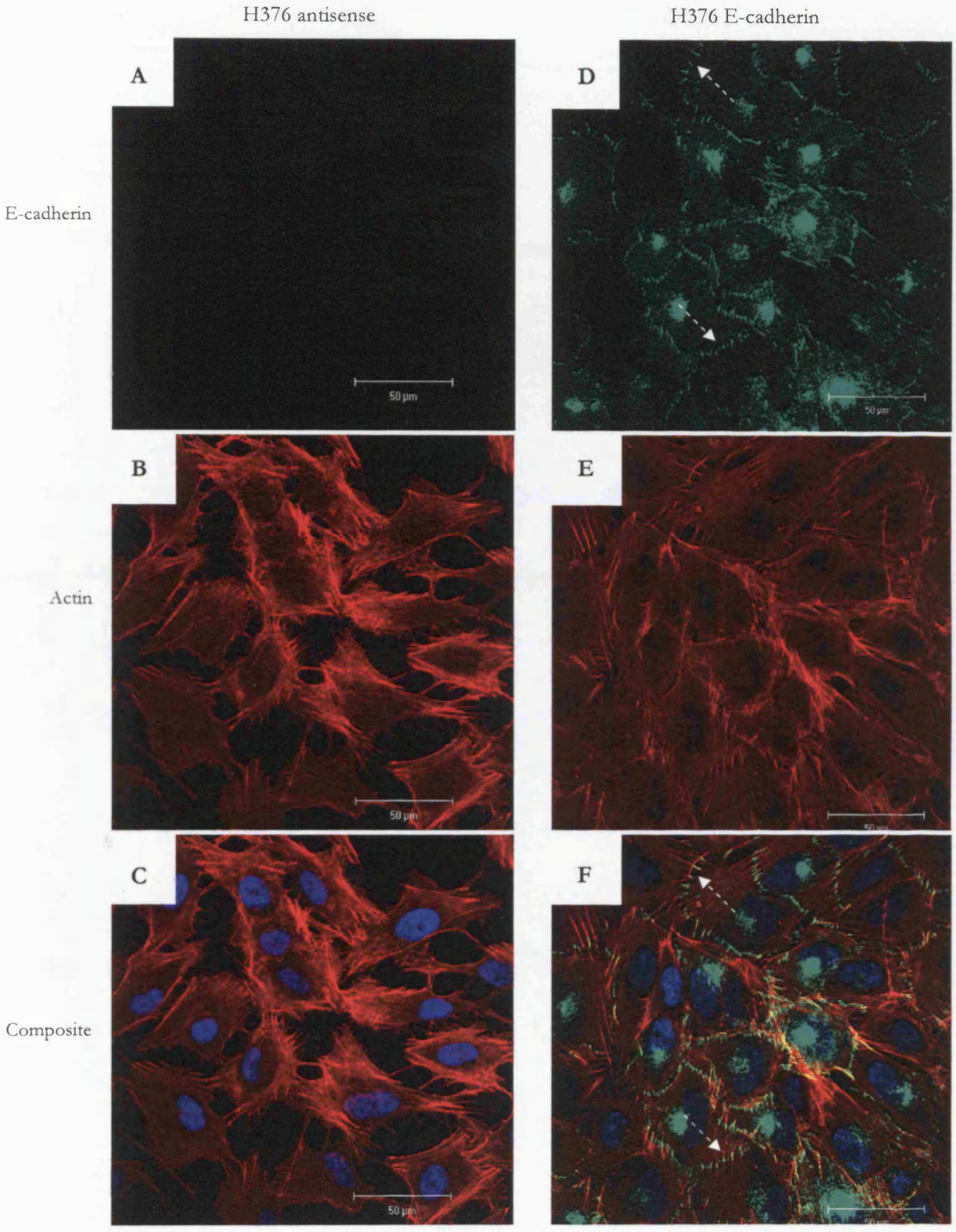

H376 antisense
H376 E-cadherin
A
D
E-cadherin
50 µm
B
E
Actin
C
F
Composite

Figure 3.9

Indirect immunostaining for E-cadherin in the H357 cell line

2 x 10^4 cells were plated onto 13mm coverslips in a 24-well plate and allowed to grow for 48 hours, stained and examined on a confocal laser scanning microscope, and images collected digitally.

In Panel A, E-Cadherin, "green", was detected by HECD-1 antibody, and appeared on the cell surface at cell-cell junctions forming a mature junction. In Panel B, Actin cytoskeleton, "red", was detected with phalloidin-TRITC, and showed that the actin filaments formed an actin belt parallel to the circumference of the cell; a feature of cells with an epithelial morphology.

Panel C, is an image of H357 cells visualised under "contrast phase" microscopy, showing typical epithelial morphological presentation.

Figure 3.9

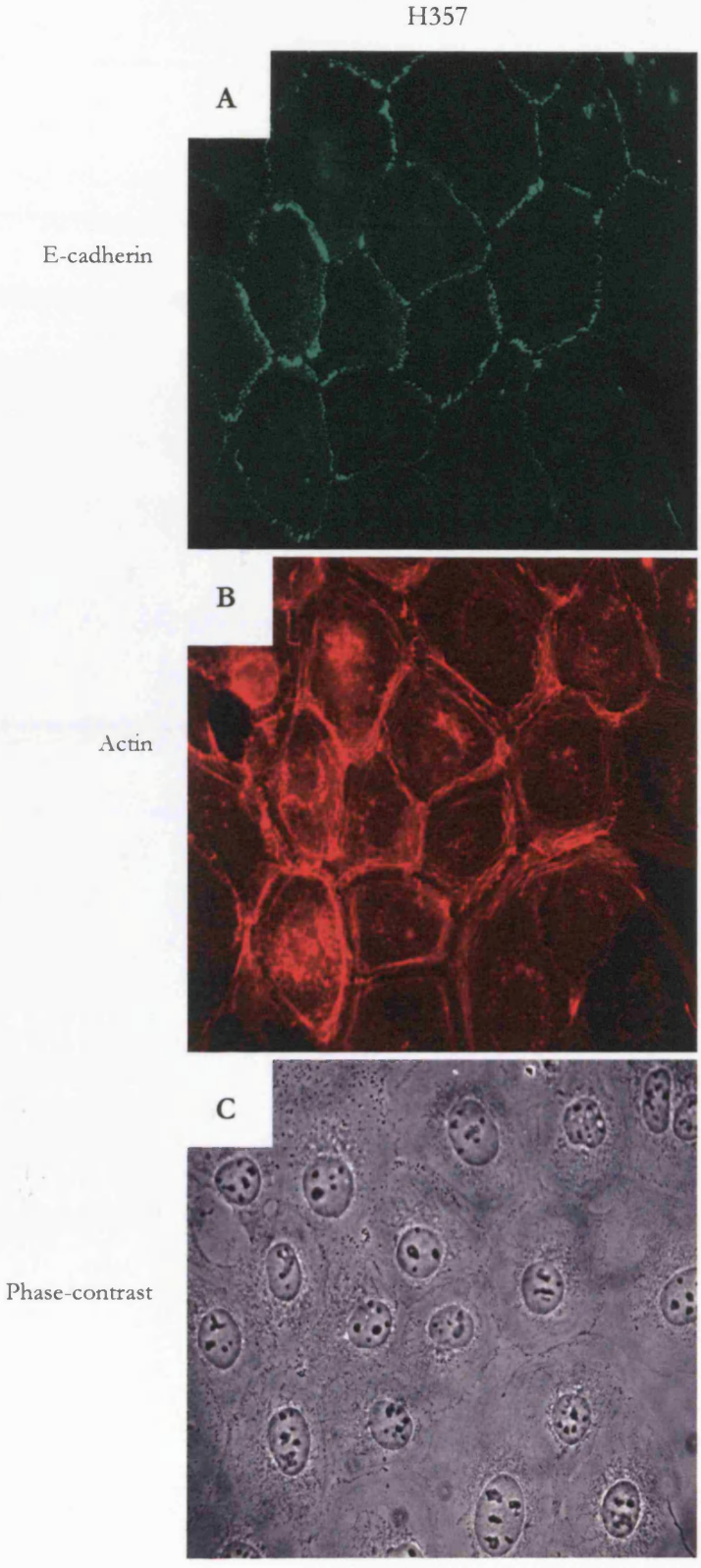


Figure 3.10

Microscopic images of the stably transfected cell lines

Under "contrast phase" microscopy the E-cadherin positive cell lines exhibited a more epithelial morphology (Panel A) than the more spindly anti-sense control cells (Panel B).

Figure 3.10

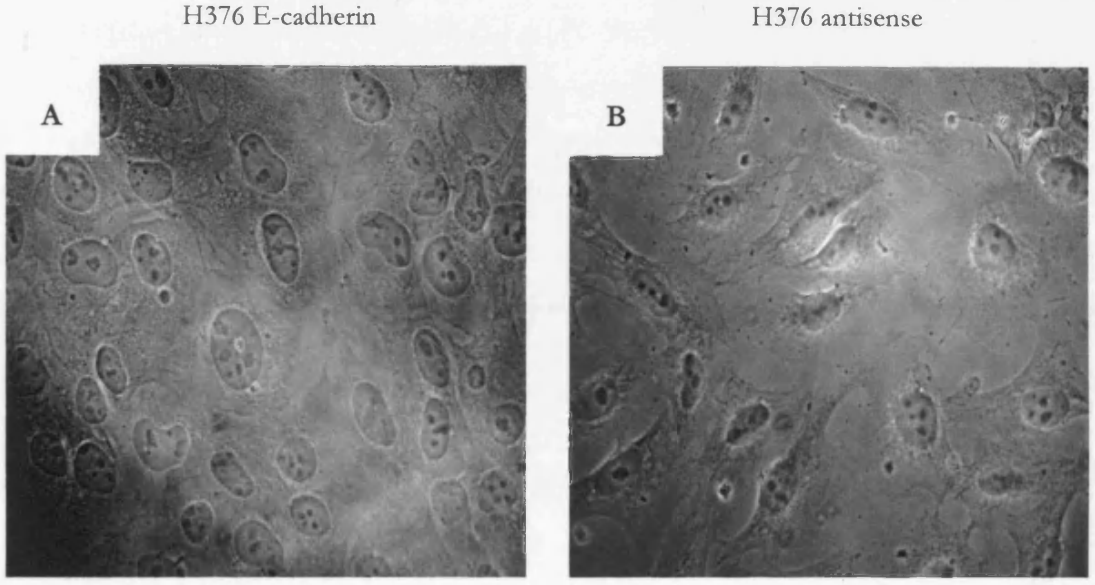


Figure 3.11

Catenin profile of the stably transfected cell lines using Western blot analysis

Equal cell numbers of each cell line were plated for 48 hours in KGM prior to lysing. Whole cell RIPA lysates were obtained and 25 μg protein/lane were resolved by SDS-PAGE and blotted onto nitrocellulose membrane. Densitometric analysis: values were obtained and corrected for loading. Error bars represent standard error of the mean.

a) Histogram obtained from results of densitometric values of three independent experiments shows that E-cadherin transfection resulted in an increase in the total level of α-catenin (p value $\leq$ 0.01). The top blot is a representative Western blot probed with monoclonal antibody to α-catenin (BD Transduction Laboratories) and shows a 102 kDa α-catenin. β-actin has been used as a loading control.

b) Histogram obtained from results of densitometric values of three independent experiments shows that E-cadherin transfection resulted in an increase in the total level of β-catenin (p value $\leq$ 0.02).The top blot is a representative Western blot probed with monoclonal antibody to β-catenin (BD Transduction Laboratories) and shows a 92 kDa β-catenin. β-actin has been used as a loading control.

c) Histogram obtained from results of densitometric values of five independent experiments shows that γ-catenin was downregulated as a consequence of E-cadherin re-expression (p value $\leq$ 0.02). The top blot is a representative Western blot probed with monoclonal antibody to γ-catenin (BD Transduction Laboratories) and shows a 82 kDa γ-catenin. β-actin has been used as a loading control.

Figure 3.11

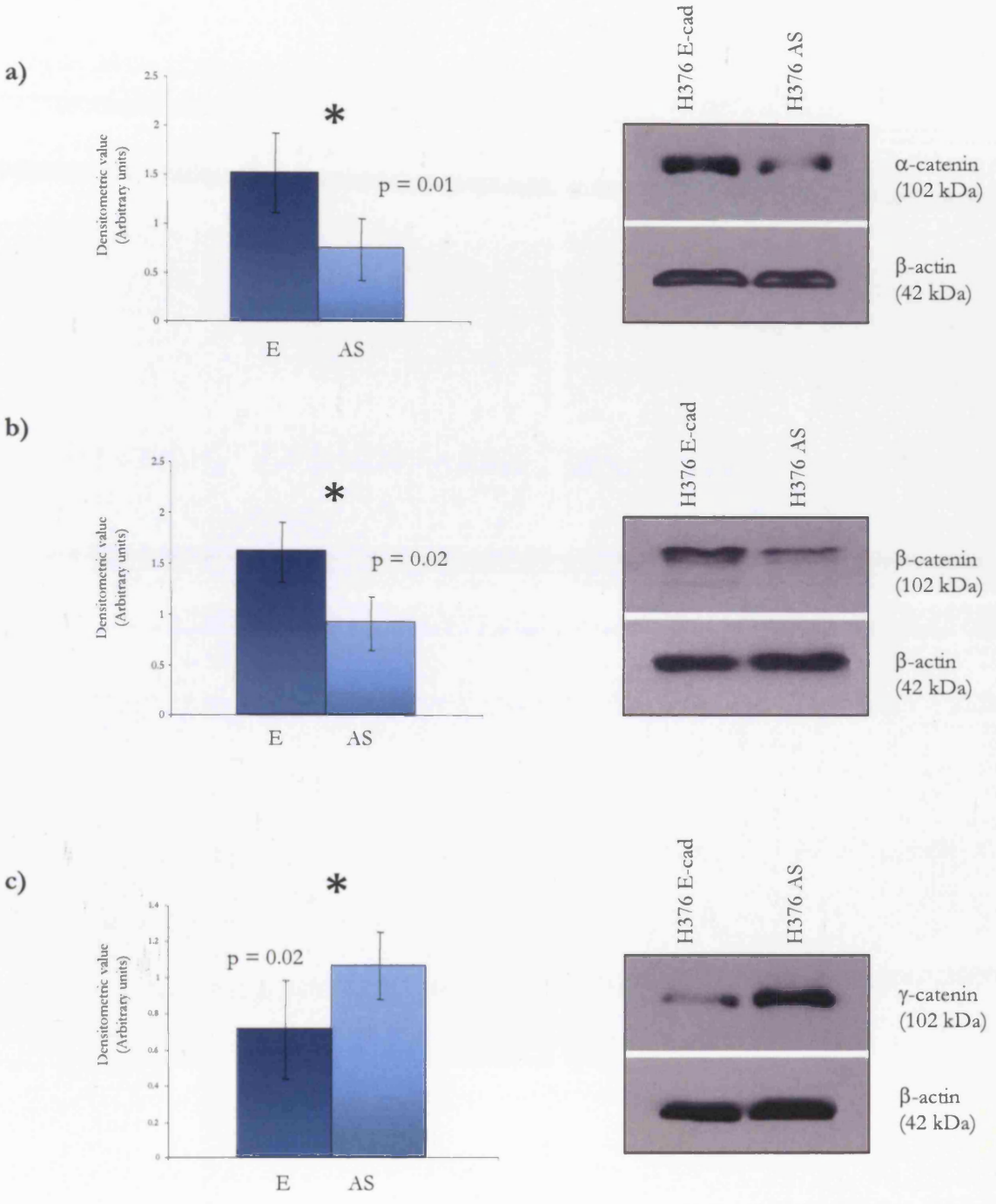

a)
Densitometric value (Arbitrary units)
2.5
2
1.5
1
0.5
0
*
p = 0.01
E
AS
H376 E-cad
H376 AS
α-catenin (102 kDa)
β-actin (42 kDa)
b)
Densitometric value (Arbitrary units)
2.5
2
1.5
1
0.5
0
*
p = 0.02
E
AS
H376 E-cad
H376 AS
β-catenin (102 kDa)
β-actin (42 kDa)
c)
Densitometric value (Arbitrary units)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
p = 0.02
E
AS
H376 E-cad
H376 AS
γ-catenin (102 kDa)
β-actin (42 kDa)

Figure 3.12

Indirect immunostaining for β- and γ-catenin in the H376 transfected cell lines

2 x 10^4 cells were plated onto 13mm coverslips in a 24-well plate and allowed to grow for 48 hours.

Panels A and B are confocal images of H376 transfected cells probed with monoclonal antibody to β-catenin (BD Transduction Laboratories) and show that H376 E-cadherin cells (Panel A) exhibit a stronger positivity for β-catenin with more β-catenin recruited to the cell-cell junctions, as compared to cells transefected with the antisense construct (Panel B).

Panels C and D are confocal images of H376 transfected cells probed with monoclonal antibody to γ-catenin (BD Transduction Laboratories) and show that H376 E-cadherin cells (Panel C) exhibit a stronger positivity for γ-catenin which was recruited to the cell membrane (apparently not consistent with the Western blot results that showed downregulation of the total γ-catenin in the E-cadherin positive cell line), as compared to cells transefected with the antisense construct (Panel D).

Figure 3.12

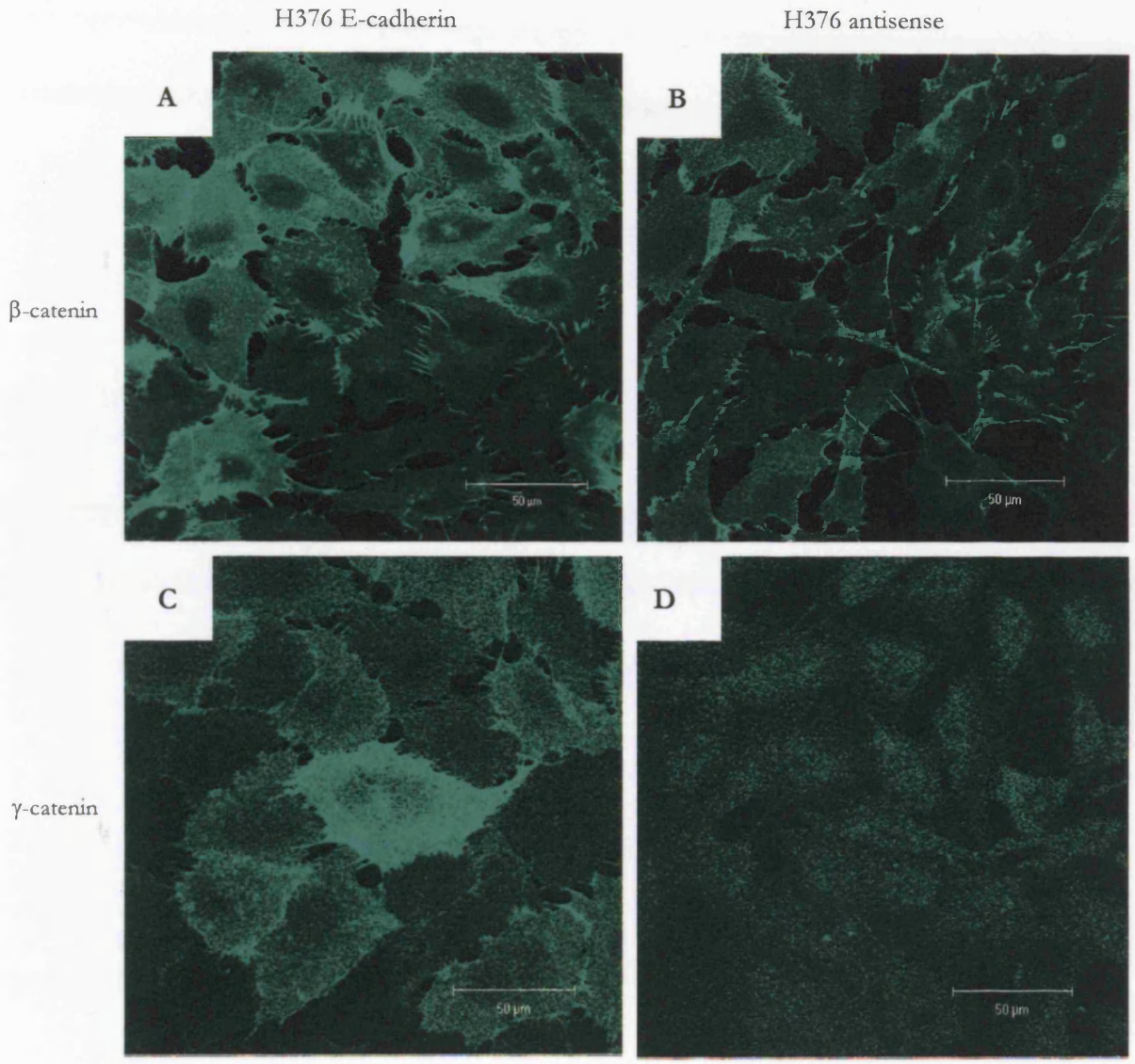

H376 E-cadherin
H376 antisense
A
B
C
D
β-catenin
γ-catenin
50 µm
50 µm
50 µm
50 µm

Figure 3.13

Expression of other Cadherins in the H376 transfected cell lines

Whole cell RIPA lysates were obtained, and equal protein was determined by BCA protein assay. Proteins (25 μg/lane) were resolved by SDS-PAGE and blotted onto nitrocellulose membranes.

The top blot was probed with monoclonal antibody to N-cadherin (BD Transduction Laboratories) and shows a 130 kDa N-cadherin band in all lanes, positive control, H376 E-cadherin and H376 antisense cell lines. With the same amount of protein loaded there appeared to be a marked downregulation of the total N-cadherin in the H376 E-cadherin cell line.

The bottom blot was probed for Hsc-70 which was used as a loading control.

Figure 3.13

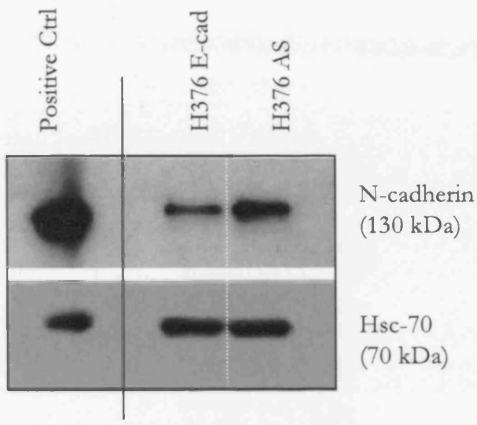

Positive Ctrl
H376 E-cad
H376 AS
N-cadherin
(130 kDa)
Hsc-70
(70 kDa)

Figure 3.14

Invasion Assay

Cell invasion was measured using Matrigel-coated polyethylene tetraphthallate (PET) membranes. Full keratinocyte growth medium was used as a chemo-attractant in the lower chamber. After 48 hours cells were detached with EDTA/trypsin and counted using the CASY counter.

A representative experiment showed that invasion was greatly inhibited (60-80%) (p value $\leq$ 0.0004) in H376 E-cadherin cell line as compared to the H376 antisense control cell line. Error bars represent standard error of the mean.

Figure 3.14

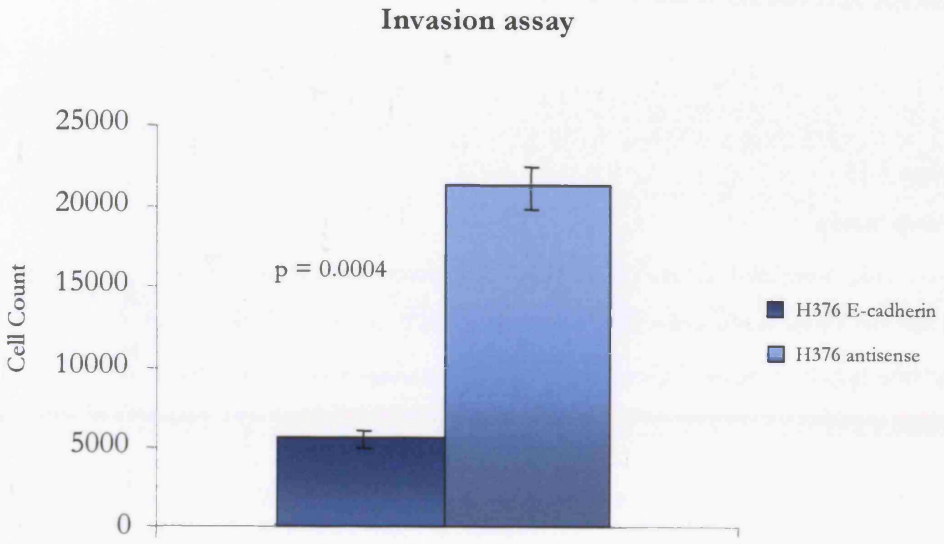

Invasion assay
25000
20000
15000
10000
5000
0
Cell Count
p = 0.0004
H376 E-cadherin
H376 antisense

Figure 3.15

Growth assay

2 x 10^4 cells were plated onto 3cm wells. Triplicate wells were counted at 5 time points up to 120 hours. Cells were counted using the CASY counter. Figure shows one representative experiment out of three. Error bars represent standard error of the mean.

Figure 3.15

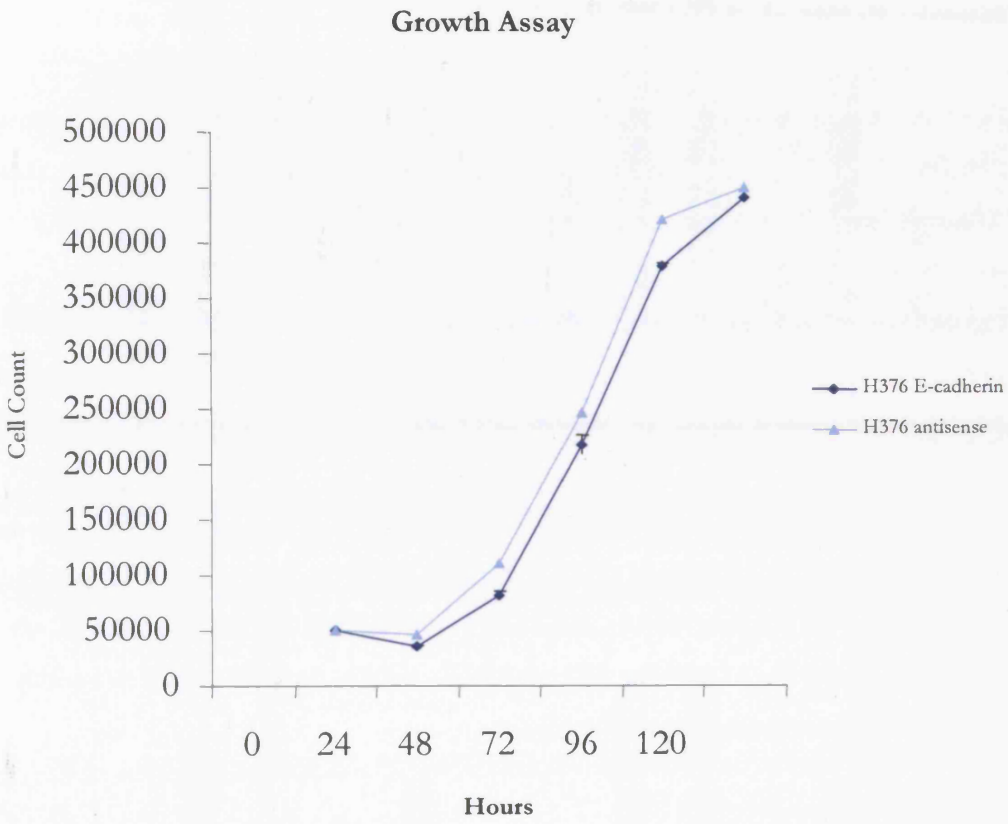

Growth Assay
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
Cell Count
0
24
48
72
96
120
Hours
H376 E-cadherin
H376 antisense

Figure 3.16

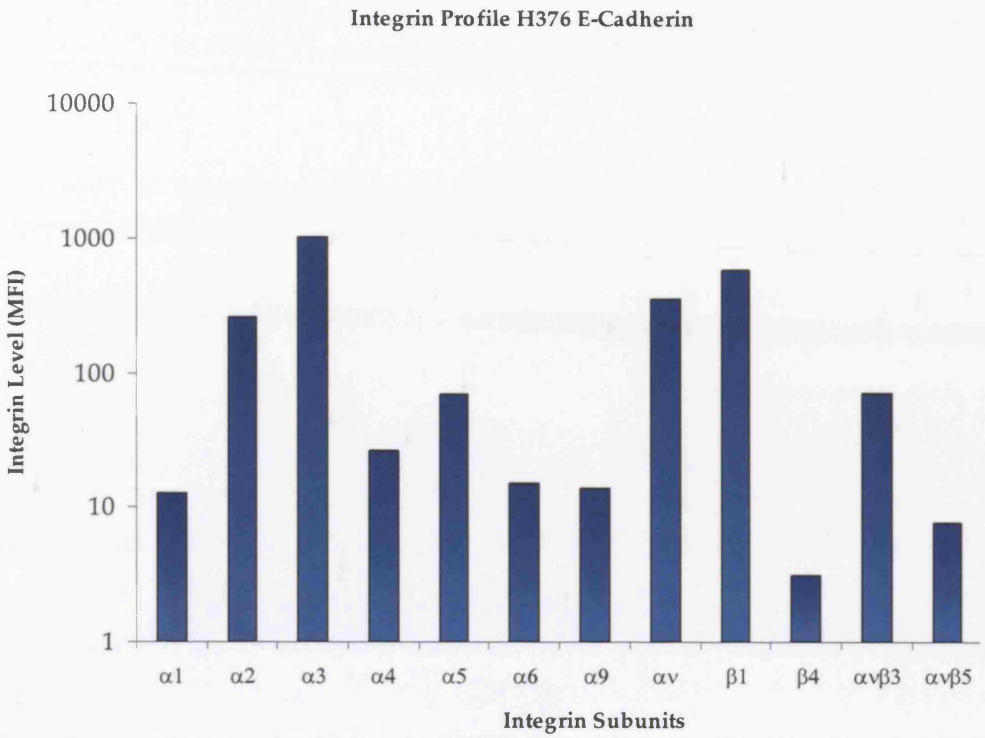
Integrin Profile of the H376 transfected cell lines (Geometric mean fluorescence histogram)

Flow cytometric analysis of expression of various integrins on the cell surface of the transfected cell lines; H376 E-cadherin and H376 antisense. Geometric mean fluorescence (arbitrary units, log scale) as measured by flow cytometry of cells labelled with anti-integrin antibodies.

Figure shows a representative experiment. The mean fluorescence intensity (MFI) of each integrin subunit in each cell line was determined by subtracting the fluorescence value of cells labelled with the appropriate IgG control antibody.

Antibody FB12 recognises the α1 subunit; P1E6 recognises the α2 subunit; P1B5 recognises the α3 subunit; 7.2 recognises the α4 subunit; P1D6 recognises the α5 subunit; GOH3 recognises the α6 subunit; Y9A8 recognises the α9 subunit; L230 recognises the αv subunit; AIIB2 recognises the β1 subunit; 3E1 recognises the β4 subunit; LM609 recognises the αvβ3 integrin; P1F6 recognises the αvβ5 integrin.

Figure 3.16


Integrin Profile H376 E-Cadherin
10000
1000
100
10
1
Integrin Level (MFI)
α1
α2
α3
α4
α5
α6
α9
αv
β1
β4
αvβ3
αvβ5
Integrin Subunits

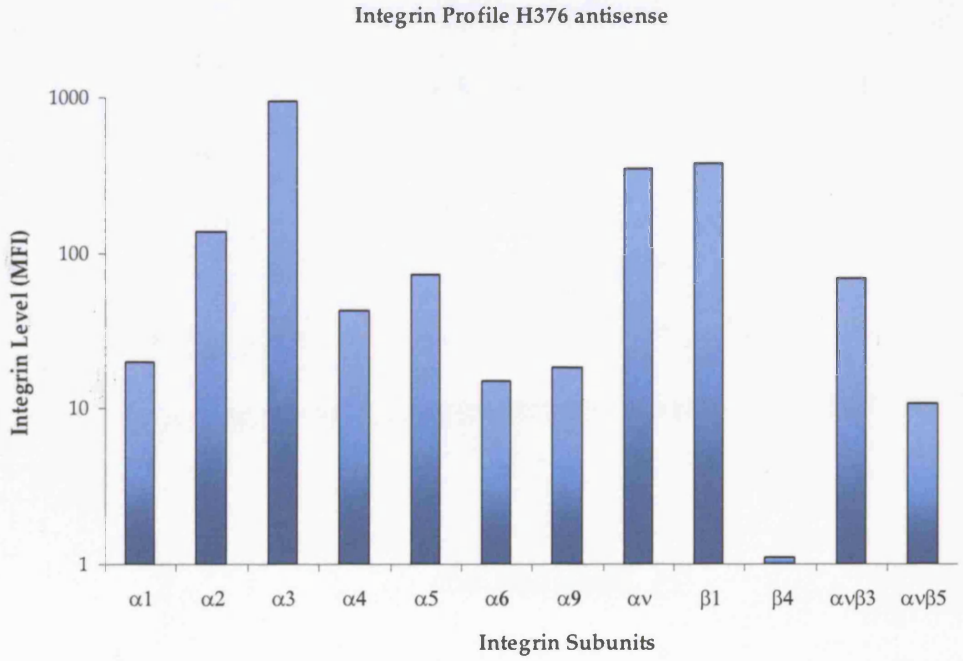

Integrin Profile H376 antisense
1000
100
10
1
Integrin Level (MFI)
α1
α2
α3
α4
α5
α6
α9
αv
β1
β4
αvβ3
αvβ5
Integrin Subunits

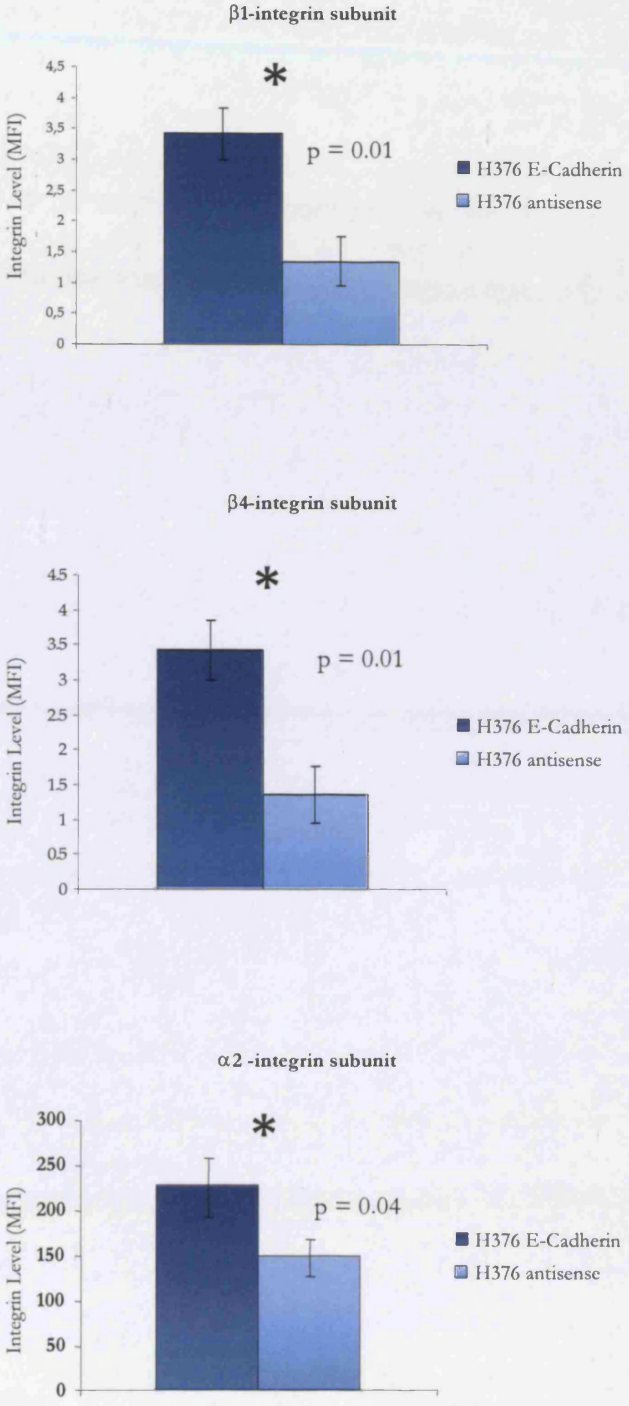
Figure 3.17

β1, β4 and α2 integrin subunit analysis in the H376 transfected cell lines (Geometric mean fluorescence histogram)

Flow cytometric analysis of expression of β1, β4 and α2 integrin subunits on the cell surface of the transfected cell lines; H376 E-cadherin and H376 antisense. Geometric mean fluorescence (arbitrary units, log scale) as measured by flow cytometry of cells labelled with anti-β1 integrin antibodies (AIIB2), anti-β4 integrin antibodies (3E1) and anti-α2 integrin antibodies (P1E6).

Figure shows histogram blotted against the mean value of three independent experiments for β1 integrin (a), β4 integrin (b) and α2 integrin (c). The mean fluorescence intensity (MFI) was determined by subtracting the fluorescence value of cells labelled with the appropriate IgG control antibody. Error bars represent standard error of the mean (sem).

Figure 3.17


β1-integrin subunit
Integrin Level (MFI)
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
*
p = 0.01
H376 E-Cadherin
H376 antisense
β4-integrin subunit
Integrin Level (MFI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
p = 0.01
H376 E-Cadherin
H376 antisense
α2 -integrin subunit
Integrin Level (MFI)
300
250
200
150
100
50
0
*
p = 0.04
H376 E-Cadherin
H376 antisense

CHAPTER 4

E-cadherin Regulation of Cell Proliferation Under 2-D or 3-D Collagen I Culture Conditions

4.1 Introduction

Tumour cells may be regarded as being like "cynical criminals" in their behaviour in that they defy the body's normal social restraints; one of these restraints being the restriction of cell growth when cells find themselves outside of their correct anatomical location. During the initial phase of the neoplastic process the transformed tumourigenic epithelial cells proliferate at accelerated rates on top of the underlying basement membrane in what, effectively, is a "two dimensional" (2-D) environment. As tumour evolution progresses the transformed epithelial cells acquire the ability to penetrate the basement membrane and invade the adjacent stromal matrix (see Figure 4.1 for an illustration of the metastatic process). The expression of this tissue-invasive phenotype occurs in conjunction with adaptation of the tumour cells to survive an abrupt transition in growth environments. No longer confined to the planar surface of the extracellular matrix (ECM), where they were located on a contiguous basement membrane, the invading cells are forced to proliferate within a dense three-dimensional (3-D) matrix composed largely of type I collagen or crosslinked fibrin (for an overview, see Hanahan and Weinberg, 2000).

In normal epithelium, the physical interactions of the epithelial cells with each other and with the underlying ECM are termed "cell-cell anchorage" and "cell-matrix anchorage", respectively (Grossmann, 2002).

Cell anchorage molecules not only act as a cellular "glue" and mechanical scaffolding to maintain the integrity of a given tissue, but also they are known to provide necessary signals that promote cell growth and survival (Meredith *et al.*, 1996; Assoian, 1997). Loss of cell

anchorage forces the detached cells to undergo programmed cell death, or apoptosis; a phenomenon found to be of relevance for development, differentiation, maintenance of tissue homeostasis and disease (Grossmann, 2002). In principle, every living cell inherits the capacity to commit suicide through different programmed cell death pathways. For normal, adherent, non-transformed cells a synergistic activation of apoptotic pathways follows loss of cell anchorage, whereas transformed tumour cells have the potential to grow in the absence of cell adhesion to the underlying ECM in an 'anchorage *in*dependent' manner; such an acquired potential allows tumour cells to grow at inappropriate sites *in vivo* as occurs during invasion and metastasis (Grossmann, 2002).

Apoptosis following loss of cell anchorage is referred to as "Anoikis"; a term adopted from Greek, meaning 'state of homelessness' (Frisch and Francis, 1994). Alternatively, the survival of tumour aggregates away from their homeland, through adhesion mediated cell-cell contact, was referred to as "Synoikis" (Shen and Kramer, 2004) (Figure 4.2).

It is believed that specific cell-cell and cell-matrix contacts regulate cell growth in epithelial cells, and that disruption of these contacts induces apoptosis (Ruoslahti and Reed, 1994; Hermiston and Gordon, 1995). On the other hand, it also has been reported that cadherin-mediated cell-cell adhesion plays a critical role in the complex network of survival signalling. There is a growing body of literature supporting the fact that E-cadherin plays a key role during the induction and progression of epithelial cancers (e.g. Cavallaro and Christofori, 2004), while its loss and malfunction contributes to increased invasiveness and proliferation in epithelial carcinomas (Margulis *et al.*, 2003). It is rather interesting that, in some tumours, cadherins seem to enhance not only cell growth but also cell survival. Engagement of detached epithelial cells via E-cadherin-mediated adhesion complexes was shown to generate anchorage-independent survival signals rendering the aggregated epithelial cellular masses

anoikis-resistant/synoikotic (Miller and Moon, 1996; Kantak and Kramer, 1998; Day *et al.*, 1999; Shen and Kramer, 2004).

The expression of this homophilic adhesion molecule, E-cadherin, has been linked to several pathways that control cell proliferation, survival and apoptosis, through the interaction with some downstream pathway effectors such as Bcl-2, Akt/PKB and ILK, and/or another cell adhesion molecule that has been reported to play an anti-apoptotic role; namely N-cadherin.

Bcl-2 (B cell leukaemia/lymphoma-2) protein:

This anti-apoptotic member of the apoptosis-regulating family of proteins (Adams and Cory, 2001), has been shown to confer a survival advantage on cells by inhibiting apoptosis. In epithelial cells, Bcl-2 expression is associated with proliferation, tissue development and morphogenesis (Lu *et al.*, 1996). High levels, or irregular patterns, of Bcl-2 expression occur in various carcinomas (Reed *et al.*, 1996; Reed, 1998; 1999). For example, Lu *et al.* (1995) showed that overexpression of Bcl-2, in a mammary epithelial cell line, increased survival of tumour cells but also promoted epithelial-mesenchymal conversion; a conversion which was accompanied by a reduction in levels of the E-cadherin molecule expressed at the cell surface (Lu *et al.*, 1995). Li *et al.* (2003) obtained similar results using MCF-7, a poorly invasive breast carcinoma cell line exhibiting a typical epithelial morphotype. They showed that Bcl-2 overexpression in this cell line led to decreased cell surface E-cadherin and disrupted the membrane localisation of all junctional markers, while interfering with junction assembly (Li *et al.*, 2003). Conversely, Sasaki *et al.* (2000) reported that re-expression of E-cadherin, in a rat breast carcinoma cell line, reduced Bcl-2 expression and resulted in the redistribution of β-catenin. This has led to the suggestion of a possible cross-talk between E-cadherin and Bcl-2 molecules (Sasaki *et al.*, 2000).

Serine/threonine kinase Akt:

Also known as protein kinase B (PKB), Akt is a proto-oncogene and a downstream effector of phosphatidylinositol 3-kinase (PI 3-kinase); an enzyme known for its vital regulatory role in diverse cellular processes, including cell changes propelling cancer progression. The three Akt isoforms (Akt1, Akt2 and Akt3) mediate many of the downstream events regulated by PI-3 kinase (PI3K) such as cell growth, cell proliferation and cell survival (Brazil *et al.*, 2002; Cantley, 2002; Vivanco and Sawyers, 2002). Furthermore, Akt/PKB activation has been reported to occur in many human epithelial cancers. For example, total Akt/PKB kinase activity is increased in non-small cell lung cancer, squamous cell carcinoma of the oral cavity as well as breast and prostate carcinoma (Nakayama *et al.*, 2001a; Sun *et al.*, 2001; Brognard *et al.*, 2001). Additionally, Akt-2 overexpression has been found to be associated with an undifferentiated histological pattern and more aggressive clinical behaviour in ovarian cancer (Bellacosa *et al.*, 1995); perhaps suggesting that Akt/PKB actually can contribute to tumour progression.

In oral squamous cell carcinoma, this kinase also was shown to induce epithelial-mesenchymal transition (EMT); a phenotypic change characterised by the downregulation of epithelial cell-specific proteins, including E-cadherin and β-catenin, and the upregulation of the mesenchymal cell-specific protein, vimentin (Grille *et al.*, 2003). This EMT was accompanied by increased motility and invasiveness of the engineered squamous carcinoma cell line (Grille *et al.*, 2003). These findings suggested that Akt/PKB kinase may be an important therapeutic target for the control of tumour cell invasion and, eventually, for the control of the metastatic behaviour of malignant cancers.

It has been reported that homophilic E-cadherin-mediated adhesion initiates the activation of PI 3-kinase followed by downstream activation of the Akt/PKB protein (Pece *et al.*, 1999; Kovacs *et al.*, 2002). Activation of Akt protein requires phosphorylation on both threonine

308 (Thr-308) and serine 473 (Ser-473). Phosphorylation of Thr-308 occurs when Akt is recruited to the cell membrane but it is not sufficient for activation of the kinase activity, which requires further phosphorylation of Ser-473 (Alessi *et al.*, 1996). Activated Akt further phosphorylates several substrates that can result in suppression of apoptosis and stimulation of cell growth (Datta *et al.*, 1999). Again the inference from these diverse studies is that there is some connection, or interaction, between E-cadherin and Akt/PKB; at least at the functional level.

Integrin Linked Kinase (ILK):

ILK is a PI-3 kinase-dependent serine/threonine kinase, originally identified as a β1 integrin-binding protein (Hannigan *et al.*, 1996) that binds to the β- cytoplasmic domains of both β1 and β3 integrins, and phosphorylates the β1 cytoplasmic domain *in vitro* (Hannigan *et al.*, 1996). It is thought to play a role in mediating interactions between integrins and the actin cytoskeleton, through interaction with multiple different proteins, in a variety of cell types (Zervas *et al.*, 2001; Wu and Dedhar, 2001; Nikolopoulos and Turner, 2001).

It has been reported that ILK is a major contributor to the phenotypic alteration of transformed epithelial cells, such that cells overexpressing ILK exhibit an induced epithelial-mesenchymal transition (Somasiri *et al.*, 2001; Lee *et al.*, 2004). Added to this role, several studies have suggested that ILK also plays a vital role in the control of cell survival (Fukuda *et al.*, 2003; Friedrich *et al.*, 2004). ILK overexpression was shown to disrupt cell-cell adhesion and has been suggested to induce anchorage-independent cell survival in the transfected cells (Hannigan *et al.*, 1996). It also has been reported that the crucial contribution of ILK to cell survival is conferred via its ability to activate Akt/PKB protein optimally which, as outlined above, is an important signalling mediator for the cell survival pathway (Persad *et al.*, 2000; Fukuda *et al.*, 2003; Troussard *et al.*, 2003; Friedrich *et al.*, 2004).

A number of studies have incriminated ILK in the regulation of E-cadherin expression (Wu *et al.*, 1998; Somasiri *et al.*, 2001; Li *et al.*, 2003; Oloumi *et al.*, 2004). ILK overexpression has been shown to result in the disruption of cell-cell adhesion, loss of E-cadherin expression and the acquisition of a fibroblastic morphotype (Hannigan *et al.*, 1996; Somasiri *et al.*, 2001; Li *et al.*, 2003). Such findings were further supported by the observation that inhibiting ILK activity in human cancer cell lines has resulted in upregulation of E-cadherin expression (Tan *et al.*, 2001). In contrast, Vespa *et al.* (2003) showed there was a substantial reduction of ILK activity associated with Ca^{2+}-induced keratinocyte differentiation, although the exogenous expression of ILK in their model system did not induce EMT or even E-cadherin degradation and loss of intercellular adhesion (Vespa *et al.*, 2003). Such findings though are in contrast to other model systems which have used different epithelial cells (Wu *et al.*, 1998; Somasiri *et al.*, 2001; Lee *et al.*, 2004) and this may be indicative of the possibility that there is considerable variation between different cell types.

The Cadherin switch:

Associated with the metastatic progression of epithelial tumour cells is the dynamic regulation of cadherin expression. Loss, or reduced expression, of E-cadherin has been detected in the majority of carcinomas, and often has been correlated with increased invasiveness and poorer survival (Dorudi *et al.*, 1993; Bringuier *et al.*, 1993; Kawakita *et al.*, 1994; Lipponen and Eskelinen, 1995; Miyazaki *et al.*, 1995; Mialhe *et al.*, 1997). On the other hand, the upregulation of other members of the cadherin family in carcinomas, particularly N-cadherin, was found to function in cancer progression and contribute to the invasive phenotype (Islam *et al.*, 1996; Tran *et al.*, 1999; Hazan *et al.*, 2000; Li *et al.*, 2001). It has been documented that, associated with the loss of E-cadherin in some human cancers, there was an increased expression of other mesenchymal cadherins, like N-cadherin and cadherin-11

(Li and Herlyn, 2000; Tomita *et al.*, 2000), thus suggesting that N-cadherin, and perhaps other mesenchymal cadherins, have an opposite effect as compared to E-cadherin. This cadherin conversion has been referred to as the "Cadherin switch"; a phenomenon thought to support the transition from a benign to an invasive, malignant tumour phenotype (Tomita *et al.*, 2000; Hazan *et al.*, 2004). This switching of cadherin subtypes recapitulates a well known phenomenon that occurs during development; where epiblast cells switch from expressing E-cadherin to expressing N-cadherin, to ingress the primitive streak (Edelman *et al.*, 1983; Hatta and Takeichi, 1986). Also, the differential expression pattern of E- and P-cadherin during hair follicle development, skin morphogenesis and tooth formation, is another example of cadherin switch in normal tissues (Hardy and Vielkind, 1996; Jamora and Fuchs, 2002).

E-cadherin and N-cadherin are both classical cadherins and, as a first impression, they might seem likely to be implicated in similar mechanisms of cell-cell adhesion. Yet, unlike E-cadherin, N-cadherin is unable to support the epithelial phenotype, and as polarity is lost, N-cadherin has been proposed to promote a dynamic adhesion state in tumour cells, allowing not only dissociation of tumour cells from the tumour mass and enhancement of invasion and migration of tumour cells, but also allowing for their interaction with fibroblasts, vascular endothelial cells, and other stromal components (Hazan *et al.*, 2000; Li and Herlyn, 2000; Tomita *et al.*, 2000). Thus, this switching of cadherin subtypes might affect tumour-host cell adhesion, tumour cell invasion and tumour cell migration.

N-cadherin also has been suggested to function as an anti-apoptotic protein. Thus Tran *et al.* (2002) demonstrated a signal transduction cascade that linked the N-cadherin-catenin adhesion complex to upregulation of the anti-apoptotic protein, Bcl-2. It also was proposed that N-cadherin-mediated homophilic adhesion, in the same model system, can initiate anti-

apoptotic signalling through the activation of Akt/PKB (Tran *et al.*, 2002); results somewhat similar to reports that E-cadherin ligation also is implicated in the regulation of the PI-3 kinase signalling pathway (Pece *et al.*, 1999; Kovacs *et al.*, 2002; Rieger-Christ *et al.*, 2004). Thus, the consensus of all these findings is that both E-cadherin and N-cadherin have the potential to activate the Akt signalling pathways.

Potential fate of the E-cadherin molecule in apoptosis:

In addition to β-catenin and plakoglobin, E-cadherin also is reported to be cleaved in epithelial cells as part of the changes which occur during the execution phase of programmed cell death; otherwise known as apoptosis. It has been suggested that simultaneous cleavage of the intracellular and extracellular domains of E-cadherin provides an highly efficient mechanism to disrupt cell-cell cohesion enabling apoptotic cells to round up and exit from the epithelium (Steinhusen *et al.*, 2001). Different types of proteolytic cleavage of the E-cadherin molecule have been documented. For example, it has been shown that caspase-3 mediates a cleavage that releases the cytoplasmic domain of the E-cadherin molecule, and that metalloproteinase sheds the extracellular domain from the cell surface during apoptosis (Steinhusen *et al.*, 2001). In support of the theory that E-cadherin may play a central role in adhesion-dependent survival and dissociation-induced apoptosis, are the findings of Vallorosi *et al.* (2000), who associated the loss of cell-cell adhesion with the rapid truncation of the β-catenin binding domain of the E-cadherin molecule. This resulted in the generation of two cleavage products, one of 97 kDa, specified as the membrane-bound fragment, and another of 35 kDa, specified as the cytoplasmic-free fragment, whose activity was shown to be correlated with reduced cell-cell adhesion (Vallorosi *et al.*, 2000).

Solid tumours usually grow as three-dimensional masses. A substantial body of literature has emphasised the importance of the physical three-dimensional nature of the ECM; a

condition more relevant to the *in vivo* milieu encountered by tissue invasive cells. The impact of this physical structure in regulating normal cell behaviour, including cell proliferation, has been considered by a number of authors (Bissell and Radisky, 2001; Cukierman *et al.*, 2001; Walpita and Hay, 2002; Grinnell, 2003).

Hotary *et al.* (2003) adopted the idea of studying this aspect of cell behaviour in the context of 3-D culture conditions. They compared tumour cell proliferation in 2-D and 3-D collagen type I cultures, and reported that the 3-D physical restraints were able to prevent proliferation in tumour cells. They found that some of these cells exhibited a compact, spherical morphotype with the actin filaments assembled at the cell periphery (Hotary *et al.*, 2003). However when such tumour cells were producing the membrane-anchored matrix metalloproteinase (MT1-MMP), they exhibited a stretched-out morphotype with actin filaments reorganised into centrally located robust stress fibres (Hotary *et al.*, 2003). In previous work Chen *et al.* (1997) showed that cell shape, and the associated organisation of the cytoskeleton, played a role in cell growth and apoptosis (Chen *et al.*, 1997). A possible interpretation of the Hotary *et al.* (2003) results would be that the capacity of tumour cells to grow is linked to their ability to alter cell shape. Such results further support the importance of utilising a 3-D microenvironment when conducting different biochemical experiments on cellular growth parameters.

Much of the data which I have discussed above actually have been derived from studies on tumour cells growing within the confines of two-dimensional tissue culture dishes. Because solid tumours usually grow as three-dimensional masses, I have been interested in understanding the impact of E-cadherin re-expression on the growth properties of my tumour cells in the context of three-dimensional collagen I culture conditions; a system more

likely to simulate the normal physiological milieu of the human biological environment than two-dimensional growth.

The main aim of this chapter was to detect any differences in the cells, in relation to E-cadherin expression, to any of the previously listed cell survival molecules and to assess such differences in the context of 3-D collagen I culture conditions.

4.2 Results

4.2.1 Analysis of expression of proteins associated with cell survival in E- cadherin transfected cell lines growing on tissue culture plastic

Cells were allowed to grow as a monolayer on serum-coated tissue culture plastic for 5 days. When cells had just reached confluency, total RIPA cell lysates were prepared, as described in Chapter 2 Section 2.3. Using the appropriate antibody concentration (Appendix II, Table II.3), Western blots for both the H376 E-cadherin cell line and its antisense counterpart (H376 antisense) were probed for proteins that have been linked to cell survival, such as Bcl-2, Akt-1, phospho-Akt, ILK and N-cadherin proteins.

Western blot analysis, using Anti-Bcl2 (clone 100) monoclonal antibody (Upstate), consistently, showed a two-fold decrease in Bcl-2 expression associated with *de novo* expression of E-cadherin in my OSCC cell lines. Figure 4.3 (a) shows a representative Western blot analysis and a histogram, obtained from results of four independent experiments, illustrating a statistically significant reduction of Bcl-2 levels in the H376 E-cadherin cell line as compared to the antisense counterpart (p value ≤ 0.004).

Using monoclonal anti-Akt-1 (clone 2H10) antibody (Cell Signaling) the Western blot results showed a slight, yet significantly different, reduction (p value ≤ 0.001), in total Akt-1 levels associated with re-expression of the E-cadherin molecule in my OSCC cell lines. Figure 4.3 (b) shows a representative Western blot and a histogram summarising data obtained from three different experiments.

At the same time, Western blot analysis also was used to evaluate the levels of phosphorylation of the Akt/PKB protein at the Thr-308 location as a consequence of the *de novo* expression of the E-cadherin molecule. There was nearly a 1.5 fold, statistically significant, increase in levels of phospho-Akt (Thr-308) in the H376 E-cadherin cell line as

compared to the antisense counterpart (p value ≤ 0.001) (Figure 4.3: c). Figure 4.3 (c) shows a representative Western blot that has been probed with monoclonal anti-phospho-Akt (Thr308) antibody (Cell Signaling), and histogram representing data of three different individual experiments.

Previous work has linked the E-cadherin molecule to ILK in that ILK overexpression was shown to result in the loss of E-cadherin expression (Somasiri *et al.*, 2001; Li *et al.*, 2003). Conversely it also was shown that ILK was reduced substantially upon Ca^{2+}-induced E-cadherin-dependent cell-cell junction assembly and keratinocyte differentiation (Vespa *et al.*, 2003). This 'cross-talk'-like interaction between the E-cadherin molecule and ILK was investigated in my OSCC model system. Western blot results, obtained from four independent experiments using an anti-ILK monoclonal antibody (BD Transduction Laboratories), consistently showed a slight reduction, that was not statistically different (p value ≤ 0.2), in ILK levels associated with the re-expression of E-cadherin in my OSCC cells (Figure 4.3: d).

Preliminary results from the previous chapter suggested that *de novo* E-cadherin expression in my OSCC cell lines resulted in the downregulation of N-cadherin; a previously observed phenomenon that has been referred to as the "Cadherin switch" (Tomita *et al.*, 2000; Hazan *et al.*, 2004). The N-cadherin molecule also has been proposed to play an anti-apoptotic role, which made me think it would be sensible to investigate the expression of this protein further in my system. Western blot results, from three independent experiments, using anti-N-cadherin antibody (BD Transduction Laboratories), consistently showed about a two-fold reduction in N-cadherin levels as a consequence of *de novo* expression of the E-cadherin molecule. Results were significantly different between the H376 E-cadherin cell line and the antisense counterpart (p value ≤ 0.03) (Figure 4.3: e).

4.2.2 E-cadherin transfected OSCC cell lines in the context of 2-D and 3-D collagen I culture conditions

4.2.2.1 Gross morphology of E-cadherin transfected cell lines in 2-D/3-D collagen I cultures

A number of papers have reported that E-cadherin positive cells adopted a rounded morphology when implanted in 3-D cultures (St Croix *et al.*, 1998; Hotary *et al.*, 2003; Shen and Kramer, 2004). Somewhat in contrast to these findings, and as observed under contrast-phase microscopy, my transfected cell lines (both the E-cadherin sense and the antisense) manifested the same morphological appearance and formed spherical aggregates when they were implanted in 3-D Matrigel cultures though not when they were implanted in 3-D collagen I cultures (Figure 4.4). In fact in the 3-D collagen I cultures my transfected cell lines exhibited a more stretched fibroblastic morphotype and formed 'net-like' colonies with strands of communicating cells (Figure 4.4). There was no apparent difference between the E-cadherin positive and E-cadherin negative cell lines; clearly indicating that expression of this molecule had no impact on the gross appearance of my cells.

4.2.2.2 Growth assay in 2-D/3-D collagen I cultures

In order to study the role of the E-cadherin molecule in modulating cell growth and survival in 3-D culture conditions the growth of my transfected cell lines (H376 E-cadherin and H376 antisense) was assessed under both 2-D and 3-D collagen I culture conditions over a 10 day period (as described in Chapter 2, Section 2.6.1.2). Results showed that under 2-D collagen I culture conditions there was a marked difference between the sense and antisense cell lines. Starting with $2x10^4$ cells, for each experimental well, H376 antisense cells were able to grow to up to 6.4×10^5 cells by day 10. In contrast, H376 E-cadherin cells grew to only 3.3

x 10^5cells within the same time (Figure 4.5). Under 3-D collagen I culture conditions, these differences were abrogated. Starting with the same cell number as in 2-D cultures ($2x10^4$ cells per experimental well), H376 E-cadherin cells grew to 7 x 10^5 cells by day 10, versus 7.9 x 10^5 cells for the H376 Antisense line (Figure 4.5).

By growing my transfected cell lines in three-dimensional culture, as compared to cells growing in two-dimensional collagen I culture, and during the first 10 days of my growth assay, I could not observe any contact-dependent growth inhibition.

Having conducted these experiments it occurred to me that the 1-2 hour incubation period, a predetermined period of time required to free all cells from 2-D and 3-D collagen I culture using 6mg/ml collagenase 1 (Worthington), might be rather harsh on the cells and also might affect the cell viability. Consequently, I tested this possibility and I found (data not shown) that for cells growing for 8 days in 2-D/3-D collagen I cultures that after 2 hours incubation with collagenase 1, only 2-5% of cell were not viable (as tested with trypan blue exclusion) in 2-D cultures, compared to 0.7-0.9% of non-viable cells growing in 3-D cultures. Accordingly I concluded that the viability of the cells was not affected dramatically by culture conditions nor by the harvesting methods.

4.2.2.3 Apoptosis and cell vitality Assay

Apoptosis was assessed in 2-D and 3-D collagen I cultures using the immunostaining-based, dUTP nick-end labelling (TUNEL) assay. Because apoptosis in adherent cultures involves the detachment of cells from the substrate, supernatant was collected from each sample and the viability of detached cells was tested using Trypan blue exclusion. Results showed that, under 2-D collagen I culture conditions, E-cadherin restoration was associated with an increased apoptotic response in the H376 E-cadherin cell line as compared to the H376 antisense control cell line. TUNEL assay results showed that re-expression of E-cadherin

increased the apoptotic response by 4.1%, p value ≤ 0.03 (Experiment I) and by 3.7%, p value ≤ 0.02 (Experiment II) (Table 4.1). Moreover, the detached H376 E-cadherin cells showed 30.9% viability as compared to 52.8% viability in the H376 antisense cells (p value ≤ 0.003), in Experiment I, similar results were obtained in Experiment II, where the H376 E-cadherin cells showed 31.3% viability as compared to 51.4% viability in the H376 antisense cells (p value ≤ 0.001) (Table 4.2). However, in the 3-D collagen I culture conditions, the apoptotic profile of both the H376 E-cadherin cell line and the H376 antisense counterpart cell line did not show any statistical significance (Experiment I, p value ≤ 0.2; Experiment II, p value ≤ 0.1) (Table 4.3), suggesting that E-cadherin restoration in 3-D collagen I conditions had no effect on the apoptotic potential of the transfected cell line.

Table 4.1: Enumeration of Apoptotic cells in 2-D collagen I cultures

		H376 E-cadherin			H376 antisense		
	Field No.	Total No. of cells/Field	No. of apoptotic cells	Apoptosis %	Total No. of cells/Field	No. of apoptotic cells	Apoptosis %
Experiment I	1	30	2	6.7	30	0	0.0
	2	40	2	5.0	30	0	0.0
	3	33	1	3.0	33	0	0.0
	4	33	0	0.0	34	0	0.0
	5	35	2	5.7	40	0	0.0
	Mean	**34.2**	**1.4**	**4.1**	**33.4**	**0**	**0.0**
Experiment II	1	41	0	0.0	28	0	0.0
	2	25	1	4.0	25	0	0.0
	3	32	2	6.3	40	0	0.0
	4	35	2	5.7	37	0	0.0
	5	28	1	3.6	33	0	0.0
	Mean	**32.2**	**1.2**	**3.7**	**32.6**	**0**	**0.0**

Table 4.2: Viability of detached cells from 2-D collagen I cultures

		H376 E-cadherin			H376 antisense		
	Sample No.	Viable cells No. (x 10^6)	Total cells No. (x 10^6)	Viability %	Viable cells No. (x 10^6)	Total cells No. (x 10^6)	Viability %
Experiment I	1	0.018	0.064	28.1	0.029	0.055	52.9
	2	0.019	0.061	31.2	0.030	0.064	47.5
	3	0.015	0.045	33.3	0.039	0.081	48.0
	4	0.013	0.044	29.6	0.031	0.066	47.1
	5	0.020	0.065	30.0	0.031	0.067	46.2
	6	0.019	0.055	34.1	0.046	0.061	75.0
	Mean	**0.019**	**0.056**	**30.9**	**0.034**	**0.066**	**52.8**
Experiment II	1	0.025	0.066	37.5	0.034	0.057	58.8
	2	0.013	0.049	27.5	0.036	0.067	53.8
	3	0.019	0.067	29.0	0.031	0.066	47.1
	4	0.013	0.044	30	0.027	0.055	48.9
	5	0.020	0.065	31.1	0.028	0.064	44.5
	6	0.018	0.055	32.7	0.045	0.081	55.0
	Mean	**0.018**	**0.058**	**31.3**	**0.033**	**0.065**	**51.4**

Table 4.3: Enumeration of Apoptotic cells in 3-D collagen I cultures

		H376 E-cadherin			H376 antisense		
	Field No.	Total No. of cells/Field	No. of apoptotic cells	Apoptosis %	Total No. of cells/Field	No. of apoptotic cells	Apoptosis %
Experiment I	1	24	2	8.3	23	4	17.4
	2	20	1	5.0	10	2	20.0
	3	34	2	5.9	17	1	5.9
	4	19	0	0.0	17	2	11.8
	5	20	1	5.0	30	1	3.3
	6	30	2	6.7	25	3	12.0
	7	23	1	4.3	35	1	2.9
	8	23	2	8.7	33	0	0.0
	Mean	**24.13**	**1.38**	**5.7**	**23.75**	**1.75**	**7.4**
Experiment II	1	22	1	4.5	33	2	6.1
	2	31	1	3.2	18	2	11.1
	3	24	2	8.3	11	1	9.1
	4	21	0	0.0	25	1	4.0
	5	19	0	0.0	15	2	13.3
	6	19	1	5.3	22	2	9.1
	7	17	2	11.8	25	2	8.0
	8	24	2	8.3	29	2	6.9
	Mean	**22.13**	**1.13**	**5.1**	**22.25**	**1.75**	**7.9**

Do any of the proteins involved in cell survival, previously shown to be affected by *de novo* E-cadherin expression in my OSCC cell line, growing on tissue culture plastic, contribute to the apoptosis-resistant phenotype?

4.2.2.4 Analysis of expression of proteins associated with cell survival in 2-D/3-D collagen I cultures

In order to analyse the proteins, discussed earlier, in the context of 2-D/3-D collagen I culture conditions, cells were allowed to grow in either condition, for 5 days. Again, total RIPA cell lysates were obtained, as described in Chapter 2 Section 2.3.2, when cells growing in 2-D collagen I culture conditions were just about confluent and when the cells growing in 3-D collagen I cultures had expanded just enough to provide sufficient protein for the subsequent analyses.

Different parameters were tested prior to monitoring protein expression in my collagen I model system, which included:

Monitoring the effect of the extended exposure time to different collagenases: Cells growing on tissue culture plastic were exposed to collagenase 1 or collagenase 3 for varying lengths of time, and the results showed that up to 2 hours exposure time, the time required to free all cells off the collagen I matrix, resulted in cells exhibiting over 95% viability (data not shown).

Monitoring the difference between collagenase 1 and 3: Results showed that there was no difference in the length of time required for the collagenase 1, or collagenase 3, solutions to dissolve the collagen I matrix. Both enzyme preparations required from 1 to 2 hours to dissolve collagen I matrix completely. Nor was there a difference in the Western blot results when either of these collagenases was used (data not shown).

Having established the working parameters I then used Western blot analysis to monitor the effect of *de novo* E-cadherin expression in the context of 2-D and 3-D collagen I cultures, in my OSCC model system, on the expression of Bcl-2, Akt-1, Phospho-Akt and ILK proteins; as discussed previously these all are proteins that have been linked to cell survival, by other investigators.

4.2.2.4.1 Effect of E-cadherin on Bcl-2 expression in 2-D/3-D collagen I cultures

De novo E-cadherin expression in my OSCC cell lines resulted in a maintained trend, as compared to the results obtained from preliminary Western blot analysis, in 2-D collagen I cultures but not in 3-D cultures, where restoration of E-cadherin expression in my transfected cell line has resulted in downregulation of total Bcl-2 levels. Figure 4.6 (b) presents a representative Western blot result, while Figure 4.6 (a) shows a histogram illustrating results obtained from four independent experiments. Data show that E-cadherin re-expression downregulated Bcl-2 levels in the H376 E-cadherin cell line, growing in 2-D collagen I cultures, as compared to the antisense counterpart (p value ≤ 0.01). However, that effect was not significantly different when cells were plated in 3-D collagen I culture (p value ≤ 0.1).

4.2.2.4.2 Effect of E-cadherin re-expression on Akt/PKB expression and activation in 2-D/3-D collagen I cultures

I have utilised my 2-D/3-D collagen I model system to determine whether E-cadherin may play a role in the activation of the PI3-K pathway. First I analysed the effect of E-cadherin re-expression on total levels of Akt/PKB protein, and secondly I examined the phosphorylation of a downstream substrate of Akt.

Figure 4.7 (b), shows a representative Western blot of total Akt-1 levels in my transfected cell lines, both in 2-D and 3-D collagen I culture conditions. While, Figure 4.7 (a) shows a histogram that illustrates results obtained from three independent experiments, showing that E-cadherin re-expression was associated with a reduction in total Akt-1 levels in the E-cadherin positive cells (H376 E-cadherin) growing in 2-D collagen I cultures. When this reduction was compared to the antisense counterpart (H374 antisense) the change, though small, was significantly different (p value ≤ 0.04). On the contrary, this downregulation was not observed in cells growing in 3-D collagen I culture conditions, where, instead, E-cadherin re-expression showed a reversal of this finding and resulted in upregulation of total Akt-1 levels. However, upregulation of total Akt-1 levels, in the transfected cell lines, appeared to be solely a consequence of their maintenance under 3-D growth conditions rather than a consequence of the *de novo* expression of E-cadherin. Thus, in 3-D collagen I culture, there was no significant difference between H376 E-cadherin and the antisense counterpart (p value ≤ 0.1) in terms of Akt-1 levels.

Western blot analysis also was used to evaluate the levels of phosphorylation of the Akt/PKB proetin at the Thr-308 location and how this varied as a consequence of the *de novo* expression of the E-cadherin molecule in 2-D versus 3-D collagen I culture conditions. Figure 4.8 (b) shows a representative Western blot of phospho-Akt (Thr-308) levels in my transfected cell lines. Figure 4.8 (a) illustrates results from three independent experiments, plotted on a histogram, showing a significant 7-fold increase in phosphorylation of Akt in the H376 E-cadherin cell line growing in 3-D collagen culture as compared to the antisense counterpart cell line (p value ≤ 0.01). Cells growing on a 2-D collagen I matrix showed no significant difference in phospho-Akt (Thr-308) levels between E-cadherin sense and antisense cells (p value ≤ 0.2).

4.2.2.4.3 Effect of E-cadherin re-expression on ILK expression in 2-D/3-D collagen I cultures

Using Western blot analysis, I showed that growing my cells in either 2-D or 3-D collagen I culture conditions was associated with the upregulation of ILK levels in E-cadherin positive cell lines as compared to the antisense control cell lines. Figure 4.9 (b) shows a representative Western blot analysis of ILK expression in my transfected cell lines growing under 2-D and 3-D collagen I culture conditions. Figure 4.9 (a) shows a histogram illustrating results obtained from four independent experiments, showing a statistically significant increase in ILK levels in H376 E-cadherin cell lines in both 2-D collagen I cultures (p value ≤ 0.05) and in 3-D collagen I cultures (p value ≤ 0.002), as compared to the antisense counterpart cell lines.

4.2.2.4.4 Effect of E-cadherin re-expression on N-cadherin expression in 2-D/3-D collagen I cultures

Western blot results showed that the downregulation of N-cadherin, as a consequence of *de novo* E-cadherin expression in my OSCC cell lines, was reversed when cells were embedded in 3-D collagen I culture. Figure 4.10 (b) illustrates a representative Western blot experiment, while Figure 4.10 (a) shows a histogram, obtained from one of two identical experiments, denoting a two-fold decrease in N-cadherin levels in the H376 E-cadherin cell line, growing on serum-coated tissue culture plastic, as compared to its antisense counterpart. These findings are consistent with the reports of others (Chen *et al.*, 2004).

On the other hand, this difference in N-cadherin expression was hardly noticeable when cells were grown atop of collagen I in 2-D cultures. In contrast to these findings, cells growing in 3-D collagen I cultures actually showed a reversal of this result such that the N-cadherin level

was increased by about 1.7 fold in the H376 E-cadherin cell line as compared to the antisense control cell line.

4.2.2.5 E-cadherin expression as a consequence of growth conditions in 2-D/3-D collagen I cultures

While conducting Western blot analysis in my model system, where I grew my transfected cell lines under three different culture conditions, (serum-coated tissue culture plastic, atop of collagen I in what I termed 2-D cultures and embedded in a collagen I milieu termed 3-D cultures), I also assessed the expression of the E-cadherin molecule. As shown in Figure 4.11 (a), using the monoclonal anti-E-cadherin antibody (HECD-1), the following features were observed:

The E-cadherin molecule appeared as double bands, a thick band of 120 kDa molecular weight and a faint band of 79 kDa molecular weight, when the H376 E-cadherin cell line was grown on serum-coated tissue culture plastic.

The E-cadherin molecule appeared as only the lower molecular weight band, 79 kDa in size when the H376 E-cadherin cell line was grown in collagen I cultures, whether under 2-D or 3-D growth conditions.

Regarding the first observation, the E-cadherin double band appearance, I investigated the cause and showed it to be as a result of the increased confluence of the cell cultures. Figure 4.11 (b) showed that, when cells were allowed to grow to only about 60-70% confluency, E-cadherin appeared as a single band of 120 kDa molecular weight. In contrast when cells were allowed to grow to 100% confluency or more I started to detect a double band of 79 kDa molecular weight.

I doubted that this would be the reason why cells growing in 2-D or 3-D collagen I cultures gave rise to the cleaved version of the E-cadherin molecule, because such cells never were allowed to grow to such high confluency before obtaining cell lysates.

In the literature, it has been reported that matrix metalloproteinases have the ability to cleave the extracellular domain of the E-cadherin molecule (Nöe *et al.*, 2001; Steinhusen *et al.*, 2001). This made me think that treating my cells with collagenase 1 or collagenase 3, in order to retrieve them from the collagen I prior to cell lysis, might contribute to this E-cadherin cleavage. In an attempt to investigate this finding, I grew my transfected cell lines on tissue culture plastic to about 80% confluency, before I treated them with either collagenase 1 or collagenase 3, for the same length of time as the cells in collagen I cultures had been exposed to, and then I obtained total RIPA cell lysates. Figure 4.11 (c) shows that cells treated with collagenase 1 or collagenase 3 expressed a cleaved E-cadherin molecule of 79 kDa molecular weight, as compared to the 120 kDa E-cadherin molecule expressed by the un-treated cells.

4.3 Discussion

In an elegantly written review, Schmeichel and Bissell (2003) described a series of experimental models for maintaining cultured cells in 3-dimensional microenvironments sufficiently long enough to make cellular and molecular biological assays possible. The models they described ranged in complexity from a monotypic 3-D culture system to multicellular 'humanised' organs *in vitro* (Schmeichel and Bissell, 2003). Apart from considering any difficulties that can be encountered by experimental intervention, a 3-D model design ideally should recapitulate both the 3-D organisation and the particular function for any given organ. Some 3-D models will, of necessity, represent compromises between the theoretical requirements and the practical realities.

The model I used to conduct my experiments consisted of a 3-D monotypic collagen I culture, that was composed of single cell type, hence the name 'monotypic', but nonetheless appeared to allow replication of the minimum unit of differentiated tissues required for tissue-specific function (Cukierman *et al.*, 2002). Because epithelial cancer cells have acquired an ability to penetrate the basement membrane and invade the sub-adjacent stromal matrix, which is largely composed of collagen I (Hanahan and Weinberg, 2000), I have chosen collagen I as my matrix substrate in order to study the effect of E-cadherin re-expression on growth and survival of OSCC within the context of 3-D physical constraints.

It has been reported that epithelial cells in 3-D culture environments were able to form nests of E-cadherin-dependent three-dimensional aggregates, which have been referred to as multicellular aggregates (MCAs) (Kantak and Kramer, 1998; St Croix *et al.*, 1998; Hotary *et al.*, 2003; Shen and Kramer, 2004). Moreover, studies of 3-D collagen I culture of cell lines that are transfected with E-cadherin cDNA showed MCAs (Jiao *et al.*, 2001). Preliminary results showed that my transfected cell lines (H376 E-cadherin and H376 antisense), regardless of their E-cadherin expression status, appeared as multicellular spherical

aggregates (MCAs) *only* when they were implanted in 3-D Matrigel culture , but *not* in 3-D collagen I cultures. When the same cells were grown in 3-D collagen I culture, they showed altered morphology and abnormal polarity, exhibiting a more stretched, fibroblastic morphotype (Figure 4.4). These results lead me to the conclusion that it is primarily the growth conditions, in particular the nature and composition of the substrate, and not expression of the E-cadherin molecule *per se* that contributed to the formation of MCAs in my cell lines. This result is compatible with the findings of Howlett *et al.* (1995) and Gudjonsson *et al.* (2002); by altering the growth conditions and allowing the same cell line to grow in an interstitial ECM, such as collagen I, as compared to cells growing in 3-D laminin-rich basement membrane substrata, these authors found, that the cells showed altered integrin expression coupled with abnormal polarity and organisation (Howlett *et al.*, 1995; Gudjonsson *et al.*, 2002). Hotary *et al.* (2003) found that when growing in 3-D collagen I cultures their cells, unless they produced MT1-MMP, manifested a compact spherical morphology, otherwise, they exhibited a "stretched-out" morphotype which was attributed to MT1-MMP activity (Hotary *et al.*, 2003). Based on their findings, I suspect that my OSCC cell line is an MT1-MMP producer, since the 3-D constrained cells appeared more like fibroblastic cells. Expression of this metalloproteinase should be considered in future work to check for the validity of this suggestion and, had I had the reagents available, I would have tested this possibility.

Also had I had more time, I would have considered comparing the results obtained from experiments conducted on 2-D/3-D collagen I model systems with results obtained from analogous experiments using Matrigel as a 2-D or 3-D substratum. Alternatively I might have considered utilising a more complex organotypic co-culture model system. Such co-cultures have been shown to allow for epithelial stratification; a differentiated appearance that replicates many of the morphological and functional features of an epithelium *in vivo* (Watt,

1988; Kopan and Fuchs, 1989; Smola *et al.*, 1998; Schmeichel and Bissell, 2003). Thus, using such culture systems, it is possible to conduct experiments to study the biological events associated with malignancy in light of the interaction of transformed cells with normal cells (Fusenig *et al.*, 1983; Fusenig *et al.*, 1994; Mackenzie, 2004; Stark *et al.*, 2004). Taking into consideration the experimental difficulties that could be encountered when trying to retrieve cells out of these cultures, for Western blot analysis, I might also have considered using a modified organotypic co-culture system. The modification that I would suggest is presented in Figure 4.12, and includes both the deposition of a laminin-rich basement membrane beneath the epithelial cells and on top of a set collagen matrix with a nylon filter placed within the collagen matrix with pores, of 2-4μm diameter, small enough to prevent the fibroblasts growing underneath from mixing with epithelial cells living above but, at the same time, large enough to allow for any soluble growth factor or signalling molecules to pass through. This would permit epithelial-fibroblastic interaction to occur yet would still allow the investigator to obtain cellular lysates from either of the two cellular subtypes. Furthermore, the epithelial cells would benefit from a more physiological interaction with the underlying laminin-rich basement membrane leaving them with the entire sub-adjacent collagen I-rich substratum to invade once they have penetrated this basement membrane.

As delineated in the Introduction of this chapter, the E-cadherin molecule, besides being involved in multiple critical cellular events during the organisation of the epithelium, also has been reported to play a vital role in controlling individual epithelial cell fate. A researcher might find two 'sets' of literature about this matter. One 'set' would support the fact that the engagement of the epithelial cells in E-cadherin dependent cell-cell complexes (or the formation of MCAs) initiates anchorage-independent survival pathways (Miller and Moon, 1996; Kantak and Kramer, 1998; Day *et al.*, 1999). In some contrast the other 'set' of

literature, suggests that formation of E-cadherin-dependent cell-cell complexes induces contact-inhibition (or density-dependent) inhibition of growth; suggesting that when epithelial cells reach confluency *in vitro*, a number of energy-demanding activities, such as growth and motility, are contact-inhibited (Chen and Obrink, 1991; Kandikonda *et al.*, 1996; Tsukatani *et al.*, 1997; Li *et al.*, 1998; St Croix *et al.*, 1998). Even more interestingly the induction of apoptosis has been reported to occur in relation to increased density, characterised by overexpression of E-cadherin and other apoptotic signalling molecules, in K-*ras* mutant cell lines (Guerrero *et al.*, 2000). However the work of Guerrero *et al.* (2000) was conducted on an NIH 3T3 cell line; a line that is well characterised to be a fibroblast and, to my surprise, they reported an increased expression of E-cadherin. However it is well known this molecule is not expressed by this subtype of cells. I suspect that perhaps the reagent they used, to blot for E-cadherin, possessed the potential to interact with a similar peptide present in a cell adhesion molecule, possibly N-cadherin, which these cells do express. This means that probably this latter report is not to be accepted completely as being correct in all its findings (Guerrero *et al.*, 2000).

As mentioned earlier, several molecules involved in the induction and control of different cell survival signalling pathways, also have been linked to the E-cadherin adhesion molecule, including Bcl-2, Akt/PKB, ILK and N-cadherin.

In an attempt to test the effect of E-cadherin re-expression, in my OSCC model system, on levels of expression of these molecules when cells were growing under 3-D collagen I cultures, I used Western blot analysis to analyse the levels of these proteins in both E-cadherin expressing cells and in their antisense control counterparts. First, I have tested the levels of these proteins when cells were growing in the traditional serum-coated tissue culture plastic dishes to provide a base line data for subsequent experiments. I then tested the effect

of *de novo* E-cadherin expression on the same survival molecules under 2-D/3-D collagen I culture conditions.

When cells were growing on serum-coated tissue culture plastic dishes, my results showed that *de novo* expression of the E-cadherin molecule in the H376 OSCC cell line resulted in downregulation of the Bcl-2 molecule. In general terms this result was consistent with the findings of Sasaki *et al.* (2000), in that these authors also observed that, upon re-expression of the E-cadherin molecule in rat breast carcinoma cells, those cells showed a downregulation of Bcl-2 levels (Sasaki *et al.*, 2000).

In order to measure the relative level of Akt phosphorylation in each cell line, the ratio of phospho-Akt (Thr-308) to the total Akt-1 levels was measured using the densitometric values of Western blots from three independent experiments (Figure 4.13). In my OSCC cell line, *de novo* E-cadherin expression showed a statistically significant increase in phosphorylation of the Akt (Thr-308) as compared to the antisense control (Figure 4.13). These results were, also, consistent with those of others, where the engagement of epithelial cells into E-cadherin-dependent adhesion complexes led to increased PI3-Kinase-dependent Akt phosphorylation (Pece *et al.*, 1999; Kovacs *et al.*, 2002; Munshi *et al.*, 2002).

My results, also, showed that in four independent experiments, there was a consistent, but not significant, downregulation of ILK as a consequence of E-cadherin re-expression in my OSCC line. This result was somewhat similar to the findings obtained by Vespa *et al.* (2003) who showed that there was a marked reduction in the ILK activity associated with the increased keratinocyte differentiation (Vespa *et al.*, 2003). Moreover, these data were further supported by the observations of others, who suggested that ILK is implicated in the regulation of E-cadherin expression (Hannigan *et al.*, 1996; Somasiri *et al.*, 2001; Tan *et al.*, 2001; Li *et al.*, 2003; Oloumi *et al.*, 2004).

Added to all this, there was a downregulation of N-cadherin, known to act as an anti-apoptotic molecule (Tran *et al.*, 2002), in my E-cadherin positive cell line as compared to the antisense control cell line.

Taken altogether, these data provide evidence that there is an interplay between the pathways regulating cell adhesion and cell death and that this type of 'cross-talk' or interaction does occur between the E-cadherin and the survival molecules.

These initial findings were then expanded by altering the tissue culture conditions, so that cells were grown on, or in, collagen I as an extracellular matrix in both 2-dimensional and 3-dimensional milieus. In these two modified culture conditions, I tested both the growth capacities and the apoptotic profile of my transfected OSCC cell lines. Results showed that, over a 10 day period, under 2-D collagen I culture conditions there were marked differences between the H376 E-cadherin sense cell line as compared to the antisense control cell line; thus E-cadherin re-expression seemed to induce contact-dependent growth suppression; quite possibly through the induction of apoptosis. However, when growing the same cells under 3-D collagen I culture conditions, these differences were abrogated, and the E-cadherin positive cell line seemed to manifest an apoptosis/anoikis-resistant phenotype. This result fits well with the results obtained from other studies, where E-cadherin has been shown to regulate anchorage-independent growth and survival pathways and where engagement of cells into E-cadherin-dependent complexes generates compensatory survival signalling (Kantak and Kramer, 1998; Shen and Kramer, 2004).

The findings, obtained from the growth and apoptosis assays, provided a good basis for me to study previously examined molecules associated with cell survival and cell death pathways, but in the context of 2-D and 3-D collagen I culture conditions.

Because growing my OSCC transfected cell lines in 2-D collagen I cultures seemed to indicate that E-cadherin re-expression triggered contact-dependent growth suppression, associated with enhanced apoptosis, this made me think that such findings might be attributable to the effect of E-cadherin on Bcl-2 and other molecules associated with cell survival. This molecule was, at first sight, apparently downregulated slightly but still it shows a significant difference between the H376 E-cadherin cell line and its antisense counterpart; a finding that mimics results obtained from experiments conducted on tissue culture plastic and agrees with previously published data (Sasaki *et al.*, 2000). However, in 3-D collagen I cultures these differences were abrogated. Based on these findings, and results obtained from the apoptosis assays, in my transfected OSCC cell line model system it seems that E-cadherin-dependent growth suppression and increased apoptosis could, possibly, be due to Bcl-2 suppression.

In 3-D, but not 2-D, collagen I cultures, there was a consistent, statistically significant, increase in the phosphorylation of the Akt (Thr-308) as a consequence of *de novo* E-cadherin expression in my OSCC (Figure 4.13). These results did not appear to be in accord with the results obtained from cells plated on tissue culture plastic, and even seemed to run counter to this scenario, but the PI3-kinase pathways are known to play a central role in many cellular processes, such as mitogenic signalling and cell survival, cytoskeletal remodelling, metabolic control and vesicular trafficking (Wymann and Pirola, 1998), so I can not know for certain if E-cadherin re-expression in my OSCC transfected cell line activates the same signalling pathways when varying the culture conditions. This could be verified by detailed examination of other key role players in each pathway.

With regard to ILK expression in my transfected cell lines, under both 2-D and 3-D collagen I culture conditions, *de novo* E-cadherin expression upregulated ILK levels in H376 E-cadherin cell line as compared to the antisense control cell line (Figure 4.13).

Because ILK is an integrin-linked kinase that has been reported to interact with both β1 and β3 integrins (Hannigan *et al.*, 1996), and knowing that the α2β1 integrin is a well characterised receptor for collagen I (Siljander *et al.*, 2004), I thought it very likely that growth in a collagen I environment could have a substantial impact on ILK expression. From comparing these results with results obtained from my transfected cells growing on tissue culture plastic, I would say that the influence of collagen I binding, with its impact upon ILK levels, is more the reason for changes in growth signals rather than a consequence of the effect of the 3-dimensional microenvironment. Results from Chapter 3 (Figure 3.17) showed that E-cadherin re-expression resulted in a consistent, though slight, upregulation of the β1-integrin subunit. Upregulation of ILK might accompany the increased β1-integrin subunit levels simultaneously as a consequence of E-cadherin re-expression in my OSCC model system; but *only* when cells are allowed to bind to collagen I, thus accounting for my observed results. I would also like to believe that E-cadherin, in the context of collagen I culture conditions, favours the ILK-related cell survival pathway in order to induce cell survival. These findings should be thoroughly studied, and should be tested in future work.

E-cadherin-dependent downregulation of N-cadherin has been observed in cells growing on tissue culture plastic. This modifying effect seems to be blocked when my transfected OSCC cells were plated atop of collagen I matrix in 2-D cultures, where the E-cadherin effect on the expression of N-cadherin seems to fade away. When cells were embedded in 3-D collagen I culture, E-cadherin re-expression, appeared to upregulate N-cadherin rather than reduce its expression.

In 3-D collagen I cultures, there was an upregulation of N-cadherin levels, an upregulation of ILK levels and an increase in Akt phosphorylation as a consequence of E-cadherin re-expression in my OSCC model system. Furthermore, E-cadherin-dependent suppression of

Bcl-2 seems to be blocked when cells were embedded in 3-D collagen I cultures, as compared to cells growing in 2-D culture conditions (both on tissue culture plastic, or atop collagen I). This seems to suggest that, under 3-D collagen I culture conditions, the E-cadherin molecule results in an apoptosis/anoikis-resistant phenotype.

Several mechanisms by which cadherins alter cell survival have been reported. In prostate and mammary epithelial cells, E-cadherin was shown to promote cell survival through the Retinoblastoma (Rb) cell-cycle control pathway (Day *et al.*, 1999). This putative mechanism was supported by the fact that E-cadherin was shown to induce cell survival through activation of the PI3-kinase/Akt pathway in the Madin-Darby canine kidney (MDCK) cell line (Pece *et al.*, 1999), In prostate carcinoma cells N-cadherin was shown to activate the same pathways; an activation which led to a subsequent elevation of Bcl-2 levels (Tran *et al.*, 2002). In human OSCC cells, E-cadherin was shown to promote aggregation-dependent cell survival through the activation of the epidermal growth factor receptor (EGFR) and the downstream MAPK pathway; a biochemical step further shown to control Bcl-2 levels (Shen and Kramer, 2004). Also, in some cell types the 'balance of life and death' will not easily 'tip towards cell death' nor will it 'tip towards cell survival' as a consequence of changes in cellular adhesion although there might be a key regulator that adjusts the balance between these extremes of cell fate. If this scenario was correct then my results might suggest that the E-cadherin molecule seems to function as just such a 'regulator' molecule. This could explain why on the one hand, E-cadherin favoured cells to undergo apoptosis through downregulation of Bcl-2 and upregulation of N-cadherin, while on the other hand, it maintained cell growth and survival through activation/phosphorylation of Akt/PKB. Because transformed cells will have altered their growth characteristics considerably though it might be more important to consolidate these results by performing similar experiments in

non-transformed, putatively 'normal' epithelial cells; this I would have done had I had more time available.

My results indicate that, in my OSCC model system, the E-cadherin molecule controls cell survival through different pathways, depending on, first, the ECM substrate and second, the encountered physical restraints provided by the third dimension of this particular type of tissue culture. When cells were growing on the conventional tissue culture plastic dishes, E-cadherin appeared to control cell survival mainly through activation of the PI3-kinase/Akt pathway. However when cells were embedded in 2-D collagen I cultures, E-cadherin was shown to activate another survival pathway, i.e. through the upregulation of ILK. Finally in 3-D collagen I cultures, E-cadherin enhanced the activation of the PI3-kinase/Akt pathway and, it seems, it might also have utilised an additional pathway to promote cell survival, through the ILK signalling pathway. This effect was augmented further by upregulation of the anti-apoptotic cell adhesion molecule, N-cadherin.

Shen and Kramer (2004) ingeniously argued that, in contrast to many tumours such as breast, colon and prostate which mostly tend to be anchorage-independent, head and neck SCCs are known generally to be anchorage-dependent (Rheinwald and Beckett, 1980). This might explain why assembly of tumour cells into MCAs provides a survival benefit in this particular type of carcinoma (Shen and Kramer, 2004).

In summary, taken all together, results in this chapter lead to the conclusion that the adhesion status of the cells may control individual cell fate, including cell survival or death by apoptosis. Furthermore, changes in cellular events observed in 2-D tissue culture conditions might not necessarily be the same when cells are growing under 3-D culture conditions. From my results it is evident that I was able to repeat and verify some of the results reported by others. However when I tried to test these findings under 3-D conditions the results often

came out diametrically opposite to those described previously in the litrature. Given that the same cell line was used throughout, it is clear that it was the growth conditions which changed the results and not the fact that I was using a different cell line from other investigators. Such results suggest a need for caution when studying tumour cell growth exclusively in monolayer cultures. Perhaps, at a fundamental level, the biological functions of individual molecules should be investigated within the context of different microenvironments, because cross-talk between cells as well as communication between cells and the extracellular matrix exert profound effects on cellular functions.

Figure 4.1

Schematic representation of the metastatic process

The metastatic process consists of multiple, highly complicated steps, all of which must be completed successfully to give rise to a metastatic tumour mass (adapted from (Marshall, 1999; Christofori and Semb, 1999) .

a) Cells in primary tumour lose their capacity for cell-cell adhesion.

b) Downregulation of E-cadherin and loss of E-cadherin-dependent cell-cell cohesiveness takes place and cells begin to dissociate and depart from the primary tumour mass.

c) Integrin-dependent proteolysis of the basement membrane and initiation of migration and invasion of tumour cells through stroma towards blood or lymph vessels.

d) Initiation of co-ordinated signalling pathways to provide the necessary signals for growth and survival of the tumour mass at distant secondary metastatic site, perhaps through the formation of E-cadherin-dependent multi cellular aggregates (MCA's).

Figure 4.1

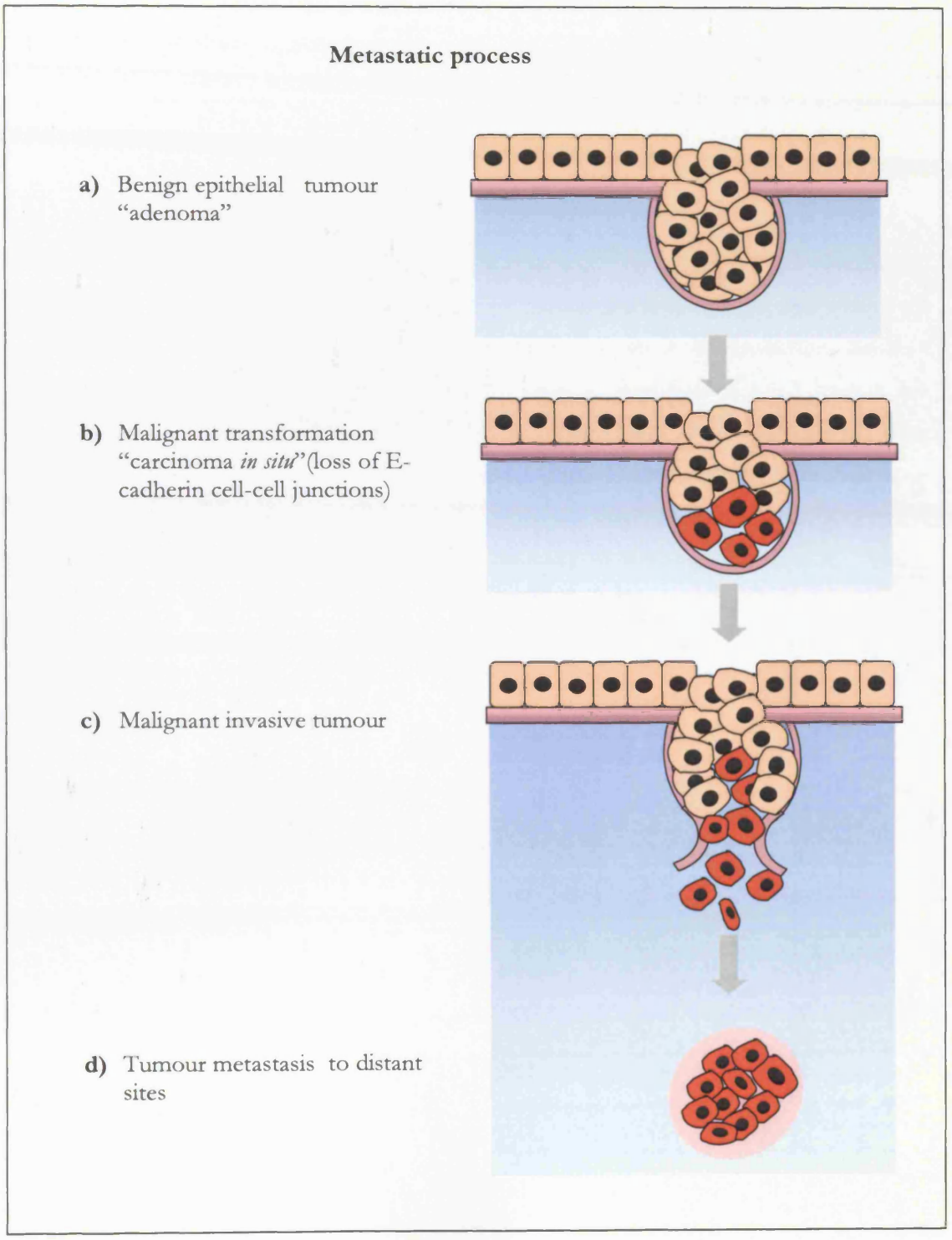

Metastatic process
a) Benign epithelial tumour "adenoma"
b) Malignant transformation "carcinoma *in situ*"(loss of E-cadherin cell-cell junctions)
c) Malignant invasive tumour
d) Tumour metastasis to distant sites

Figure 4.2

Cell adhesion-mediated cell survival

Cell-cell and cell-matrix interactions anchor the epithelial cells and provide necessary signals for survival. Loss of both leads to rapid initiation of cell death through "anoikis". Tumour cells survive this loss of anchorage through formation of organised colonies through a process termed "synoikis" (Shen and Kramer, 2004).

Figure 4.2

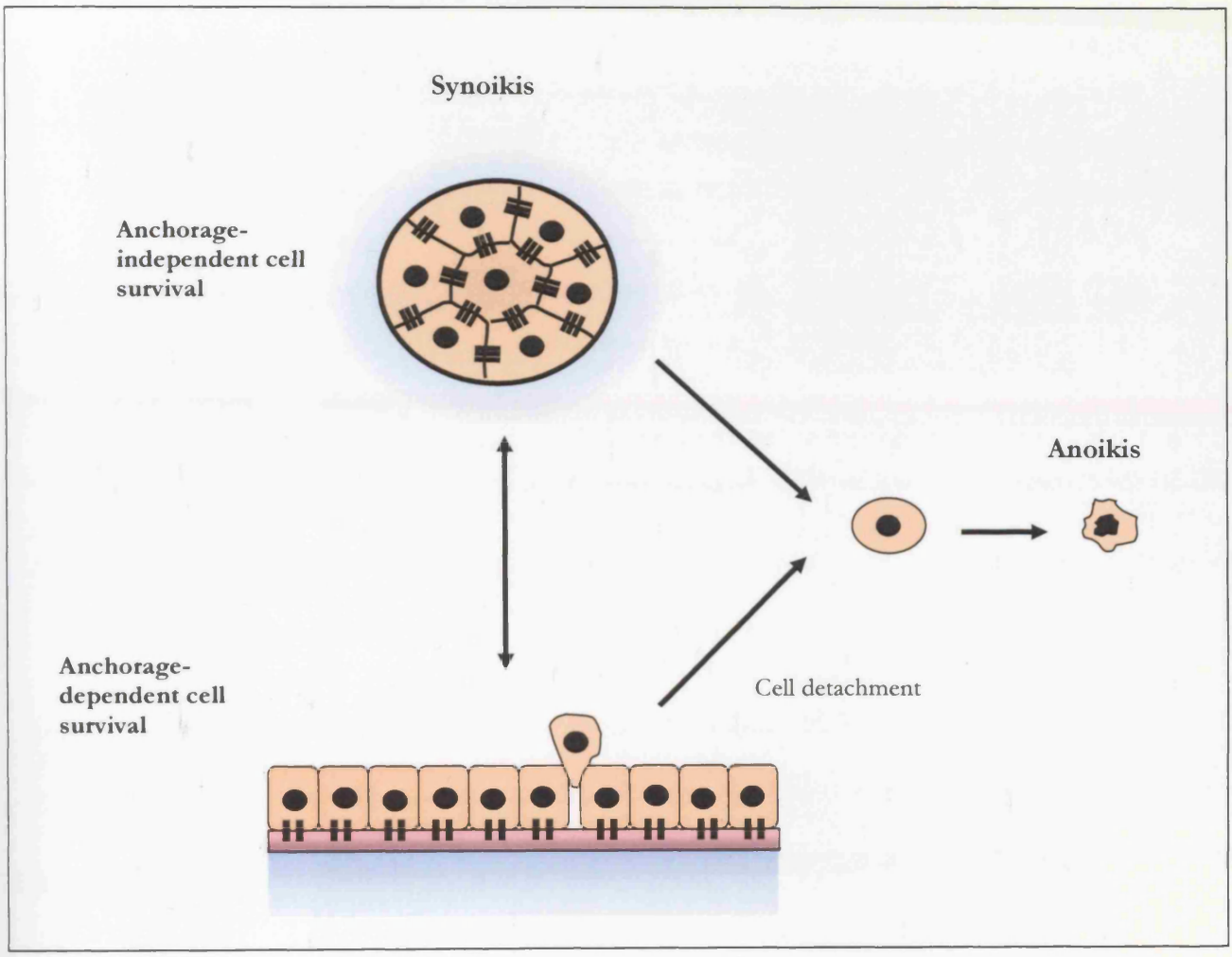

Synoikis
Anchorage-independent cell survival
Anoikis
Anchorage-dependent cell survival
Cell detachment

Figure 4.3

Western blot analysis of cell survival proteins in E-cadherin transfected cell lines growing on tissue culture plastic dishes

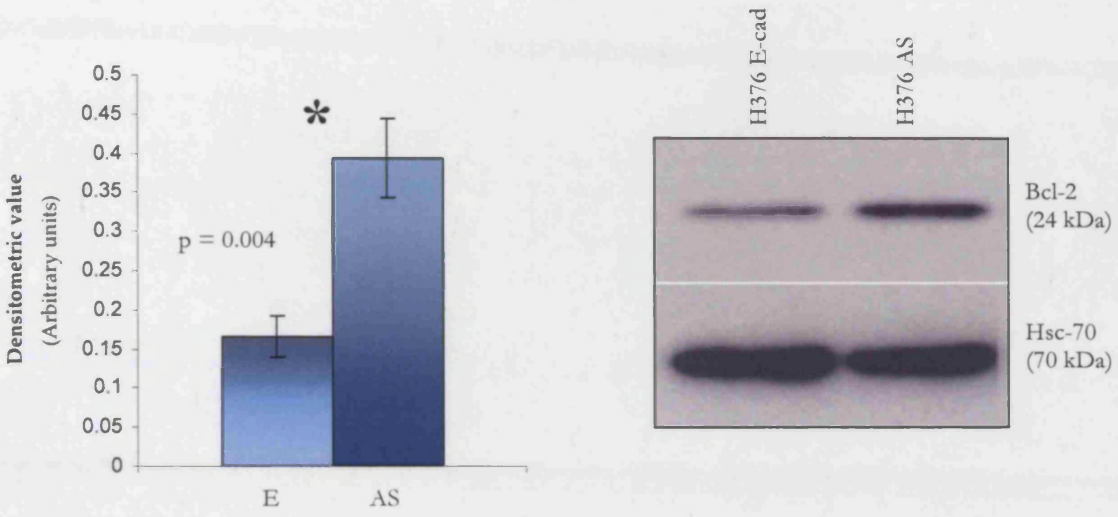
Equal cell numbers for each cell line were plated on serum-coated tissue culture T-75 flasks for 48 hours, in selection medium, prior to lysis. Whole cell RIPA lysates were obtained, and equal protein was determined by the BCA protein assay. Proteins (25μg/lane) were resolved by 12.5% SDS-PAGE and blotted onto nitrocellulose membrane.

a) Bcl-2 levels. Results are representative of four independent experiments. Student's t test showed a p value ≤ 0.004 between H376 E-cadherin (H376 E-cad) and H376 antisense (H376 AS) cell lines.

b) Total Akt-1 levels. Results are representative of three independent experiments. Student's t test showed a p value ≤ 0.001 between H376 E-cadherin (H376 E-cad) and H376 antisense (H376 AS) cell lines.

Figure 4.3

a) Bcl-2 Blots



b) Akt-1 Blots

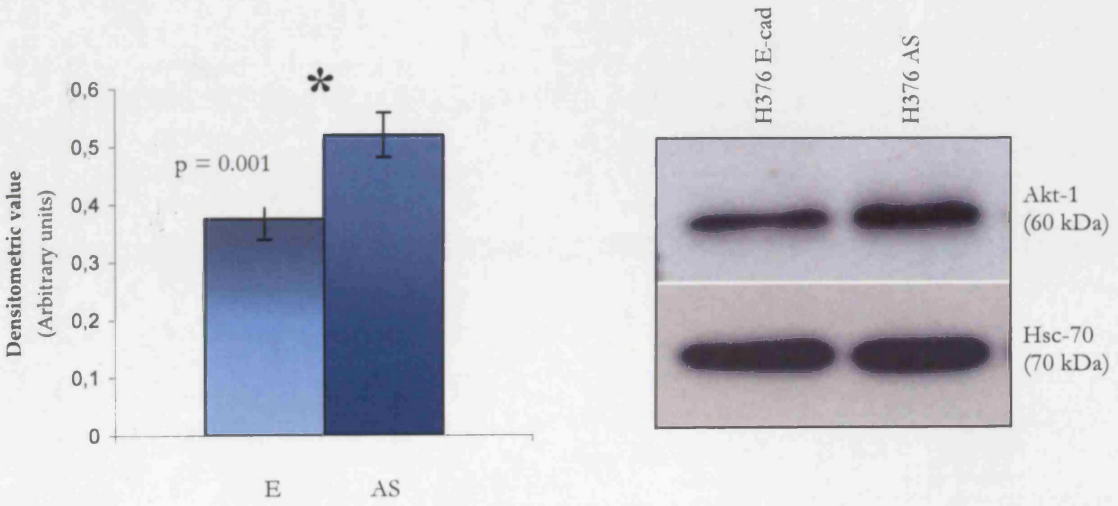


Figure 4.3 (cont.)

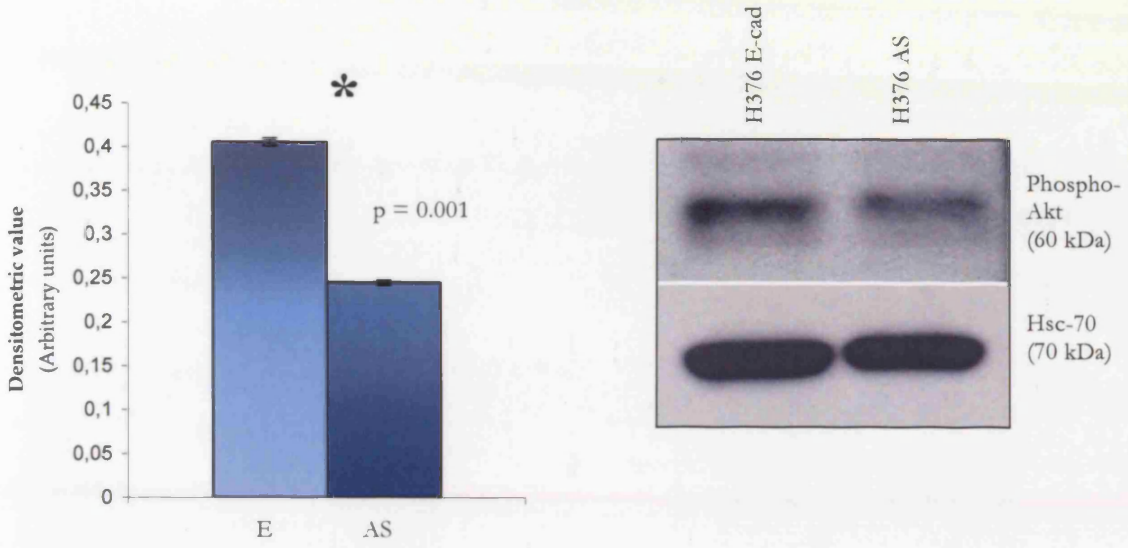
Western blot analysis of cell survival proteins in E-cadherin transfected cell lines growing on tissue culture plastic dishes.

c) Phospho-Akt (Thr-308) levels. Results are representative of three independent experiments. Student's t test showed a p value ≤ 0.001 between H376 E-cadherin (H376 E-cad) and H376 antisense (H376 AS) cell lines.

d) ILK levels. Results are representative of four independent experiments. Student's t test showed a p value ≤ 0.2 between H376 E-cadherin (H376 E-cad) and H376 antisense (H376 AS) cell lines.

Figure 4.3 (cont.)

c) phospho-Akt Blots



d) ILK Blots

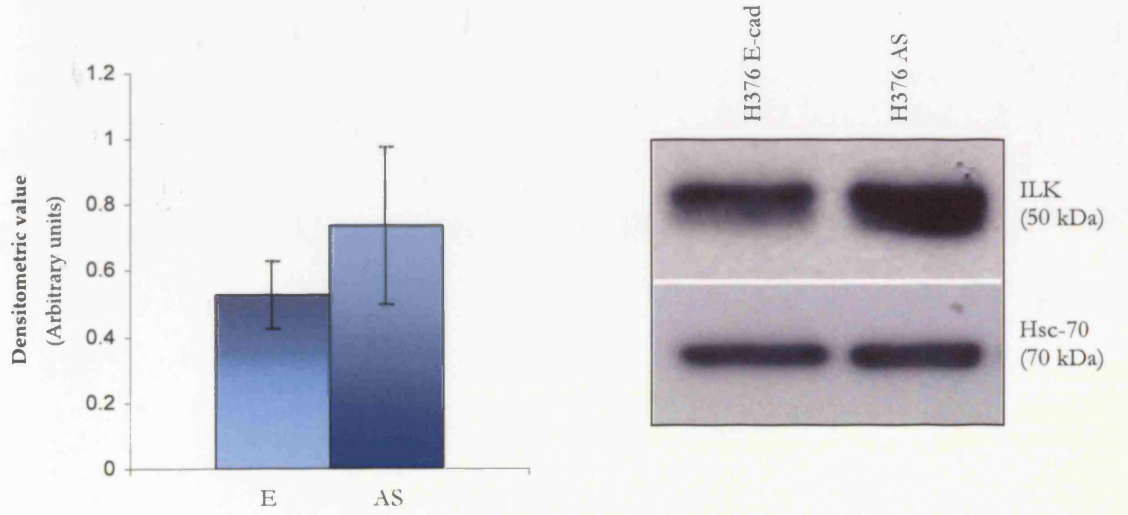


Figure 4.3 (cont.)

Western blot analysis of cell survival proteins in E-cadherin transfected cell lines growing on tissue culture plastic dishes.

e) N-cadherin levels. Results are representative of three independent experiments. Student's t test showed a p value ≤ 0.03 between H376 E-cadherin (H376 E-cad) and H376 antisense (H376 AS) cell lines.

Figure 4.3 (cont.)

e) N-cadherin Blots

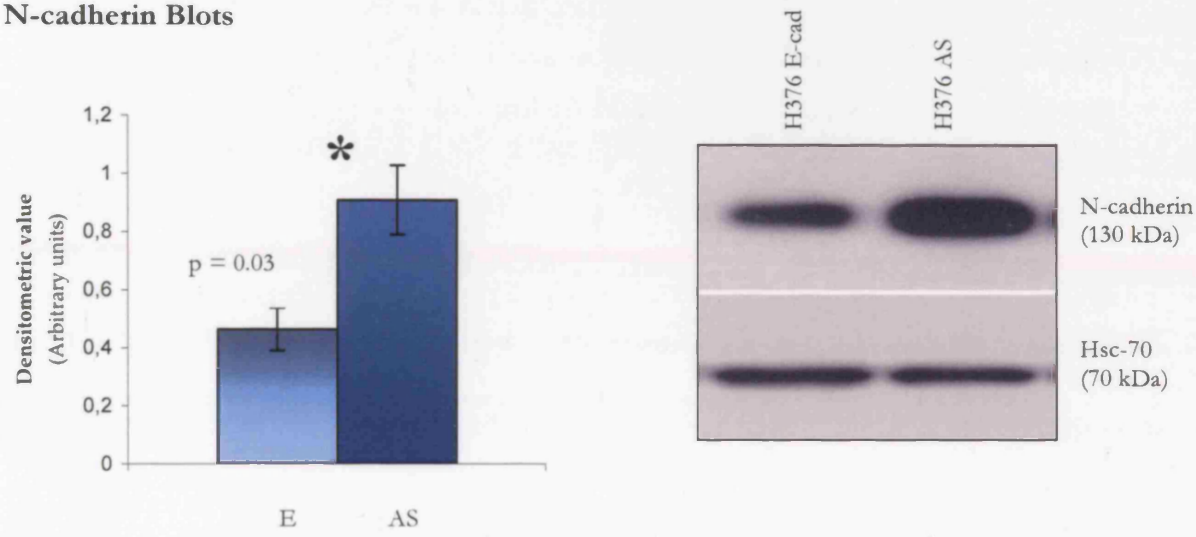


Figure 4.4

Gross appearance of E-cadherin transfected cell lines growing in 3-D cultures

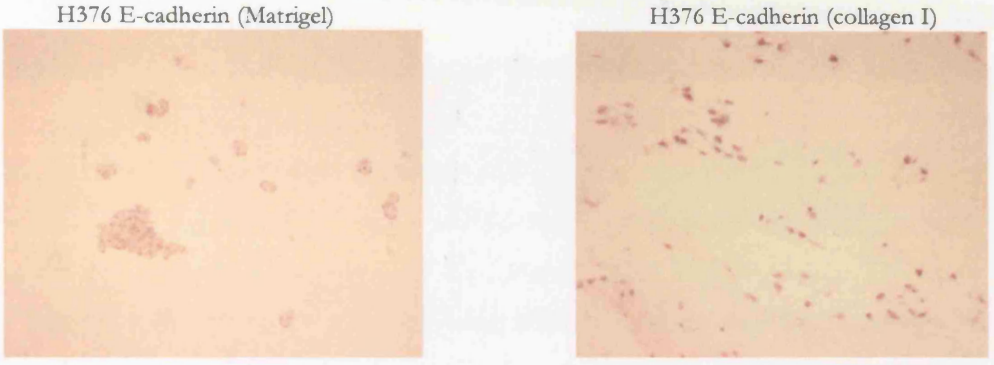
Cells were embedded in either Matrigel or collagen I 3-D culture as described in Chapter 2, Section 2.3.2 and allowed to grow for 8 days, then cells were fixed using formol saline and were embedded in paraffin. Parrafin sections were stained with HandE stain by Histopathology lab (Cancer Research UK Central Cell Services). Cells were viewed under contrast phase microscopy.

a) E-cadherin transfected cells (H376 E-cadherin) growing in 3-D Matrigel culture formed spherical multicellular aggregates (left), while the same cell line formed 'net-like' colonies and exhibited a fibroblastic morphology when grown in collagen I 3-D cultures (right).

b) Schematic representation of E-cadherin transfected cells in both cultures.

Figure 4.4

a)


H376 E-cadherin (Matrigel)
H376 E-cadherin (collagen I)

b)

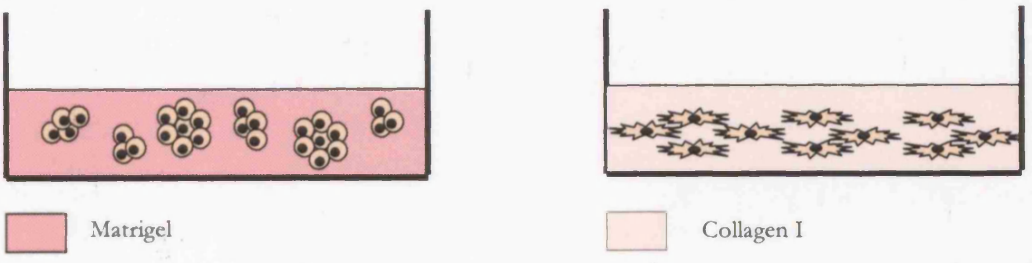

Matrigel
Collagen I

Figure 4.5

Growth curves of E-cadherin transfected cell lines growing in 2-D and 3-D collagen I cultures

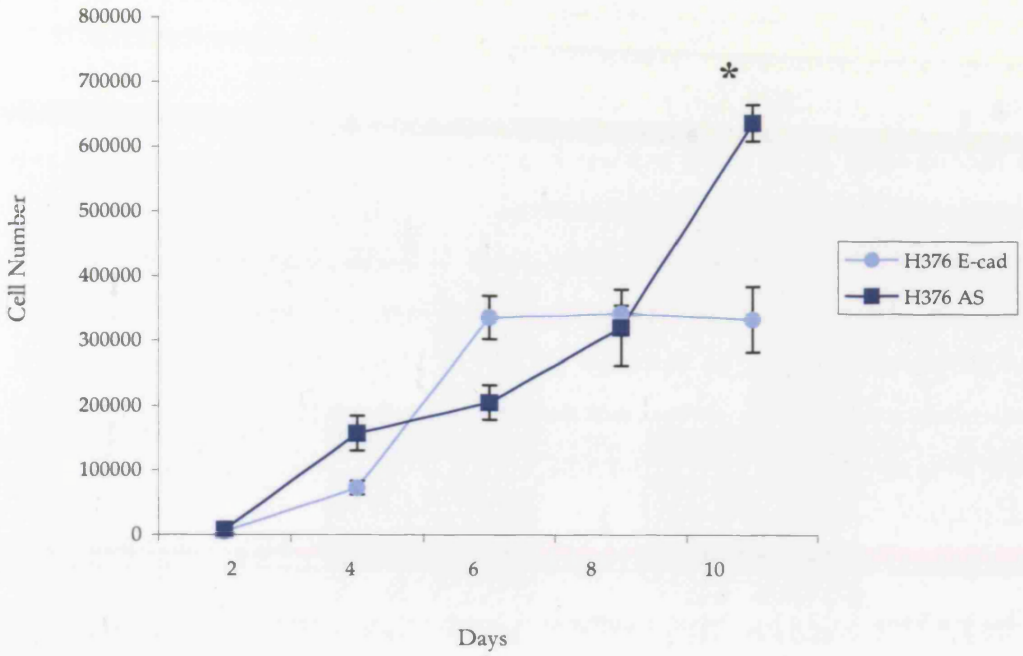
Transfected cell lines (H376 E-cadherin and H376 antisense) were plated, in triplicates, at an initial density of $2x10^4$ cell/well, either atop 2-D or within 3-D collagen I cultures, and allowed to grow for 10 days. At 2-day intervals, cells were harvested after treatment with 6 mg/ml collagenase 1 and trypsin, then counted using an haemocytometer. Cell viability was determined using "Trypan blue exclusion" and results were plotted into a growth curve histogram.

a) Growth curve of cells growing in 2-D collagen I culture

b) Growth curve of cells growing in 3-D collagen I culture

Figure 4.5

a)


800000
700000
600000
500000
400000
300000
200000
100000
0
Cell Number
*
H376 E-cad
H376 AS
2
4
6
8
10
Days

b)

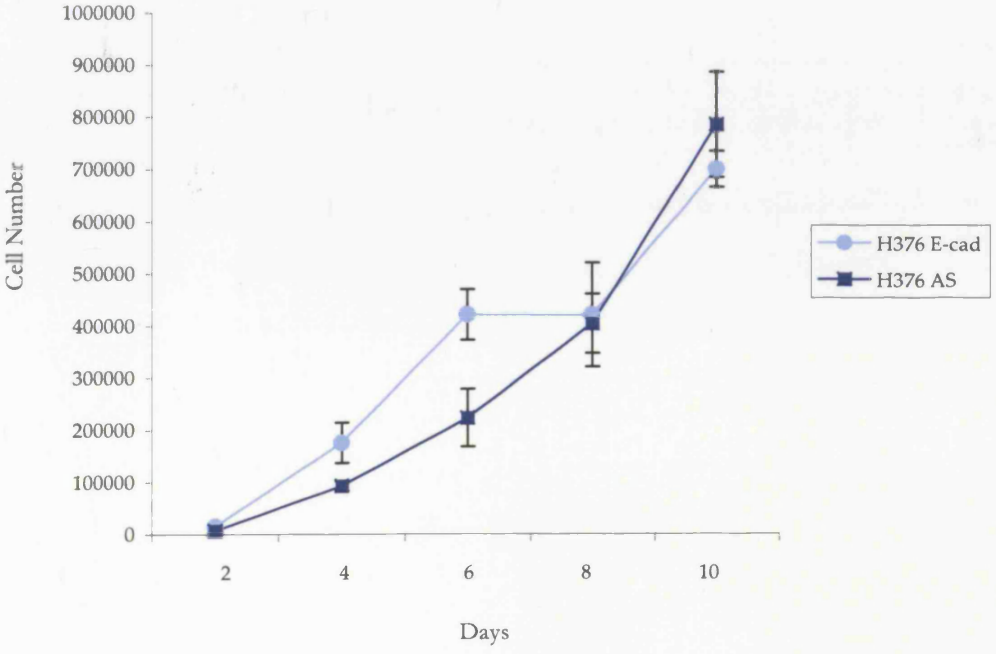

1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
Cell Number
H376 E-cad
H376 AS
2
4
6
8
10
Days

Figure 4.6

Western blot analysis of the Bcl-2 cell survival protein in E-cadherin transfected cell lines growing in 2-D and 3-D collagen I cultures

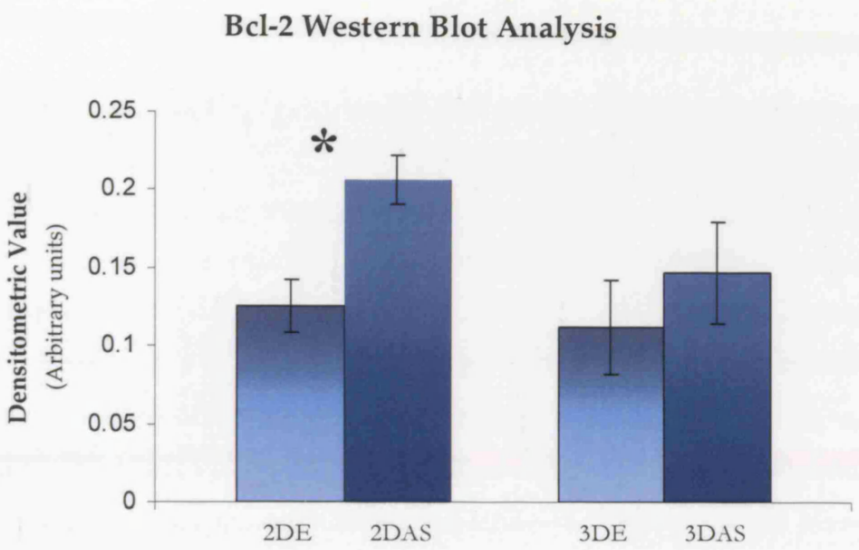
Equal numbers of cells ($6x10^4$ cell/well) were plated in 6-well plates, either atop 2-D or within 3-D collagen I cultures, and allowed to grow for 5 days. Cells were retrieved from the collagen I culture prior to lysis, as described in Chapter 2, Section 2.3.2. Whole cell RIPA lysates were obtained, and equal protein was determined by the BCA protein assay. Proteins (25μg/lane) were resolved by 12.5% SDS-PAGE and blotted onto nitrocellulose membrane.

a) Bcl-2 levels. Results are from densitometric values from four independent Western blot analyses. In 2-D collagen I cultures, E-cadherin transfection (2DE) resulted in downregulation of Bcl-2 relative to the antisense counterpart (2DAS) (p value $\leq$ 0.02). This effect was abrogated when cells were embedded in 3-D collagen I cultures.

b) Representative Western blot analysis from one of four independent experiments used to generate the graph in Figure a). Heat Shock Protein (Hsc-70) has been used as a loading control.

Figure 4.6

a)


Bcl-2 Western Blot Analysis
Densitometric Value
(Arbitrary units)
0.25
0.2
0.15
0.1
0.05
0
*
2DE
2DAS
3DE
3DAS

b)

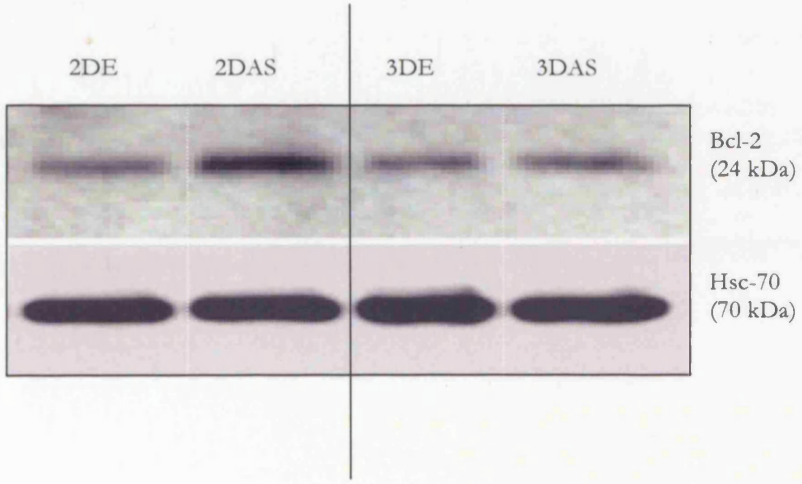

2DE
2DAS
3DE
3DAS
Bcl-2
(24 kDa)
Hsc-70
(70 kDa)

Figure 4.7

Western blot analysis of the Akt-1 cell survival protein in E-cadherin transfected cell lines growing in 2-D and 3-D collagen I cultures

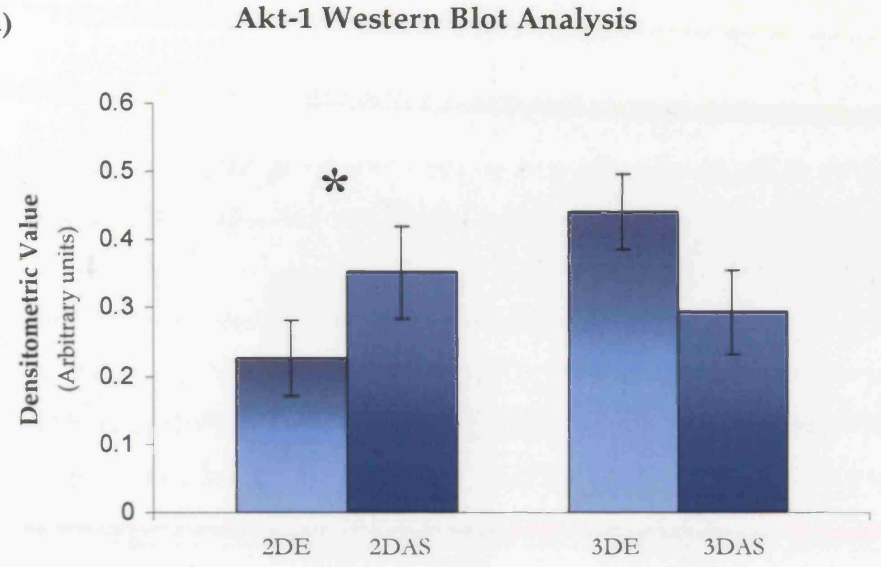
Equal numbers of cells ($6x10^4$ cell/well) were plated in 6-well plates, either atop 2-D or within 3-D collagen I cultures, and allowed to grow for 5 days. Cells were retrieved from the collagen I culture prior to lysis, as described in Chapter 2, Section 2.3.2. Whole cell RIPA lysates were obtained, and equal protein was determined by the BCA protein assay. Proteins (25μg/lane) were resolved by 12.5% SDS-PAGE and blotted onto nitrocellulose membrane.

a) Akt-1 levels. Results are from densitometric values from three independent Western blot analyses. In 2-D collagen I cultures, E-cadherin transfection (2DE) resulted in downregulation of Akt-1 relative to the antisense counterpart (2DAS) (p value $\leq$ 0.04). This effect was abrogated when cells were embedded in 3-D collagen I cultures.

b) Representative Western blot analysis from one of three independent experiments used to generate the graph in Figure a). Heat Shock Protein (Hsc-70) has been used as a loading control.

Figure 4.7

a)


Akt-1 Western Blot Analysis
Densitometric Value
(Arbitrary units)
0.6
0.5
0.4
0.3
0.2
0.1
0
*
2DE
2DAS
3DE
3DAS

b)

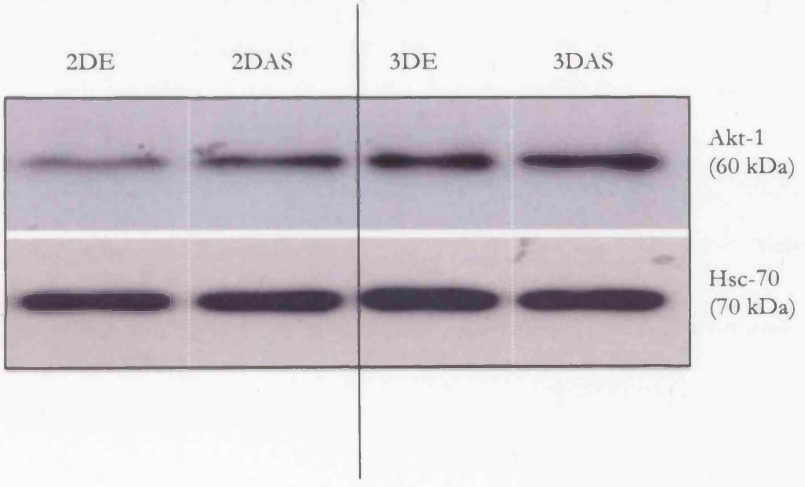

2DE
2DAS
3DE
3DAS
Akt-1
(60 kDa)
Hsc-70
(70 kDa)

Figure 4.8

Western blot analysis of the phosphorylation of Akt cell survival protein at threonine-308 in E-cadherin transfected cell lines growing in 2-D and 3-D collagen I cultures

Equal numbers of cells ($6x10^4$ cell/well) were plated in 6-well plates, either atop 2-D or within 3-D collagen I cultures, and allowed to grow for 5 days. Cells were retrieved from the collagen I culture prior to lysis, as described in Chapter 2, Section 2.3.2. Whole cell RIPA lysates were obtained, and equal protein was determined by the BCA protein assay. Proteins (25μg/lane) were resolved by 12.5% SDS-PAGE and blotted onto nitrocellulose membrane.

a) Phosphor-Akt (Thr-308) levels. Results are from densitometric values from three independent Western blot analyses. In 3-D collagen I cultures, E-cadherin transfection (3DE) resulted in substantial upregulation of phosphor-Akt (Thr-308) levels relative to the antisense counterpart (3DAS) (p value ≤ 0.02). This difference was abrogated when cells were grown on top of 2-D collagen I matrix.

b) Representative Western blot analysis from one of three independent experiments used to generate the graph in Figure a). Heat Shock Protein (Hsc-70) has been used as a loading control.

Figure 4.8

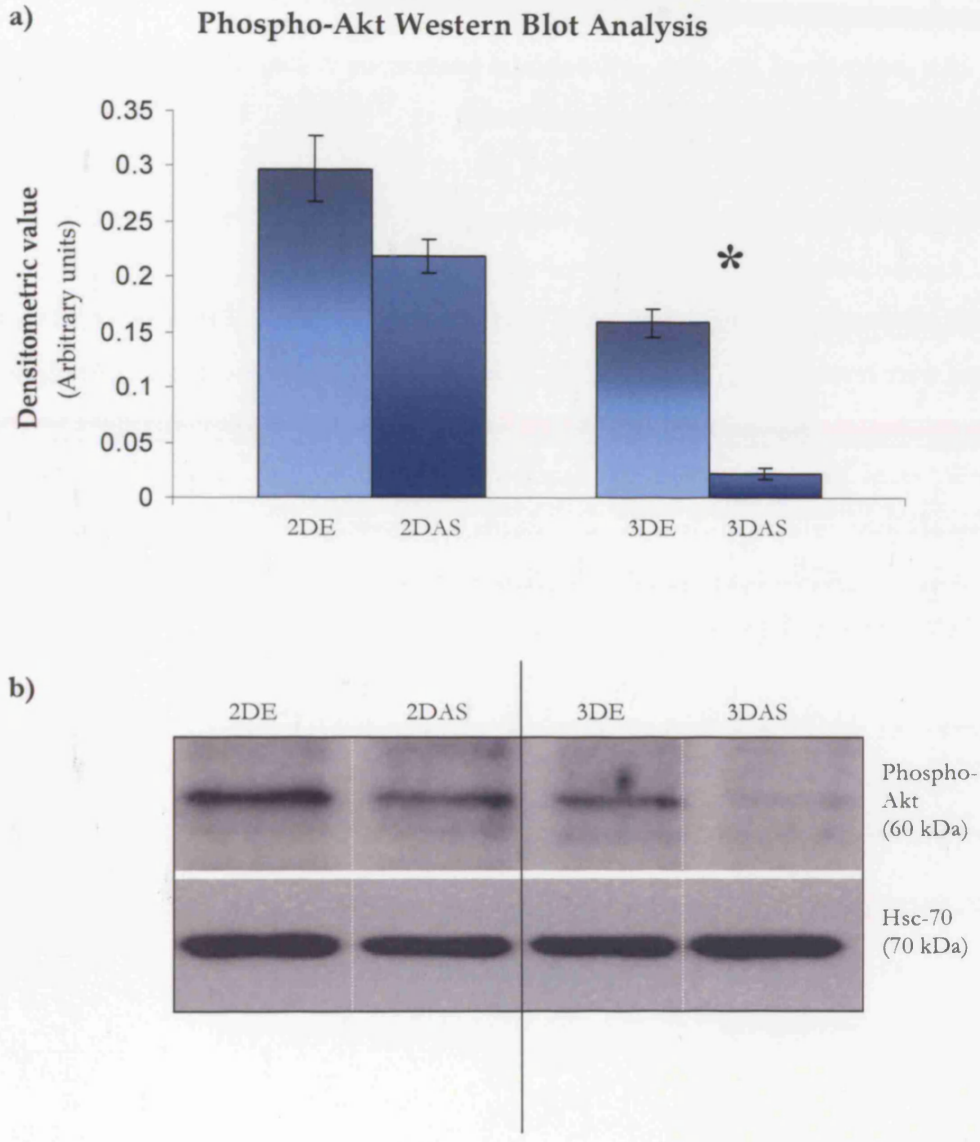

a)
Phospho-Akt Western Blot Analysis
Densitometric value
(Arbitrary units)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
2DE
2DAS
3DE
3DAS
b)
2DE
2DAS
3DE
3DAS
Phospho-
Akt
(60 kDa)
Hsc-70
(70 kDa)

Figure 4.9

Western blot analysis of the ILK cell survival protein in E-cadherin transfected cell lines growing in 2-D and 3-D collagen I cultures

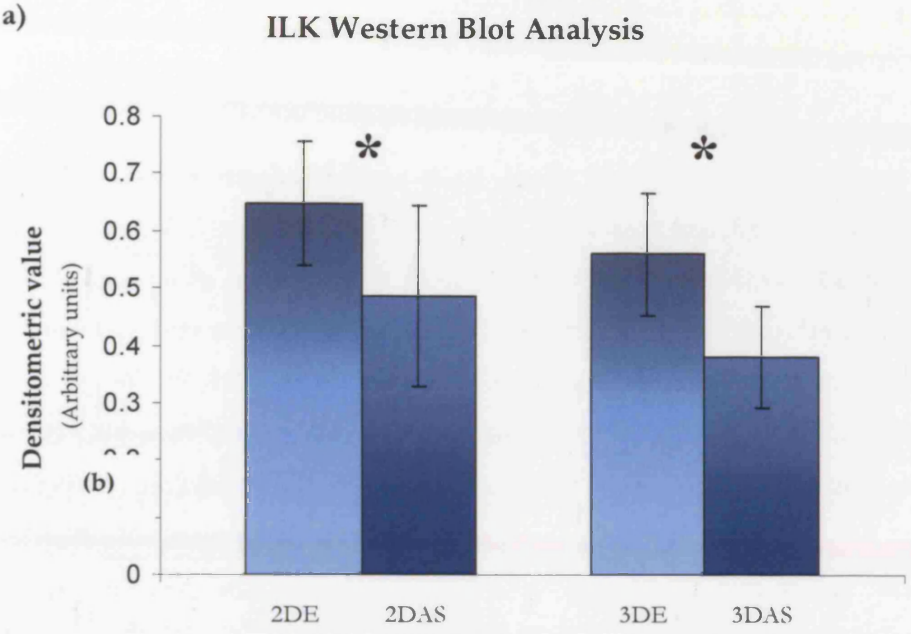
Equal numbers of cells ($6x10^4$ cell/well) were plated in 6-well plates, either atop 2-D or within 3-D collagen I cultures, and allowed to grow for 5 days. Cells were retrieved from the collagen I culture prior to lysis, as described in Chapter 2, Section 2.3.2. Whole cell RIPA lysates were obtained, and equal protein was determined by the BCA protein assay. Proteins (25μg/lane) were resolved by 12.5% SDS-PAGE and blotted onto nitrocellulose membrane.

a) ILK levels. Results are from densitometric values from four independent Western blot analyses. In both 2-D and 3-D collagen I cultures, E-cadherin transfection (2DE and 3DE) resulted in upregulation of ILK levels relative to the antisense counterpart (2DAS and 3DAS) (p value $\leq$ 0.05 and 0.002 respectively).

b) Representative Western blot analysis from one of four independent experiments used to generate the graph in Figure a). Heat Shock Protein (Hsc-70) has been used as a loading control.

Figure 4.9

a)


ILK Western Blot Analysis
Densitometric value
(Arbitrary units)
0.8
0.7
0.6
0.5
0.4
0.3
(b)
0
*
*
2DE
2DAS
3DE
3DAS

b)

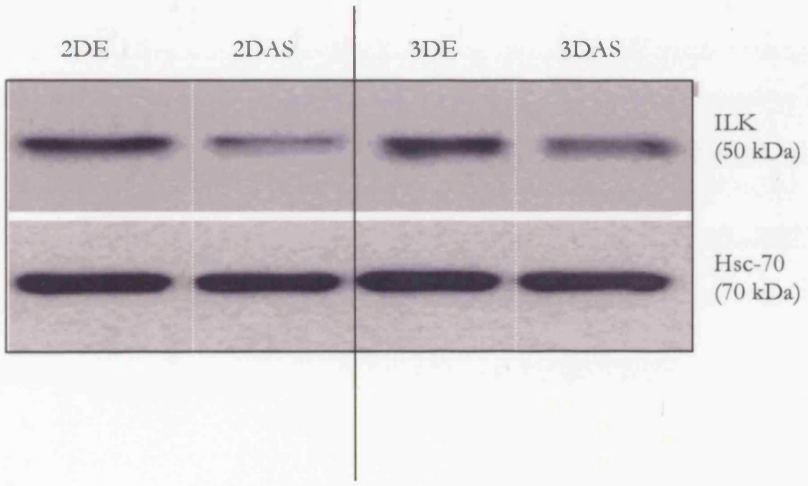

2DE
2DAS
3DE
3DAS
ILK
(50 kDa)
Hsc-70
(70 kDa)

Figure 4.10

Western blot analysis of N-cadherin levels in E-cadherin transfected cell lines growing in 2-D and 3-D collagen I cultures

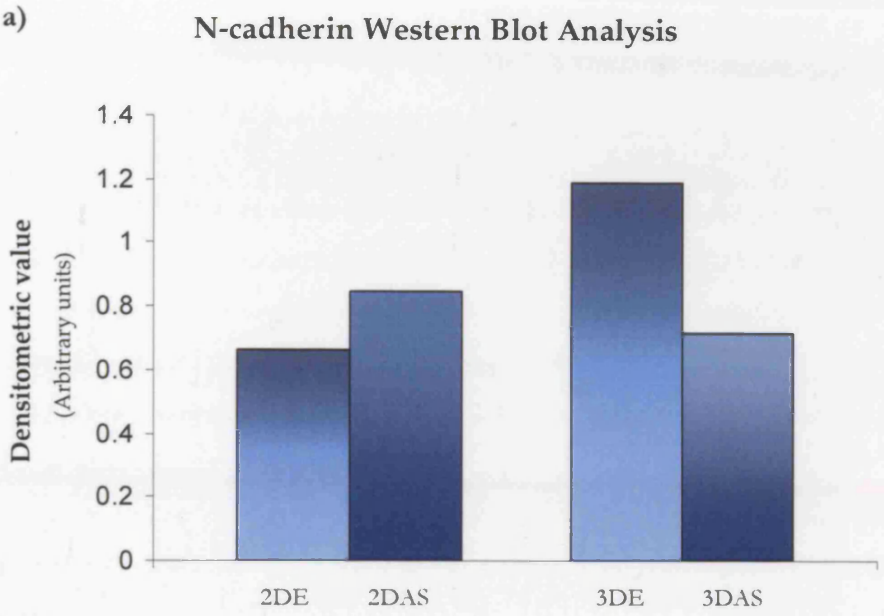
Equal numbers of cells ($6x10^4$ cell/well) were plated in 6-well plates, either atop 2-D or within 3-D collagen I cultures, and allowed to grow for 5 days. Cells were retrieved from the collagen I culture prior to lysis, as described in Chapter 2, Section 2.3.2. Whole cell RIPA lysates were obtained, and equal protein was determined by the BCA protein assay. Proteins (25μg/lane) were resolved by 12.5% SDS-PAGE and blotted onto nitrocellulose membrane.

a) N-cadherin levels. Result represents one of two Western blot analyses. In 3-D collagen I cultures, E-cadherin transfection (3DE) resulted in upregulation of N-cadherin levels relative to the antisense counterpart (3DAS). This effect was not evident between cells growing in 2-D collagen I cultures.

b) Representative Western blot analysis from one of two independent experiments used to generate the graph in Figure a). Heat Shock Protein (Hsc-70) has been used as a loading control.

Figure 4.10

a)



b)

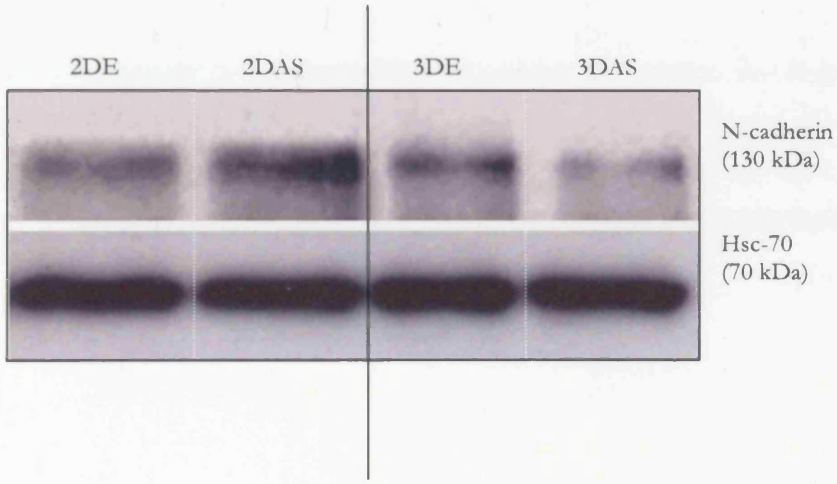


Figure 4.11

E-cadherin expression as a consequence of growth conditions in 2-D/3-D collagen I cultures

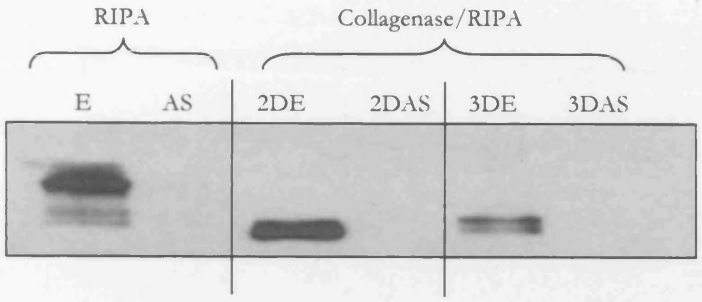
a) Equal cell numbers, of transfected cell lines H376 E-cadherin and H376 antisense, were grown on tissue culture plastic (E and AS), on 2-D collagen I (2DE and 2DAS) and within 3-D collagen I matrix (3DE and 3DAS), and allowed to grow for 5 days. Cells growing on tissue culture plastic were retrieved as described in Chapter 2, Section 2.3.1, while cells growing on 2-D and 3-D collagen I cultures were retrieved from the collagen I culture prior to lysis, as described in Chapter 2, Section 2.3.2. Western blot analysis shows E-cadherin expression in all three culture conditions.

b) Transfected cell lines H376 E-cadherin and H376 antisense (E and AS) were grown, for 48 hours up to different confluencies, on tissue culture plastic. Total RIPA cell lysated were obtained and assessed for expression of E-cadherin molecule.

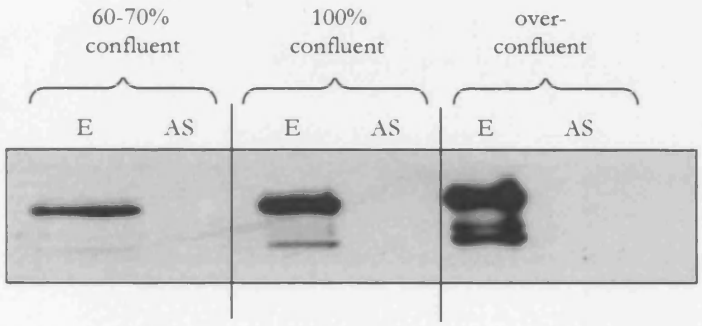
c) Equal cell numbers, of transfected cell lines H376 E-cadherin and H376 antisense, were grown on tissue culture plastic (E and AS). Cells were treated, with either collagenase 1 or collagenase 3 (Worthington Biochemical Corporation), prior to lysis. Total RIPA cell lysates were assessed for E-cadherin expression using Western blot analysis.

Figure 4.11

a)



b)



c)

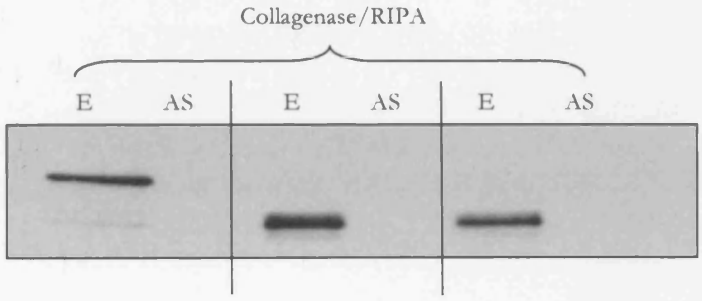


Figure 4.12

Proposed model of a modified organotypic co-culture

A model adapted from the organotypic co-culture system described by Fusenig *et al.* (1983), being modified to be used to extract cellular protein from the cultured cell populations. The modifications include: separating the collagen-embedded fibroblast population from being mixed with the epithelial cells via the use of a nylon filter and the addition of a separate laminin-rich basement membrane (as illustrated in Figure).

Figure 4.12

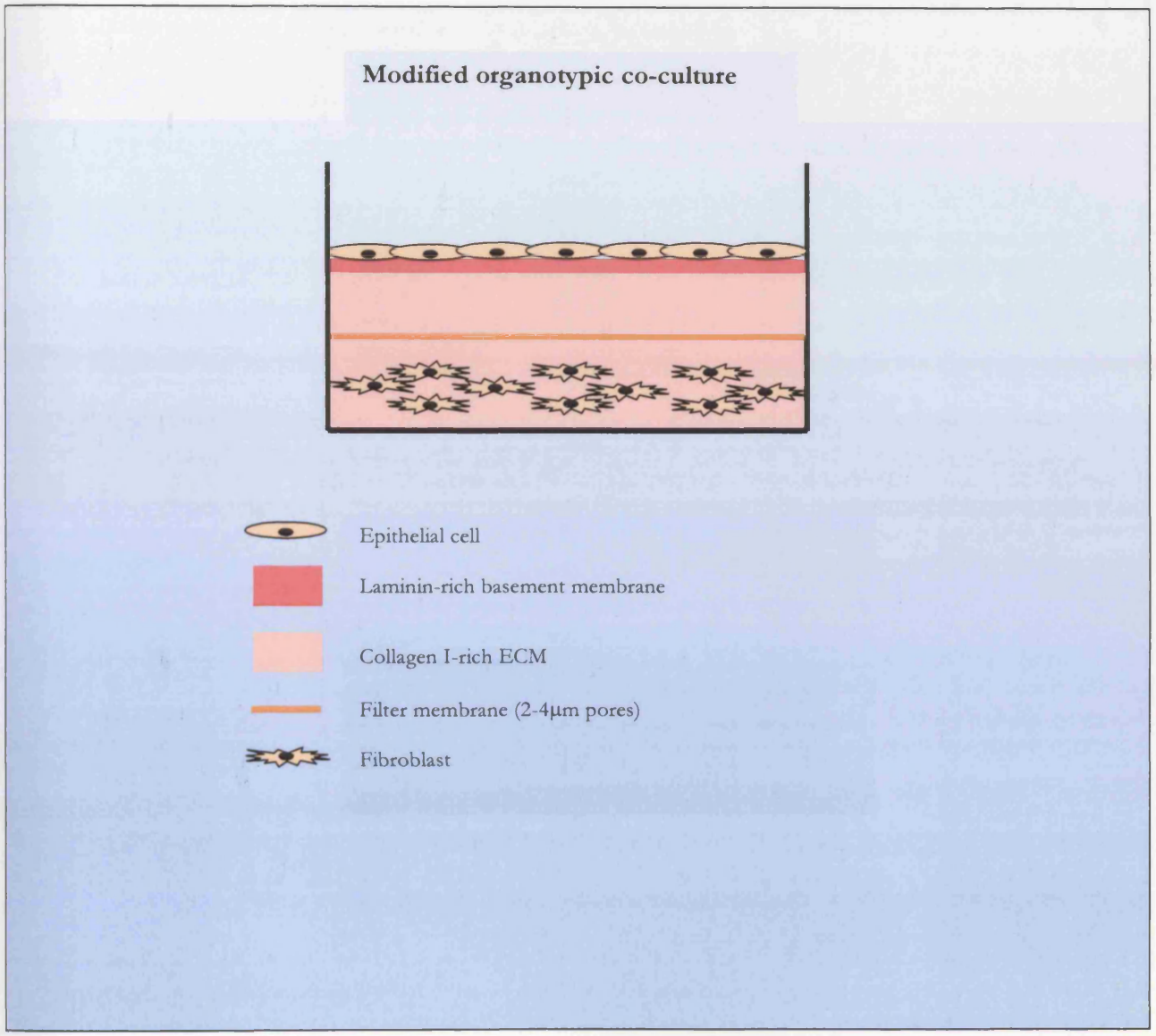
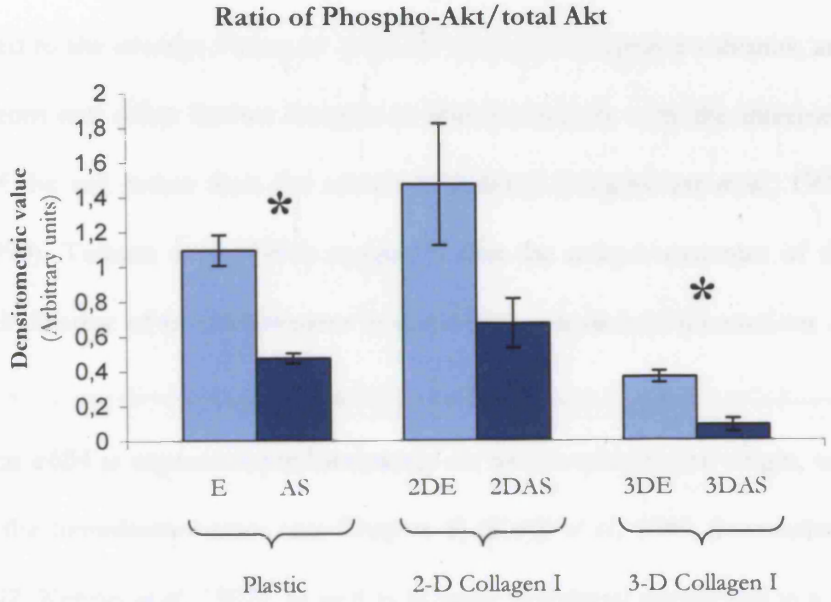

Modified organotypic co-culture
Epithelial cell
Laminin-rich basement membrane
Collagen I-rich ECM
Filter membrane (2-4μm pores)
Fibroblast

Figure 4.13

Level of phosphorylation of Akt in E-cadherin transfected cell lines growing on tissue culture plastic, 2-D collagen I and in 3-D collagen I cultures

Histogram representing the ratio of phospho-Akt/total Akt. In cultures where cells were growing on serum-coated plastic, E-cadherin expressing cells (E) showed a substantial increase of phosphorylation of Akt (Thr-308) as compared to the antisense control cell line (AS) (p value $\leq$ 0.01). In 3-D collagen I cultures, E-cadherin transfection (3DE), also resulted in increased phosphorylation of Akt (Thr-308) levels relative to the antisense counterpart (3DAS) (p value $\leq$ 0.05). This difference was abrogated when cells were grown on top of 2-D collagen I matrix (p value $\leq$ 0.1).

Figure 4.13


Ratio of Phospho-Akt/total Akt
Densitometric value
(Arbitrary units)
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
*
E
AS
2DE
2DAS
3DE
3DAS
Plastic
2-D Collagen I
3-D Collagen I

CHAPTER 5

Cross-Talk between Integrin α6β4 and E-cadherin

5.1 Introduction

The α6- and β4-integrin subunits are transmembrane proteins with the cytoplasmic domain of the β4-integrin subunit being unusually large. It is composed of about 1000 amino acids, as compared to the average 50 amino acids for the other integrin β subunits, and is distinctly different from any other known integrin in that it interacts with the intermediate filament network of the cell rather than the actin cytoskeleton (Hogervorst *et al.*, 1990; Suzuki and Naitoh, 1990). Tamura *et al.* (1990) suggested that the unique structure of the β4-integrin subunit is indicative of its involvement in distinctive cytoskeletal interactions (Tamura *et al.*, 1990).

The integrin α6β4 is expressed predominantly on tissues of epithelial origin, where it can be located in the hemidesmosomes (see Chapter 6) (Kajiji *et al.*, 1989; Sonnenberg *et al.*, 1990; Hynes, 1992; Kennel *et al.*, 1992), as well as in some peripheral nerves and in a few subsets of endothelial cells (Sonnenberg *et al.*, 1990). Stratified epithelia and cultured keratinocytes adhering to basement membrane substrates were also found to express α6β4 (De Luca *et al.*, 1990; Marchisio *et al.*, 1991; Jones *et al.*, 1993). Although hemidesmosomes occur only in cells that express α6β4 integrins, not all of the cells expressing α6β4 integrin possess hemidesmosomes (Owaribe *et al.*, 1990; Sonnenberg *et al.*, 1990; Natali *et al.*, 1992).

Loss of this pattern of expression is seen in carcinomas *in situ* and there is a consistent alteration involving α6β4 integrin expression in carcinomas in general (Natali *et al.*, 1992; Carico *et al.*, 1993; Juliano and Varner, 1993). Both decreased and increased expression of the α6β4 integrin have been observed in epithelial tumours (Gomez *et al.*, 1992; Tennenbaum *et al.*, 1992; Tennenbaum *et al.*, 1993; Carico *et al.*, 1993; Downer *et al.*, 1993; Juliano and

Varner, 1993; Cress *et al.*, 1995; Liebert and Seigne, 1996; Rabinovitz and Mercurio, 1996; Allen *et al.*, 1998; Schofield *et al.*, 1998; Lo *et al.*, 2001). In head and neck squamous cell carcinoma, some workers have reported an increase in α6β4 expression (Wolf *et al.*, 1990; Carey *et al.*, 1992), while others have observed reduced expression of α6β4 in poorly differentiated oral squamous cell carcinomas (Downer *et al.*, 1993; Jones *et al.*, 1993).

Loss of α6β4 in OSCC was found to be most evident at the leading edge of tumours and in the poorly differentiated lesions (Jones *et al.*, 1993). It also has been reported that the decrease in α6β4 integrin levels observed in carcinoma was not a part of a general alteration of other integrin levels but rather was specific for this particular integrin (Witkowski *et al.*, 2000).

The biochemical changes behind the altered expression of the α6β4 integrin, during tumour progression, in carcinomas are unknown. Witkowski *et al.* (2000) have investigated the expression of α6β4 in a multi-step mouse model of skin carcinogenesis comprised of normal keratinocytes, benign papillomas and malignant undifferentiated carcinoma cells. Results revealed that there was about a 75% reduction in the surface expression of α6β4 integrin in the undifferentiated carcinoma cells as compared with the normal keratinocytes and papilloma cell lines (Witkowski *et al.*, 2000). They also showed that the remaining α6β4 integrin heterodimers lost their polarised expression and were distributed diffusely around the cell membrane in carcinoma cells and showed about a 2.5-fold increase in turnover as compared with the turnover in normal keratinocytes (Witkowski *et al.*, 2000).

The loss of both hemidesmosome-associated structures and basal cell differentiation has been associated with early stromal invasion in prostate cancer (Bonkhoff, 1998) indicating that such associations are not restricted to tumours of a single lineage.

Recent studies suggest that the α6β4 integrin plays a critical role in transducing signals from the extracellular environment to the interior of the cell, significantly modulating the

organisation of the cytoskeleton, cell proliferation, apoptosis and differentiation (Borradori and Sonnenberg, 1999). Other studies indicate that the α6β4 integrin can function in migration through its ability to promote the formation and stabilisation of motility structures (Rabinovitz and Mercurio, 1997). Geuijen and Sonnenberg (2002) found that α6β4 integrin induces hemidesmosome formation and slowed down migration of epithelial cells, in response to wound healing when the β4-integrin subunit was allowed to bind to its ligand, laminin-5, through the interaction of β4 with plectin (Geuijen and Sonnenberg, 2002). Conversely, it was found that, in carcinoma cells, the α6β4 integrin promotes cell migration and invasion (Shaw *et al.*, 1997). Thus suggesting that α6β4 may play a binary, and apparently paradoxical, role both in maintaining the normal epithelial cell's stationary status and in promoting its migration after transformation.

The formation of hemidesmosomes, as well as cadherin-dependent adherens junctions, is dependent upon extracellular Ca^{2+} ions (Trinkaus-Randall and Gipson, 1984; Braga, 2000), possibly indicating a relationship between the divalent-dependent α6β4 integrin and the cadherin proteins (Collins *et al.*, 1991). Because of their need for co-ordinated changes during neoplastic transformation I considered it possible that there might be a connection/cross-talk between E-cadherin and α6β4 integrin in epithelial cells and that these interactions might play a role in carcinoma development and prognosis.

Results from Chapter 3 were suggestive for the existence of a possible "cross-talk" interaction between E-cadherin and β4-integrin. The H376 OSCC line showed a low surface level of β4 expression (Prime *et al.*, 1990). This cell line had been transfected previously with cDNA for the human β4-integrin subunit (Jones *et al.*, 1996a) to investigate the role of this molecule in the behaviour of malignant keratinocytes. As assessed by FACS analysis (Jones J., PhD thesis, University of London, 1996) the derived Clone B6 showed a substantial level

of β4-integrin subunit expression. This line, which I used, is referred to as the "H376β4" cell line. IA2 cells were transfected with the empty vector and were used as a negative control (Jones J., PhD thesis, University of London, 1996).

The aim was to use these matched cell lines, expressing and not expressing β4-integrin, and transfect them with my E-cadherin plasmid to produce stable transfectants that could then be used to study the possible cross-talk interactions between E-cadherin and the β4-integrin subunit.

5.2 Results

5.2.1 Characterisation of the H376β4 and IA2 primary cell lines

The H376β4 and IA2 cell lines were examined for the presence and the expression of both E-cadherin and the β4-integrin subunit using FACS analysis and Western blotting. Figures 5.1 and 5.2 showed that H376β4 and IA2 lines were both, E-cadherin negative. Moreover, only the H376β4 cell line, but neither the IA2 nor the H376 parental cell lines, expressed the β4-integrin subunit.

Knowing that both the H376β4 and the IA2 cell lines express the necessary catenins (data not shown), they became suitable candidates for transfection with E-cadherin cDNA.

5.2.2 Establishment of E-cadherin/β4-integrin positive stable transfectants (Introduction of E-cadherin cDNA into the H376β4 cell line)

The H376β4, as well as the IA2, cell lines were stably transfected with the E-cadherin sense and antisense constructs using FuGENE™ 6 Transfection reagent (Roche) to generate four transfected cell lines: H376β4 E-cadherin cell line, H376β4 antisense control cell line, IA2 E-cadherin cell line and IA2 antisense cell line (Chapter 2, Figure 2.3). As with my previous transfection studies (Chapter 3) the E-cadherin expressing cells also were selected by two rounds of magnetic bead sorting and the resulting cell populations were grown in puromycin-supplemented KGM "Selection Medium", with a concentration of 0.5 μg/ml; a value previously determined with preliminary "kill curve" experiments. Again, the isolated positive colonies, capable of growing in this selection medium, were pooled as a means of avoiding clonal heterogeneity.

Over the next 12 months of continuous culture expression of the molecules of interest were checked at different times on a routine basis using both FACS and Western blot analysis.

Representative results are illustrated in Figure 5.3, where the stable cell lines "H376β4 E-cadherin" and "IA2 E-cadherin" expressed high levels of E-cadherin as compared to their antisense control counterpart cell lines, "H376β4 antisense" and "IA2 antisense", which were negative for E-cadherin.

5.2.3 Characterisation of stably transfected cell lines

All transfected cell lines were characterised for the expression of catenins, other cadherins, and integrins. Characterisation was carried out in exactly the same way as previously conducted on H376 E-cadherin and H376 antisense transfected cell lines. Similar results were obtained, where α- and β-catenin upregulation and γ-catenin downregulation appeared concomitant with the *de novo* E-cadherin expression, as compared with the negative antisense control (Appendix IV). The E-cadherin positive clones showed a marked downregulation of N-cadherin (Appendix IV) and an integrin profile presented similarly as the H376 E-cadherin cell line, when each clone was compared to its antisense control (Appendix IV).

5.2.4 Effect of E-cadherin re-expression on the total levels of β4-integrin subunit in the transfected cell lines

Results from Chapter 3 had shown that E-cadherin re-expression in the H376 OSCC cell line resulted in about a 2.5-fold increase in the surface β4-integrin levels in the H376 E-cadherin cell line as compared to the antisense control counterpart (p value ≤ 0.01) (Chapter 3, Figure 17). Consistent with this result, when similar experiments were conducted on the new stable transfectants, both the H376β4 E-cadherin and IA2 E-cadherin cell lines showed an upregulation in the cell surface levels of the β4-integrin as a consequence of *de novo* E-cadherin expression. Figure 5.4 shows histograms illustrating results from three independent FACS experiments, using anti-β4-integrin (clone 3E1) monoclonal antibody (CHEMICON®

international), where there was about a 5-fold increase in the levels of β4-integrin subunit in the IA2 E-cadherin cell line as compared to the IA2 antisense control (p value ≤ 0.04). In the H376β4 E-cadherin cell line there was a statistically highly significant 17-fold increase in the levels of β4-integrin as a consequence of E-cadherin re-expression (p value ≤ 0.003).

The effect of E-cadherin re-expression on the total levels of the β4-integrin was further investigated using Western blot analysis.

Western blot results, using anti-β4-integrin (clone 7) monoclonal antibody (BD Transduction Laboratories), showed that, upon re-expression of E-cadherin there was about a 6-fold increase in the total β4-integrin levels in the H376β4 E-cadherin cell line as compared to the H376β4 primary cell line (Figure 5.3). Moreover, *de novo* E-cadherin expression resulted in the re-expression of the β4-integrin in the IA2 E-cadherin cell line as compared to the IA2 primary cell line and to the antisense negative control (Figure 5.4).

FACS and Western blot analyses however showed that the H376β4 antisense cell line, unexpectedly, did not express β4. This was a repeatable observation that I considered might be because this particular cell line was the most difficult to select for using 'puromycin'. After being transfected with the E-cadherin antisense construct, it could have been that the previously introduced β4-integrin subunit was lost during the subsequent selection procedure. Alternatively it could be that the E-cadherin 'antisense' cDNA acts as a "silencing gene" though I have performed no experiments to address these issues directly.

Moreover, despite continuous effort, I could not obtain a similar result with the H376 E-cadherin cell line. Contrary to the FACS results presented in Figure 3.17 (Chapter 3), the H376 E-cadherin cell line was found to be negative for the β4-integrin subunit when tested with Western blotting. A few possibilities were considered in an attempt to interpret this finding:

First it may have been that the signal was very weak and that more protein needed to be loaded before detecting any signal. Consequently other Western blots were carried out with 50μg of protein being loaded per lane; but still no β4-integrin was detected in relation to the H376 E-cadherin cell line (data not shown).

Second it was considered possible that this cell line, transfected with E-cadherin constructs more than a year before this Western blot experiment was carried out and grown in selection medium throughout, may have had its entire integrin profile changed. However on going back to an early passage, recovered from the liquid nitrogen freezer, I still could not detect any signal (data not shown).

Third it was thought possible that the results I obtained might be a consequence of unexpected technical problems. However it was a repeatable finding (three independent experiments), with both the H376 and the IA2 cell line.

Lastly it was considered possible that I might, inadvertently, have contaminated this particular cell population with another cell line. To rule out the possibility of contamination, DNA fingerprinting of the various lines was carried out next.

In Western blots, there appeared to be cleavage or processing of the β4-integrin, as it was represented by double bands; 200 and 185 kDa, which have been previously reported in this particular cell line (Jones J., PhD thesis, University of London, 1996) and in other cell lines (Giancotti *et al.*, 1992; Potts *et al.*, 1994; Witkowski *et al.*, 2000).

5.2.5 DNA fingerprinting of the transfected cell lines

To exclude the possibility of cell contamination and to ensure that all the transfected cell line clones originated from the same parental cell line (the H376 OSSC line), DNA fingerprinting was carried out by the Cell Production Unit at Cancer Reasearch-UK, Clare Hall. DNA was

collected from all clones and "fingerprinted" by this department. As shown in Figure 5.5, all clones came from the same origin.

5.2.6 Immunocytochemical assessment of β4-integrin expression in the H376β4 E-cadherin OSCC cell line

J. Jones reported that the β4-integrin subunit transfected into the H376 OSCC cell line failed, together with the endogenously expressed α6-integrin, to form stable anchoring contacts (SACs) (Jones *et al.*, 1996a). She claimed that the inability of α6β4 integrin to be expressed as SACs, in her transfected cell lines, might be attributable to the low level of expression of α6β4 integrin as compared to the levels expressed by normal keratinocytes (Jones J., PhD thesis, University of London, 1996). *De novo* E-cadherin expression into the H376β4 cell line resulted in a significant increase in α6β4 integrin (as assessed by FACS analysis and Western blot, Figure 5.4) and thus the ability of the transfected cell line to present β4-integrin at SACs was checked using indirect immunocytochemical staining. The H376β4 E-cadherin and CA1 cell lines were plated on laminin-5-coated 13mm coverslips and allowed to grow for 48 hours in full KGM medium. Cells were fixed with 4% formaldehyde, permeabilised using Triton X-100 and stained for β4-integrin subunit using anti-β4 integrin (clone 7) monoclonal antibody (BD Transduction Laboratories). Images were taken using confocal laser scanning microscopy.

Compared to the CA1 cell line the β4-integrin subunit in the H376β4 E-cadherin cell line failed to form the highly organised SAC-like structures. Rather it assembled into a reticular-like cytoplasmic network patterned throughout the entire cell (Figure 5.6).

Another difference was observed in the nuclei of the H376β4 E-cadherin cell line. They exhibited a lobular appearance and, when staining with anti-β4-integrin monoclonal antibody, β4-integrin was shown to localise between those lobules (Figure 5.6).

5.2.7 Immunocytochemical localisation of E-cadherin in transfected lines

For indirect immunofluorescence experiments, H376 E-cadherin, H376β4 E-cadherin and IA2 E-cadherin cell lines were grown on coverslips for 48 hours in order to allow for the formation of mature adherens junctions. Cells were fixed with 4% formaldehyde, permeabilised using Triton X-100 and stained for E-cadherin using HECD-1 antibody. Antibodies were visualised with fluorochromes linked to second layer antibodies. The actin cytoskeleton and nuclei were counterstained in some experiments.

Images from all transfected cell lines were taken using confocal laser scanning microscopy. In Figures 5.7, 5.8 and 5.9 (the Panels A), all E-cadherin positive cell lines (the H376 E-cadherin, H376β4 E-cadherin and IA2 E-cadherin cell lines) exhibited strong cell surface positivity for E-cadherin, as compared to their negative antisense counterparts (images not shown). However, there were marked morphological differences, in terms of E-cadherin localisation, between the three cell lines. Thus the H376 E-cadherin cell line manifested what I term "zipper-like" appearance at sites of cell-cell contact (Figure 5.7; structures designated by arrows) indicative of relative immaturity. In contrast the H376β4 E-cadherin cell line had a more mature "straight line" contact (Figure 5.8; arrows). I considered that the appearance of the IA2 E-cadherin cell line also was consistent with the "zipper-like" appearance of immature junctions (Figure 5.9; arrows).

I also observed changes in the actin organisation, such that the H376β4 E-cadherin cell line showed disrupted actin filaments and less stress fibres as compared to both the H376 E-cadherin cell line and the IA2 E-cadherin cell line. In both these latter lines the actin filaments re-distributed into bundles co-localising with the cell surface E-cadherin molecule (Figures 5.7, 5.8 and 5.9, Panels B and C).

Taken together, these morphological changes suggest that the β4-integrin might be involved in the maturation of the E-cadherin-dependent cell-cell junction.

Because the initial aim of this Chapter was to detect any sort of E-cadherin/β4-integrin cross-talk interaction, based on results from Sections 5.2.3 and 5.2.4, I decided to carry out the rest of my experiments with only 2 cell lines, the H376 E-cadherin and the H376β4 E-cadherin lines. The fact that they had been proven to originate from the same parental cell line, and that the only difference between them appeared to be the lack of the β4-integrin subunit in the H376 E-cadherin cell line, meant I considered they would provide a good model to study the effect of β4-integrin on the maturation of the E-cadherin-dependent cell-cell junction.

5.3 Discussion

The primary H376 OSCC cell line was shown to express low surface levels of the β4-integrin subunit (Sugiyama *et al.*, 1993) and was characterised as being E-cadherin negative (Chapter 3). Later the H376 cell line was transfected with human β4-integrin cDNA by J. Jones (Jones *et al.*, 1996a) and the H376β4 (G418-resistant clone 6) stable transfectant was shown to express substantial levels of β4-integrin subunit at the cell surface (Jones J., PhD thesis, University of London, 1996). However these levels still were less than those expressed by normal keratinocytes (Sugiyama *et al.*, 1993). The IA2 cell line was used as a negative control since it had been transfected with the empty vector only (Jones *et al.*, 1996a). FACS and Western blot analyses showed that the primary H376 and IA2 cell lines were negative for the β4-integrin subunit, while the H376β4 cell line was confirmed as positive (Figure 5.1). E-cadherin sense and antisense cDNA was stably transfected into all three primary cell lines (H376, H376β4 and IA2) and as a consequence of *de novo* E-cadherin, three main findings occurred:

1) Failure of the β4-integrin subunit to assemble into hemidesmosome-like structures in β4-integrin-transfected cell lines.
2) Limited to large, yet always significant, increases in the basal level of β4-integrin expression in all three E-cadherin positive transfectants.
3) Occurrence of a mature form of E-cadherin-dependent adherens junctions in the H376β4 E-cadherin cell line as compared to the zipper-like cell-cell contacts in the H376 E-cadherin (β4-negative) cell line.

The β4-integrin subunit dimerises with the α6- integrin subunit and plays an important role in the assembly of hemidesmosomes (Jones *et al.*, 1991) such that antibodies directed against α6β4 integrin inhibited hemidesmosome assembly (Jones *et al.*, 1991; Kurpakus *et al.*, 1991).

In the literature, cultured keratinocytes expressing α6β4 integrin have been reported as manifesting two different hemidesmosome-like structures. First, the hemidesomosomal proteins formed patch-like structures, which were referred to as "SACs" (Carter *et al.*, 1990), or they assembled into lines which were referred to as "cat-paw arrays" (Tsuruta *et al.*, 2003). In the primary H376β4 line the β4-integrin subunit failed to assemble into either structure; rather it assembled into reticular network-like arrays, as appeared in Figure 5.6; a result previously described by J. Jones (Jones *et al.*, 1996a), such an arrangement I found, mostly, to mimic the microscopic presentation of the keratin intermediate filament system (Ghosh *et al.*, 2002).

J. Jones claimed that the inability of the transfected cell line to show hemidesmosomal SACs may be due to its inability to express normal levels of β4 integrin. *De novo* E-cadherin expression in this particular cell line, "H376β4", resulted in markedly increased β4-integrin levels, as established by FACS and Western blot analyses (Figure 5.4) but this elevation of the β4-integrin level did not alter the ability of the transfected cell line to assemble hemidesmosomal SAC-like structures.

It is known that the α6β4 integrin binds the cell to ECM via the laminin-5 protein (Niessen *et al.*, 1994) and such binding triggers clustering of other hemidesmosomal proteins and induces hemidesmosome formation (Mainiero *et al.*, 1995; Spinardi *et al.*, 1995). However, Nievers *et al.* (1998) showed that β4-integrin alone was able to induce hemidesmosome assembly in a ligand-independent manner (Nievers *et al.*, 1998). The ability of the H376β4 E-cadherin cell line to form β4-integrin-dependent hemidesmosomal structures after binding to laminin-5 was tested with the cells plated atop laminin-5-coated cover slips. Using indirect immunofluorescence, confocal images showed that β4-integrin-to-laminin-binding did not induce hemidesmosomal structure formation in this particular cell line (Figure 5.6).

Classical hemidesmosomes are formed by clustering of multiple transmembrane and cytoplasmic proteins. α6β4 integrin together with the bullous pemphigoid antigen 180 (BP180 or BPAG-2) constitute the transmembrane component of the hemidesmosome (Jones *et al.*, 1991; Li *et al.*, 1993). In the cytoplasm, α6β4 integrin and BP180 are associated with the cytoplasmic components of the hemidesmosome which include the HD1/plectin protein (Hieda *et al.*, 1992), the bullous pemphigoid antigen 230 (BP230 or BPAG-1) (Tamai *et al.*, 1993) and the intermediate filament associated protein (IFAP300) (Skalli *et al.*, 1994) (see Chapter 6; Figure 6.1 for an illustration). The presence or absence of these proteins, together with their distribution, should be monitored in my H376β4 E-cadherin cell line since the lack of any of these accessory proteins might provide an explanation for the failure of this line to form SACs.

The α6β4 integrin is expressed in various epithelial tissues that are controlled by Epidermal Growth Factor (EGF) and it has been reported that activation of EGF receptors causes disassembly of the hemidesmosomes by obstructing the association of the activated α6β4 integrin with the cytoskeleton (Mainiero *et al.*, 1996). My OSCC cell was grown in KGM enriched with EGF and this too might explain why *de novo* β4 expression in this particular cell line failed to result in it being organised into hemidesmosomal structures.

It is known that in order to build the protective layer that is stratified squamous epithelium, epithelial cells must be bound tightly to each other and collectively they must adhere firmly to the underlying basement membrane.

In general, cell-cell and cell-ECM adhesion are mediated by different adhesion receptor systems among which cadherin and integrins, respectively, play a major role (Juliano, 2002). Adherens junctions, with E-cadherin being the main adhesive receptor, in conjunction with the actin cytoskeleton, "zip" the apposing cell surfaces together in an homophilic manner

and seal them into a cohesive well-protected sheet of cells (Vasioukhin and Fuchs, 2001). Concurrently the hemidesmosomes, harbouring the major α6β4 epithelial integrin (laminin receptor), connect the intermediate filament system to laminin-5; a major constituent of the basement membrane (Fuchs *et al.*, 1997; van der Flier and Sonnenberg, 2001; Watt, 2002).

In tandem with the E-cadherin-mediated intercellular adhesion, the α6β4 integrin is known to contribute to the development of a stable adhesion phenotype with a non-motile morphotype (Carter *et al.*, 1990; Fukata *et al.*, 1999b).

It seems logical to think that these two major epithelial adhesive receptors complement each other in allowing a stable integration of cells into a multicellular tissue.

In the literature there is little solid evidence to suggest that such "cross-talk" does exist. Thus Hintermann *et al.* (2005) provided proof that the α6β4 integrin possesses the ability to upregulate E-cadherin-mediated cell-cell adhesion through the activation of erbB2 (Hintermann *et al.*, 2005). On the other hand, disruption of α6β4-dependent hemidesmosomal cell anchorage was shown to trigger a rapid loss of E-cadherin from cell-cell contacts (Fouquet *et al.*, 2004).

In this Chapter I have provided preliminary evidence that E-cadherin and β4-integrin 'cross-talk'. *De novo* E-cadherin expression in all transfected cell lines resulted in an increase in the expression of the β4-integrin subunit. In Chapter 3, H376 E-cadherin transfectants showed about a 2.5-fold increase in β4-integrin levels. The increase was, although apparently relatively trivial, statistically significant (p value ≤ 0.01) (Chapter 3, Figure 3.21). Two years later when I used these same cell lines again these results were not-repeatable (which could be attributable to a potential artefactual change in integrin expression as a consequence of the selection procedure and the continuous propagation of the tumour cell line). However, re-expression of E-cadherin in an other two primary cell lines, the H376β4 and IA2 lines, which were proved, using DNA finger-printing, to originate from the same parental cell line

as well as the H376 cell line, resulted in the same effect. Accordingly there was about a 5-fold increase in the levels of β4-integrin subunit in the IA2 E-cadherin cell line as compared to the IA2 antisense control (p value $\leq$ 0.04). In the H376β4 E-cadherin cell line there was a significant 17-fold increase in the levels of β4-integrin as a consequence of E-cadherin re-expression (p value $\leq$ 0.003) (Figure 5.4). This independent experiment supports my first observation that restoration of E-cadherin expression can lead to an increase in the basal surface levels of the β4-integrin.

In Chapter 3, I showed that restoration of E-cadherin into the H376 OSCC cell line resulted in the formation of E-cadherin-dependent cell-cell contacts. Visual evaluation, under the confocal microscope, led me to conclude that these were incompletely formed cell-cell contacts in that they appeared as stitched "zipper-like" contacts rather than straight line cell-cell contacts. These "zipper-like" cell-cell contacts represented an intermediate step in adherens junction formation preceding the full merger of the neighbouring cell membrane into a single line, as described by Vasioukhin *et al.* (2000). Suprisingly, this was not the case when E-cadherin cDNA was introduced into the H376β4 cell line. The resultant transfected cell line, the H376β4 E-cadherin line, exhibited typical mature E-cadherin-dependent cell-cell contacts (Figures 5.7 and 5.8). Moreover, under the microscope, compared to the H376 E-cadherin transfectants, the H376β4 E-cadherin cell line, certainly, exhibited a more epithelial morphotype.

It is known that the re-expression of the β4-integrin subunit has rescued the epithelial phenotype through restoration of the ability of the epithelial cells to form hemidesmosomes (Gagnoux-Palacios *et al.*, 1997; Schaapveld *et al.*, 1998; van der Neut *et al.*, 1999) suggesting that some of these changes might be mediated through E-cadherin activity.

In conclusion, there is increasing evidence that molecules mediating cell-cell and cell-ECM adhesion "cross-talk" to each other. In OSCC, loss or perturbed expression of both the E-cadherin molecule and epithelial integrins are closely related (Bagutti *et al.*, 1998). In cultured keratinocytes it has been shown that inhibition of E-cadherin-mediated cell-cell adhesion resulted in a decrease in integrin expression by proliferating cells and their *de novo* expression by differentiating cells (Hodivala and Watt, 1994; Zhu and Watt, 1996). In this Chapter I provide evidence that restoration of the E-cadherin molecule in this particular OSCC cell line, a cell line that was proved to normally lack E-cadherin, resulted in an increase in the expression of the β4-integrin subunit. There also is a strong suggestion that the β4-integrin subunit is involved in the maturation of E-cadherin-dependent cell-cell adherens junctions. This latter observation is investigated more comprehensively in the next Chapter.

Figure 5.1

Detection of E-cadherin and β4-integrin expression in the parental cell lines using FACS analysis

For E-cadherin detection, cells were harvested as described in Chapter 2, Section 2.2.1.1. FACS results showed E-cadherin surface expression of the parental cell lines, the black histogram represents the negative control labelled with Mouse IgG1 negative control (DAKO), while the blue histogram represents the positive cell population expressing E-cadherin labelled with anti-E-cadherin antibody "HECD-1". H357 cell line was used as a positive control and shown to expresses high levels of E-cadherin, while H376 cell line was used as a negative control and was shown to be E-cadherin negative. Both H376β4 and IA2 cell lines were characterised as E-cadherin negative.

For β4-integrin detection, cells were harvested as described in Chapter 2, Section 2.2.1.2. FACS results showed β4-integrin surface expression of the parental cell lines, the black histogram represents the negative control labelled with Mouse IgG1 negative control (DAKO), while the red histogram represents the positive cell population expressing β4-integrin labelled with anti-β4-integrin antibody "3E1". H357 cell line was used as a positive control and shown to express high levels of β4-integrin. Both H376 and IA2 cell lines were found negative for β4-integrin subunit, while H376β4 cell lines appeared positive.

Figure 5.1

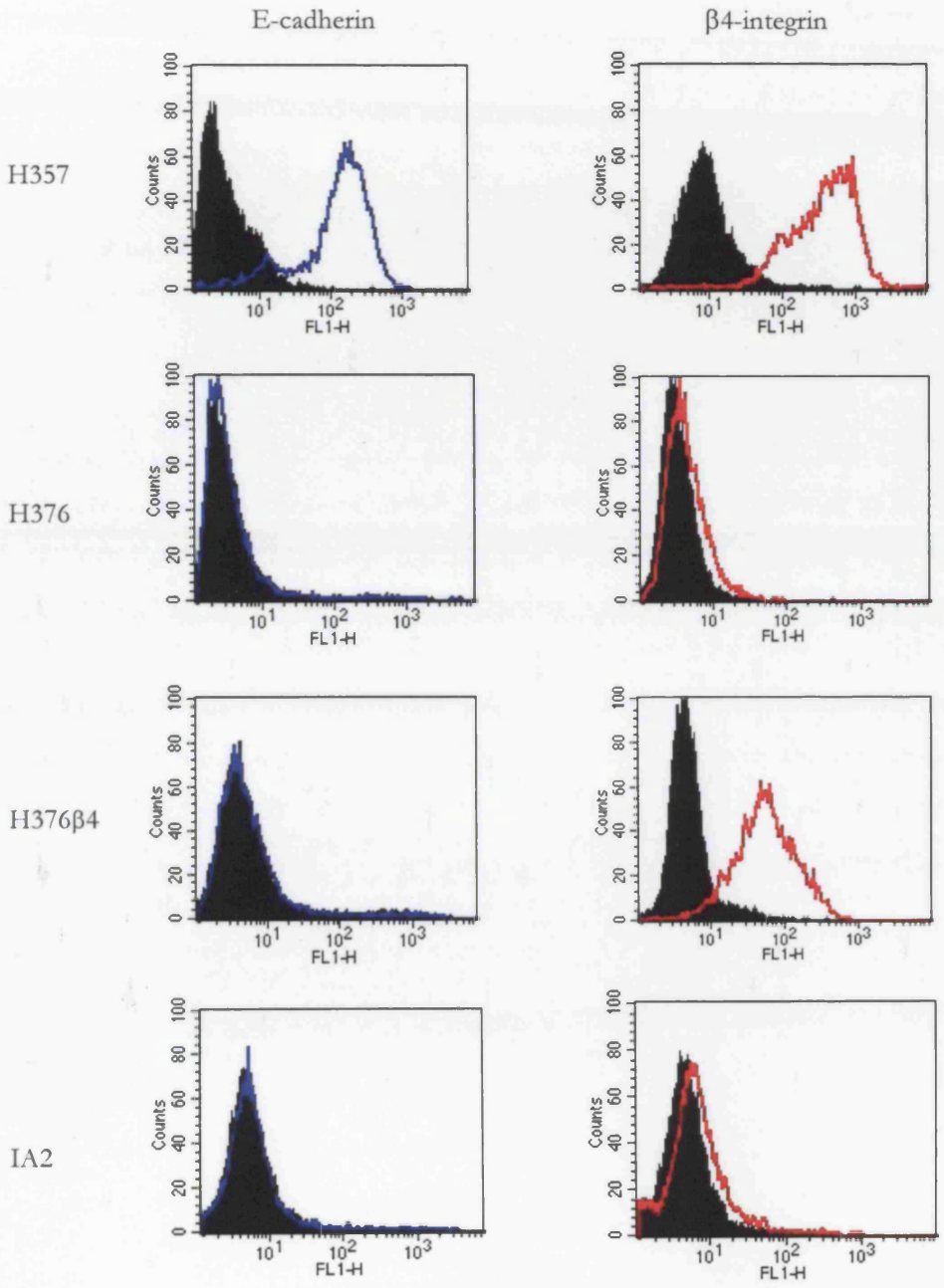


Figure 5.2

Detection of E-cadherin and β4-integrin expression in the parental cell lines using Western blot analysis

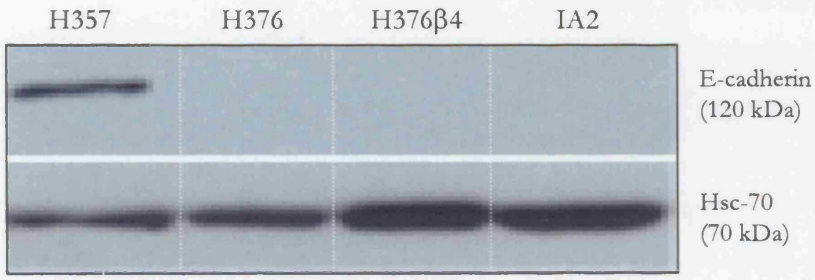
Equal cell numbers of each cell line were plated for 48 hours in KGM prior to lysing. Whole cell RIPA lysates were obtained, and equal protein was determined by BCA protein assay. Proteins (25 μg/lane) were resolved by SDS-PAGE and blotted onto nitrocellulose membrane.

a) For E-cadherin detection, the blot was probed using HECD-1 antibody and shows a 120 kDa band in relation to H357 line "positive control", whereas H376, H376β4 and IA2 parental cell lines were negative for E-cadherin. The bottom blot was probed for Hsc-70 and used as a loading control.

b) For β4-integrin detection, the blot was probed using "clone 7" anti-β4-integrin monoclonal antibody and shows a 200 kDa band in relation to the H376β4 cell line, only. In contrast, both H376 and IA2 parental cell lines were negative for β4-integrin. The bottom blot was probed for Hsc-70 and used as a loading control.

Figure 5.2

a)


H357
H376
H376β4
IA2
E-cadherin
(120 kDa)
Hsc-70
(70 kDa)

b)

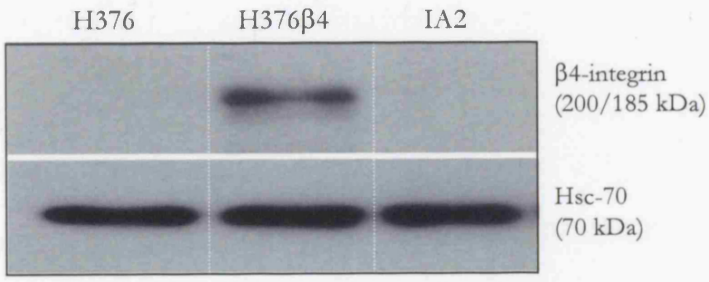

H376
H376β4
IA2
β4-integrin
(200/185 kDa)
Hsc-70
(70 kDa)

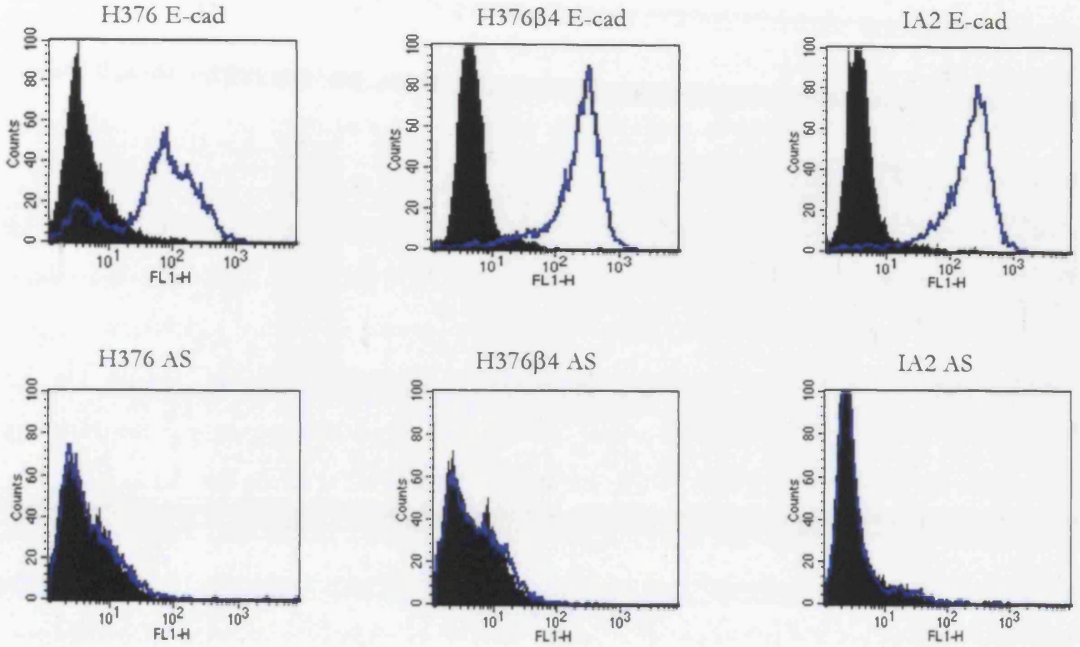
Figure 5.3

Determination of E-cadherin expression in stably transfected cell lines using FACS analysis and Western blotting

FACS analysis: Cells were allowed to grow to confluency then harvested using the E-cadherin preservation method (Chapter 2, Section 2.2.1.1). FACS results showed E-cadherin surface expression of the transfected cell lines, the black histogram represents the negative control labelled with Mouse IgG1 negative control (DAKO), while the blue histogram represents the positive cell population expressing E-cadherin labelled with HECD-1 antibody. H376 antisense, H376β4 antisense and IA2 antisense cell lines showed no expression of E-cadherin while the H376 E-cadherin, H376β4 E-cadherin and IA2 E-cadherin cell lines expressed high levels of E-cadherin.

Western blot analysis: Whole cell RIPA lysates (25 μg/lane) were resolved by SDS-PAGE and blotted onto nitrocellulose membranes. The top blot was probed for E-cadherin using HECD-1 antibody and shows a 120 kDa band in relation to the H376 E-cadherin, H376β4 E-cadherin and IA2 E-cadherin cell lines transfected with the E-cadherin sense construct. In contrast cell lines transfected with the anti-sense construct (H376 antisense, H376β4 antisense and IA2 antisense) were negative for E-cadherin. The bottom blot was probed for Hsc-70 which was used as a loading control.

Figure 5.3


H376 E-cad
H376β4 E-cad
IA2 E-cad
H376 AS
H376β4 AS
IA2 AS
Counts
FL1-H

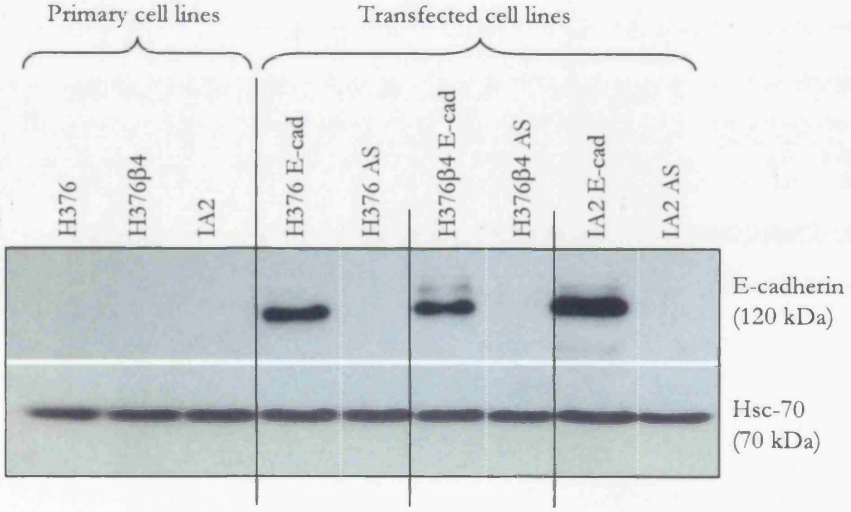

Primary cell lines
Transfected cell lines
H376
H376β4
IA2
H376 E-cad
H376 AS
H376β4 E-cad
H376β4 AS
IA2 E-cad
IA2 AS
E-cadherin
(120 kDa)
Hsc-70
(70 kDa)

Figure 5.4

Determination of β4-integrin expression in stably transfected cell lines using FACS analysis and Western blotting

FACS analysis: Surface expression of the β4-integrin subunit in the transfected cell lines; H376β4 E-cadherin, H376β4 antisense, IA2 E-cadherin and IA2 antisense, was assessed using anti-β4 integrin antibodies (3E1). Geometric mean fluorescence (arbitrary units, log scale) as measured by flow cytometry was used to plot the histogram. Figure 5.4 shows histogram plotted against the mean value of three independent experiments for β4-integrin. The mean fluorescence intensity (MFI) was determined by subtracting the fluorescence value of cells labelled with the appropriate IgG control antibody. Error bars represent standard error of the mean (sem). Results showed that *de novo* E-cadherin expression in transfected cell lines had resulted in a statistically significant increase in surface expression of β4-integrin as compared to the antisense control cell lines.

Western blot analysis: Whole cell RIPA lysates (25 μg/lane) were resolved by SDS-PAGE and blotted onto nitrocellulose membranes. The top blot was probed for β4-integrin using "clone 7" anti-β 4-integrin monoclonal antibody and shows a 200 kDa band in relation to the H376β4 parental cell line, as well as the H376β4 E-cadherin cell line and the IA2 E-cadherin cell line. The bottom blot was probed for Hsc-70 which was used as a loading control. The β4-integrin was represented by double bands of 200 kDa and 185 kDa molecular weight.

Figure 5.4

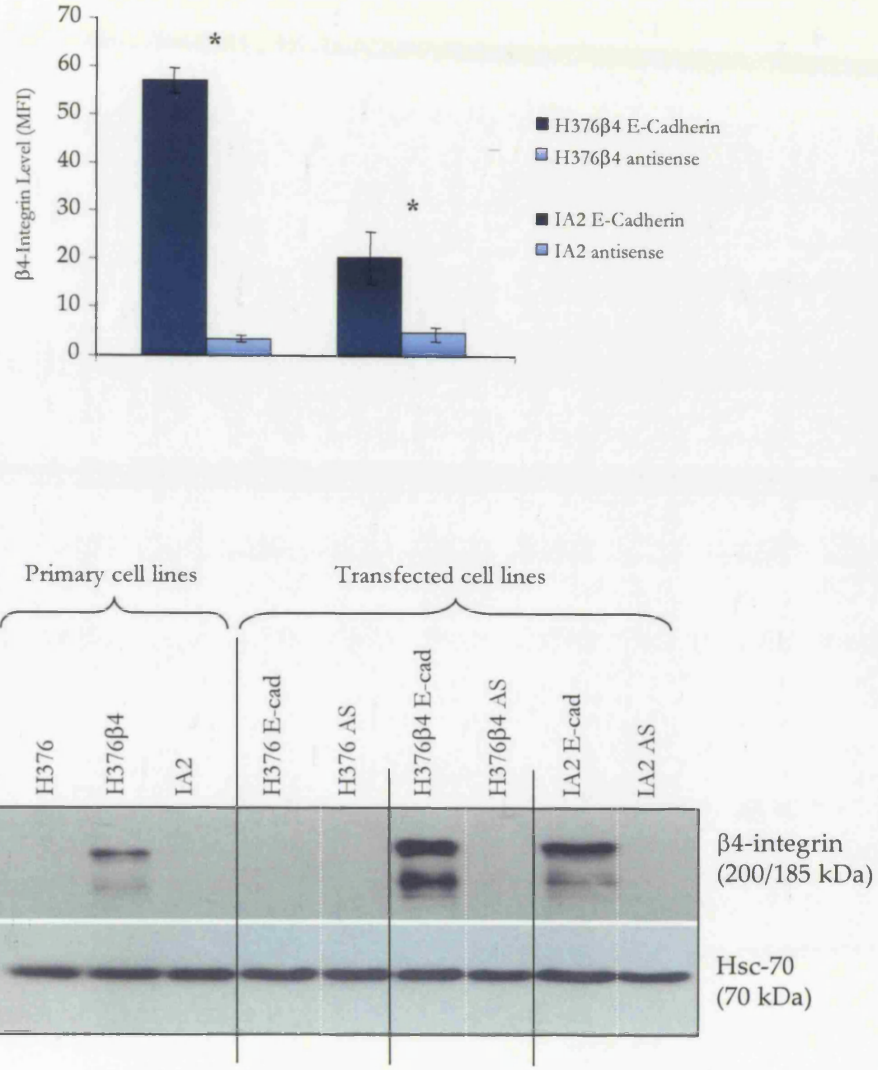

β4-Integrin Level (MFI)
70
60
50
40
30
20
10
0
*
*
H376β4 E-Cadherin
H376β4 antisense
IA2 E-Cadherin
IA2 antisense
Primary cell lines
Transfected cell lines
H376
H376β4
IA2
H376 E-cad
H376 AS
H376β4 E-cad
H376β4 AS
IA2 E-cad
IA2 AS
β4-integrin
(200/185 kDa)
Hsc-70
(70 kDa)

Figure 5.5
DNA fingerprinting

Figure 5.5

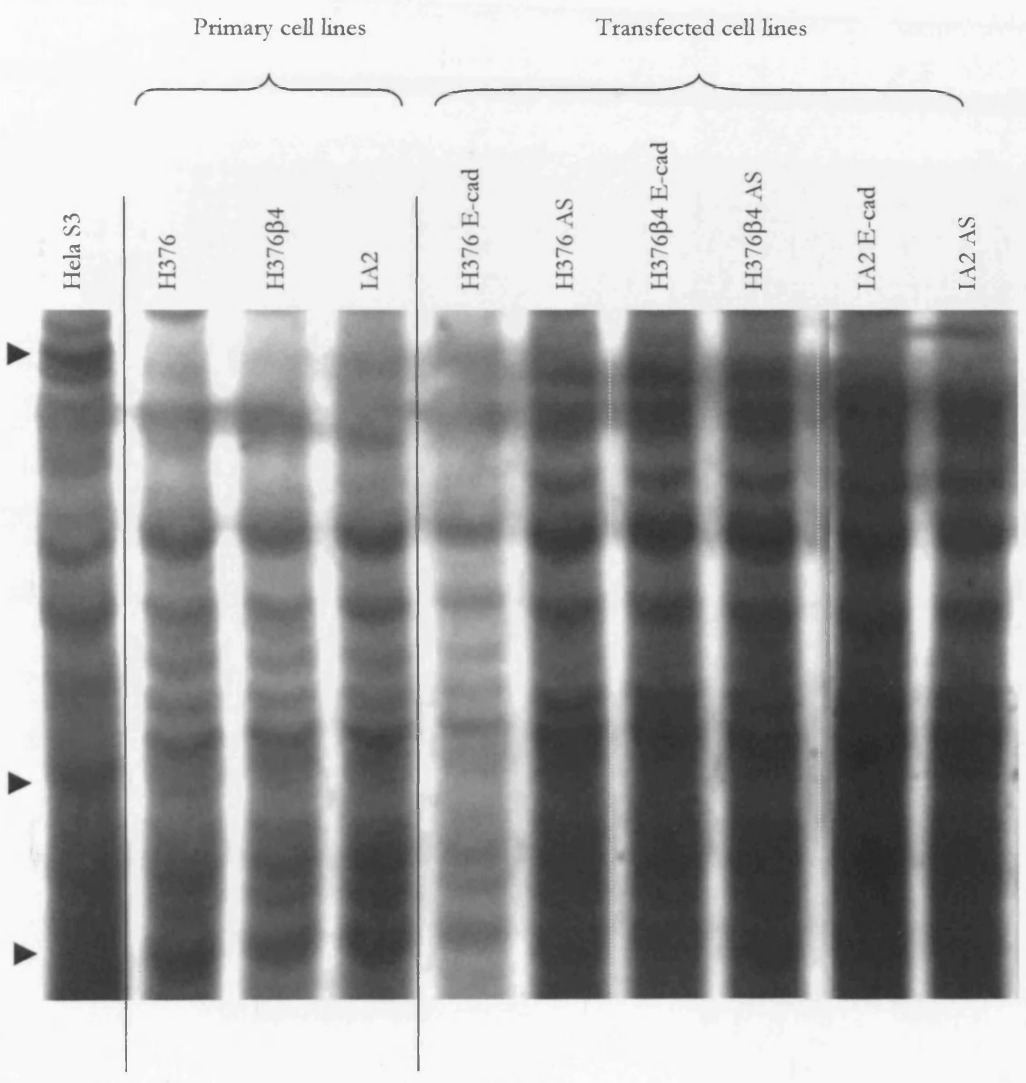

Primary cell lines
Transfected cell lines
Hela S3
H376
H376β4
IA2
H376 E-cad
H376 AS
H376β4 E-cad
H376β4 AS
IA2 E-cad
IA2 AS

Figure 5.6

Immunocytochemical assessment of β4-integrin expression in OSCC cell lines

2 x 10^4 cells were plated onto laminin-5-coated 13mm coverslips in a 24-well plate and allowed to grow for 48 hours. Cells were examined on a confocal laser scanning microscope, and images collected digitally.

Panels A and C are confocal images of H376β4 E-cadherin cell line. Panels B and D are confocal images of CA1 cell line.

Panels A and B show β4-integrin, "green", detected by anti-β4-integrin monoclonal antibody (BD Transduction Laboratories). Panel A shows reticular distribution of β4-integrin in H376β4 E-cadherin transfectans and Panel B the β4-integrin SAC-like structures.

Panels C and D are composite confocal images showing β4-integrin, "green", actin cytoskeleton, "red", detected with phalloidin-TRITC and nuclei, "blue", detected with DAPI.

Figure 5.6

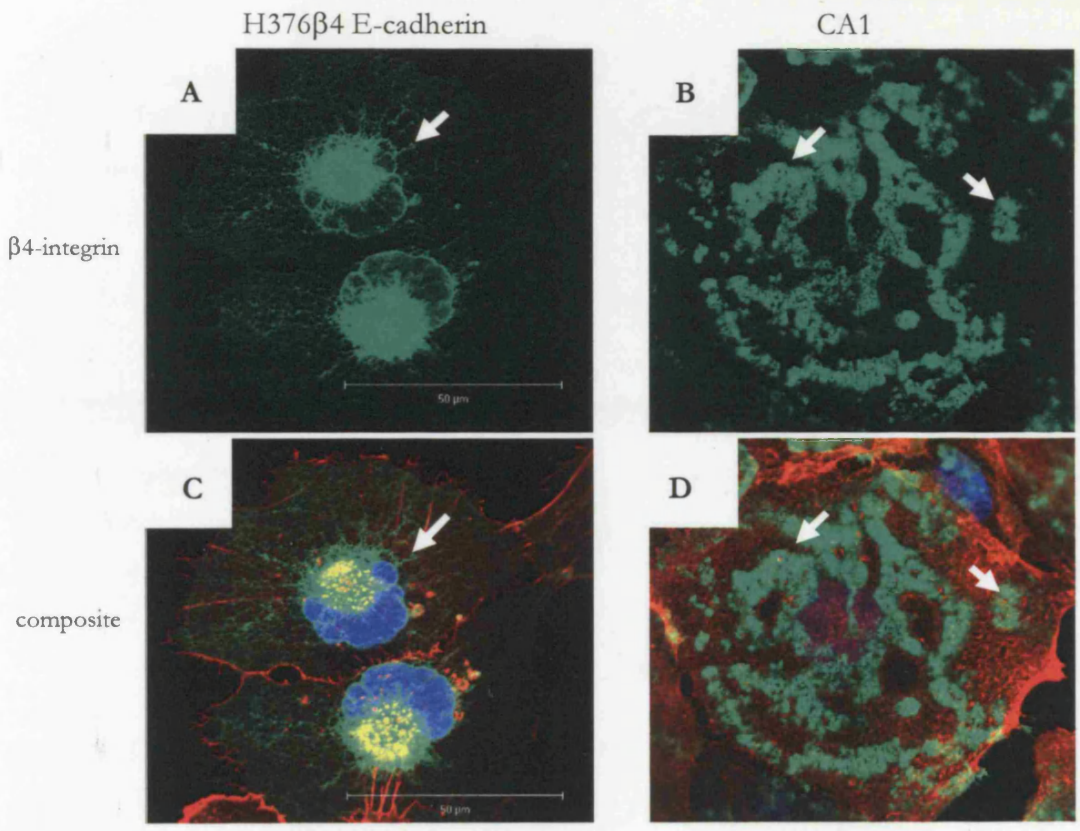


Figure 5.7

Determination of E-cadherin expression in H376 E-cadherin transfected cell line

2×10^4 cells were plated onto 13mm coverslips in a 24-well plate and allowed to grow for 48 hours with selection medium. Cells were examined on a confocal laser scanning microscope, and images collected digitally.

In Panel A, E-Cadherin, "green", was detected by HECD-1 antibody and showed E-cadherin-dependent "zipper-like" cell-cell contacts (as denoted with open arrow).

In Panel B, actin cytoskeleton, "red", was detected with phalloidin-TRITC, and showed that the actin filaments co-localised with E-cadherin and redistributed into stress fibres.

Panel C is composite confocal a image showing E-cadherin, "green", actin cytoskeleton, "red", and nuclei, "blue", detected with DAPI.

Figure 5.7

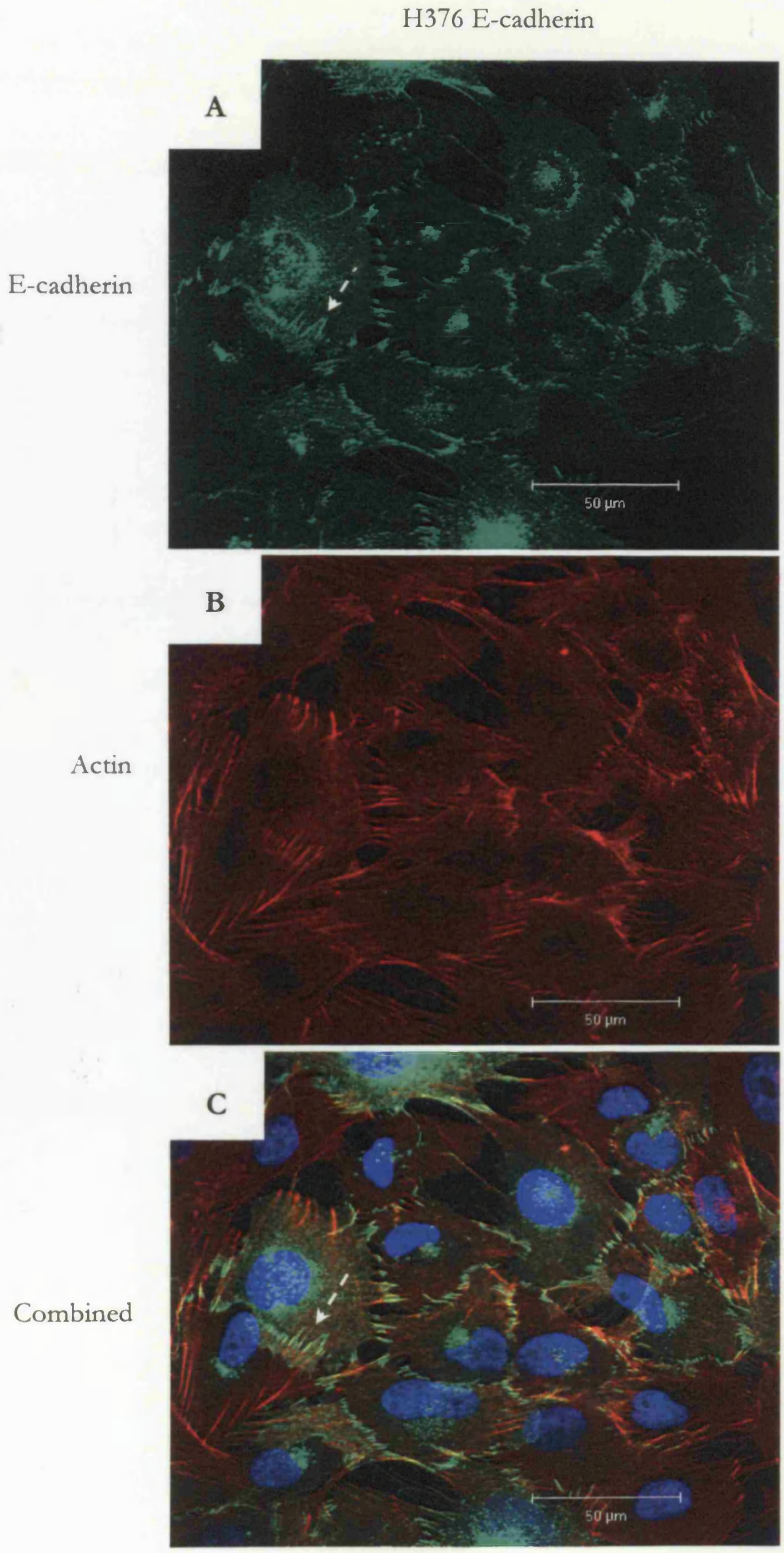

H376 E-cadherin
A
E-cadherin
50 µm
B
Actin
50 µm
C
Combined
50 µm

Figure 5.8

Determination of E-cadherin expression in H376β4 E-cadherin transfected cell line

2 x 10^4 cells were plated onto 13mm coverslips in a 24-well plate and allowed to grow for 48 hours with selection medium. Cells were examined on a confocal laser scanning microscope, and images collected digitally.

In Panel A, E-Cadherin, "green", was detected by HECD-1 antibody and showed E-cadherin-dependent "straight-line" cell-cell contacts, "green",(as denoted with arrows).

In Panel B, actin cytoskeleton, "red", was detected with phalloidin-TRITC, and showed redistribution of the actin filaments.

Panel C is composite confocal a image showing E-cadherin, "green", actin cytoskeleton, "red", and nuclei, "blue", detected with DAPI.

Figure 5.8

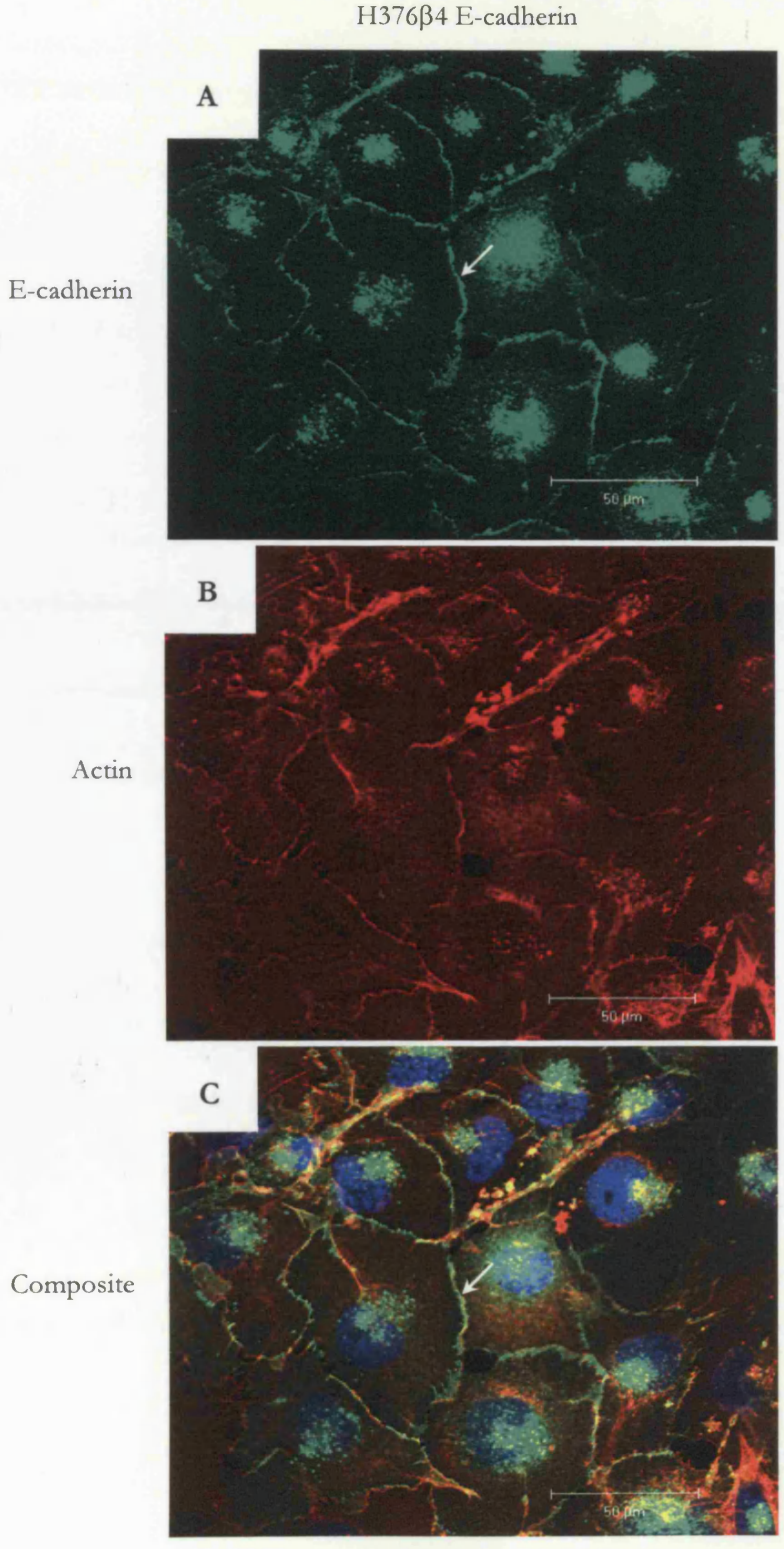

H376β4 E-cadherin
A
E-cadherin
50 µm
B
Actin
50 µm
C
Composite
50 µm

Figure 5.9

Determination of E-cadherin expression in IA2 E-cadherin transfected cell line

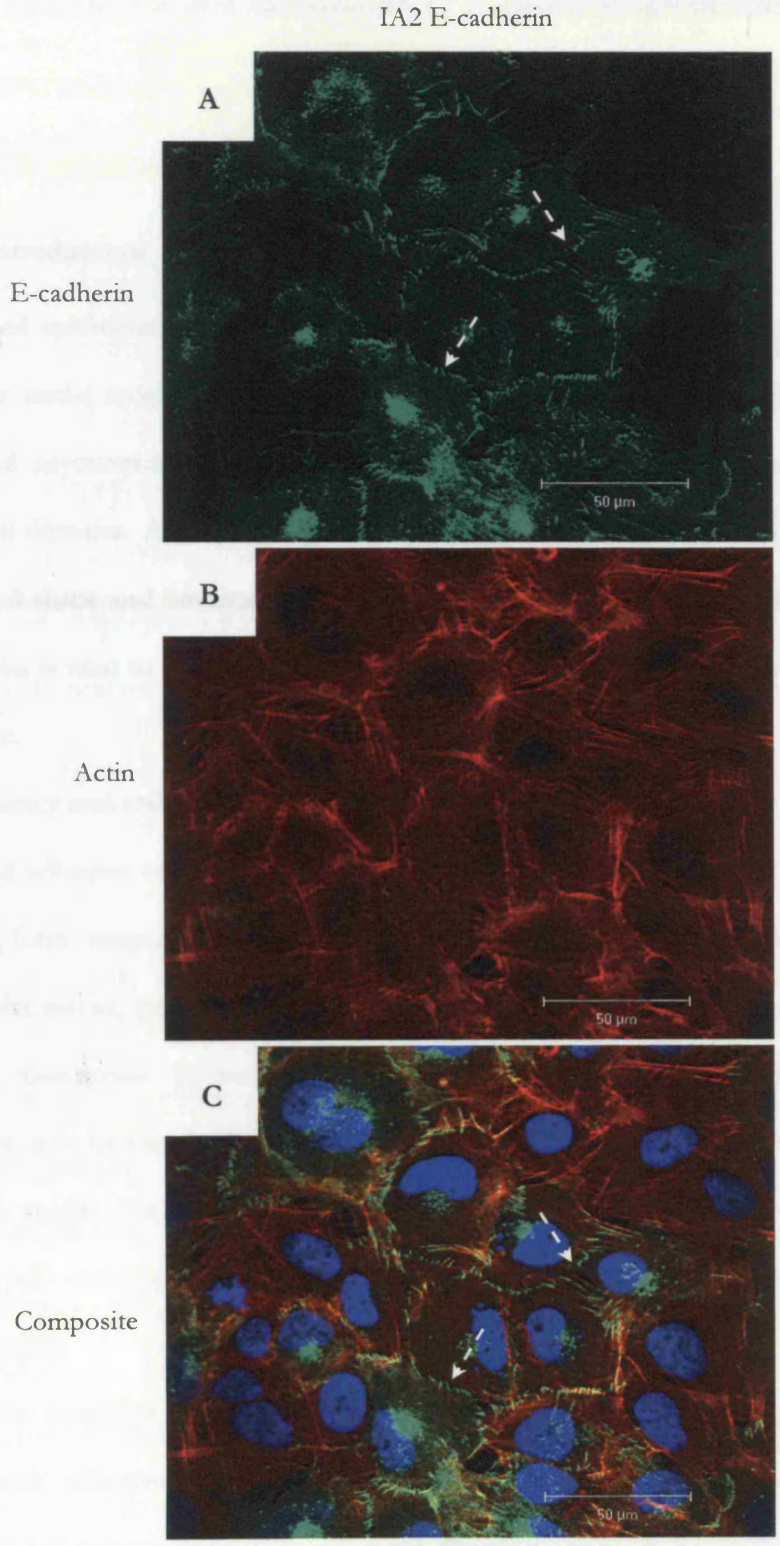
2 x 10^4 cells were plated onto 13mm coverslips in a 24-well plate and allowed to grow for 48 hours with selection medium. Cells were examined on a confocal laser scanning microscope, and images collected digitally.

In Panel A, E-Cadherin, "green", was detected by HECD-1 antibody and showed E-cadherin-dependent "zipper-like" cell-cell contacts (as denoted with open arrow).

In Panel B, actin cytoskeleton, "red", was detected with phalloidin-TRITC, and showed that the actin filaments co-localised with E-cadherin and redistributed into stress fibres.

Panel C is composite confocal a image showing E-cadherin, "green", actin cytoskeleton, "red", and nuclei, "blue", detected with DAPI.

Figure 5.9


IA2 E-cadherin
A
E-cadherin
50 µm
B
Actin
50 µm
C
Composite
50 µm

CHAPTER 6

β4-integrin and Maturation of E-cadherin-Dependent Adherens Junctions

6.1 Introduction

A polarised epithelium is composed of epithelial cells that possess a cuboidal morphology with their nuclei localised at the basal aspect of the cell, and with their cellular proteins distributed asymmetrically along well-defined membrane domains; the apical and the basolateral domains. A polarised epithelial phenotype provides one of the primary examples of how cell shape and function essentially are dependent on each other. The maintenance of polarisation is vital to ensure an intact structural integrity and physiological function of the epithelium.

The efficiency and stability of this epithelial barrier is maintained through the interaction of specialised adhesive complexes that either interact with similar structures on neighbouring cells, to form intercellular junctions (Nelson, 2003), or interact with the underlying extracellular matrix, through hemidesmosomes, to anchor the overlying epithelial cells to the basement membrane (Borradori and Sonnenberg, 1999) (Figure 6.1). These adhesive complexes are involved directly in the organisation of the cytoskeleton and provide important spatial cues to generate cell polarity and enable cells to sense and respond to adjacent cells and to the surrounding environment (Borradori and Sonnenberg, 1999; Nelson, 2003).

Intercellular junctions include: tight junctions, adherens junctions, and desmosomes. The cadherin-rich adherens junctions and desmosomes provide tight adhesion and mediate strong cell-cell contact while tight junctions, the most apical of these junctional complexes,

seal the epithelial cells together rendering the epithelial front impermeable to ions and small solutes (Fleming *et al.*, 2000).

Cadherin-dependent Adherens 'Cell-Cell' Junctions:

Adherens junctions are formed by cadherin molecules that mediate calcium-dependent cell-cell adhesion. They have a common structural organisation, and their junctional complexes consist of extracellular components, transmembrane components and cytoplasmic domains that interact with adaptor proteins, collectively known as the catenins, which all together assemble to form a cytoplasmic cell adhesion complex (CCC), that provides a direct, or an indirect, link to the actin cytoskeleton (Christofori and Semb, 1999).

Epithelial cells express at least two types of classical cadherin molecules, E- and P-cadherin. In contrast to other cadherin molecules expressed in other cell types, adhesion mediated by E-cadherin triggers the development of epithelial morphology (Gumbiner, 1996). Loss of E-cadherin function, either through inhibition by different experimental methods or naturally, such as during tumourigenesis, has been shown to result in the loss of the epithelial morphotype (Gumbiner, 1996; Christofori and Semb, 1999).

Although cell-cell adhesion might, at first, seem static it is in fact a dynamic process and the reorganisation of these adhesion complexes triggers a wide range of vital physiological processes, starting from the early days of embryonic development and continuing throughout tissue development (Takeichi, 1995; Adams and Nelson, 1998; Gumbiner, 2000; Tepass *et al.*, 2000). Similar reorganisation also is implicated during pathophysiological processes such as wound-healing and tumour metastasis (Fukata and Kaibuchi, 2001). Cellular polarity in earliest stages of vertebrate development is thought to be mediated by E-cadherin (Wheelock and Johnson, 2003b).

Cell-cell adhesion complexes rearrange constantly by remodelling of the cadherin-catenin complexes. It has been estimated that half of the E-cadherin molecules in any individual cell are engaged with the actin cytoskeleton to form cell-cell adhesion complexes, while the remainder remain in the cytoplasm, unattached (Sako *et al.*, 1998).

The α6β4-integrin-dependent Hemidesmosomal 'Cell-ECM' Junctions:

The hemidesmosome is a complex structure, consisting of several proteins that assemble collectively to form an attachment site for the intermediate (Keratin) filament network in epithelial cells and contributes to the attachment of epithelial cells to the underlying basement membrane (Jones *et al.*, 1991; Borradori and Sonnenberg, 1999). The integrin α6β4 is an essential component of the hemidesmosome (Stepp *et al.*, 1990; Jones *et al.*, 1998; Borradori and Sonnenberg, 1999) which uses as a substrate laminin-5 anchoring filaments to anchor the epithelial cells tightly to the underlying basement membrane (Carter *et al.*, 1991; Rousselle *et al.*, 1991; Geuijen and Sonnenberg, 2002).

Recent studies suggest that the α6β4 integrin plays a critical role in transducing signals from the extracellular environment to the interior of the cell; significantly modulating the organisation of the cytoskeleton, cell proliferation, apoptosis and differentiation (Borradori and Sonnenberg, 1999). The bullous pemphigoid antigen BP180 (BPAG-2) hemidesmosomal component, also known as collagen XVII (Jones *et al.*, 1998), interacts directly with the α6β4 integrin and was found to play a role in establishing cell-cell interaction via actinin, a component of the actin cytoskeleton; thus suggesting a "cross-talk" interaction between the cell-ECM and cell-cell adhesion systems (Gonzalez *et al.*, 2001).

Formation of the cadherin-dependent cell-cell junctions:

Cadherin molecules exist as parallel dimers at the surface of expressing cells (Chapter 1, Figure 1.2); a phenomenon thought to be mediated solely by the extracellular domains of the cadherin molecules; additionally Ca^{2+} ions are thought not to play a role in this dimerisation process (Chitaev and Troyanovsky, 1998).

Cadherin-dependent cell-cell adhesion is an highly organised process that consists of sequential steps of increasing complexity (Figure 6.2), that contribute to the generation of the polarised, fully differentiated, epithelial cell (Braga, 2000; 2002):

Step 1 (Homophilic binding): In the presence of Ca^{2+} ions, an 'homophilic interaction', between cadherin dimers on neighbouring cells, is initiated. This is followed by:-

Step 2 (Clustering): Clustering of the adhesion complexes at sites of cell-cell contacts.

Step 3 (Stabilisation of the adhesion complexes): Adhesion molecules cluster at cell-cell contacts and are further stabilised through the formation of the CCC and its subsequent interaction with the cytoskeletal proteins. Homophilic binding generates weak adhesive forces that are further strengthened via clustering of the adhesion complexes and the cytoskeletal interaction (Gumbiner, 1996). However, association with the actin filaments was shown not to affect the homophilic binding, *per se*, of the cadherin molecules (Yap *et al.*, 1997).

Step 4 (Remodelling of the cytoskeleton): Remodelling and reorganisation of the cytoskeleton, with the recruitment of new actin to the junction, follows and results in the generation of the necessary mechanical forces that are utilised for epithelial compaction and polarisation.

Step 5 (Generation of a polarised epithelial phenotype): Simultaneously, three other key processes take place: change in cell shape, formation of other adhesive structures, and

formation of distinct cell membrane domains, that collectively contribute to the formation of a polarised epithelial phenotype.

The actual machinery for the development of cell-cell adhesion and regulation of the polarised epithelial phenotype is still far from being completely understood. Recently, the signalling pathway regulated by Rho small GTPases has emerged as a key regulator that controls the cell membrane and actin cytoskeleton dynamics (Van Aelst and D'Souza-Schorey, 1997; Kaibuchi *et al.*, 1999; Bishop and Hall, 2000; Braga, 2000). Three members of the Rho small GTPases family (Rho, Rac, and Cdc42) have been implicated comprehensively in the process of epithelial morphogenesis and differentiation (Eaton *et al.*, 1996; Strutt *et al.*, 1997; Raich *et al.*, 1999; Stappenbeck and Gordon, 2000). It also has been shown that these members of the Rho small GTPases family play a major role in the regulation of adherens junctions and the development of epithelia (Takaishi *et al.*, 1997; Kodama *et al.*, 1999; Braga, 1999; Fukata and Kaibuchi, 2001).

Following identification of the regulator and effector molecules of the small GTPases subfamily, signalling pathways running both ways, from cadherin-dependent adhesion complexes to Rho GTPases and from Rho GTPases back to cadherin adhesion complexes, are beginning to be clarified (Fukata and Kaibuchi, 2001; Braga, 2002).

Rho GTPases act like molecular switches; they cycle between an inactive, GDP-bound variant, and an active, GTP-bound variant. There are several mechanisms by which Rho family GTPases can affect the E-cadherin-dependent cell-cell junctions. For example, Rho GTPases have been reported to directly affect E-cadherin-catenin complex. When Rac1 and Cdc42 are inactive their effector protein, IQGAP1, binds to both the E-cadherin cytoplasmic domain and to β-catenin, apparently causing a subsequent dissociation of the α-catenin/actin from the E-cadherin-catenin complex and loss of cell adhesion (Kuroda *et al.*, 1998; Fukata *et*

al., 1999a). On the other hand, when Rac1 and Cdc42 are active, they interact with IQGAP1; therefore preventing its interaction with the E-cadherin-catenin complex (Figure 6.3). Interchange between the active and inactive variants of Rho GTPases family proteins is tightly regulated by associated protein. The exchange of bound GDP for GTP and the subsequent activation of the GTPases is controlled by Guanine nucleotide exchange factors (GEFs), while the inactivation is stimulated by intrinsic hydrolysis of GTP by GTPase-activating proteins (GAPs) (for review see: Van Aelst and D'Souza-Schorey, 1997). Tiam-1 is a Rac-specific GEF and is one important regulator of Rho GTPases functions in tumour cells (Mertens *et al.*, 2003). It has been reported that Tiam-1 expression promotes cadherin-mediated adhesion of fibroblasts and epithelial cells (Hordijk *et al.*, 1997; Sander *et al.*, 1998; Engers *et al.*, 2001). Knock-down of Tiam-1, using siRNA technology, in MDCK cells resulted in the acquisition of a migratory and mesenchymal phenotype, with disruption of the E-cadherin-dependent cell-cell contacts (Malliri *et al.*, 2004).

Results from the previous Chapter suggested that maturation of E-cadherin-dependent cell-cell junctions, in this particular OSCC cell line, was dependent upon the presence of the β4-integrin subunit. In this Chapter I decided to investigate this finding by conducting further experiments on just two cell lines, the H376 E-cadherin and the H376β4 E-cadherin lines. The fact that they had been proven to originate from the same parental cell line and that the only difference between them appeared to be the lack of the β4-integrin subunit in the H376 E-cadherin cell line made them a good model with which to study the effect of β4-integrin on the maturation of the E-cadherin-dependent cell-cell junction.

6.2 Results

6.2.1 Effect of the β4-integrin subunit on the maturation of the E-cadherin-dependent adherens junction

Using confocal microscopy the nature of the E-cadherin-dependent cell-cell junctions, in both the H376 E-cadherin and the H376β4 E-cadherin cell lines, was investigated carefully. Figure 6.4 shows a close-up of both E-cadherin-dependent cell-cell contacts. In the H376 E-cadherin cell line the E-cadherin molecules appeared to accumulate at the cell-cell border as punctata forming a "zipper-like" adhesion contact that failed to fuse into a linear contact. In contrast, E-cadherin-dependent cell-cell junctions in the H376β4E-cadherin cell line appeared as a "straight line" contact. This result suggests that the β4-integrin subunit plays a role in the maturation of the E-cadherin-dependent adherence junction, by allowing the E-cadherin punctata to fully merge into a straight-line cell-cell contact. Attached (Appendix IV) is a CD with a movie illustrating 3-D images of both types of adhesion contacts.

6.2.2 β4-integrin knock-down in the H376β4 E-cadherin transfected cell line

Results from the immunocytochemical staining experiments suggested that the β4-integrin subunit contributes to the maturation of the E-cadherin-dependent cell-cell contacts. If this suggestion was true, then knock-down of β4-integrin should result in disruption of these cell-cell contacts. To test this hypothesis β4-integrin gene silencing was achieved using RNA interference (RNAi) technology on the H376β4 E-cadherin transfected cell line. In these studies β4-integrin mRNA was silenced specifically and β4-integrin expression was, consequently, inhibited. A pool of synthetic small interfering (si) double stranded RNA sequences (Dharmacon) was used to knock-down β4 to test if this could convert the cell-cell contact from a mature straight-line into an immature zipper-like contact. As described in

Chapter 2, Section 2.5.2, cells were grown in 6-well plates in an antibiotic-free KGM medium. When they were 40% confluent, they were transfected with either β4-integrin RNAi complex or scrambled oligonucleotides (positive control) using Oligofectamine™ transfection reagent (Invitrogen). Post-transfection, cells were allowed to grow for 48 hours before the efficiency of the knock-down was tested using FACS analysis and Western blotting. Two positive controls were incorporated, the non-treated cells growing for 4 hours in OptiMEM before being replaced with full KGM medium and the cells being transfected with a scrambled oligonucleotide. FACS results, from five independent experiments, showed about a 93% knock-down as compared to the positive controls (Figure 6.5), where Western blot analysis, from five independent experiments, showed about a 90% knock-down (Figure 6.5). Concomitant with this expected reduction in β4 protein, there was a significant downregulation of E-cadherin by about 67% (Figure 6.5).

6.2.3 E-cadherin-dependent cell-cell junction formation as a consequence of β4-integrin knock-down

Indirect immunostaining was used to study the effect of the β4 knock-down on the integrity of the E-cadherin-dependent cell-cell contacts. For each knock-down experiment OptiMEM and scrambled control were used. β4-integrin knock-down was, first, carried out in 6-well plates (as described above). 24 hours post-transfection, cells were harvested and plated on top of 13mm coverslips in 24-well plates and allowed to grow for another 24 hours in full KGM medium. 48 hours post-transfection, cells were fixed using 4% formaldehyde, permeabilised with Triton X-100 then stained for E-cadherin with HECD-1 monoclonal antibody. Results showed that, compared to the positive control cells, when β4-integrin was knocked-out there was a disruption of the cell-cell junctions. Thus where the straight-line

cell-cell contacts had been observed previously they now were reverted back to the less mature, zipper-like junctions with more stress fibres co-localising with the surviving E-cadherin molecules (Figure 6.6). Furthermore, as a consequence of β4-integrin knock-down, the H376β4 E-cadherin cell line exhibited what appeared to be a more fibroblastic phenotype as compared to their original, and the control, epithelial morphology (Figure 6.6). Perhaps even more interestingly lobulated nuclei were reverted back to an oval shape when β4-integrin was knocked-out indicating that β4-integrin was accountable for these changes in appearance detected in the previous chapter (see Chapter 5, Section 5.2.6)

6.2.4 β4-integrin knock-down in the CA1 OSCC cell line

I also studied this phenomenon in another cell line; the CA1 OSCC cell line (a kind gift of Professor Ian Mackenzie). As mentioned earlier, this particular cell line showed well-formed, SAC-like hemidesmosomal structures when it was stained for the β4-integrin subunit (Chapter 5, Figure 5.5). RNAi-mediated depletion of β4-integrin was carried out on the CA1 cell line in exactly same way as described above. FACS and Western blot analyses confirmed an efficient β4-integrin knock-down of about 96% (Figure 6.7). Concomitantly there was about a 42% downregulation of the E-cadherin molecule which occurred when the β4-integrin subunit was knocked down (Figure 6.7). However, there was little difference in cell-cell contact morphology as compared to the OptiMEM and scrambled control, with only 5-10% of the total population showing altered cell morphology and cell-cell contact (Figure 6.8). Unfortunately, this experiment was conducted only once, so this phenomenon could not be generalised as occurring on other epithelial cells. Ideally I would have liked to test a range of other β4-expressing cell lines, including normal epithelial cells, and this is something I would have done had I had more time available.

6.2.5 In the H376β4 E-cadherin OSCC cell line the β4-integrin subunit induces the maturation of E-cadherin-dependent cell-cell contact in a Rac1-dependent manner

De novo β4-integrin expression in the H376β4 E-cadherin cell line resulted in actin remodelling; thus this cytoskeletal protein was transformed, from bundles of stress fibres co-localising with the E-cadherin surface molecules in the H376 E-cadherin transfectants, into apparently disintegrated structures in the H376β4 E-cadherin cell line (see Chapter 5, Figures 5.7 and 5.8).

Members of the Rho small GTPases family (Rho, Rac, and Cdc42) have been identified as regulators of actin cytoskeleton dynamics (Braga, 2000). It was reported that the activation of Rac results in the formation of more intermingled membrane junctioning between neighbouring cells (Takaishi *et al.*, 1997; Stoffler *et al.*, 1998; Kodama *et al.*, 1999). Therefore, I thought that the Rho small GTPases, particularly Rac, most likely were serious contenders to be the down-stream effectors of β4-integrin.

Endogenous Rac1 levels were assessed using indirect immunostaining. Cells were plated on top of 13mm coverslips and allowed to grow for 48 hours, until they reached confluency, in full KGM medium. Rac1 expression was stained for using human anti-Rac1 monoclonal antibody (Transduction Laboratories). Results showed that Rac1 was expressed in abundance in the cytoplasm of the H376 E-cadherin cell line, but not at cell-cell contacts, denoting the apparent failure of E-cadherin alone to recruit Rac1 to the cell-cell junctions (Figure 6.9). In contrast, Rac1 in the H376β4 E-cadherin cell line was localised both in the nuclei and at the cell-cell junctions; a shift in localisation that appeared to have occurred as a consequence of the *de novo* expression of E-cadherin in the H376β4 cell line (Figure 6.9).

Knock-down of the β4-integrin subunit resulted in the translocation of Rac1 from the cell-cell contacts, and from the nuclei, to the cytoplasm (Figure 6.10). Furthermore, in almost 30% of the cells, Rac1 accumulated into polarised large vesicles located near the cell membrane at the leading edge of the cell (designated by arrows, Figure 6.10).

The effect of levels of β4-integrin subunit on the translocation of Rac1 was tested in the CA1 OSCC cell line and here also endogenous Rac1 was found to be located at the cell-cell junctions and in the nuclei (Figure 6.10). When the β4-integrin subunit was silenced using RNAi, the Rac1 translocated into the cytoplasm and to the free cell border (Figure 6.10).

Taken altogether, these results suggest that the β4-integrin might affect the maturation of the E-cadherin-dependent cell-cell adhesion through activation and/or recruitment of Rac1 to the site of the cell-cell junction. Also, this result might well suggest that Rac1, in the H376 E-cadherin cell line, is expressed in an 'inactive' state, while in the H376β4 E-cadherin cell line it is expressed as an 'active' variant of Rac1.

6.2.6 Pull-down activity assay

Rac1 and Cdc42 activity in H376E-cadherin was assessed using the GST-PAK-CRIB pull-down assay (Chapter 2, Section 2.6.4). Active Rac1 and Cdc42 were pulled down with the GST-PAK-CRIB fusion protein. Amounts of active versus total proteins were assessed with Western blot analysis using human monoclonal antibodies against Rac1 and Cdc42 (Transduction Laboratories). *De novo* E-cadherin expression in H376 E-cadherin cell line resulted in inactivation of Rac1 as compared to the antisense negative control (Figure 6.11). This finding supports the previous results that the H376 E-cadherin cell line expresses an inactive variant of Rac1. In contrast E-cadherin re-expression has resulted in the activation of Cdc42 as compared to the antisense control cell line (Figure 6.11). This result was

consistent with the work of others who concluded that E-cadherin-mediated cell-cell adhesion activates Cdc42 (Kim *et al.*, 2000).

With the H376β4 E-cadherin cell line the results were inconclusive, as there was no activity detected, neither in relation to both the H376β4 E-cadherin cell line nor with the β4-knock-down control (data not shown). I suspect this was because a large fraction of Rac1 activity was not detected because it was intra-nuclear. I suggest that this particular assay should be repeated using another lysis buffer to include the nuclear proteins.

6.2.7 Introduction of constitutively-active and dominant-negative Rac1 into H376 E-cadherin and H376β4E-cadherin OSCC cell lines

To confirm that activation of Rac1 is accountable for the maturation of the E-cadherin-dependent cell-cell junction, dominant-negative (Rac^{N17}) or constitutively-active Rac1 (Rac^{V12}) variants were overexpressed transiently in both H376 E-cadherin and H376β4 E-cadherin cell lines, using FuGENE™ 6 Transfection reagent (Roche) and transfectants were analysed using indirect immunostaining. Figure 6.12 shows a representative illustration of the H376 E-cadherin cell line transfected with the constitutively-active Rac1 (Rac^{V12}) construct. In the H376 E-cadherin cell line, contrary to the endogenously expressed cytoplasmic Rac1, the GFP-tagged-Rac^{V12} translocated to either the cell-cell junction, or to the nucleus or to both locations (Green: Figure 6.12) and these re-localisations have been associated with the maturation of the E-cadherin-dependent cell-cell contacts (Figure 6.12, Red: designated with solid arrow), whereas negative cells still showed the immature zipper-like cell-cell junctions (Figure 6.12, Red: designated with open arrow). Interestingly, activation of Rac1 in the H376 E-cadherin cell line has lead to the occurrence of a fan-shaped cells (Figure 6.12); a phenotype previously described by Sander *et al.* (1998), where expression of constitutively

active Tiam1/Rac in MDCK cells contributed to the development of a fan-shaped motile phenotype in cells when grown on collagen I, whereas this effect was abrogated when cells were grown on laminin-1 or fibronectin (Sander *et al.*, 1998).

Likewise the GFP-tagged-Rac^{V12}, in the H376β4 E-cadherin line also was translocated to the cell-cell junction and to the nucleus (Figure 6.13); a similar localisation to that observed with the endogenously expressed Rac1 in this cell line, supporting the previous suggestion that the H376β4E-cadherin cell line does, indeed, express an active variant of Rac1. In contrast to its effect in the H376 E-cadherin cell line, over expression of Rac^{V12} in the H376β4E-cadherin cell line did not result in altered cell morphology and the transfected cells remained epithelial in shape (Figure 6.13).

To further prove that activation of Rac1 contributes to the maturation of E-cadherin-dependent cell-cell contacts in this particular cell line a dominant-negative (Rac^{N17}) construct was introduced into the H376β4 E-cadherin cell line where it was shown to result in the disruption of the original straight line contact and contributed to the development of the immature zipper-like E-cadherin-dependent cell-cell contacts (Figure 6.13).

6.3 Discussion

Results from Chapter 3 and 5, when taken together provide evidence that, at least in this particular OSCC cell line, E-cadherin can cross-talk to β4-integrin. *De novo* E-cadherin expression in all transfected cell lines resulted in an increase in the expression of the β4-integrin subunit. Moreover, visual assessment of the resultant E-cadherin cell-cell adherens junctions revealed there were marked differences between the ones which existed in the H376 E-cadherin cell line as compared to the ones expressed in the H376β4E-cadherin line. It appears therefore that the presence of β4-integrin in the H376β4E-cadherin cell line has contributed to:

a) the formation of a mature phenotype of E-cadherin-dependent cell-cell junctions.
b) the disruption of actin stress-fibres formed in the H376 E-cadherin cell line.
c) the development of a typical epithelial morphotype as compared to the less epithelial H376 E-cadherin cells.

In this Chapter I have provided evidence that the β4-integrin is accountable for all of the above changes. Silencing of β4-integrin mRNA and the consequent downregulation of β4-integrin, resulted in the disruption of cell-cell adherens junctions and contributed to the reappearance of E-cadherin-dependent zipper-like contacts together with the formation of stress fibres that co-localised with the E-cadherin molecules (Figure 6.4). Moreover, the knock-down of expression of the β4-integrin subunit in the H376β4 E-cadherin cell line has resulted in the reversion of the appearance of these cells from a more epithelial appearance into a more fibroblastic phenotype (Figure 6.6). Additionally, knock-down of β4-integrin resulted in a significant (p value $\leq$ 0.04 and 0.04) 67% downregulation of E-cadherin (Figure 6.5). This result is strongly suggestive of the existence of an interactive cross-talk between E-cadherin and β4-integrin. In general terms, my findings further support the observations by

Hintermann *et al.* (2005), where they report that α6β4 integrin increased E-cadherin-dependent cell-cell adhesion (Hintermann *et al.*, 2005).

In an attempt to find out if this phenomenon could be generalised to another epithelial cell line, knock-down of β4-integrin by RNAi was carried out in the CA1 OSCC cell line. Results from a single experiment showed that there was about 5-10% of the total cell population with altered cell-cell contacts and altered morphology (Figure 6.8) and about a 42% reduction in total levels of E-cadherin. Considering that this particular cell line showed well-formed SAC-like hemidesmosomal structures, when they were immunostained indirectly with anti-β4-integrin antibody (Figure 6.8), perhaps this is why knock-down of β4-integrin did not have a similar impact as in my previous model. Unfortunately, this experiment was conducted only once rendering the results intriguing rather than totally unequivocal. Nonetheless it does appear that the phenomenon of β4-integrin/E-cadherin crosstalk is applicable to more than just the model system utilised here.

Had I had more time available I would have repeated these experiments on CA1 cells and extended them to other cell lines, including normal epithelial cells.

Members of the Rho small GTPases family (Rho, Rac, and Cdc42) have been categorised as regulators of actin cytoskeleton dynamics (Braga, 2000). They were shown to play a significant role in adherens junction formation (Fukata and Kaibuchi, 2001; Braga, 2002) and are the most likely candidates to govern the phenomenon under study here. As mentioned above, activation of Rac was shown to result in the formation of a more intermingled membrane between neighbouring cells (Takaishi *et al.*, 1997; Stoffler *et al.*, 1998; Kodama *et al.*, 1999). These data suggest that, in this particular cell line; the H376 OSCC, the effect of β4-integrin upon the maturation of the E-cadherin-dependent cell-cell junction could, possibly, be mediated by Rac1.

Results showed that Rac1 was expressed in abundance in the cytoplasm of the H376 E-cadherin cell line. In contrast, Rac1 was concentrated at cell-cell contacts and in the nuclei of the H376β4 E-cadherin cell line (Figure 6.9). This result suggested that β4-integrin is associated with the translocation of Rac1. Knock-down of β4-integrin subunit, from the H376β4 E-cadherin cell line, resulted in the translocation of Rac1 from the cell-cell contacts, and from the nuclei, to the cytoplasm (Figure 6.10). Furthermore, in almost 30% of the cells, Rac1 accumulated into polarised, large vesicles located near the cell membrane at the leading edge of the cell (Figure 6.10; structures designated by arrows). Similarly, the specific knock-down of the β4-integrin subunit, from the CA1 cell line, also resulted in the translocation of Rac1 from the regions of cell-cell contact and the nuclei into the cytoplasm (Figure 6.10; structures designated by arrows). These results suggest that the β4-integrin might affect the maturation of the E-cadherin-dependent cell-cell adhesion through recruitment of Rac1 to the sites of cell-cell junctions.

Because activation of Rac was shown to result in the formation of a more intermingled membrane between neighbouring cells (Takaishi *et al.*, 1997; Stoffler *et al.*, 1998; Kodama *et al.*, 1999). I considered it possible that Rac1, in the H376 E-cadherin cell line, might be expressed in an 'inactive' state, while the H376β4 E-cadherin cell line might express an 'active' variant of Rac1. Pull-down activity assays showed that *de novo* expression of E-cadherin in the H376 E-cadherin cell line resulted in the inactivation of Rac1, as compared to the antisense control cell line (Figure 6.11); a finding in support of the previous suggestion. This however, is somewhat in contradiction to previous suggestions that cell-cell junction formation, mediated by cadherins, is strictly required for Rac recruitment and activation, and that the activation of Rac by itself cannot bypass this requirement (Braga *et al.*, 1999;

Nakagawa *et al.*, 2001). In my model system, E-cadherin restoration had, instead, lead to the activation of Cdc42 (Figure 6.11); a result consistent with the findings of Kim *et al.* (2000).
It has been reported that, depending on the Rho GTPase effector involved, actin remodelling builds different structures. Activation of the Rho GTPase, Cdc42, was shown to induce the formation of extended, linear, hair-like actin bundles. In contrast, Rac and Rho were shown to induce assembly of circumferential actin and to result in cell spreading (Etienne-Manneville and Hall, 2002). Thus it might be that the formation of actin stress-fibres in the H376 E-cadherin cell line is attributable to the activation of Cdc42 as a consequence of *de novo* E-cadherin expression.
Certainly it appears that, upon adhesion, cadherin receptors signal to other molecules that co-operate with Rac for the localised actin requirement at cell-cell junctions. Could it be that in this particular cell line; the H376 OSCC, that this 'other' molecule might be the β4-integrin subunit?

As mentioned earlier I had hypothesised that the H376β4 E-cadherin cell line might express an 'active' variant of Rac1. Unfortunately with the H376β4 E-cadherin cell line the results of the Rac1 pull-down assay were inconclusive, as there was no activity detected neither in the H376β4 E-cadherin cell line nor after the β4-knock-down control (Data not shown). I suspect, in retrospect, that this was because a large fraction of Rac1 activity was located in an intra-nuclear position. I suggest that this assay should be repeated but using a lysis buffer that would include the nuclear proteins.

I have, however, shown that activation of Rac1 was, indeed, required for the maturation of E-cadherin-dependent cell-cell contacts in the H376 E-cadherin cell line. Using the constitutively-active Rac1 (Rac^{V12}), it appeared that cells which overexpressed the active

variant exhibited a mature, straight E-cadherin-dependent, cell-cell contact as compared to those cells that did not express the Rac^{V12}. Moreover, Rac^{V12} was expressed at sites of cell-cell contact, as well as in the nuclei (Figure 6.12). Interestingly, expression of Rac^{V12} contributed to the occurrence of fan-shaped cells (Figure 6.12); a phenotype previously described by Sander *et al.* (1998), after the expression of constitutively active Tiam1/Rac in MDCK cells. In these authors' experiments such fan-shaped, motile cells occurred only when cells were grown on collagen I, whereas this effect was abrogated when cells were grown on laminin-1 or fibronectin (Sander *et al.*, 1998). Since α6β4 integrin is a known receptor for laminin-1 (Niessen *et al.*, 1994) and since the MDCK cell line is known to express β4-integrin, I am tempted to suggest that the changes observed by Sander *et al.* (1998) might be attributable to the α6β4 integrin. When both the H376 E-cadherin and the H376β4E-cadherin cell lines were transfected with the constitutively-active Rac1 (Rac^{V12}), only the H376 E-cadherin cell line, which lacks the β4-integrin subunit, showed these fan-shaped cells.

Similarly in the H376β4 E-cadherin cell line, the constitutively-active Rac1 (Rac^{V12}) also was translocated to the cell-cell junctions and to the nucleus (Figure 6.13); a similar localisation to the endogenously expressed Rac1 in this cell line. However, in contrast to the H376 E-cadherin line the expression of the Rac^{V12} did not appear to transform any of the cells into fan-shaped variants; instead, they all retained their epithelial morphology.

From the literature, the effects of Rho small GTPases in epithelial cells seem to be contradictory, since they have been shown to be required both for cell migration and also for cell-cell adhesion (Sander *et al.*, 1998; Sander *et al.*, 1999). This leads me to suggest that Tiam1/Rac-induced inhibition of migration on laminin-1 is mediated via β4-integrin. The absence of β4-integrin in the H376 E-cadherin cell line has shifted the cellular response to produce the fan-shaped cells.

Certainly it appears that, in this particular OSCC cell line, E-cadherin and β4-integrin 'cross-talk' to each other and it is possible that the β4-integrin might be a key role player in the maturation of E-cadherin-dependent adherens junction through either changes of location or change of activity status of the member of the Rho small GTPase family, Rac1.

Figure 6.1

Diagrammatic illustration of the hemidesmosomal components

Hemidesmosomes consist of two cytoplasmic cytoskeleton linkers; the 230 kDa human autoantigen named BP230 (BPAG-1) and Plectin, and two transmembrane proteins; the 180 kDa human autoantigen named BP180 (BPAG-2) and the $\alpha 6\beta 4$ integrin (Figure adapted from Cox and Hardin, 2004).

Figure 6.1

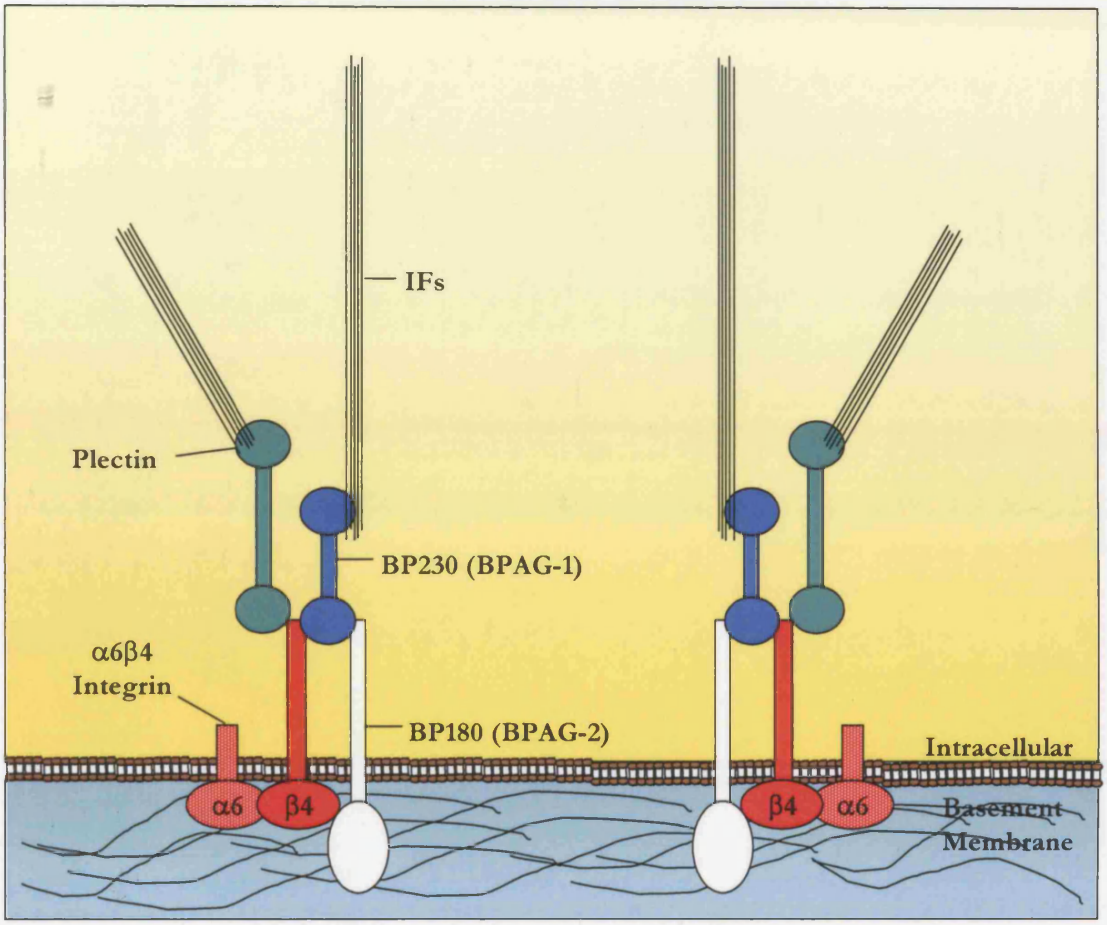
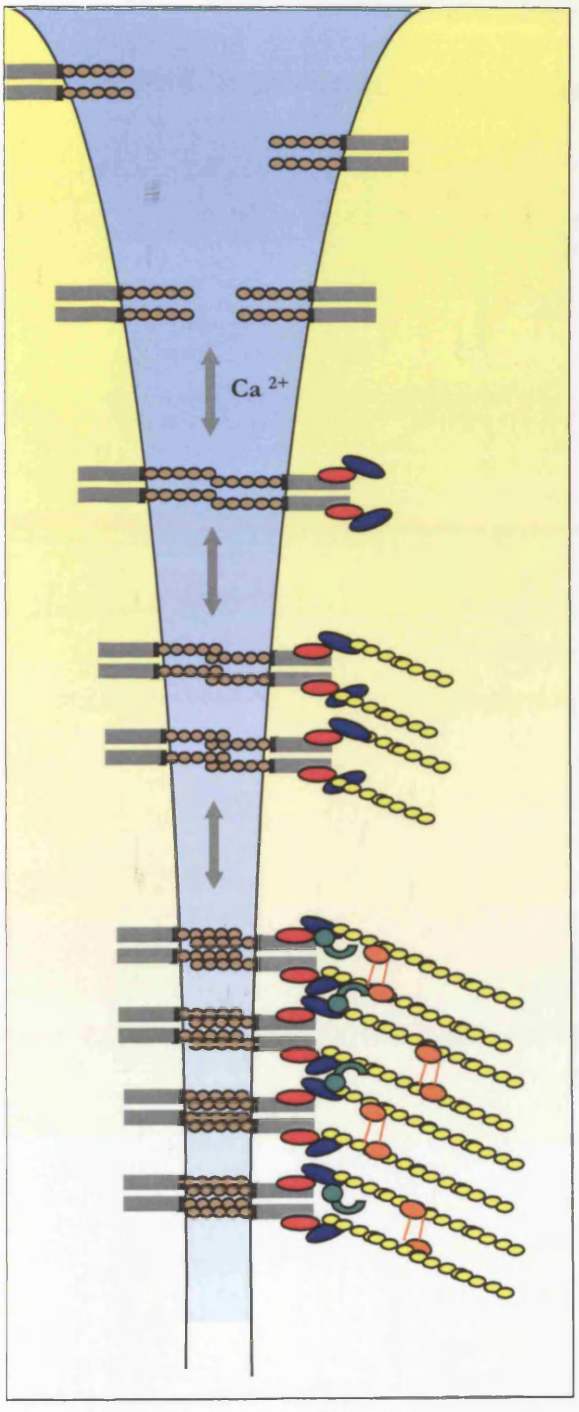

IFs
Plectin
BP230 (BPAG-1)
α6β4
Integrin
BP180 (BPAG-2)
Intracellular
α6
β4
β4
α6
Basement
Membrane

Figure 6.2

Diagrammatic illustration of steps in the formation of E-cadherin-dependent cell-cell junctions and epithelial polarisation

Formation of cadherin-mediated cell-cell contacts and the development of the epithelial morphotype is a complex process, which is believed to be under the control of several signalling pathways; one of most important being mediated by the Rho family GTPases (Figure adapted from Braga, 2000).

Figure 6.2



Cadherin-mediated adhesion and steps for the generation of Epithelial phenotype

Dimerisation of cadherin molecules

Ca^{2+}

1) Homophilic binding

2) Clustering

Recycling cadherins

3) Stabilisation of cell-cell adhesion + engagement of actin filaments

4) Recruitment of actin + remodelling of the cytoskeleton

Formation of adhesive structures

Change in cell shape

Formation of distinct domains

5) Polarised epithelial phenotype

Figure 6.3

Rho GTPases and the development of the E-cadherin-dependent cell-cell junctions

Digramatic illustration demonstrating the interchange between the active (GTP-bound) and inactive (GDP-bound) variants of the Rho GTPases family proteins (Rac1 and Cdc42). Tiam-1, Rac-specific GEF, regulates the switch between the two variants. Inactivation of Rho GTPases releases IQGAP1 effector protein which competes for binding with the β-catenin/E-cadherin complex and results in the dissociation of α-catenin/actin complex and the subsequent disruption of cell-cell contacts.

Figure 6.3

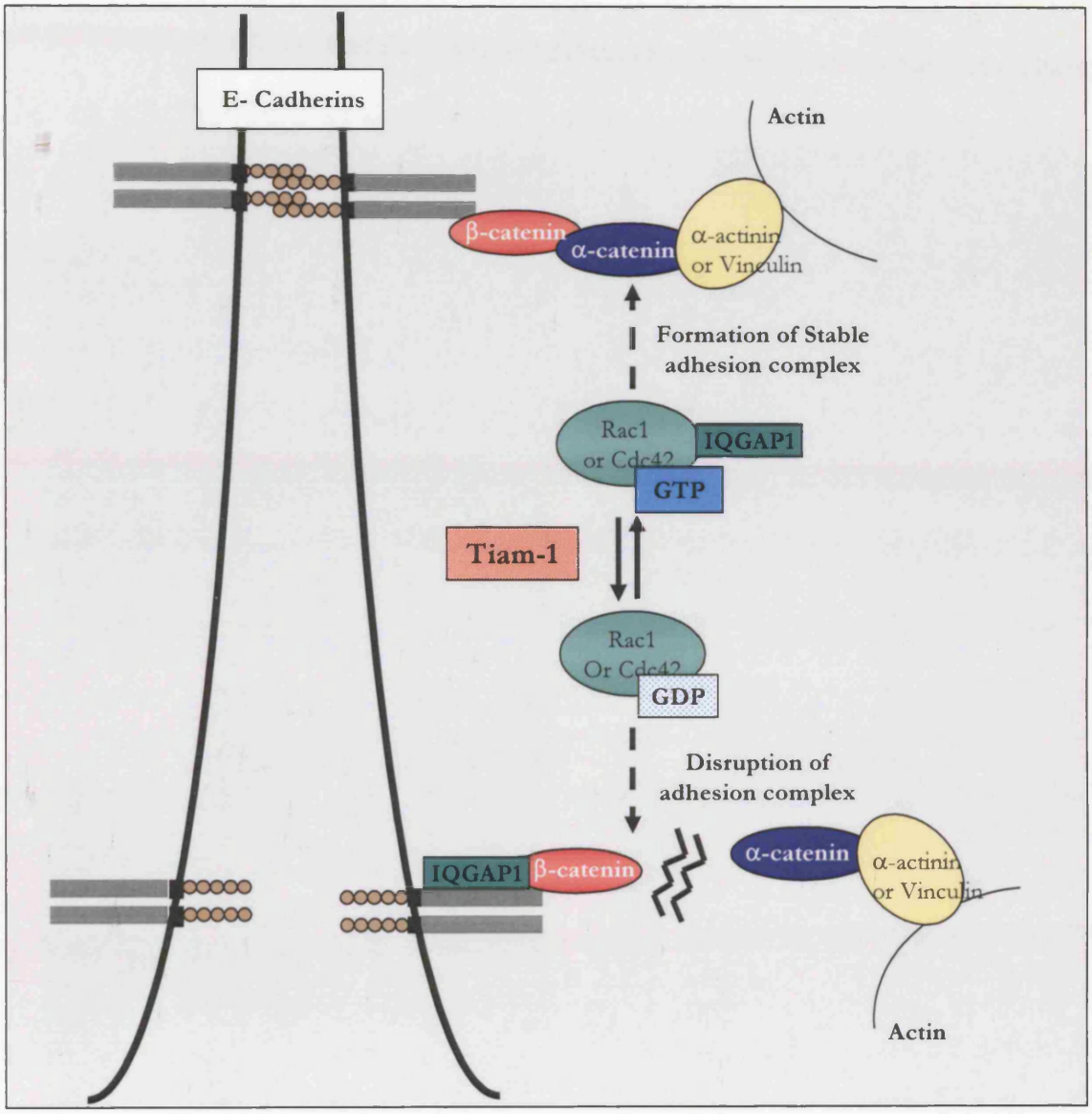

E- Cadherins
Actin
β-catenin
α-catenin
α-actinin
or Vinculin
Formation of Stable
adhesion complex
Rac1
or Cdc42
IQGAP1
GTP
Tiam-1
Rac1
Or Cdc42
GDP
Disruption of
adhesion complex
IQGAP1
β-catenin
α-catenin
α-actinin
or Vinculin
Actin

Figure 6.4

Immunocytochemical assessment of E-cadherin-dependent cell-cell junctions in transfected OSCC cell lines

2 x 10^4 cells were plated onto 13mm coverslips in a 24-well plate and allowed to grow for 48 hours with selection medium. Cells were examined on a confocal laser scanning microscope, and images collected digitally.

Panels A and C are confocal images of H376 E-cadherin cell line.

Panels B and D are confocal images of H376β4 E-cadherin cell line.

In Panels A and B, E-Cadherin, "green", was detected by HECD-1 antibody and showed the the E-cadherin-dependent "zipper-like" cell-cell contacts in H376 E-cadherin cell line (Panel A: denoted with open arrow) as compared to the E-cadherin-dependent "straight-line" cell-cell contacts in H376β4 E-cadherin cell line (Panel B: denoted with solid arrow).

Panels C and D are composite confocal images showing E-cadherin, "green", actin cytoskeleton, "red", was detected with phalloidin-TRITC and nuclei, "blue", were detected with DAPI.

Figure 6.4

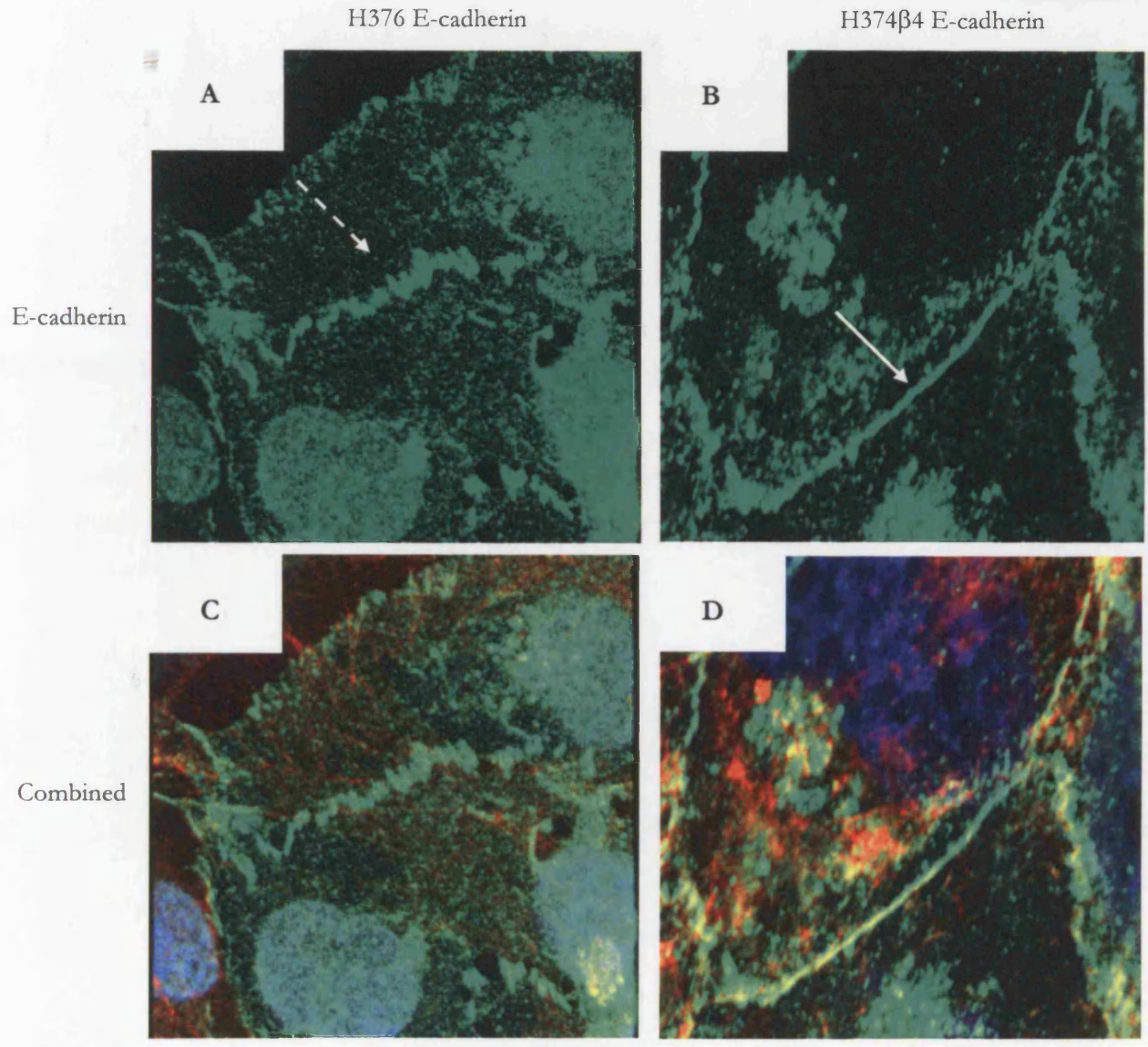


Figure 6.5

β4-integrin knock-down in the H376β4 E-cadherin OSCC cell line

RNA interference (RNAi) technology was used to knock-down β4-integrin expression in H376β4 E-cadherin cell line. Expression of β4-integrin was assessed 48 hours post-transfection using FACS and Western blot analyses.

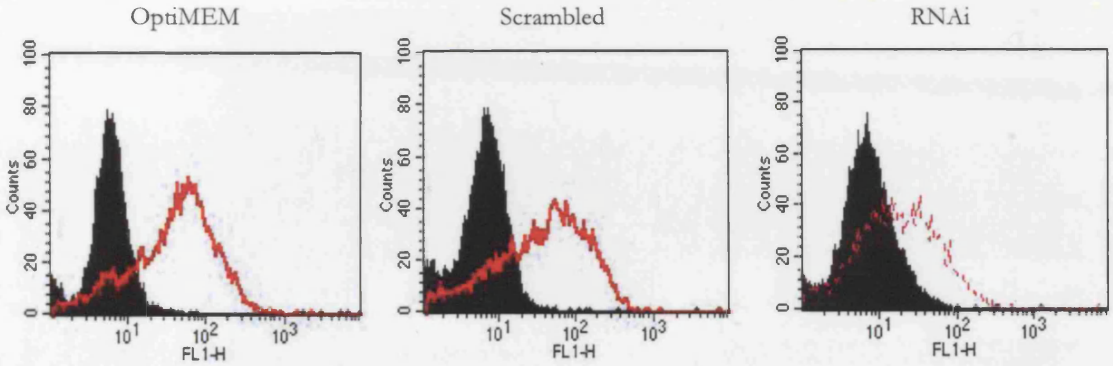
For β4-integrin detection, cells were harvested as described in Chapter 2, Section 2.2.1.2. Figure (a) shows a representative flow cytometry experiment, of five independent experiments, with about 80% reduction in the level of β4-integrin surface expression, labelled with anti-β4-integrin antibody "3E1", as compared with the non-treated OptiMEM control and the scrambled control.

For β4-integrin expression, 25 μg protein/lane were resolved by SDS-PAGE and blotted onto nitrocellulose membrane. The blot was probed using "clone 7" anti-β4-integrin monoclonal antibody. A histogram of data collected from five independent experiments shows about 90% reduction of β4-integrin expression in the RNAi lane compared to the OptiMEM and scrambled control lanes (p value $\leq$ 0.02 when compared to the OptiMEM control; p value $\leq$ 0.002 when compared to Scrambled control).

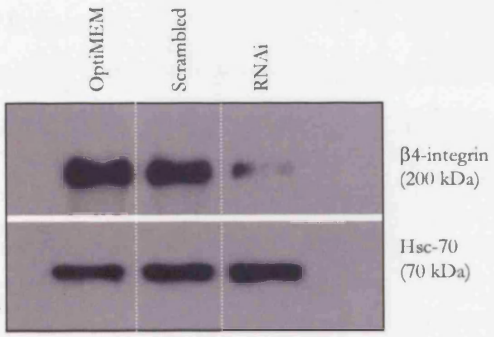
For E-cadherin detection, the blot was probed using HECD-1 antibody. A histogram of data collected from five independent experiments shows a concomitant 67% reduction of E-cadherin expression in the RNAi lane compared to the OptiMEM and scrambled control lanes (p value $\leq$ 0.04 when compared to the OptiMEM control; p value $\leq$ 0.04 when compared to Scrambled control).

Figure 6.5

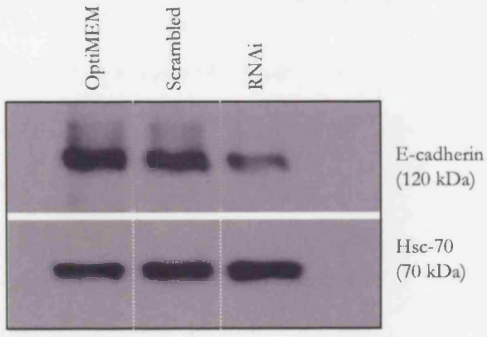
a)

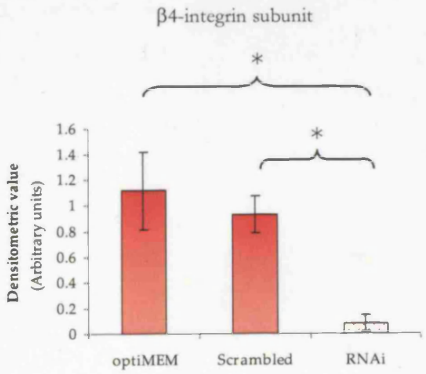


b)



c)





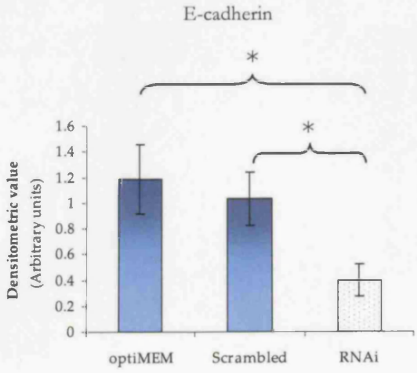


Figure 6.6

Effect of β4-integrin knock-down on the maturation of E-cadherin-dependent cell-cell contacts in the H376β4 E-cadherin OSCC cell line

Expression of β4-integrin and E-cadherin was assessed using indirect immunostaining.

Panels A, D, G and J are confocal images of the H376β4 E-cadherin/OptiMEM control cell line.

Panels B, E, H and K are confocal images of the H376β4 E-cadherin/Scrambled control cell line.

Panels C, F, I and L are confocal images of the H376β4 E-cadherin/RNAi cell line. In Panels A-C: β4-integrin, "green", was detected by clone 7 antibody and showed level of reduction of β4-integrin expression upon knock-down using RNAi (Panel C) as compared with the OptiMEM and scrambled control lines (Panels A and B).

Panels D-F are composite confocal images of β4-integrin, "green", actin cytoskeleton, "red", was detected with phalloidin-TRITC and nuclei, "blue", were detected with DAPI.

In Panels G-I: E-Cadherin, "green", was detected by HECD-1 antibody and showed disruption of E-cadherin-dependent cell-cell contacts as a consequence of β4-integrin knock-out (Panel I) as compared with the fully-formed, straight-line E-cadherin-dependent cell-cell contacts in the OptiMEM and scrambled control lines (Panels G and H).

Panels J-L are composite confocal images of E-cadherin "green", actin cytoskeleton, "red", was detected with phalloidin-TRITC and nuclei, "blue", were detected with DAPI. Panel L showed redistribution of the actin filaments into stress fibres colocalising with E-cadherin molecule.

Figure 6.6

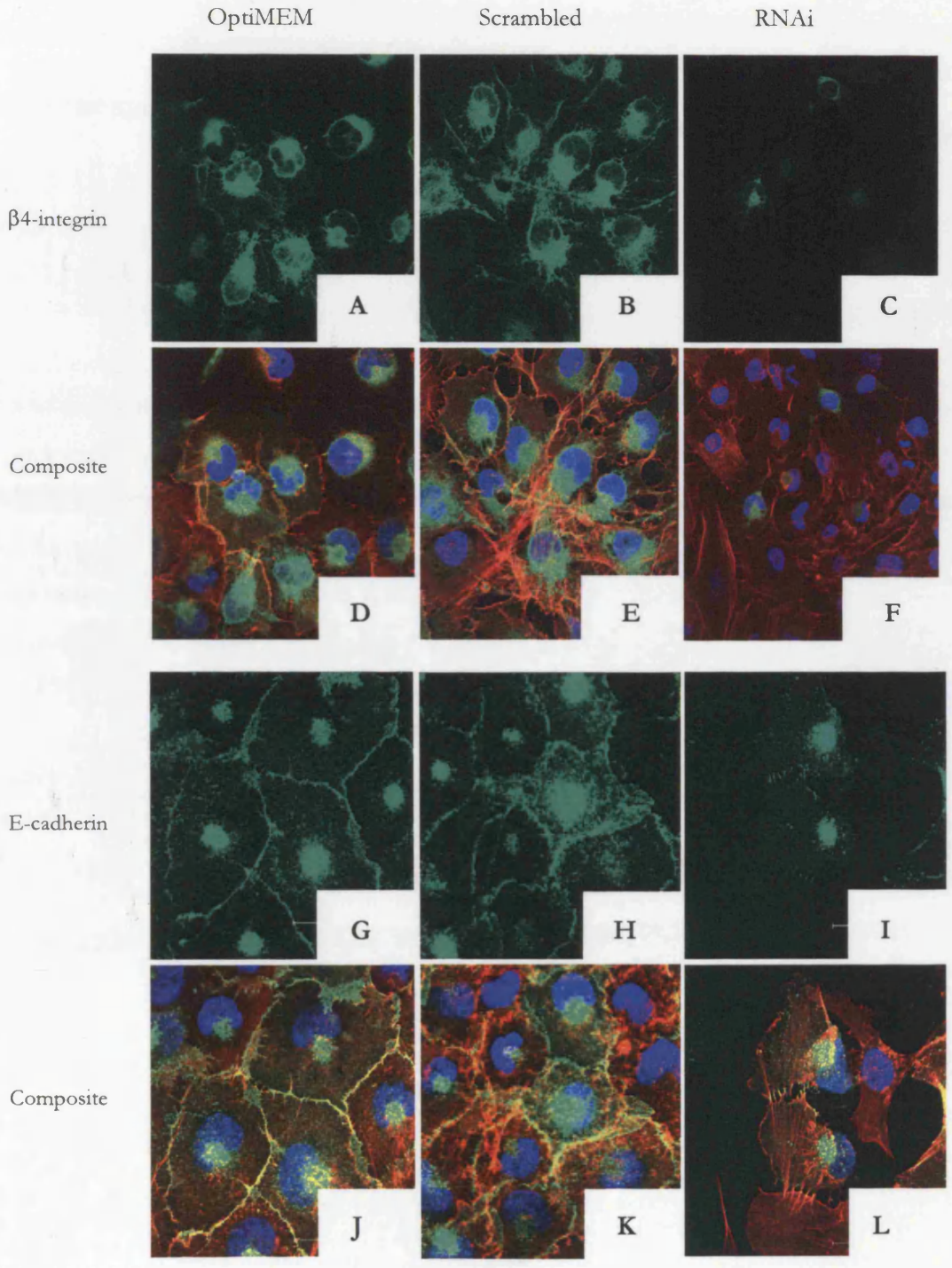

OptiMEM
Scrambled
RNAi
β4-integrin
Composite
E-cadherin
Composite
A
B
C
D
E
F
G
H
I
J
K
L

Figure 6.7

β4-integrin knock-down in the CA1 OSCC cell line

RNA interference (RNAi) technology was used to knock-down β4-integrin expression in CA1 cell line. Expression of β4-integrin was assessed 48 hours post-transfection using FACS and Western blot analyses.

For β4-integrin detection, cells were harvested as described in Chapter 2, Section 2.2.1.2. Figure (a) shows a flow cytometry experiment with about 96% reduction in the level of β4-integrin surface expression, labelled with anti-β4-integrin antibody "3E1", as compared with the non-treated OptiMEM control and the scrambled control.

For β4-integrin expression, 25 μg protein/lane were resolved by SDS-PAGE and blotted onto nitrocellulose membrane. The blot was probed using "clone 7" anti-β4-integrin monoclonal antibody. A histogram, of data collected from a single experiment, shows about 100% reduction of β4-integrin expression in the RNAi lane compared to the OptiMEM and scrambled control lanes.

For E-cadherin detection, the blot was probed using HECD-1 antibody. A histogram, of data collected from a single experiment, shows a concomitant 42% reduction of E-cadherin expression in the RNAi lane compared to the OptiMEM and scrambled control lanes.

Figure 6.7

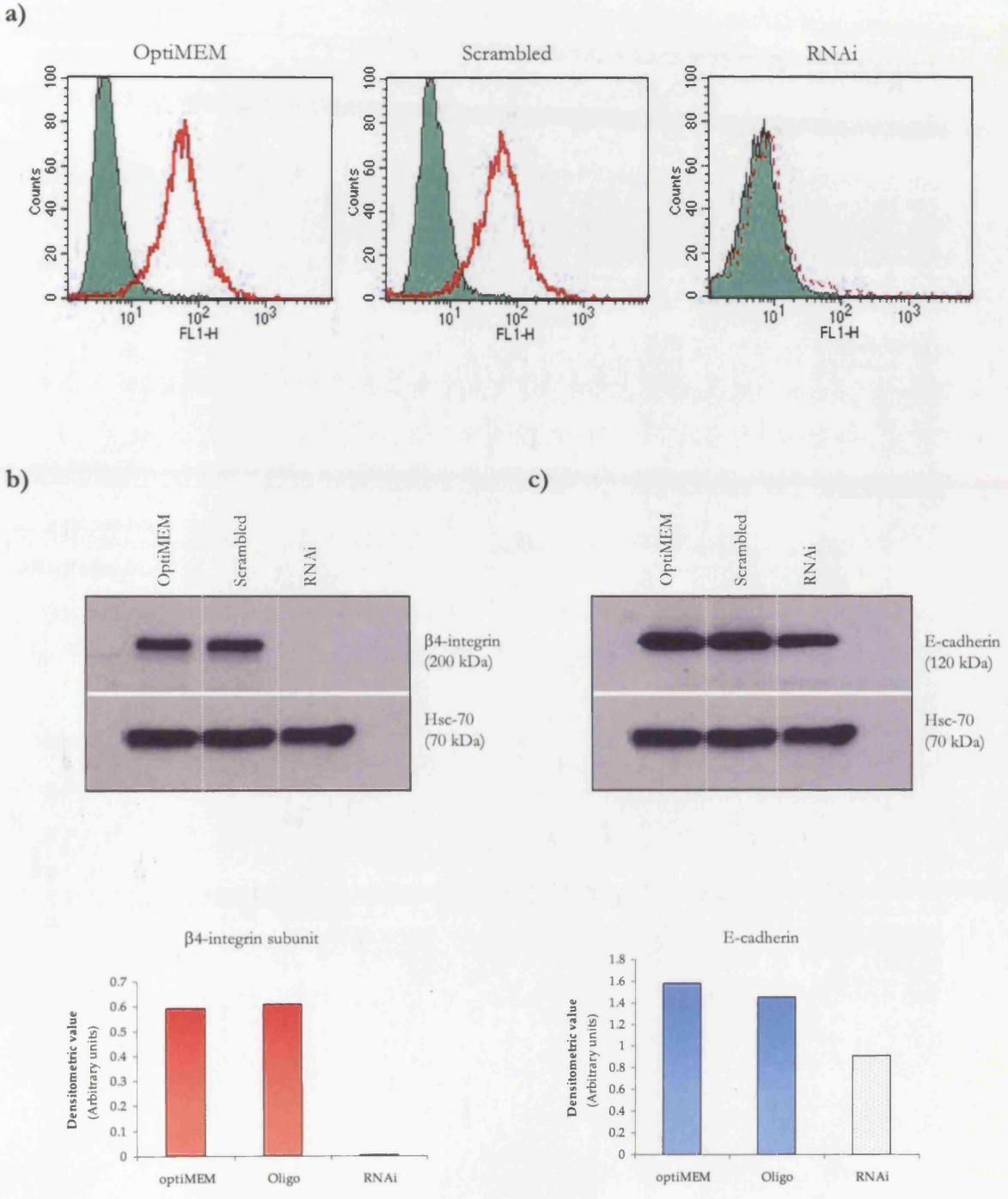

a)
OptiMEM
Scrambled
RNAi
Counts
FL1-H
b)
c)
OptiMEM
Scrambled
RNAi
β4-integrin
(200 kDa)
Hsc-70
(70 kDa)
E-cadherin
(120 kDa)
Hsc-70
(70 kDa)
β4-integrin subunit
E-cadherin
Densitometric value
(Arbitrary units)
optiMEM
Oligo
RNAi

Figure 6.8

Effect of β4-integrin knock-down on the maturation of E-cadherin-dependent cell-cell contacts in the CA1 OSCC cell line

Expression of β4-integrin and E-cadherin was assessed, 48 hours post-transfection, using indirect immunostaining. Cells were examined on a confocal laser scanning microscope, and images collected digitally.

Panels A, D, G and J are confocal images of the CA1/OptiMEM control cell line.

Panels B, E, H and K are confocal images of the CA1/Scrambled control cell line.

Panels C, F, I and L are confocal images of the CA1/RNAi cell line.

In Panels A-C: β4-integrin, "green", was detected by clone 7 antibody and showed level of reduction of β4-integrin expression upon knock-down using RNAi (Panel C) as compared with the OptiMEM and scrambled control lines (Panels A and B).

Panels D-F are composite confocal images of β4-integrin, "green", actin cytoskeleton, "red", was detected with phalloidin-TRITC and nuclei, "blue", were detected with DAPI.

In Panels G-I: E-Cadherin, "green", was detected by HECD-1 antibody. Panel I represents about 5-10% of the total population and shows disruption of E-cadherin-dependent cell-cell contacts.

Panels J-L are composite confocal images of E-cadherin "green", actin cytoskeleton, "red", was detected with phalloidin-TRITC and nuclei, "blue", were detected with DAPI.

Figure 6.8

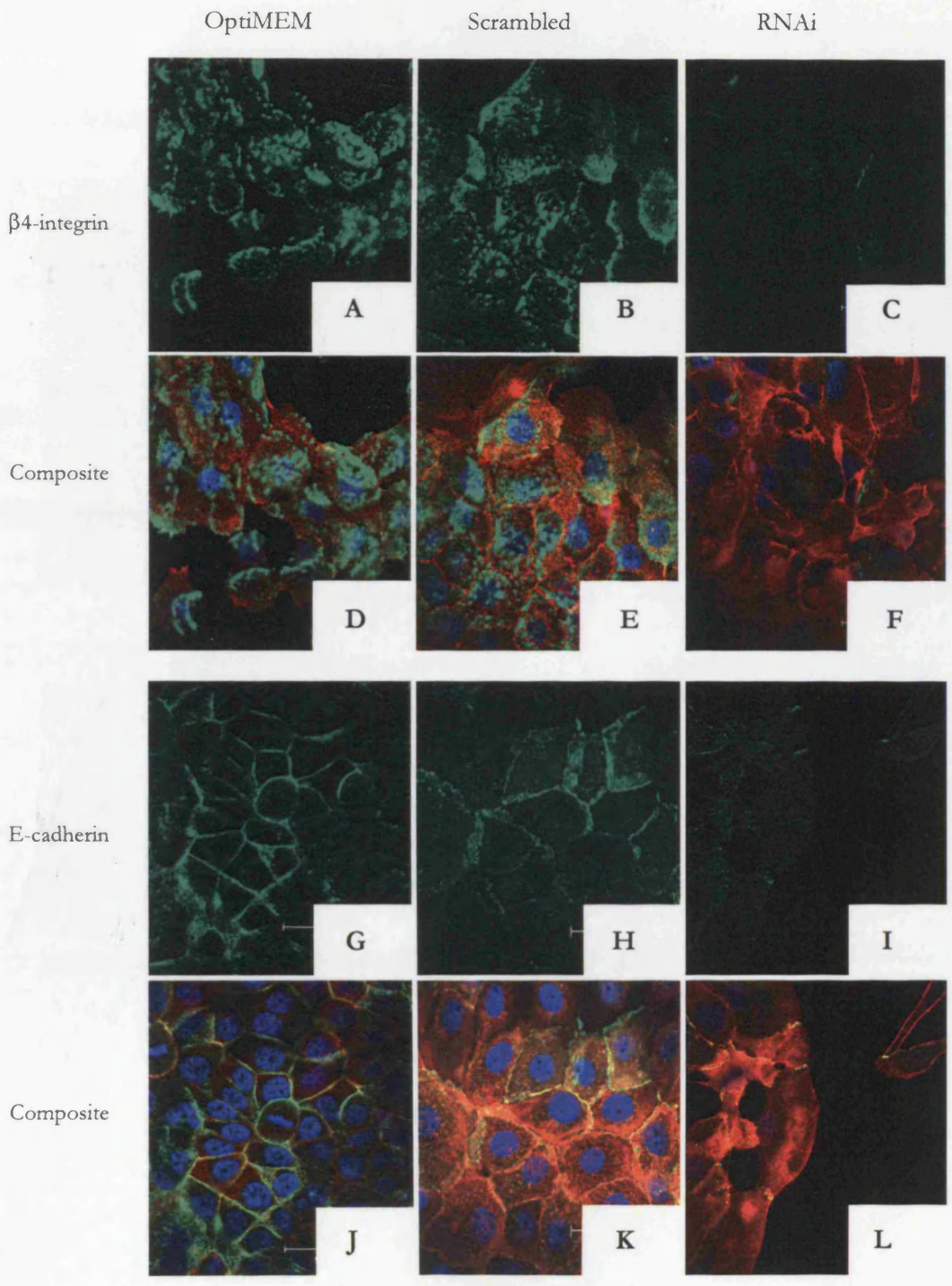

OptiMEM
Scrambled
RNAi
β4-integrin
Composite
E-cadherin
Composite
A
B
C
D
E
F
G
H
I
J
K
L

Figure 6.9

Assessment of endogenous Rac1 expression in H367 E-cadherin and H376β4 E-cadherin OSCC cell lines

2 x 10^4 cells were plated onto 13mm coverslips in a 24-well plate and allowed to grow for 48 hours with selection medium.

Expression of endogenous Rac1 and E-cadherin was assessed using indirect immunostaining. Cells were examined on a confocal laser scanning microscope, and images collected digitally.

Panels A and C are confocal images of the H376 E-cadherin cell line.

Panels B and D are confocal images of the H376β4 E-cadherin cell line.

In Panels A and B: Rac1, "green", was detected by anti-Rac1 "clone 23A8" monoclonal antibody (Upstate Cell Signaling Solutions).

Panels C and D are composite confocal images of Rac1, "green", actin cytoskeleton, "red", was detected with phalloidin-TRITC and nuclei, "blue", were detected with DAPI.

Panels A and B show expression of Rac1 in the H376 E-cadherin cell line, with Rac1 being expressed in abundance in the cytoplasm.

Panels C and D show expression of Rac1 in the H376β4 E-cadherin cell line, with Rac1 being expressed at the nucleus, the cytoplasm and at the cell-cell junctions.

Figure 6.9

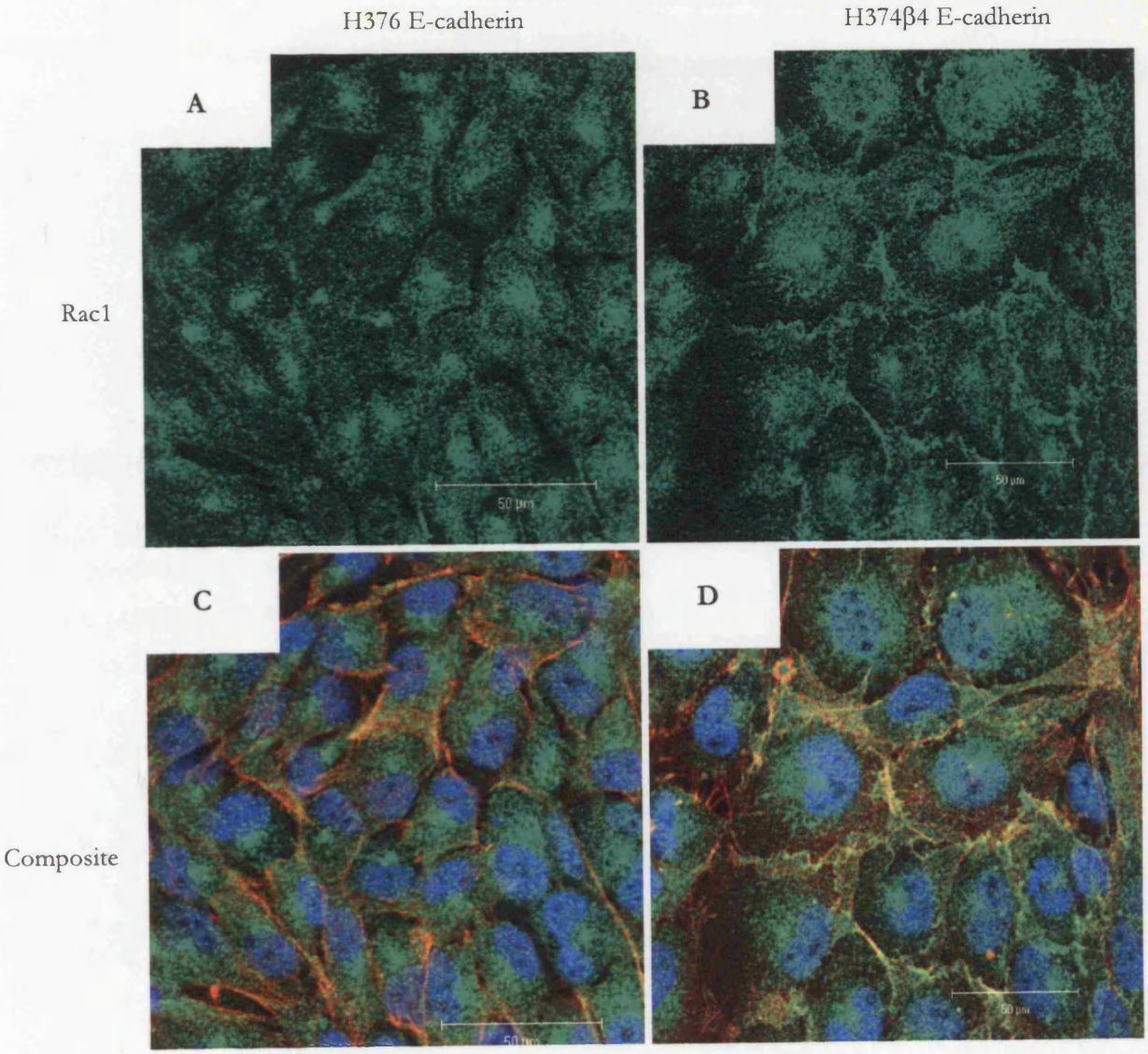

H376 E-cadherin
H374β4 E-cadherin
A
B
Rac1
50 μm
50 μm
C
D
Composite
50 μm
50 μm

Figure 6.10

Effect of β4-integrin knock-down on localisation of endogenous Rac1 in H376β4 E-cadherin and CA1 OSCC cell lines

Expression and localisation of Rac1 was assessed, 48 hours post-transfection, using indirect immunostaining. Cells were examined on a confocal laser scanning microscope, and images collected digitally.

Panels A-F are confocal images of the H376β4 E-cadherin cell line.

Panels G-L are confocal images of CA1 cell line.

In Panels A-C and G-I: Rac1, "green", was detected by -Rac1 (clone 23A8) monoclonal antibody.

Panels D-F and J-L are composite confocal images of Rac1, "green", actin cytoskeleton, "red", was detected with phalloidin-TRITC and nuclei, "blue", were detected with DAPI.

Panels A-F show H376β4 E-cadherin cell line with Rac1 expressed predominantly in the nuclei and at the cell-cell junction in OptiMEM and scrambled control panels (Panels A and B). Upon β4-integrin knock down, Rac1 was relocalised to the cell periphery at the leading edge of the cells (Panel C) forming vesicle-like structures (Panel C: designated by arrows).

Panels G-L show CA1 cell line with Rac1 expressed predominantly in the nuclei and at the cell-cell junction in OptiMEM and scrambled control panels (Panels G and H). Upon β4-integrin knock down, Rac1 was relocalised to the cell periphery at the leading edge of the cells (Panel C: designated by arrows).

Figure 6.10

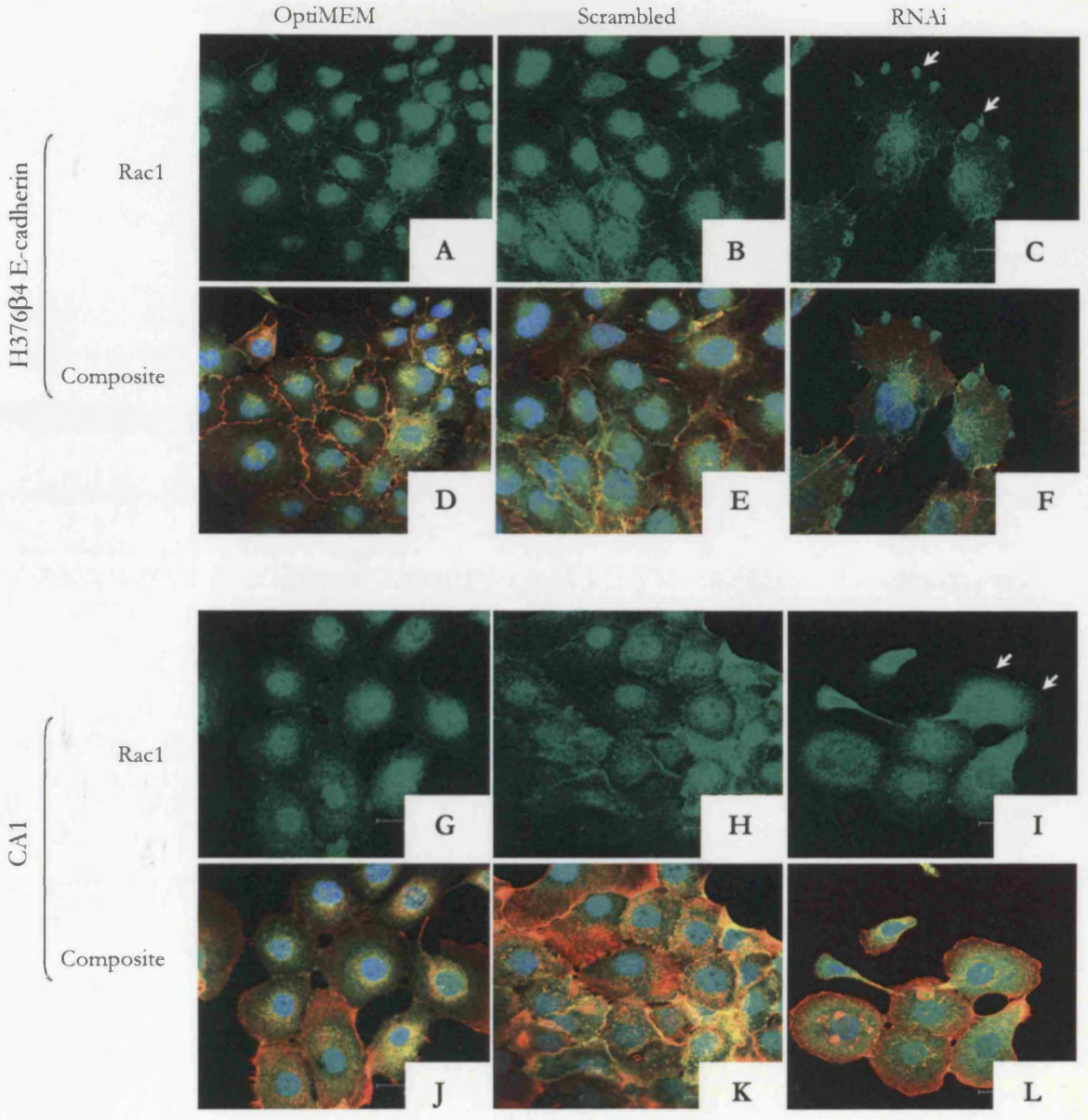

OptiMEM
Scrambled
RNAi
H376β4 E-cadherin
Rac1
Composite
A
B
C
D
E
F
CA1
Rac1
Composite
G
H
I
J
K
L

Figure 6.11

Pull-down of activated Rac1 and Cdc42

Activated Rac1-GTP level was determined, in H376 E-cadherin cell line, by GST pull-down assay using the PAK binding domain (PBD), followed by Western blot analysis. Data shown are representative of two independent experiments.

Activated/total Rac1 blotted using anti-Rac1 (clone 23A8) monoclonal antibody (Upstate).

Activated/total Cdc42 blotted using anti-Cdc42 (clone 3707) monoclonal antibodies (CHEMICON® international).

Figure 6.11

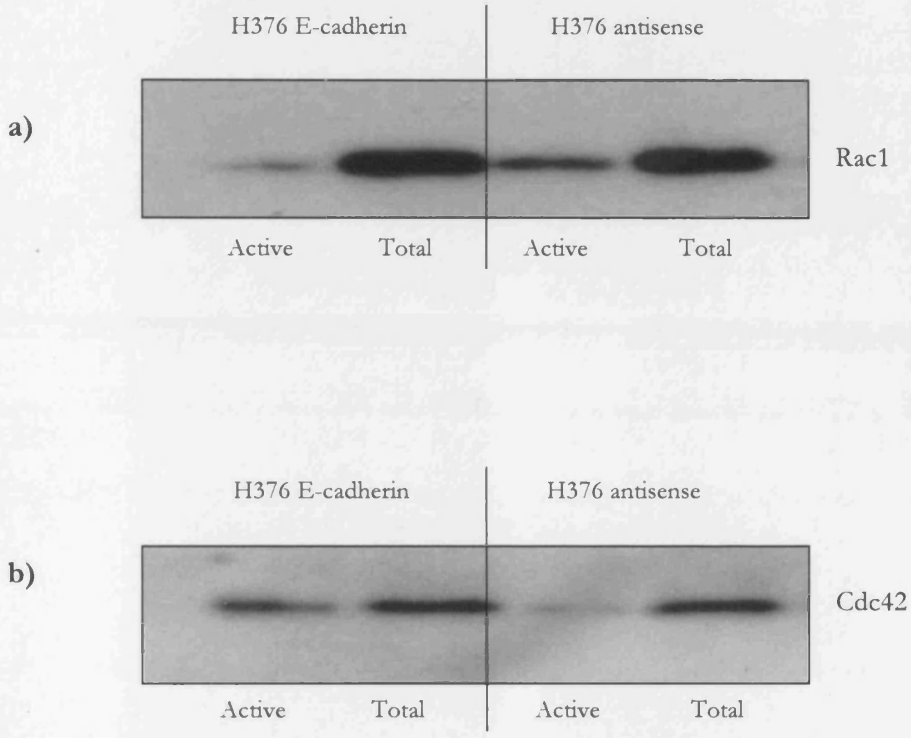

a)
H376 E-cadherin
H376 antisense
Rac1
Active
Total
Active
Total
b)
H376 E-cadherin
H376 antisense
Cdc42
Active
Total
Active
Total

Figure 6.12

Expression of constitutively-active Rac1 (Rac^{V12}) in H376 E-cadherin OSCC cell line

Constitutively-active Rac1 (Rac^{V12}-GFP) was introduced into H376 E-cadherin cell line. Expression and effect of Rac^{V12} was assessed 48 hours post-transfection using indirect immunostaining.

In Panels A and D: Rac^{V12}-GFP appearing "green".

In Panels B and E: E-cadherin, "red", was detected with HECD-1 antibody coupled to Cy3-anti-mouse secondary layer.

Panels C and F are composite confocal images of Rac^{V12}-GFP, "green", E-cadherin "red", actin cytoskeleton, "blue", was detected with Cy5 and nuclei, "yellow", were detected with DAPI.

Panels A-C show that H376 E-cadherin cells expressing Rac^{V12}-GFP (Panel A) showed more mature E-cadherin-dependent cell-cell contacts (Panel B: designated by solid arrows) compared with the immature 'zipper-like' contacts in cells that did not express Rac^{V12}-GFP (Panel B: designated by open arrows).

Panels D-F show that H376 E-cadherin cells expressing Rac^{V12}-GFP adopted a fan-shaped morphology.

Figure 6.12

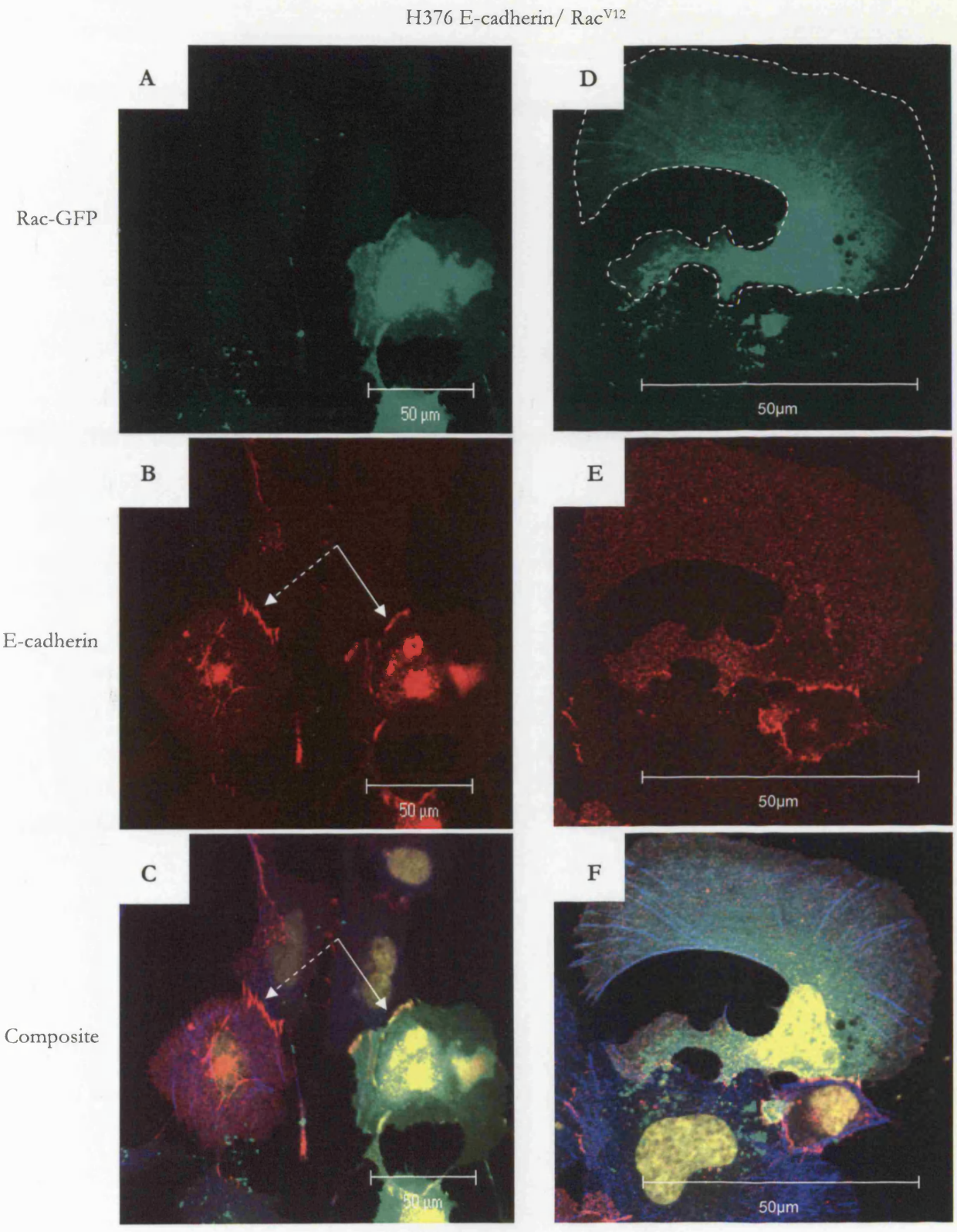

H376 E-cadherin/ Rac^{V12}
A
D
Rac-GFP
50 µm
50µm
B
E
E-cadherin
50 µm
50µm
C
F
Composite
50µm

Figure 6.13

Expression of constitutively-active Rac1 (Rac^{V12}) and dominant-negative (Rac^{N17}) in H376β4 E-cadherin OSCC cell line

Constitutively-active Rac1 (Rac^{V12}-GFP) or dominant-negative (Rac^{N17}-GFP) were introduced into H376β4 E-cadherin cell line. Expression and effect of Rac^{V12} or Rac^{N17} was assessed 48 hours post-transfection using indirect immunostaining.

Panels A-C are confocal images of the H376β4 E-cadherin cell line transfected with Rac^{V12}-GFP.

Panels D-F are confocal images of the H376β4 E-cadherin cell line transfected with Rac^{N17}-GFP.

In Panels A and D: Rac^{V12}-GFP and Rac^{N17}-GFP appearing "green".

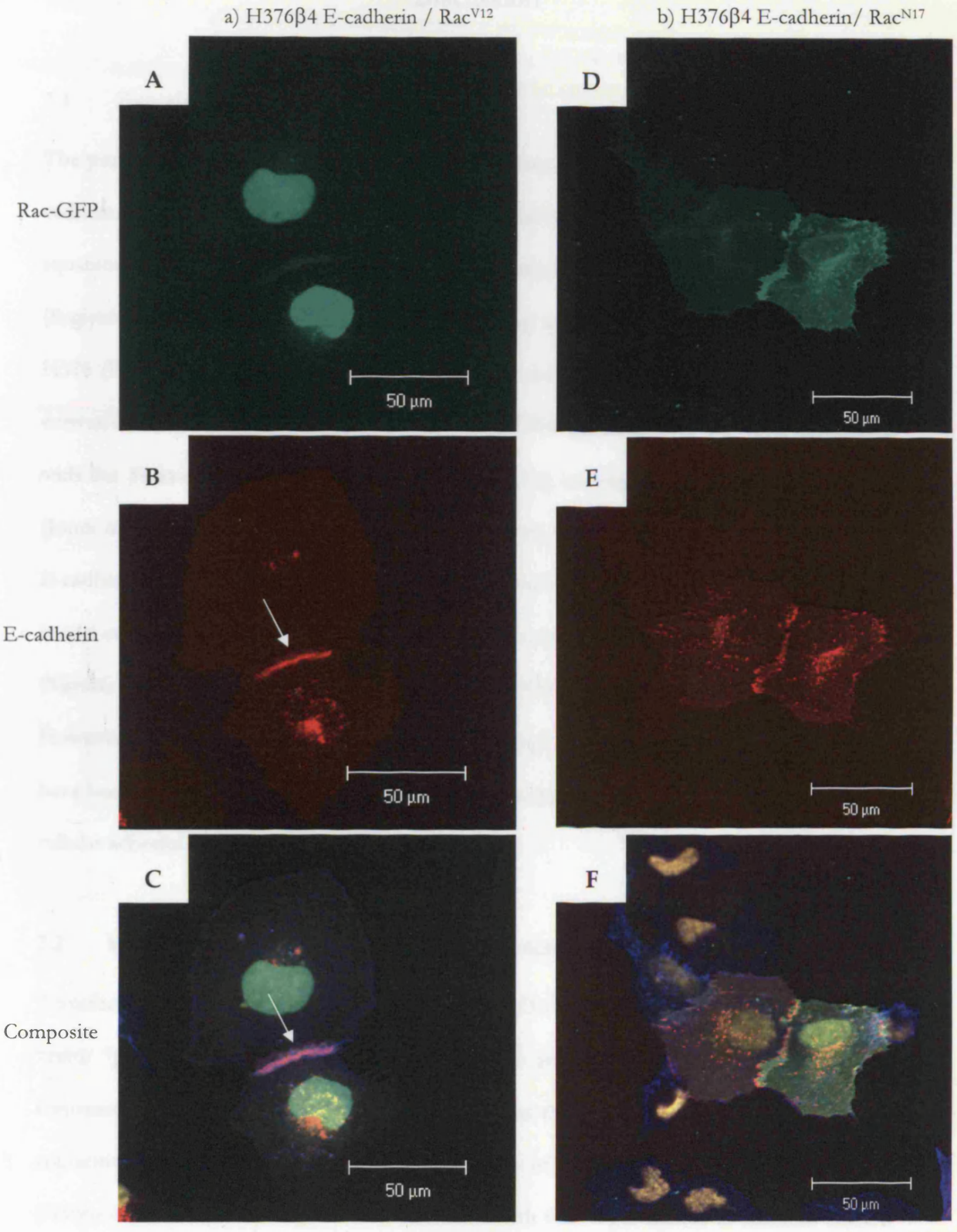
In Panels B and E: E-cadherin, "red", was detected with HECD-1 antibody coupled to Cy3-anti-mouse secondary layer.

Panels C and F are composite confocal images of Rac-GFP, "green", E-cadherin, "red", actin cytoskeleton, "blue", was detected with Cy5 and nuclei, "yellow", were detected with DAPI.

Panels A-C show that H376β4 E-cadherin cells expressing Rac^{V12}-GFP (Panel A) showed mature E-cadherin-dependent cell-cell contacts (Panel B: designated by solid arrows) with a typical epithelial morphology.

Panels D-F show that H376 E-cadherin cells expressing Rac^{N17}-GFP (Panel D) showed disrupted E-cadherin-dependent cell-cell contact (Panel E: designated by open arrow).

Figure 6.13


a) H376β4 E-cadherin / Rac^{V12}
b) H376β4 E-cadherin/ Rac^{N17}
A
D
Rac-GFP
50 µm
50 µm
B
E
E-cadherin
50 µm
50 µm
C
F
Composite
50 µm
50 µm

CHAPTER 7
Discussion

7.1 Experimental approach

The purpose of this study was to investigate the effect of expression and function of the E-cadherin cell-cell adhesion molecule on the behaviour of epithelial cells derived from an oral squamous cell carcinoma lacking endogenous expression of this particular molecule (Sugiyama *et al.*, 1993). Three related starting cell lines were used to commence this study, the H376 (Prime *et al.*, 1990), the H376β4 and the IA2 cell lines; the latter two cell lines being derivatives of the H376 cell line, with the H376β4 line previously having been transfected with the β4-integrin human cDNA and the IA2 being its negative, or vector only, control (Jones *et al.*, 1996a). All three cell lines were transfected with constructs expressing full-length E-cadherin cDNA. On other occasions when E-cadherin cDNA was transfected into other model cell line systems it was shown to have a profound effect on subsequent cell behaviour (Miyaki, 1995; Hermiston *et al.*, 1996; Gottardi *et al.*, 2001; Jiao *et al.*, 2001; Kohya *et al.*, 2003). However, as far as I could ascertain, in OSCC studies on E-cadherin re-expression largely have been unsuccessful with few, if any, documented instances of successful manipulation of cellular adhesion and migration characteristics.

7.2 Production of E-cadherin positive stable clones

Transfection of recipient cells with the E-cadherin cDNA was performed using a bicistronic vector "pIRES-puro", with both the drug-resistance gene and the desired gene of interest contained within a single transcription cassette; thus the clones that survived selection in antibiotic-containing medium expressed high levels of the transcribed protein of interest (Hobbs *et al.*, 1998). Cells were transfected either with full-length human E-cadherin cDNA

in the sense orientation or with the full-length human E-cadherin cDNA in the antisense orientation

In order to produce the stable E-cadherin positive clones, transfected cells were put through a vigorous two rounds of a magnetic bead sorting procedure (to select for high levels of cell-surface expression of E-cadherin) and then were grown in the drug-selection medium. However, the antisense control clones were selected using drug-selection medium only.

E-cadherin was highly expressed in all positive lines and clearly was able to induce morphological changes in the OSCC cells similar to those features exhibited by other epithelial cells expressing E-cadherin. Furthermore, I showed that E-cadherin in these cells was as capable of inhibiting invasion as has been shown in other cells (Vleminckx *et al.*, 1991; Takeichi *et al.*, 1993; Moersig *et al.*, 2002). Moreover, the endogenous β-catenin protein was upregulated and redistributed to the cell-cell junctions as a consequence of *de novo* E-cadherin expression in my OSCC model system, suggesting that the relationship of E-cadherin and β-catenin was restored back to what might be considered a normal epithelial phenotype. Similar findings were obtained with α-catenin, in terms of its patterns of re-distribution, but not with γ-catenin which was, instead, dowregulated.

7.3 Effect of E-cadherin re-expression on the differentiation of an OSCC cell line

Because of the fact that the primary H376 cell line was characterised previously as a low expresser of involucrin (Sugiyama *et al.*, 1993) and because all transfected cell lines were negative for this particular differentiation marker I concluded that the restoration of E-cadherin expression had no apparent effect on the differentiation capacity of the variant H376 cell lines; a similar finding was established by Jones *et al.* (1996) where *de novo* expression of the β4-integrin subunit into the H376 cell line was found not sufficient to induce terminal differentiation in this particular OSCC cell line (Jones *et al.*, 1996a).

7.4 Effect of E-cadherin re-expression on expression of other cadherins

N-cadherin is a mesenchymal cadherin (Li and Herlyn, 2000; Tomita *et al.*, 2000). Upregulation of N-cadherin in tumours of epithelial origin has been associated with a poorly differentiated appearance (Islam *et al.*, 1996; Nieman *et al.*, 1999; Hazan *et al.*, 2000; Tomita *et al.*, 2000). All three OSCC primary cell lines used in this study were characterised as being negative for E-cadherin expression but were strongly positive for N-cadherin expression. Reciprocal downregulation of N-cadherin was observed upon re-expression of E-cadherin in these cell lines; simulating the phenomenon of the "cadherin switch", previously described as both a normal and a pathologic condition (Hatta and Takeichi, 1986; Hazan *et al.*, 2000). Similar observations were made by Li *et al.* (1998) where the introduction of L-CAM, a functional chicken homologue of E-cadherin, into an OSCC cell line resulted in the downregulation of N-cadherin and a subsequent loss of the epithelial phenotype (Li *et al.*, 1998). This result suggests strongly that E-cadherin is accountable for reversion to the epithelial phenotype.

7.5 Effect of E-cadherin re-expression on the invasive potential of OSCC

Loss of E-cadherin expression, or function, has been one of the major hallmarks of malignant epithelial cancers and has been associated with increased invasion and metastasis of low- or non-expressing tumours (Mareel *et al.*, 1991; Birchmeier and Behrens, 1994). The precise role of E-cadherin in modulating tumour invasion is still unclear though it most likely relates to the removal of cells from the constraints of cell-cell adhesion; though it has rather been attributed to the abnormal expression of N-cadherin rather than loss of E-cadherin (Nieman *et al.*, 1999). *De novo* E-cadherin expression in my OSCC model system resulted in a marked inhibition of invasion, as well as a substantial downregulation of N-cadherin. These results are suggestive of the possibility that, in this particular model system, E-cadherin might

have abrogated invasiveness through downregulation of N-cadherin expression as much as it restricted cells through enhanced cell-cell interactions.

7.6 Growth and survival in 3-D collagen I cultures

Tumour cell invasion and mestastasis are three-dimensional processes. When transformed epithelial cells escape their normal anatomical location they are forced to proliferate within the dense three-dimensional collagen I-rich extracellular milieu; an environment which might be very different from their usual situation on a basement membrane.

I tried to mimic the conditions faced by proliferating, metastasising epithelial tumour cells by growing my transfected OSCC cell lines (H376 E-cadherin and H376 antisense cell lines) in a 3-D collagen I culture and, for direct comparison, under collagen I 2-D conditions; these latter conditions previously have been used by Hotary and colleagues (2003). By providing these various culture conditions I sought to test the effect of *de novo* E-cadherin expression, in my model system, on tumour cell growth and survival. Under 2-D growth conditions, restoration of E-cadherin suppressed tumour cell proliferation, suggesting that cancer growth suppression in the 2-D environment is an E-cadherin-dependent process. This type of result has been reported previously in the literature (Tsukatani *et al.*, 1997; Li *et al.*, 1998). However, I found it difficult to detect any effect of E-cadherin on inhibition of cell growth in a 3-D environment; a result unlike those of others whose studies have used normal epithelial cells and reported reduction in their growth capacities (Takahashi and Suzuki, 1996; Kandikonda *et al.*, 1996) or even others who also have used transformed cell lines (St Croix *et al.*, 1998). My results might even suggest that expression of E-cadherin in a 3-D collagen I culture conferred a growth advantage on this particular OSCC cell line. Bcl-2 is an anti-apoptotic protein that protects cells from undergoing programmed cell death or apoptosis (Adams and Cory, 2001). Abrogation of cell growth, as a consequence of E-cadherin restoration in 2-D

cultures, was shown to be due to increased apoptosis. A similar observation was made by (Sasatomi *et al.*, 1996). Restoration of expression of the E-cadherin molecule has been reported to reduce the expression of Bcl-2 (Sasaki *et al.*, 2000). In my model system, similar results were obtained when cells were growing under 2-D culture conditions however, in contrast, this effect was abrogated when cells were allowed to grow in 3-D collagen I cultures. I have interpreted these findings to suggest that, in 2-D culture systems, E-cadherin was able to confer a density-inhibition of growth on my OSCC cell line by downregulation of Bcl-2 causing a subsequent induction of apoptosis. However, in 3-D cultures, E-cadherin seems almost to give preference to the development of an apoptosis-resistant phenotype; this, perhaps, might be due to a higher exposure of the embedded cells to survival signals mediated by other signalling pathways, such as integrin-ECM interactions (Meredith *et al.*, 1993). This suggestion was supported further by the fact that, in 3-D collagen I culture, E-cadherin re-expression concomitantly upregulated N-cadherin, phospho-Akt and ILK; all molecules that have been known for their anti-apoptotic effects in a range of cell types (Datta *et al.*, 1999; Tran *et al.*, 2002; Friedrich *et al.*, 2004).

The fact that, in certain tumours, partial E-cadherin function is maintained and is not selected against, despite its ability to inhibit growth under certain conditions implies that some level of E-cadherin function may be advantageous to tumour cells. For example, expression of E-cadherin has been shown to protect cells from undergoing apoptosis or programmed cell death (Hermiston and Gordon, 1995). Thus, in addition to growing more slowly (Navarro *et al.*, 1991; Miyaki *et al.*, 1995) epithelial tumours with functional E-cadherin may also be resistant to apoptosis induced by environmental stress (Helmlinger *et al.*, 1997) or cytotoxic anticancer therapy (Dimanche-Boitrel *et al.*, 1994; St Croix and Kerbel, 1997).

Upregulation of ILK in the E-cadherin positive OSCC cell lines was observed in both 2-D and 3-D collagen I culture conditions and, most likely, could be attributed to the

upregulation of the β1-integrin (as shown in Chapter 3, Section 3.2.10), a known collagen I receptor and ILK-linker protein, as a consequence of *de novo* E-cadherin expression in my OSCC model system.

Taken all together, these findings might suggest different mechanisms by which E-cadherin could provide an anti-apoptotic activity, and therefore mediate resistance to different therapeutic agents to cancer cells growing as 3-D multicellular aggregated masses *in vitro* and *in vivo*. This phenomenon previously has been described as "multicellular resistance" (Green *et al.*, 2002), where formation of E-cadherin-dependent MCAs contributed to the development of therapeutic resistance. Knowing the underlying mechanisms which control this phenomenon might provide a clue as to what combination of therapeutic agents could be used in order to inhibit the growth and expansion of tumours once they have defeated their physical restaraints and expanded into the collagen I based surroundings.

7.7 E-cadherin/β-4 integrin cross-talk

In my OSCC model system I provided evidence that these two epithelial adhesion molecules, E-cadherin and β4-integrin, do indeed cross-talk to each other. *De novo* E-cadherin expression in all three variant cell lines resulted in varying degrees of upregulation or re-expression of the β4-integrin (results from Chapter 3 and 5). On the other hand, *de novo* β4-integrin expression in the H376β4 E-cadherin cell line resulted in the maturation of the E-cadherin dependent cell-cell contacts accompanied by derangement of the actin cytoskeleton of the cell and development of an epithelial morphotype. In order to prove that β4-integrin was accountable for these changes, knock-down of β4-inetgrin was carried out using RNAi technology. The subsequent downregulation of β4 resulted in the reversion of the previous findings; where the E-cadherin-dependent cell-cell contacts were disrupted and showed a zipper-like appearance with the actin filaments reorganised into stress fibres. Moreover, there

was down regulation of E-cadherin molecules. The effect of β4-integrin on E-cadherin-dependent adherence junction formation was tested in another cell line, the CA1 OSCC cell line. Similar results were obtained but only in 5-10% of the total population that showed alteration in the E-cadherin-dependent cell-cell junctions. E-cadherin was also subsequently downregulated as a result of β4 knock down. These findings strongly support the idea that the β4-integrin is accountable for these changes, at least in this particular OSCC cell line. In general terms, my findings further support the observations by Hintermann *et al.* (2005), where they report that α6β4 integrin increased E-cadherin-dependent cell-cell adhesion (Hintermann *et al.*, 2005).

In an attempt to establish how β4-integrin plays its role, members of the Rho small GTPases family (Rho, Rac, and Cdc42), which have been shown to play a significant role in adherens junction formation (Fukata and Kaibuchi, 2001; Braga, 2002) were examined and manipulated. Rac1 has been thought of as a major role player in the formation of a more intermingled membrane between neighbouring cells (Takaishi *et al.*, 1997; Stoffler *et al.*, 1998; Kodama *et al.*, 1999). Also β4-integrin has been reported to signal via Rac1 (Russell *et al.*, 2003). Thus, the maturation of the E-cadherin-dependent cell-cell junction, via β4-integrin, could possibly be mediated by Rac1.

Results showed that β4-integrin was accountable for, at least, the re-localisation of Rac1 to the nuclei and areas of cell-cell junctions; an effect that was confirmed using RNAi technology to knock-down β4-integrin. Downregulation of β4-integrin has resulted in the mobilisation of Rac1 from nuclei and cell-cell junctions back to the cytoplasm and to the cell periphery at the leading edge. Here Rac1 was found in large distinct vesicular structure. Likewise, knock-down of β4-integrin in the CA1 cell line also resulted in the translocation of Rac1 from the nuclei to the cell periphery at the leading edge of the cell. Tiam-1 is an

important regulator of Rac1 activity (Mertens *et al.*, 2003) and could possibly be the downstream effector of the β4-integrin in this phenomenon.

Constitutively active Rac1 (Rac^{V12}) was shown to localise at the nuclei and at the cell-cell junctions of the transfected cells; a similar distribution to the endogenously active Rac1 in the H376β4 E-cadherin, but not in the H376 E-cadherin, cell line. These results might be suggestive of the possibility that β4-integrin might also contribute to the maturation of the adherens junction through the activation of Rac1. However, results of pull-down assays were inconclusive; which I suspect might be ascribed to the fact that a large fraction of active Rac1 is expressed in the nucleus and thus require nuclear lysis prior to detection.

Another critical observation was that, upon introduction of the constitutively active Rac1 into both the H376 E-cadherin and the H376β4 E-cadherin cell lines, only the H376 E-cadherin cell line acquired a fan-shaped morphotype (described as motile by Sander *et al.*, 1998) but not the H376β4 E-cadherin cell line. Occurrence of such a morphotype was abrogated when cells were allowed to grow on laminin-1 matrix, as compared to cells growing on a collagen I matrix (Sander *et al.*, 1998). The fact that β4-integrin is a known laminin-1 receptor (Niessen *et al.*, 1994) is strongly suggestive that these changes might be strongly linked to the expression of β4-integrin.

Perhaps the activation pathway is defective in the H376 E-cadherin cell line and it requires β4-integrin in order to mediate this process.

In summary, these results suggest that β4-integrin expression in the H376β4 E-cadherin cell line is accountable for the maturation of the E-cadherin dependent cell-cell junctions and for the development of the epithelial morphotype, possibly signalling through the Rac1 member of the Rho family GTPases. Activation of Rac1 owing to β4-integrin expression, with the possibility that β4-integrin instigates its actions through Tiam-1, should be investigated in-depth in future work.

Results from this study, and results previously reported by Jones *et al.* (1996 b), showed that neither the re-expression of E-cadherin nor of β4-integrin produced differentiation in these cell lines. However they resulted in the formation of a more mature E-cadherin-dependent cell-cell junction; such a junction is vital for integrity and function of the epithelial basal cell layer. These results work in tandem with the old notion that cells committed to terminal differentiation become less adherent to the basement membrane as well as to one another (Watt, 1984). There appear to be cell-type-specific differences in how junctions are formed and it may well be that there are cadherin-specific differences. What is certain is that collaborative matrix- and intercellular adhesion regulation is a prerequisite for tissue rearrangements during morphogenesis and tissue remodelling (Marsden and DeSimone, 2003) and it highly likely that similar mechanisms are operative in cancer cell growth and spread.

This thesis has generated more questions than it has provided answers but, there are many observations that can provide a good foundation for future research and the model system used herein will provides an ideal model to study the role of E-cadherin in OSCC.

APPENDIX I

Media, solutions and buffers

1. Tissue culture media and solutions

Phosphate-Buffered Saline (PBSA) (Cancer Research UK Central Cell Services):

NaCl	8g
KCl	0.25g
Na_2HPO_4	0.25g
KH_2PO_4	0.25g

Made up to 1 litre with distilled water, titrated to pH 7.2 and autoclaved.

PBSABC consists of PBS supplemented with 1mM $CaCl_2$ (solution B) and 1mM $MgCl_2$ (solution C)

Standard Keratinocyte Growth Medium (KGM):

α MEM
10% Foetal Calf Serum (Globepharm, Surrey)
100 IU/1penicillin
100μg/1streptomycin
$1.8x10^{-4}$M adenine
5μg/ml insulin
$1x10^{-8}$M cholera toxin
0.5μg/ml hydrocortisone
10ng/ml epidermal growth factor (Sigma)

Selection Medium is KGM supplemented with 0.5μg/ml Puromycin (Sigma)

2. Western Blotting

RIPA Lysis Buffer (2x):

2% Triton X-100	1ml
2% NaDOC	1g
0.2% SDS	0.5ml
316mM NaCl	6.32ml
20mM Tris pH 7.3	1ml
20mM EGTA	200µl
2mM Na3VO4	5ml
10mM NaF	0.5ml

Protease inhibitor (1:100) added fresh at time of use

Protease Inhibitor:

PMSF (10mg/ml)	1:100
Aprotinin (2mg/ml)	1:50
Leupeptin (10mg/ml)	1:5000

SDS-polyacrylamide gels

Resolving Gel (10ml)

	8%	**10%**	**12.5%**	**15%**
ddH_2O	4.6ml	4.0ml	3.3ml	2.3ml
Acrylamide Solution	2.7ml	3.3ml	4.0ml	5.0ml
1.5M Tris pH 8.8	2.5ml	2.5ml	2.5ml	2.5ml
20% SDS	50µl	50µl	50µl	50µl
10% APS	100µl	100µl	100µl	100µl
TEMED	6µ	4µl	4µl	4µl

* APS = Ammonium persulphate

Stacking Gel (5ml)

	4%
ddH_2O	3.0ml
Acrylamide Solution	0.7ml
0.5M Tris pH 6.8	1.25ml
20% SDS	25µl
10% APS	20µl
TEMED	10µl

Running Buffer (x1) (500ml)

Tris base	1.51g
Glycine	7.3g
20% SDS	2.5ml

Fill up to 500ml with Distilled H_2O

Transfer Buffer (x1) (500ml)

Tris base	0.73g
Glycine	3.6g
Methanol	100ml

Fill up to 500ml with Distilled H_2O

Western Blot Blocking Buffer 1 (100ml)

Tween 20	50µl
Marvel skimmed milk powder	5g
PBSA	100ml

Western Blot Blocking Buffer 2 (100ml)

Tween 20	50µl
BSA	1g
TBS	100ml

Western Blot Blocking Buffer 3 (100ml)

Tween 20	50µl
Marvel skimmed milk powder	5g
TBS	100ml

Western Blot Blocking Buffer 4 (100ml)

Tween 20	50µl
Marvel skimmed milk powder	3g
PBSA	100ml

Western Blot Wash Buffer 1 (100ml)

Tween 20	250µl
PBS	500ml

Western Blot Wash Buffer 2 (100ml)

Tween 20	250µl
TBS	500ml

3. Molecular Biology

2% Agarose Gel

Agarose	0.5g
ddH_2O	22.5ml

Heat to 56°C until agarose dissolved, then add

TAE buffer (10x)	2.5ml
Ethidium bromide (10 μg/ml)	1.25μl

DNA Loading Buffer (10ml)

Bromophenol blue	25mg
Xylene cyanol FF	25mg
100% Glycerol	3ml
ddH_2O	7ml

4. Pull-down assay

Lysis Buffer (20ml)

50 mM Tris-HCl, pH 7.5	1ml (1 M stock)
1% Triton X-100	0.2ml
0.5% sodium deoxycolate	0.1g
0.1% SDS	0.2ml (10% stock)
10 mM $MgCl_2$	0.2ml (1 M stock)
150 mM NaCl	0.6ml (5 M stock)
ddH_2O	17.7

Leupeptin, pepstatin, Pefabloc

PMSF (to 2 mM) and 1:1000 protease inhibitor mixture added fresh at time of assay.

Washing buffer (100ml)

50 mM Tris-HCl, pH 7.5	5ml (1 M stock)
1% Triton X-100	1ml
10 mM $MgCl_2$	1ml (1 M stock)
150 mM NaCl	3ml (5 M stock)
ddH_2O	17.7ml

Leupeptin, pepstatin, Pefabloc and PMFS added fresh at time

Laemmli Buffer (4 x reducing sample buffer)

1 M Tris-HCl, pH 6.8	2.5ml
20% SDS	2ml
Glycerol	4ml
Bromophenol blue	0.042g
Beta mercaptoethanol	2ml

APPENDIX II

List of Antibodies

Table II.1: Primary antibodies used in FACS analysis

Antibody	Clone	Concentration	Dilution	Source
Anti-E-cadherin	HECD-1 "Mouse"	1.8 mg/ml	1:200	M.Takeichi
Anti-α1 integrin	FB12 "Mouse"	1 mg/ml	1:100	CHEMICON® International
Anti-α2 integrin	P1E6 "Mouse"	1 mg/ml	1:100	CHEMICON® International
Anti-α3 integrin	PIB5 "Mouse"	1 mg/ml	1:100	CHEMICON® International
Anti-α4 integrin	7.2 "Mouse"	1.8 mg/ml	1:200	Made in-lab by J.F.Marshall
Anti-α5 integrin	P1D6 "Mouse"	1 mg/ml	1:100	CHEMICON® International
Anti-α6 integrin	GoH3 "Rat"	1 mg/ml	1:100	SeroTec, Oxford, UK
Anti-α9 integrin	Y9A8 "Mouse"	1 mg/ml	1:100	CHEMICON® International
Anti-αv integrin	L230 "Mouse"	1.8 mg/ml	1:200	ATCC, Middlesex, UK
Anti-β1 integrin	AIIB2 "Rat"	0.5 mg/ml	1:50	DSHB, IA, UK
Anti-β4 integrin	3E1 "Mouse"	1 mg/ml	1:100	CHEMICON® International
Anti-αvβ3 integrin	LM609 "Mouse"	1 mg/ml	1:100	CHEMICON® International
Anti-αvβ5 integrin	P1F6 "Mouse"	4.1 mg/ml	1:400	CHEMICON® International

* DSHB= Developmental Studies Hybridoma Bank, the University of Iowa

Table II.2: Primary antibodies used in indirect immunocytochemical staining

Antibody	Clone	Concentration	Dilution	Source
Anti-E-cadherin	HECD-1 "Mouse"	1.8 mg/ml	1:200	M.Takeichi
Anti-β-catenin	14 "Mouse"	250 µl/ml	1:100	BD Transduction Laboratories
Anti-γ-catenin	15 "Mouse"	250 µl/ml	1:200	BD Transduction Laboratories
Anti-β4 integrin	7 "Mouse"	250 µl/ml	1:50	BD Transduction Laboratories
Anti-Rac1	23A8 "Mouse"		1:50	Upstate Cell Signaling Solutions
Alexa Fluor ® 647 goat anti-mouse	Secodary antibody	2 mg/ml	1:400	Molecular Probes
Cy3-anti-mouse	Secondary Antibody			Stratech Scientific Ltd. Cambridgeshire,UK

Table II.3: Primary and secondary antibodies used in Western Blot analysis

Antibody	Clone	Molecular Weight	Dilution	Source
Anti-E-cadherin	HECD-1 "Mouse"	120 kDa	1:500	M.Takeichi
Anti-N-cadherin	32 "Mouse"	130 kDa	1:500	BD Transduction Laboratories
Anti-P-cadherin	56 "Mouse"	120 kDa	1:250	BD Transduction Laboratories
Anti-desmoglein	62 "Mouse"	165 kDa	1:500	BD Transduction Laboratories
Anti-α-catenin	5 "Mouse"	102 kDa	1:250	BD Transduction Laboratories
Anti-β-catenin	14 "Mouse"	92 kDa	1:500	BD Transduction Laboratories
Anti-γ-catenin	15 "Mouse"	82 kDa	1:1000	BD Transduction Laboratories
Anti-p120-catenin	98 "Mouse"	120 kDa	1:500	BD Transduction Laboratories
Anti-β4 integrin	7 "Mouse"	200 kDa	1:250	BD Transduction Laboratories
Anti-Involucrin	SY5 "Mouse"	120 kDa	1:500	Abcam Ltd., Cambridge, UK
Anti-Cytokeratin 14	B429	50 kDa	1:100	Abcam Ltd.

	"Mouse"			
Anti-Bcl-2	100 "Mouse"	26 kDa	1:500	Upstate Cell Signalling Solutions
Anti-Akt-1	2H10 "Mouse"	60 kDa	1:1000	Cell Signaling
Anti-phospho-Akt (Thr 308)	- "Rabbit"	60 kDa	1:1000	Cell Signaling
Anti-ILK	3 "Mouse"	50 kDa	1:500	BD Transduction Laboratories
Anti-Rac1	23A8 "Mouse"	24 kDa	1:500	Upstate Cell Signalling Solutions
Anti-Cdc42	3707 "Mouse"	24 kDa	1:250	CHEMICON® International
Anti-β-actin	AC-15 "Mouse"	42 kDa	1:2000	Sigma-Aldrich
Heat shock protein "Hsc-70"	B-6 "Mouse"	70 kDa	1:2000	Santa Cruz Biotechnology, UK

APPENDIX III

1. E-cadherin sequencing primers

Primers were obtained from the Cancer Research UK Oligonucleotide Synthesis Laboratory at Clare Hall

	Sequence	M.W	%GC	Annealing Temp.
E1	GCGCCCGGGCTCGAGTCGGCGTCCCCGGCCAGCCATGG	11722	81.58%	80.6°C
E2	GCGCCCGGGCTCGAGGCATTTCCCAGCACATGGGTCTGG	12064	69.23%	76.0°C
E3	GACTGGGTTATTCCTCCCATCAGC	7342	54.17%	59.0°C
E4	GACTGGGTTATTCCTCCCATCAGC	7984	53.85%	61.0°C
E5	CCGGACACTGGTGCCATTTCCACTCG	7945	61.54%	64.2°C
E6	GCTTTGCTAATTCTGATTCTGCTGC	7667	44.00%	55.9°C

2. siGENOME SMARTpool

siRNA oligonucleotides were provided as 4 duplexes, 5nmol each (Dharmacon RNA technologies, Chicago, IL, USA)

siGENOME duplex (1)
Sense sequence
G.G.A.A.A.G.A.G.C.U.G.C.A.G.G.U.G.A.A.U.U
Antisense sequence
5'-P.U.U.C.A.C.C.U.G.C.A.G.C.U.C.U.U.U.C.C.U.U

siGENOME duplex (2)
Sense sequence
G.A.G.A.G.C.A.G.C.U.U.C.C.A.A.A.U.C.A.U.U
Antisense sequence
5'-P.U.G.A.U.U.U.G.G.A.A.G.C.U.G.C.U.C.U.C.U.U

siGENOME duplex (3)
Sense sequence
C.G.A.C.U.A.C.A.C.U.A.U.U.G.G.A.U.U.U.U.U
Antisense sequence
5'-P.A.A.A.U.C.C.A.A.U.A.G.U.G.U.A.G.U.C.G.U.U

siGENOME duplex (4)

Sense sequence

C.A.G.A.A.G.A.U.G.U.G.G.A.U.G.A.G.U.U.U.U

Antisense sequence

5'-P.A.A.C.U.C.A.U.C.C.A.C.A.U.C.U.U.C.U.G.U.U

Scrambled sequence

Sense sequence

C.A.G.C.G.A.C.U.A.C.A.C.C.A.A.U.A.G.dT.dT

Antisense sequence

U.C.U.A.U.U.G.G.U.G.U.A.G.U.C.G.C.U.G.dT.dT

APPENDIX IV

Results

1. Sequencing results

The nucleic acid sequence of the isolated E-cadherin gene was matched with the E-cadherin gene sequence template, obtained from EMBL/Genbank Data Libraries under the accession number Z13009, using sequence matching software, Lalign Query, supplied by the GeneStream network server (GeneStream network server IGH, Motpellier, France).

Point mutations, resulting from substitution of Cytosine (C) with Thymidine (T), were detected at two different locations:

a) Using E4 primer

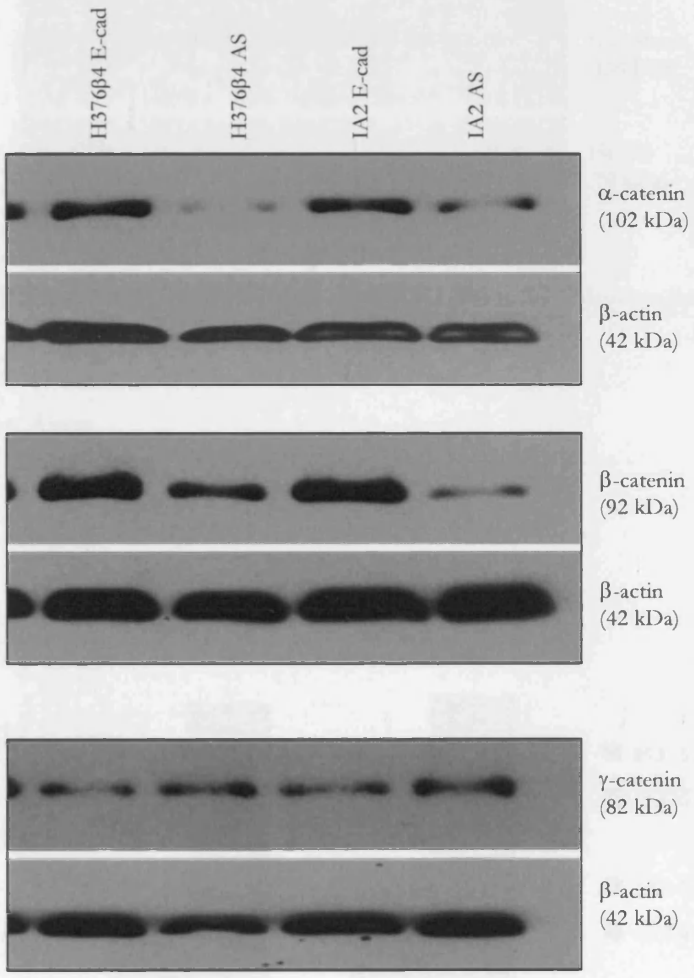
```
1330       1340       1350       1360       1370       1380
170828 TGATGCTGATGCCCCCAATACCCCAGCGTGGGAGGCTGTATACACCATATTGAATGATGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      TGATGCTGATGCCCCCAATACCCCAGCGTGGGAGGCTGTATACACCATATTGAATGATGA
     160        170        180        190        200        210

               1390       1400       1410       1420       1430       1440
170828 TGGTGGACAATTTGTCGTCACCACAAATCCAGTGAACAACGATGGCATTTTGAAAACAGC
       :::::::::::::::::::::::::::::::::::: :::::::::::::::::::::::::
_      TGGTGGACAATTTGTCGTCACCACAAATCCAGTGAATAACGATGGCATTTTGAAAACAGC
     220        230        240        250        260        270
```

b) Using E6 primer

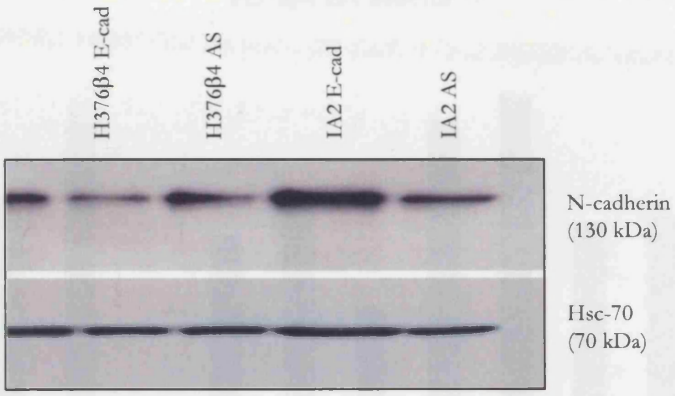
```
2280       2290       2300       2310       2320       2330
356931 TGTTTCTTCGGAGGAGAGCGGTGGTCAAAGAGCCCTTACTGCCCCCAGAGGATGACACCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      TGTTTCTTCGGAGGAGAGCGGTGGTCAAAGAGCCCTTACTGCCCCCAGAGGATGACACCC
        560        570        580        590        600        610

    2340       2350       2360       2370       2380       2390
356931 GGGACAACGTTTATTACTATGATGAAGAAGGAGGCGGAGAAGAGGACCAGGACTTTGACT
       ::::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      GGGACAATGTTTATTACTATGATGAAGAAGGAGGCGGAGAAGAGGACCAGGACTTTGACT
        620        630        640        650        660        670
```

2. Characterisation of H376β4 and IA2 transfected cell lines

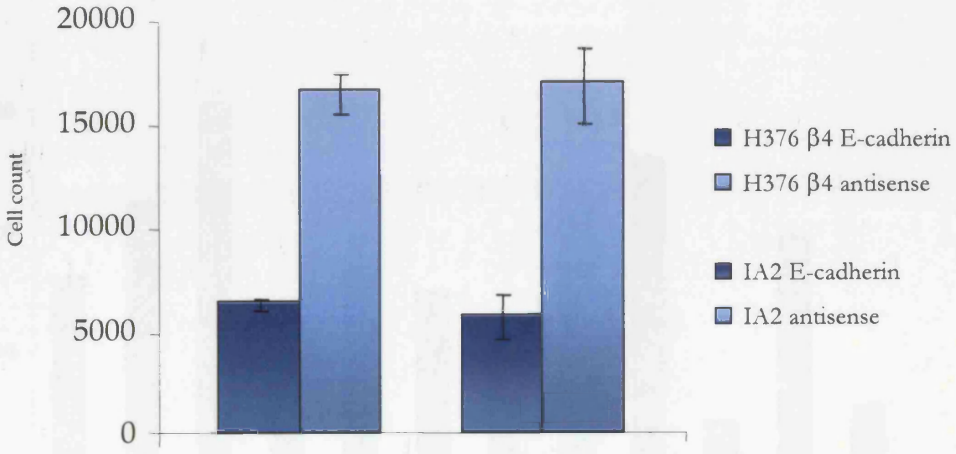
a) Catenin profile



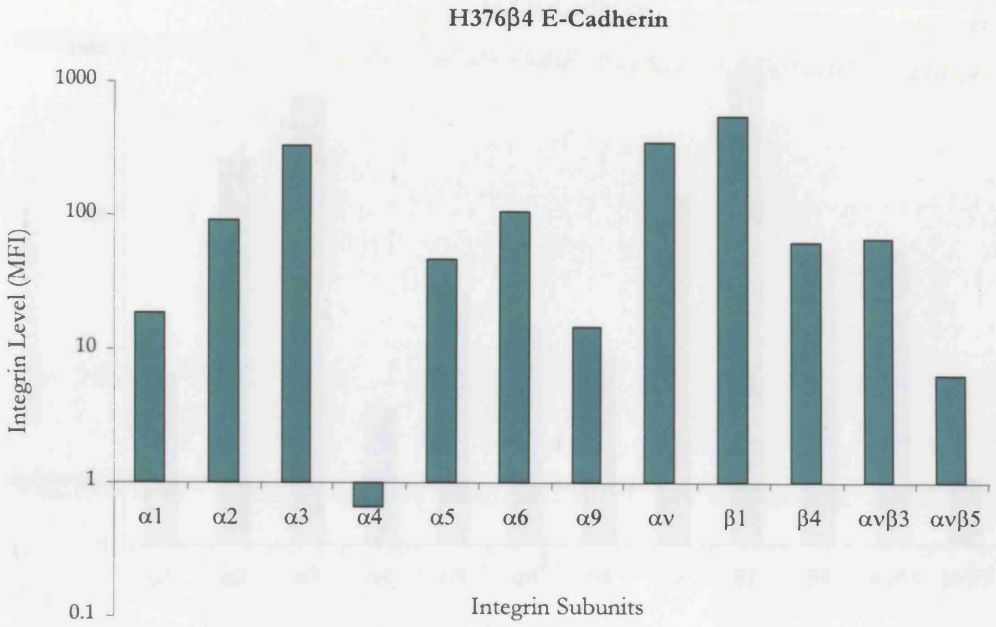
b) N-cadherin profile

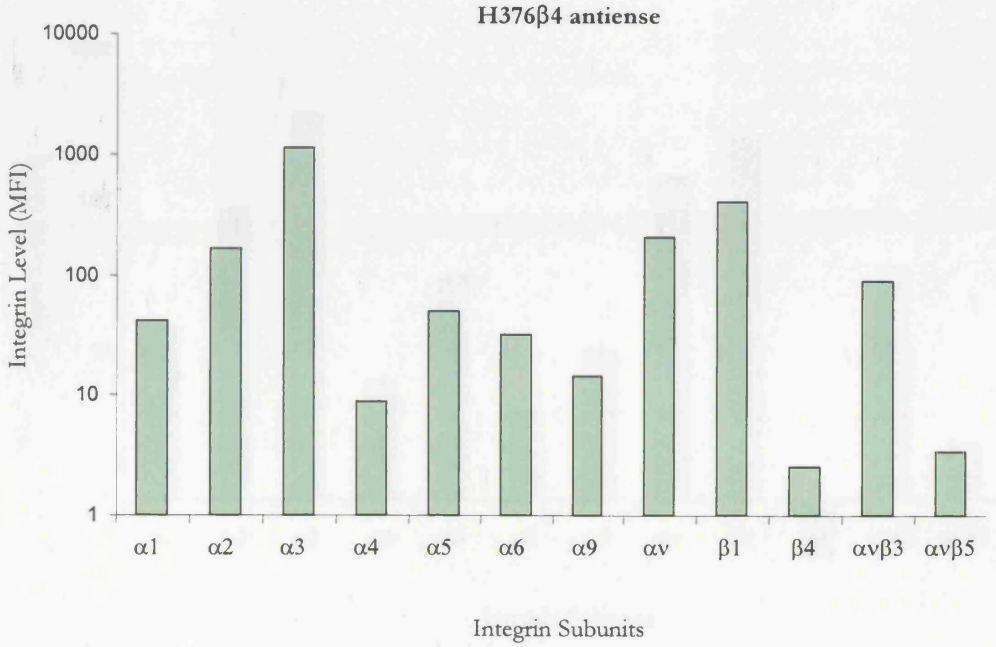


c) Invasion Assay

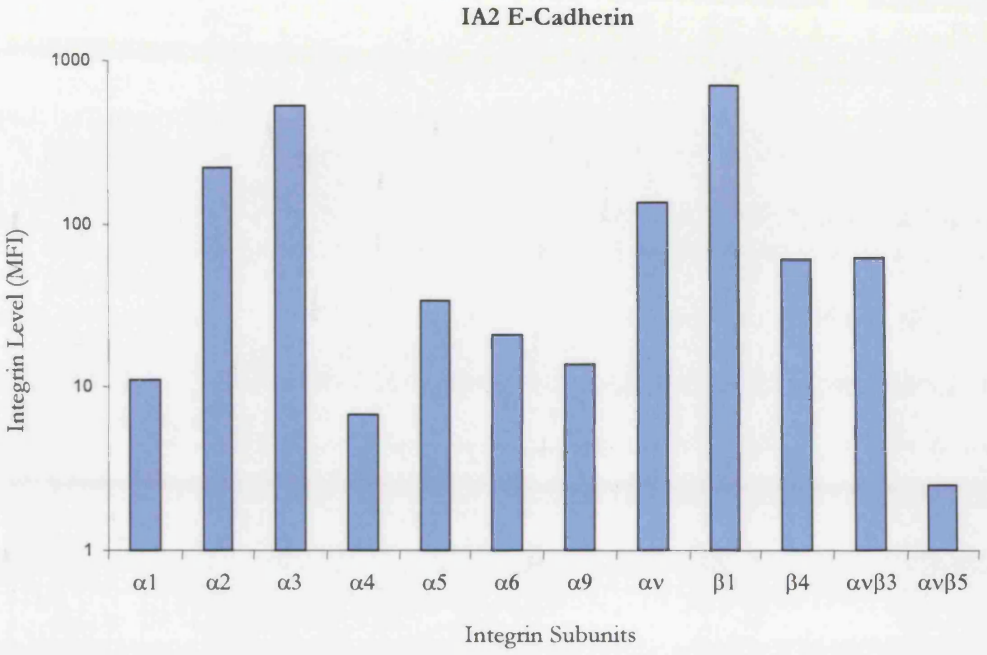


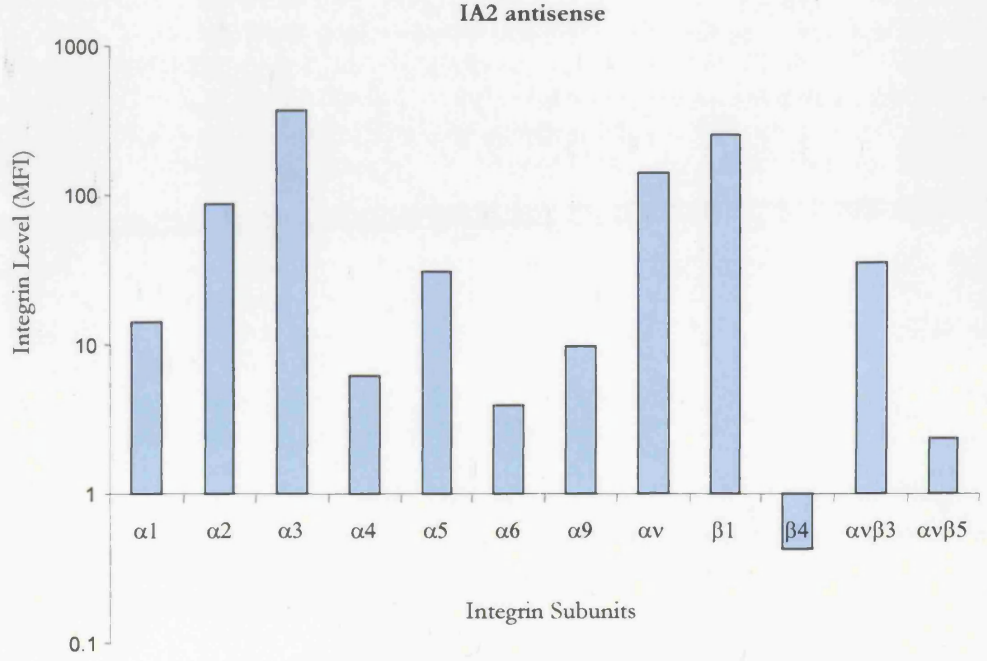
d) Integrin profile





d) Integrin profile (Cont.)





3. **3-D presentation of E-cadherin-dependent-cell-cell junctions in H376 E-cadherin and H376β4 E-cadherin OSCC cell lines**

See attached CD

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