THE ROLE OF ADHERENS JUNCTION PROTEINS IN THE INITIATION OF OVARIAN CANCER

by

Colleen Wu

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Department of Anatomy

The University of British Columbia
Vancouver, Canada
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ABSTRACT

Cells of the human ovarian surface epithelium (OSE) are plastic and uncommitted. Specifically, OSE cells do not express E-cadherin and they can shift between epithelial and stationary or mesenchymal and migratory phenotypes. In contrast, well differentiated, low grade ovarian carcinomas, which are thought to arise from the OSE, express E-cadherin and they display a committed epithelial phenotype. E-cadherin expression and stationary epithelial commitment are first observed in OSE-derived inclusion cysts and clefts which are thought to be weakly pre-neoplastic. Therefore, I determined if forced E-cadherin expression in OSE cells was capable of inducing this commitment to a stationary epithelial phenotype that is a hallmark of the earliest stages of ovarian carcinoma formation.

High levels of forced E-cadherin expression in an immortalized human OSE cell line achieved by stable transfection induced the localization of E-cadherin, β-catenin, and f-actin to sites of cell-cell contact. High levels E-cadherin also resulted in an increase in the amount of β-catenin associated with the cytoskeleton formation and a decrease in LEF-1/β-catenin signaling. Taken together, these data indicate that high levels of forced E-cadherin expression induced the formation of functional epithelial junctions in immortalized OSE cells. In addition, these cells had migratory capabilities in three-dimensional basement membrane gels compared to controls and low E-cadherin

expressors. This strongly suggests that E-cadherin is capable of inducing a stationary epithelial phenotype in human OSE cells.

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LIST OF ABBREVIATIONS

APC	Adenomatous polyposis coli
BSA	Bovine serum albumin
CMV	cytomegalovirus
DAPI	4'-6-diamidine-2-phenyl indole
DOC	Sodium deoxycholate
DSP	3,3-Dithio-bis(sulfosuccinimidyl)propionate;DTSSP;sulfo-DSP
EC	E-cadherin
ECL	Enhanced chemiluminescence
EMT	Epithelial to mesenchymal transformation
GSK-3β	Glycogen synthase kinase-3β
HSV	Herpes simplex virus
IOSE	Immortalized ovarian surface epithelial
LEF-1	Lymphoid Enhancer Factor-1
MET	Mesenchymal to epithelial transformation
NGS	Normal goat serum
OSE	Ovarian surface epithelium
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulphate
PBS	Phosphate buffered saline
PMSF	Phenylmethyl-sulfonyl fluoride
TBS	Tris-buffered saline
pc	Post crisis
TCF	T Cell Factor

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INTRODUCTION

1.1 Ovarian Surface Epithelium

In the western world, epithelial ovarian carcinoma is the second most prevalent and the most lethal of all gynecological cancers. Due to the lack of early detection and lack of reliable early screening methods, ovarian cancer is most often detected when it has spread beyond the ovaries and the prognosis is poor. The evolution of ovarian carcinoma is not well understood and there is currently no definitive experimental model for the early progression of the disease. In addition, the pre-malignant phase or phases for ovarian carcinoma have not been clearly defined. Thus, the early detection and treatment of ovarian cancer is extremely problematic (Hoskins et al., 1995).

Factors that appear to reduce the risk of epithelial ovarian carcinoma are 1) pregnancy, 2) the use of oral contraceptives, and 3) late menarche or early menopause. All these factors involve an inhibition of ovulation, leading to the hypothesis that repeated rupture and wound repair of the ovary during ovulation leads to an increased risk that cells of the ovary will undergo a malignant transformation (Tortolero-Luna and Mitchell 1995). Currently, the majority of ovarian carcinomas are believed to arise form the ovarian surface epithelium (OSE) which covers the surface of the ovary thus, making these cells an integral component in the rupture and wound repair that occurs throughout the ovulation period.

The OSE is a simple squamous to cuboidal epithelium that covers the surface of the ovary. Interestingly, the cells of the OSE display characteristics of both epithelial and mesenchymal cells. Cells of the OSE express keratin; a marker of epithelial cells; however, cells of the OSE also express markers of mesenchymal cells such as the intermediate filament vimentin as well as stromal collagen and matrix metalloproteases. This bipotent nature of the OSE may be in part explained by its tissue of origin. The OSE is developed as an embryonic specialization of the coelomic epithelium which in turn is mesodermally derived. Functionally, the epithelial/mesenchymal plasticity of the OSE allows for cells to migrate and repair the post-ovulatory damage in cortex of the ovary (Auresperg et al., 2001). Thus the resting OSE is epithelial, and during wound repair, the OSE becomes mesenchyme-like in order to migrate and repair post ovulatory damage.

The ceolomic epithelium, from which the OSE is derived, also gives rise to lining epithelium of the Mullerian ducts which form the fallopian tubes, uterus and endocervix. Interestingly, ovarian carcinomas are classified into three catagories based on their phenotypic similarity to the Mullerian-derived epithelia: serous carcinomas resemble the epithelium of the fallopian tube; endometriod adenocarcinomas resemble the uterine endometrium; and mucininous adenocarcinomas resemble the endocervix (Barber 1993, Blackledge and Chan 1986). This suggests that neoplastic transformation of the OSE involves the acquisition of a differentiated Mullerian epithelial phenotype.

Increasing evidence indicates that the majority of ovarian carcinomas are likely to be derived from the OSE (Feeley et al., 2001, Scully 1995b). For example, contralateral ovaries from women who developed ovarian cancer post surgery present with dysplasia and early stage carcinomas present in the surface epithelium (Scully 1995b). Recently, using an avian retroviral system in a mouse to introduce mutations specifically in the ovarian surface epithelium or in the ovarian stroma and ovarian surface epithelium, Orsulic and collegues were able to demonstrate that subsequent tumor formation came solely from the ovarian surface epithelium and not from the underlying stroma (Orsulic et al., 2002). Other evidence that suggests that ovarian carcinomas originate in the OSE comes from the observation that cells trapped within ovarian inclusion cysts and inclusion clefts, which are OSE derived, may be weakly pre-neoplastic. Specifically, cells within these structures often differentiate to further resemble cells of the Mullerian duct-derived epithelia and begin to express CA-125, both of which are hallmarks of ovarian carcinoma (Salazar et al., 1996). Also, tubal metaplasia, which is considered a pre-malignant lesion, occurs 10 times more frequently in inclusion cysts than in the normal OSE. Finally, contralateral ovaries in patients with unilateral ovarian cancer have increased numbers of surface inclusion clefts and inclusion cysts (Tresserra et al., 1998, Mittal et al., 1993). Taken together, this evidence suggests that ovarian carcinomas arise

in the OSE and that metaplastic OSE cells trapped within inclusion cysts and clefts may be early precursors to ovarian carcinoma.

As indicated above, pre-neoplastic cystic epithelia also acquire characteristics of committed Mullerian duct-derived epithelia. Another characteristic that is shared by pre-neo-plastic cysts and differentiated ovarian carcinomas is the presence of E-cadherin, an epithelial cell adhesive molecule that is not expressed in bipotent undifferentiated OSE cells. Thus, these pre-neoplastic cystic epithelia are likely to be stationary given that they are cohesive and express adherens junction protein, E-cadherin.

1.2 Role of Adherens Junction Proteins in the Progression of Ovarian Cancer

Cellular adhesion is required for tissue morphogenesis, and regulates many cellular processes such as motility, proliferation, and survival. Many junctions are involved in maintaining intercellular adhesion, one of which is the adherens junction. In epithelial tissues the adherens junction is involved in maintaining tissue architecture and cell polarity and can limit cell motility and proliferation (Takeichi 1991). In its simplest form, the adherens junction can be thought to be composed of three key components: the E-cadherin receptors; the catenin intracellular bridging proteins, and the f-actin portion of the cytoskeleton.

E-cadherin

E-cadherin,the major epithelial cadherin, initiates adherens junction formation. Calcium-dependant homophillic binding between E-cadherin extracellular domains is critical not only for cell sorting necessary during normal tissue development, but also for maintaining tissue integrity. Thus, loss of E-cadherin contributes to many pathological conditions (Birchmeier and Behrens 1994). For example, with the respect to cancer, E-cadherin is often considered to be a late stage tumor suppressor. Loss of E-cadherin function either through mutation, in CDH1, the E-cadherin CDH gene, or through repressed transcriptional activity can result in increased tumor cell motility and invasiveness (Hirohashi et al., 1998, Berx et al., 1995, Vleminckx et al., 1991, Behrens et al., 1989). Conversely, transfection of metastatic tumor cells with an E-cadherin cDNA prevents invasiveness (Frixen et al., 1991).

E-cadherin is expressed in most epithelial cells. A notable exception is the human OSE which does not express the cell adhesion molecule (Davies et al., 1998, Maines-Bandiera and Auersperg 1997, Sundfeldt et al., 1997). These cells, which are mesodermally-derived, are also capable of becoming highly motile. In contrast, however, the OSE-derived cells that line inclusion clefts and cysts do express E-cadherin (Maines-Bandiera and Auersperg 1997). They are also stationary, columnar, highly polarized and secretory (Scully 1995b). As a result, cystic cells often secrete CA-125 which is a high molecular weight glycoprotein that is a normal differentiation marker of Mullerian duct-

derived epithelia. Importantly, E-cadherin expression, production of CA-125 and the acquisition of morphologies that mimic Mullerian-derived epithelia are all prevalent in early stage ovarian carcinomas (Rritsche and Bast 1998, Bast et al., 1995). These data suggest that the ectopic expression of E-cadherin that first occurs in weakly preneoplastic cystic cells may be a pre-requisite for the well-differentiated epithelial phenotype that is a hallmark of ovarian carcinoma formation.

In most carcinomas, including those of the ovary, a loss of E-cadherin expression or function is correlated with increased invasiveness during the later stages of metastatic progression (Fujioka et al., 2001). However, in addition to initiating epithelial differentiation, the ectopic upregulation of E-cadherin expression that occurs during the earliest stages of ovarian carcinoma formation could also confer a selective advantage given the unusual route of metastasis in this tumor type. Specifically, the initial route of metastatic dissemination is most often not through the ovarian stroma and vasculature. Instead tumor nodules are exfoliated from the ovarian surface where they float free in the peritoneal cavity before they attach to secondary sites on the peritoneal wall. Thus, ovarian carcinoma nodules must be capable of escaping anoikic apoptosis during the free floating stage. In other systems E-cadherin-mediated cell-cell adhesion has been shown to suppress this method of apoptosis (Pece and Gutkind 2000, Kantak and Kramer 1998).

In its mature form, E-cadherin is 120 kDa transmembrane glycoprotein consisting of highly conserved carboxy terminal cytodomain, a single-pass transmembrane domain, and an extracellular domain consisting of five tandomly repeated ectodomains which are responsible for calcium dependant homophillic binding (Yap et al., 1997). The subdomain furthest from the cell membrane contains a histidine-alanine-valine (HAV) sequence which is essential for cellular adhesion as inhibition of this sequence with blocking peptides results in a decrease in cellular aggregation in MDCK cells (Noe et al., 1999). This sequence also confers the specificity of the homophillic interaction between cadherins (Nose et al 1990). Although the ectodomains confer homophillic binding, stable cell adhesion requires the cytoplasmic tail and associated proteins (Ozawa et al., 1990, Nagafuchi and Takeichi 1988)

β-catenin

β-catenin was originally identified as a junctional protein associated with E-cadherin at the adherens junction. As a component of the adherens junction, β-catenin links E-cadherin to the actin cytoskeleton via its association with α-catenin (Nagafuchi 2002). However, β-catenin not only plays an important role in the formation and maintenance of cellular junctions, but cytoplasmic β-catenin also functions as a signaling molecule. Specifically, cytoplasmic β-catenin binds to the TCF/LEF-1 family of transcription factors and this β-catenin/TCF/LEF-1 complex is able to translocate to the

nucleus where it binds to DNA and activates transcription of specific target genes such as myc and cyclin D1 (Tetsu and McCormick 1999, He et al., 1998, Behrens et al., 1996, Huber et al., 1996, Molenaar et al., 1996).

Cytoplasmic β-catenin levels are tightly regulated through its association with adenomatous polyposis coli (APC), glyocogen synthase kinase 3β (GSK-3β), and Axin (Behrens et al.,1998, Ikeda et al., 1998, Sakanaka et al., 1998, Rubinfed et al., 1996). The presence of β-catenin within this protein complex results in its phosphorylation which in turn targets it for proteosomeal degradation (Aberle et al., 1997). However, certain signals, such as Wnt, inhibit this degradation (Porfiri et al., 1997). For example, Wnt-mediated signaling inhibits β-catenin degradation by activation of dishevelled which inhibits GSK-3β (Kikuchi 2000).

Dysregulation of cytoplasmic β-catenin levels has been implicated in human tumors and cancer cell lines. For instance, mutations in APC which result in the inability of APC to phosphorylate and degrade cytoplasmic β-catenin have been found in colon carcinomas (Korinek et al., 1997, Morin et al., 1997). Mutations in β-catenin itself which results in the inability for it to become phosphorylated have also been found in melonama cell lines (Rubinfeld et al., 1997 Morin et al.,1997). Dysregulation of the β-catenin signaling pathway has also been found in ovarian carcinomas. For example, deregulation of the β-catenin signaling pathway has been implicated in late stage endometriod ovarian

carcinomas (Saegusa et al., 2001, Wu et al., 2001, Palacios et al., 1998). There is also evidence which suggests that upregulated LEF-1 activity is present in some ovarian carcinoma cell lines (Furlong and Morin 2000). Thus, it is not unreasonable to speculate that an upregulation of β-catenin/LEF-1/TCF pathway may play a role in the late stage metastatic ovarian carcinoma.

It has been proposed that 2 pools of β-catenin exist with in a cell, one pool which is involved in maintaining cellular adhesion by binding to E-cadherin at the adherens junction, and another "free" cytoplasmic pool which has the ability to bind to TCF-1/LEF-1 transcription factors and act as a transcriptional activator (Gottardi and Gumbiner 2001). The central region of β-catenin consists of 12 armadillo repeats which are involved in the binding of β-catenin to other proteins. Both E-cadherin and the LEF-1 family of transcription factors bind competitively to β-catenin in this region (Huber and Weiss 2001, Huber et al., 1997, Orsulic et al., 1999). It has been proposed that E-cadherin can depress LEF-1 activity by sequestering β-catenin, thus decreasing the amount of β-catenin available to translocate into the nucleus (Gottardi et al., 2001, Sadot et al., 1998, Faggatto et al.,1996). Therefore, the upregulation of E-cadherin expression that occurs in early ovarian carcinoma formation may downregulate β-catenin/LEF-1 activity.

LEF-1, a protein that belongs to the HMG box family of transcription factors (Clevers and Grosscheld 1996), is known to play a critical role in the epithelial-mesenchymal transformations. For example, the β-catenin/LEF-1 pathway plays a critical role in cellular movements during gastrulation and elevated LEF-1 activity has been correlated with epithelial to mesenchymal transformations in cell culture (Kim and Hay 2002, Moon and Kimelman 1998, van Genderen et al., 1994). Also, one of the target genes for LEF-1 activation during development is the mesenchyme-inducing siamoisis gene (Nelson et al., 1998). Thus, it is possible that a decrease in LEF-1 signaling activity could contribute to a mesenchymal to epithelial transformation that is seen in early stages of ovarian carcinoma formation.

1.3 Hypotheses

Cells of the normal human OSE do not express E-cadherin and have both epithelial and mesenchymal characteristics. This plasticity of the OSE allows for cells to migrate and repair post-ovulatory damage. Although the normal OSE does not express E-cadherin, cells within inclusion cysts and clefts do express the cell adhesion molecule, and it has been proposed that these cells may be weakly pre-neoplastic. Early stage, well differentiated ovarian carcinomas also express E-cadherin and they are invariably well-

differentiated and epithelial. As ovarian carcinomas progress, they often loose E-cadherin and once again exhibit mesenchymal characteristics which are associated with an invasive metastatic phenotype.

Therefore, I hypothesize that:

1) E-cadherin-mediated adherens junction formation occurs in well differentiated ovarian carcinomas that are stationary and epithelial but not in non-tumorigenic human OSE cells or in poorly differentiated ovarian carcinoma cells that are migratory and mesenchymal.

2) β-catenin/LEF-1 activity will be low in well-differentiated ovarian carcinoma cells but high in both OSE cells and in poorly differentiated ovarian carcinoma cells.

3) Forced E-cadherin expression will initiate adherens junction formation, decrease β-catenin/LEF-1 activity and result in a stationary epithelial phenotype in non-tumorigenic OSE cells.

To test hypotheses #1 and #2, I characterized the adherens junction status and ß-catenin/LEF-1 activity in: the extended life span IOSE-29 cell line; the well differentiated OVCAR-3 serous ovarian carcinoma cell line; and the poorly differentiated SKOV-3 serous ovarian carcinoma cell line. To test hypothesis #3, I characterized individual clones that were derived from a pool of the immortal IOSE-80pc cell line that had been stably transfected with a wild type mouse E-cadherin cDNA.

MATERIALS AND METHODS

2.1 Cell Culture

All cell lines were grown in 1:1 mixture of 199/105 (Sigma) supplemented with 5% fetal bovine serum (FBS) (Hyclone) and 50μg/μl of gentamycin (Sigma). Transfected cells were maintained under selection media: 199/105 supplemented with 5% FBS, 50μg/μl of gentamycin, and 400μg/μl of G418 (Sigma).

2.2 Generation of Cell Lines

IOSE-80 post crisis (from N. Auersperg) cells were transfected with mouse E-cadherin cDNA (from M. Sasaki) and were cloned by limited dilution and individual clones were selected. IOSE-80pc IRES cells were generated by incubating a 50% confluent 100mm dish with transfection solution (30ug of pIRES plasmid (from M. Sasaki), and 30μl of DMRIE-C (Invitrogen) in 199/105 base media). Cells were incubated with transfection solution for 3 hours. After the incubation period cells were rinsed three times with 199/105 base media and grown in 199/105 supplemented with 5% FBS for 48 hours. Transfected cells were then maintained under selection media (see above).

2.3 Immunofluorescence Microscopy

Cells were cultured on glass coverslips with 199/105 base media supplemented with 5% FBS and 50 μg/μl of gentamycin until fully confluent. For E-cadherin and β-catenin staining, cells were rinsed three times with PBS and then fixed with ice cold methanol for

20 minutes at 20°C. Cells were blocked with 1% (w/v) bovine serum albumin (BSA) (Fisher), 10% normal goat serum (v/v) (NGS) (Jackson ImmunoResearch) in phosphate buffered saline ph 7.4 (PBS) for 30 minutes at room temperature. To assess the presence of β-catenin or E-cadherin, cells were incubated with either 25 μg/μl of mouse anti-β-catenin antibody (Transduction Laboratories) or 25μg/μl of mouse anti-E-cadherin antibody (Transduction Laboratories) or 25ug/ul of mouse IgG (negative control) (Jackson ImmunoResearch) in 1% BSA in PBS for 1 hour at room temperature. Binding of primary antibodies was visualized by incubation of cells with 75μg/μl Fluorescein-conjugated AffiniPure goat anti-mouse IgG (H+L) (Jackson ImmunoResearch) in 1% BSA for 1 hour at room temperature. Immunofluoresent staining was visualized with a Zeiss Axiophot microscope.

For Large T, confluent coverslips were rinsed three times with PBS and fixed with ice cold methanol for 20 minutes at -20°C. Cells were postfixed with an ice cold 1:1 acetone:methanol (v/v) mixture for 5 minutes at -20°C. Coverslips were allowed to air dry. Coverslips were then blocked with 1% BSA, 10% BSA in PBS for 30 minutes at room temperature. Binding of primary antibodies was visualized by incubation of cells with 1μg/ml of mouse anti-Tag antibody (Oncogene Science) or 1ug/ul of mouse IgG (negative control) (Jackson ImmunoResearch) for 1 hour at room temperature. Binding of primary antibodies was visualized by incubation of cells with 75μg/μl Fluorescein-

conjugated AffiniPure goat anti-mouse IgG(H+L) (Jackson ImmunoResearch) in 1% BSA for 1 hour at room temperature. Immunofluoresent staining was visualized with a Zeiss Axiophot microscope.

For f-actin, confluent coverslips were rinsed three times with PBS and fixed with 3.7% paraformaldehyde (Fisher) for 15 minutes at room temperature. Cells were postfixed with acetone for 5 minutes at -20°C and then allowed to air dry. F-actin was assessed with rhodamine phalloidin (Molecular Probes) and visualized with a Zeiss Axiophot microscope.

2.4 Western Blot Analysis

a) Whole Cell Lysates

For whole cell lysates, 100mm confluent dishes were rinsed three times with ice cold 199/105 base media. 200ul of RIPA buffer (150mM NaCl, 50mM Tris pH 7.4, 5mM EDTA, 5.0% NP-40, 1.0% sodium deoxycholate (DOC) and 0.1% sodium dodecyl sulphate (SDS), aprotinin, leupeptin, phenylmethyl-sulfonyl floride (PMSF)) was added to the 100mm dish. Cells were collected by scraping 100mm dishes with a cell lifter and were transferred to 1.5 ml centrifuge tubes. Cells were incubated in RIPA buffer on ice for 10 minutes and then centrifuged at 13,000g at 4°C for 10 minutes. Supernatants were collected and assayed for protein concentrations according to manufacturer's instructions (BioRad). 2.5 μg (β-catenin)/ 40 μg (E-cadherin) of total protein was loaded and

separated on a 10% SDS-poly acrylamide gel electrophoresis (PAGE) gels and then transferred on to PDVF (BioRad) membrane. Membranes were blocked with 4% bovine serum albumin (BSA) in Tris buffered saline (TBS)-Tween-20 for 6 hous at 4°C. Membranes were probed with 2.5ug/ml of mouse anti-β-catenin (Transduction Laboratories) or 2.5ug/ml mouse anti-E-cadherin (Transduction Laboratories in 1% BSA in TBS-Tween-20 overnight at 4°C. Blots were incubated with 0.4 μg/ml of anti-mouse horse radish peroxidase (Jackson Immuno Research) in 1% BSA in TBS-Tween-20 for 1 hour at room temperature. Antibody binding was visualized by enhanced chemiluminesence (ECL) (Amersham Pharcmacia) according to manufacturer's instructions.

b) Cytoskeletal Extractions

For cytoskeletal fractions, 100mm confluent dishes were rinsed three times with 199/105 base media and then treaded with 200μg/ml of the cross linker (DSP) (Chemicon) for 20 minutes at room temperature. Cells were rinsed two times with 199/105 base media and quenched with 50mM glycine for 5 minutes at room temperature. Cells were then incubated on ice with constant rotation for 20 minutes in cytoskeletal buffer (50mM NaCl, 10mM Pipes pH 6.8, 3mM $MgCl_2$, 300mM Sucorse, 0.5% Triton-X 100 1.2 mM PMSF, 10μg/ml aprotinin) (Hinck et al., 1994) to extract the Triton-X 100 insoluble proteins. Dishes were then scraped on ice and lysates were transferred into 1.7ml tubes.

Lysates were spun down at 14,000 rpm for 10 minutes at 4°C the supernatant (soluble fraction) and the pellet (insoluble fraction) were separated. SDS IP buffer (15mM Tris pH 7.5, 5mM EDTA, 2.5mM EGTA, 1% SDS) was added to the pellets and the pellets were boiled for 10 min at 100°C. Protein assays were preformed on both fractions (BioRad) according to manufacturer's instructions. Western blot analysis (see above) were then performed using 2.5μg of protein (β-catenin) or 7ug of protein (E-cadherin) from the insoluble fraction were used to determine the amounts of cytoskeletal-associated proteins. 3μg of protein from the insoluble and soluble fractions of cell lysates were also probed with goat anti-vimentin antibody (Accurate Chemical & Scientific Corporation) to determine if cytoskeletal extractions were successful.

2.5 Affinity Precipitaions

Preparation of GST-E-cadherin Fusion Protein

E coli DH5 cells transformed with the vector containing the E-cadherin gluathione-S-transferase fusion protein (E-cad-GST) (Aberle et al., 1994) was used to inoculate 20ml LB medium supplemented with 50μg/μl of ampicillin. The culture was incubated overnight at 37°C and then used to inoculate 1 liter of LB medium supplemented with 50μg/μl of ampicillin. This was grown for 4 hours at 37°C. IPTG (0.1mM final) was added to allow for protein expression. *E. coli* DH5 transformed bacteria were induced for four hours at 37°C. Bacteria was collected by centrifugation at 5000rpm at 4°C and the

pellet was lysed for 30 minutes on ice with lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 1% Trition-X 100, 1mg/ml lysozyme, 0.1mg/ml DNASE I, leupeptin, aprotinin, PMSF). Lysed bacteria were spun down at 13,000 rpm at 4°C and pellets were discarded. 80ul of glutationone sepharose bead slurry (Pharmacia) was added to 500ul of bacterial supernatant to isolate E-cad-GST fusion protein. Beads were washed three times with wash buffer (25mM Tris pH 7.5, 150mM NaCl, 0.1% triton-X 100, leupeptin, aprotinin, PMSF). Precleared lysates (see below) were then added to the E-cad-GST fusion protein bound to glutatione-sepharose beads.

Affinity Pull Down

100mm confluent dishes were lysed in PBS containing 0.1% Triton-X 100 sodium floride, sodium vanadate, leupeptin, aprotinin, and PMSF. Cell lysates were nomalized (BioRad) (1000ug of protein) and were depleted of endogenous glutothione-S-trnasferase by incubation of lysates with 80µl of glutothione-sepharose bead slurry (Pharmacia) for 1h at 4°C. Precleared lysates were then incubated with E-cad-GST fusion bound to glutathione-sepharose beads (see above). Protein complexes were precipitated by incubation for 1 hour at 4°C. Protein complexes were collected by centrifugation at 13,000rpm at 4°C. Beads were washed five times with wash buffer (25mM Tris pH 7.5, 150mM NaCl, and 0.1% Triton-X 100) and protein was eluted by boiling beads in

Lamelli sample buffer. Samples were then resolved by SDS-PAGE and subjected to Western blot analysis (see above).

2.6 Reporter Gene Assays

1 x 10^5 cells were plated overnight in a 6 well dish in 199/105 supplemented with 5% FBS and 50μg/μl of gentamycin. Cells were rinsed three times with 199/105 before a three hour incubation with the transfection solution which consisted of: 1) 2μg of TOPFLASH or FOPFLASH (van de Wetering et al., 1997) (LEF-1 promoter assays), or 2) 250μg of the -178-+17 region of the E-cadherin promoter fused to firefly luciferase gene (E-cadherin promoter assays) (Behrens et al., 1991), and 2.5ul of DRMIE-C (Gibco) in 199/105 supplemented with 2% FBS. To control for transfection efficiencies, 2.0ng of a *Renilla* luciferase gene construct containing either a cytomegalovirus (CMV) or herpes simplex virus (HSV) promoter was included in each experiment. After three hours cells were rinsed three times with 199/105 and incubated for 48 hours with 199/105 supplemented with 5% FBS. Cells were lysed with 1X Passive Lysis Buffer (Promega) and lysates were subjected to a Dual Luciferese Assay (Promega) according to manufacturers instructions. Luciferease activity was measured with a Berthold luminometer. For LEF-1 promoter activity, numbers are given as a relative activation compared to cells transfected with the FOPFLASH-firefly luciferase reporter.

Results

3.1 Characterization of the IOSE-29, OVCAR-3 and SKOV-3 Cell Lines

Localization of Adherens Junctions Proteins in IOSE-29, OVCAR-3 and SKOV-3 Cell Lines

When they were originally maintained in monolayer culture, the OSE-29 cell strain underwent a characteristic epithelial to mesenchymal transformation. After transfection with simian virus-40 derived large T antigen the mesenchymal aspects of the phenotype were maintained (Auersperg et al., 1999). Thus, the resulting IOSE-29 cells exhibit a typical bipolar fibroblastic appearance. In contrast, the OVCAR-3 cell line, which was derived from a well differentiated serous adenocarcinoma, displays epithelial characteristics. OVCAR-3 cells form cohesive contacts with neighboring cells and grow in a cobblestone pattern characteristic of epithelial cells. Interestingly, the SKOV-3 cell line, which was derived from a de-differentiated serous adenocarcinoma, displays a fibroblastic morphology that is comparable to the IOSE-29. These different culture characteristics very broadly mimic the situation *in vivo*: normal OSE cells, which are mesodermally derived, retain a number of mesenchymal characteristics which become very apparent during migratory wound healing after ovulation (ie IOSE-29); differentiated ovarian carcinoma cells lose mesenchymal characteristics and exhibit a restricted, often glandular, epithelial phenotype (ie OVCAR-3); poorly differentiated

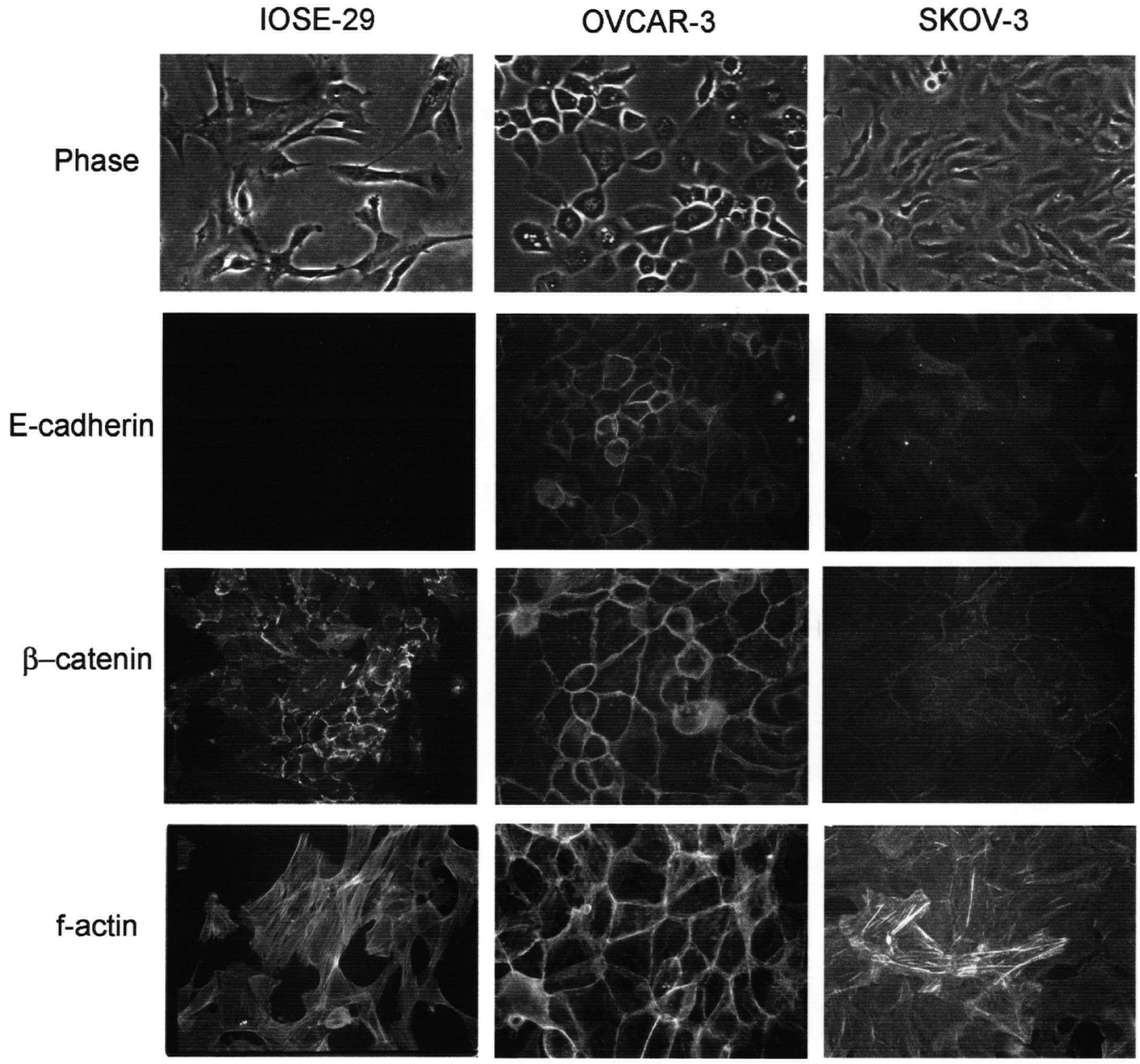
ovarian carcinoma cells once again exhibit mesenchymal characteristics which are associated with an invasive, metastatic phenotype (ie SKOV-3).

A key regulator of phenotypic switching between mesenchymal and epithelial phenotypes is the adherens junction. Therefore, we initially characterized the location of three adherens junction proteins, E-cadherin, β-catenin and f-actin, by immunofluorescence microscopy in the ISOE-29, OVCAR-3, and SKOV-3 cells. IOSE-29 cells showed a complete lack of E-cadherin while the junctional protein is present continuously at sites of cell-cell contact in OVCAR-3 cells. While E-cadherin was also present in SKOV-3 cells the staining intensity was low and most of the protein was not present at sites of cell-cell contact. In IOSE-29 cells most of the β-catenin was diffusely located throughout the cell, although a small amount of the linker protein was present at sites of cell-cell contact. In contrast, the OVCAR-3 cell line showed uniform continuous junctional β-catenin with little cytoplasmic staining. In the SKOV-3 cell line the β-catenin staining was intermediate; the junctional protein was only weakly associated at sites of cell-cell contact. Amongst the three lines only the OVCAR-3 cells had cortical actin that was associated with sites of cell contact. In contrast f-actin in both IOSE-29 and SKOV-3 cells was predominately found in non-junctional cytoplasmic stress fibres. Taken together, these data suggest that adherens junctions are not present in non-

tumorigenic IOSE-29 cells, are present and functional in differentiated OVCAR-3 cells, and are not functional in de-differentiated SKOV-3 cells.

Figure 1. Immunofluorescence Staining of IOSE-29, OVCAR-3, and SKOV-3 Cell Lines

Immunofluoresence staining for adherens junction proteins, E-cadherin, β-catenin, and f-actin was performed on IOSE-29, OVCAR-3, and SKOV-3 cell lines. IOSE-29 cells completely lack E-cadherin whereas E-cadherin is located at sites of cell-cell interaction in OVCAR-3 cells. SKOV-3 cell also express E-cadherin however, the great majority of the protein is not located at sites of cell-cell contact. β-catenin is present in all three cell lines however only in the OVCAR-3 cells is β-catenin strongly associated with cell-cell junctions. Only in the OVCAR-3 cells is f-actin associated with sites of cell contact. F-actin is present as non-junctional stress fibers in both IOSE-29 and SKOV-3 cells (X250; different fields).


IOSE-29
OVCAR-3
SKOV-3
Phase
E-cadherin
β–catenin
f-actin

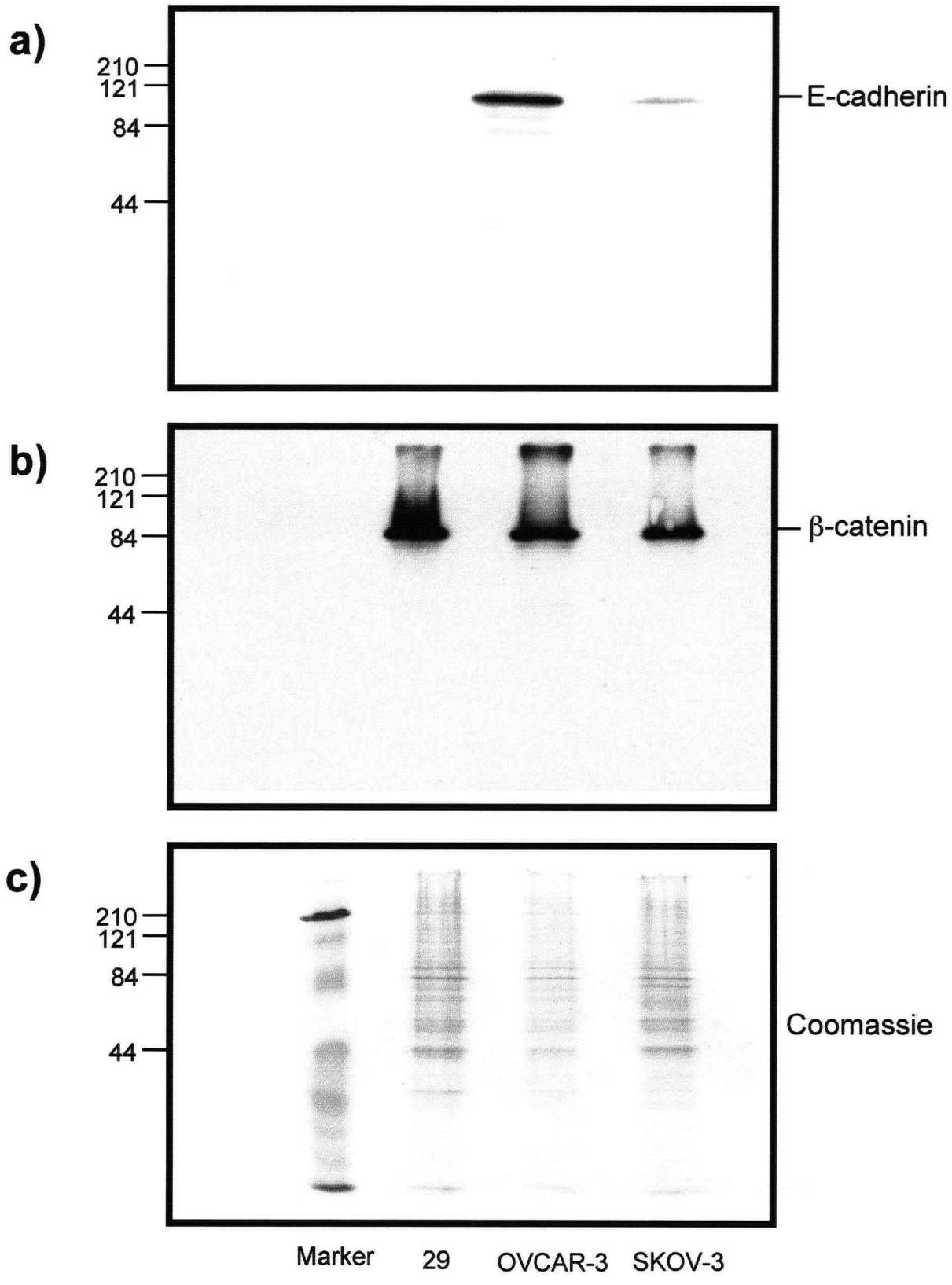
Steady State Levels of Adherens Junction Proteins in IOSE-29, OVCAR-3 and SKOV-3 Cell Lines

To confirm the differences in steady-state levels of adherens junction proteins implied by immunofluoresence staining (see Fig 1 above), Western blot analysis was performed on total cell lysates from the IOSE-29, OVCAR-3, and SKOV-3 cell lines. As is the case in the ovarian surface epithelium *in vivo*, there was a complete lack of E-cadherin in the IOSE-29 cell line (Fig 2a). In contrast, the OVCAR-3 cell line expressed high levels of the 120kD E-cadherin protein. While the SKOV-3 cell line also expressed E-cadherin, steady state levels were much lower than that observed in OVCAR-3 cells which agrees with the decreased intensity observed in immunofluorescence.

Total cell lysates were also probed for ß-catenin (Fig 2b). All three cell lines contained very similar amounts of this 94kDa protein. Thus, the differences in steady state E-cadherin levels do not dictate similar differences in total amounts of ß-catenin.

Figure 2. Western Blot Analysis of Whole Cell Lysates From IOSE-29, OVCAR-3, and SKOV-3 Cell Lines

Western blot analysis was performed on whole cell lysates from ISOE-29, OVCAR-3, and SKOV-3 cell lines. Blots were probed for E-cadherin and β-catenin. a) Although differences in E-cadherin expression were observed between the three cell lines with the OVCAR-3 cells exhibiting the most E-cadherin expression, b) no differences were detected in steady state levels of β-catenin between the three cell lines. c) Coomassie staining was performed to ensure equal protein loading between samples. Data shown is representative of three independent experiments.


a)
210
121
84
44
E-cadherin
b)
210
121
84
44
β-catenin
c)
210
121
84
44
Coomassie
Marker
29
OVCAR-3
SKOV-3

Comparison of E-cadherin Promoter Activity Between IOSE-29, OVCAR-3 and SKOV-3 Cell Lines

In vivo, normal OSE cells do not contain the E-cadherin protein which likely reflects their mesodermal origin (Davies et al., 1998, Maines-Bandiera and Auesperg 1997, 1998, Sundfeldt et al., 1997). In contrast, well differentiated ovarian carcinomas that arise from the OSE do contain the junctional protein but it is decreased or lost again as the tumors progress to a less differentiated state with increased metastatic potential (Fujioka et al., 2001). As demonstrated above, these changes in E-cadherin protein levels are mimicked in the IOSE-29 (normal immortalized OSE), OVCAR-3 (differentiated carcinoma) and SKOV-3 (poorly differentiated carcinoma) cell lines in vitro. To determine if the differences in steady-state levels of E-cadherin are due to changes in transcriptional regulation we assessed the activation state of the E-cadherin promoter in the three cell lines. This was achieved by transient transfection of a -178 to + 17 fragment of the mouse E-cadherin promoter fused to the luciferase reporter gene (Behrens et al.,1991). This fragment contains the palindromic 'E-box' which is a critical determinant of E-cadherin induction in epithelial cells and repression in mesenchymal cells (Batlle et al., 2000, Behrens et al 1991).

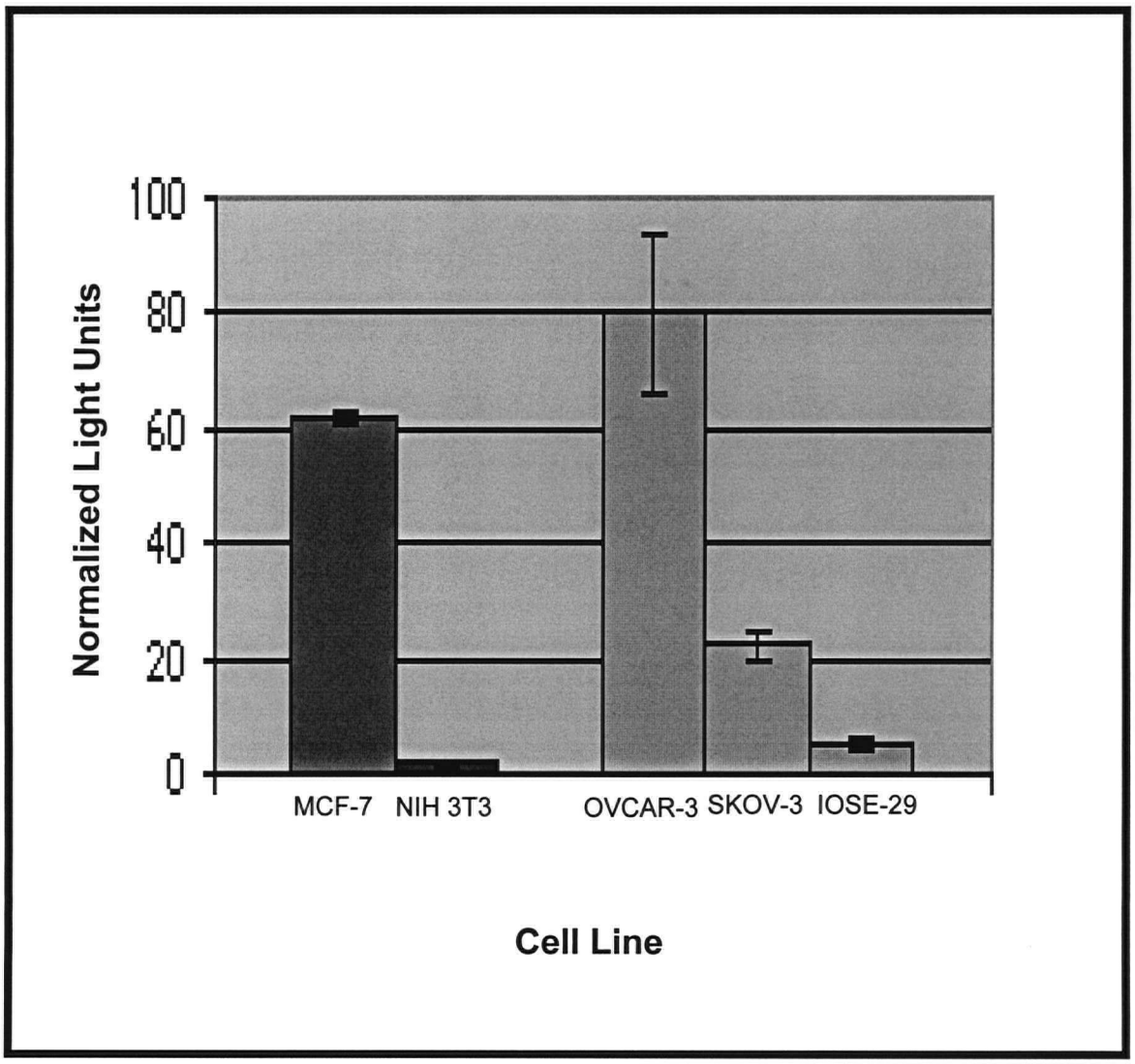
MCF-7 breast carcinoma cells, which are epithelial and express E-cadherin, were used as positive controls. NIH3T3 fibroblasts, which are mesenchymal and do not

express E-cadherin were used as negative controls (Behrens et al., 1991). The fact that MCF-7 cells exhibited considerable E-cadherin promoter activity while 3T3 cells exhibited very little activity indicated that the assay was valid (Fig. 3). IOSE-29 cells (E-cadherin protein negative) cells had a very low promoter activity that was comparable to the 3T3 cell negative control. OVCAR-3 cells (E-cadherin positive) had the highest promoter activity amongst the three experimental lines. SKOV-3 (E-cadherin protein intermediate) had a promoter activity that was intermediate between the other two experimental lines. Therefore, there is a positive correlation between the steady state levels of E-cadherin protein and E-cadherin promoter activity (Fig. 3). This suggests that the differences in E-cadherin protein levels observed amongst the experimental lines are due, at least in part, to differences in transcriptional regulation.

Figure 3. E-cadherin Promoter Activity in IOSE-29, OVCAR-3, and SKOV-3 Cell Lines

IOSE-29, OVCAR-3, and SKOV-3 cell lines were transiently transfected with a -178 to +92 fragment of the E-cadherin promoter fused to the firefly luciferase reporter gene. Transfection efficiencies were normalized by assessing the activity of a second reporter, *Renilla* luciferase, which is under control of the constituively active CMV promoter. The OVCAR-3 cell line exhibited three fold higher E- cadherin promoter activity compared to that observed in SKOV-3 and IOSE-29 cell lines. MCF-7 breast carcinoma cells and NIH-3T3 fibroblasts were used as positive and negative controls respectively. The data are representative of three independent transfections performed in triplicate for each sample.

E-cadherin Promoter Activity



Comparison of Cytoskeletal Associated β-catenin in IOSE-29, OVCAR-3, and SKOV-3 Cell Lines

Because ß-catenin becomes physically linked to the f-actin when it is associated with E-cadherin in adherens junctions (Nagafuchi 2001), we next asked if the differences in E-cadherin levels and location amongst the three lines was associated with similar differences in the fraction of ß-catenin associated with the cytoskeleton.

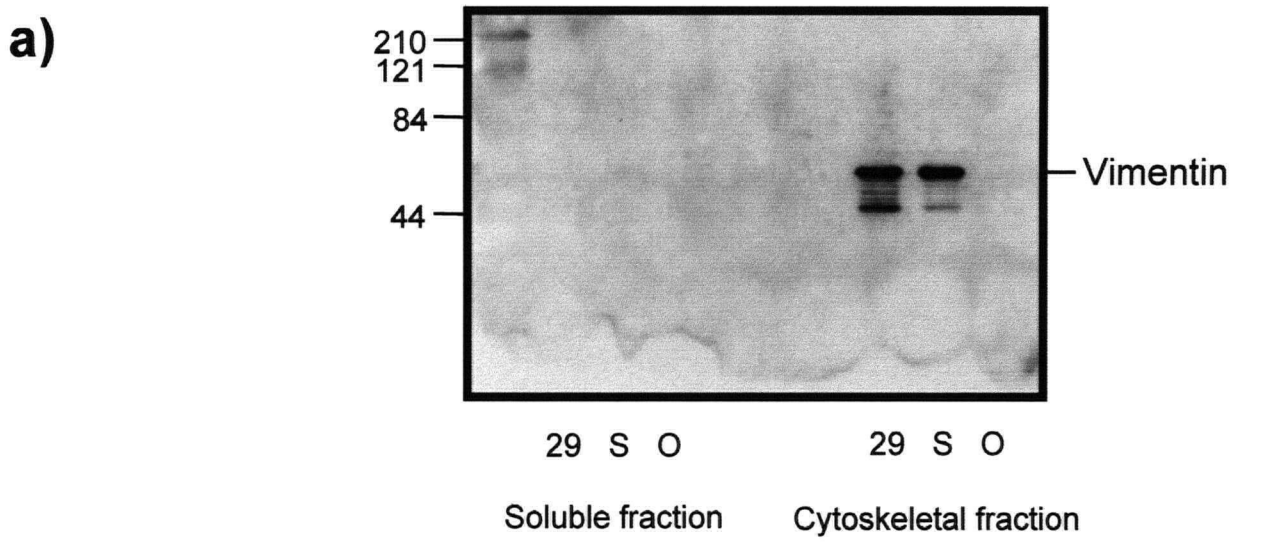
Cytoskeletal fractionation was achieved by lysing the cells in 0.5% Triton (Hinck et al., 1994). The insoluble fraction of this lysate contained the cytoskeleton. To confirm efficient separation, the two fractions were probed for the mesenchymal intermediate filament protein vimentin (Fig 4a). Clearly, all of the vimentin was found in the cytoskeletal fraction of IOSE-29 and SKOV-3 lysates. No vimentin was found in either OVCAR-3 fraction, which was expected given that these epithelial cells do not express vimentin (data not shown).

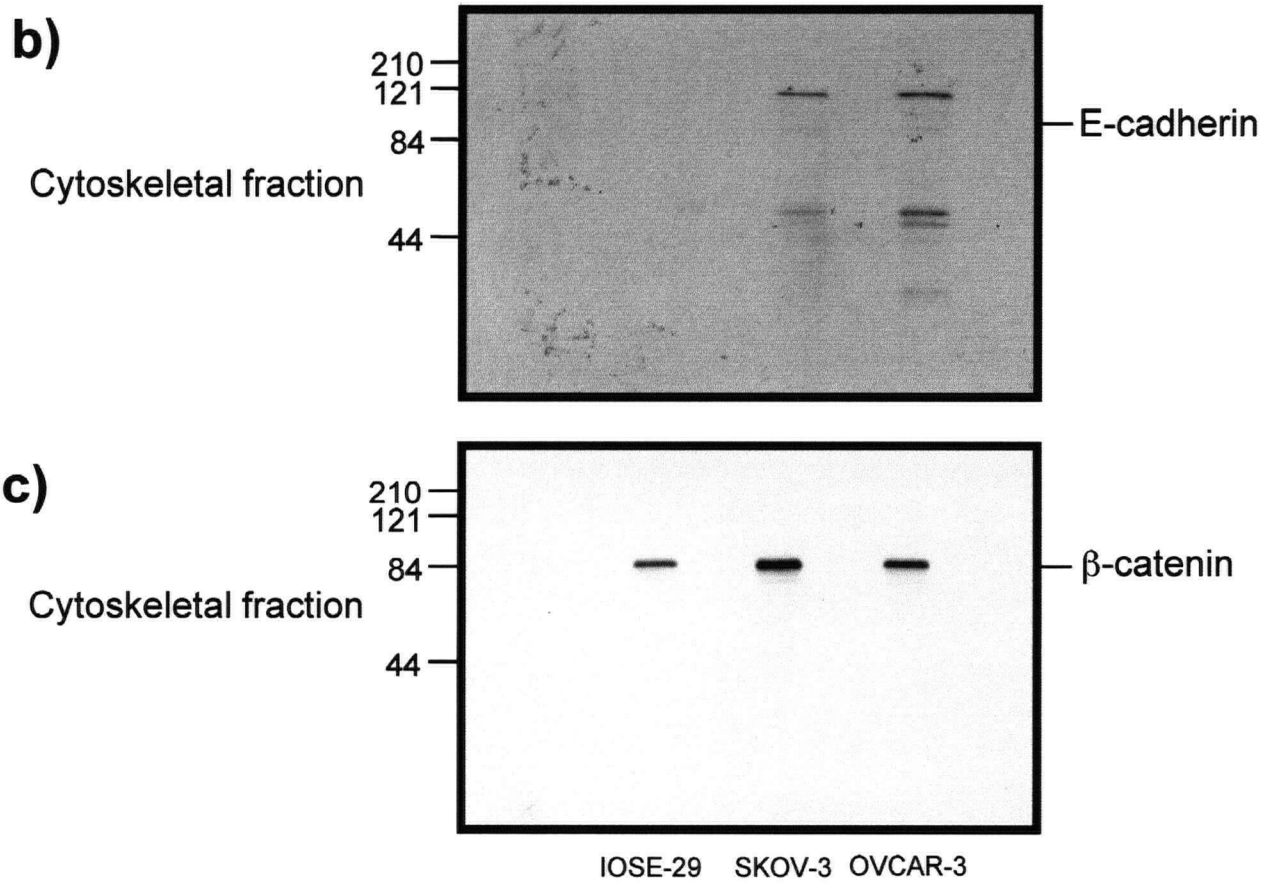
Immunofluorescence data suggested that OVCAR-3 well differentiated carcinoma cells form adherens junctions while IOSE-29 cells do not (see Fig 1 above). This was confirmed by the cytoskeletal fractionation data. OVCAR-3 clearly had considerable cytoskeletal/junctional E-cadherin while IOSE-29 cells had none (Fig 4b). In addition, OVCAR-3 had significantly more cytoskeletal/junctional ß-catenin than did IOSE-29 cells (Fig 4c).

Immunofluorescence data suggested that SKOV-3 poorly differentiated carcinoma cells formed incomplete adherens junctions. This tentative conclusion was partially confirmed by the cytoskeletal fractionation experiments. SKOV-3 cells had less cytoskeletal E-cadherin than did OVCAR-3 cells. However, SKOV-3 cells had considerably more cytoskeletal β-catenin than did OVCAR-3 cells. The latter result was somewhat surprising and suggests that E-cadherin-independent complexing of β-catenin to the cytoskeleton must be occuring in SKOV-3 cells. This could be achieved by N-cadherin which is expressed in ovarian carcinomas, but unlike E-cadherin may promote mesenchymal migratrion rather than epithelial cohesiveness (Kim et al., 2000, Wong et al.,1999).

Figure 4. Cytoskeletal Associated E-cadherin and β-catenin in IOSE-29, OVCAR-3, and SKOV-3 Cell Lines

Cytoskeletal fractionation was achieved by lysing the IOSE-29, OVCAR-3, and SKOV-3 cell lines with 0.5% Triton-X 100. Both the Triton-X 100 insoluble and soluble portions of the lysates were resolved by SDS-PAGE and analyzed by Western blotting using anti-vimentin. a) The majority of vimentin was located in the insoluble portion of the cell lysates thus confirming cytoskeletal fractionation of the cell lysates was achieved. b) The IOSE-29 cells completely lack E-cadherin. OVCAR-3 cells have more cytoskeletal associated E-cadherin compared to the SKOV-3 cells. c) OVCAR-3 cells have an increased amount of cytoskeletal associated β-catenin when compared to that observed in the IOSE-29 cells. The SKOV-3 cells have more cytoskeletal associated β-catenin than both the IOSE-29 and OVCAR-3 cells. Data shown is representative of independent separate experiments.


a)
210
121
84
44
Vimentin
29 S O
29 S O
Soluble fraction
Cytoskeletal fraction


b)
210
121
84
Cytoskeletal fraction
44
E-cadherin
c)
210
121
84
Cytoskeletal fraction
44
β-catenin
IOSE-29 SKOV-3 OVCAR-3

"Free" Cytosolic β-catenin In the IOSE-29, OVCAR-3, and SKOV-3 Cell Lines

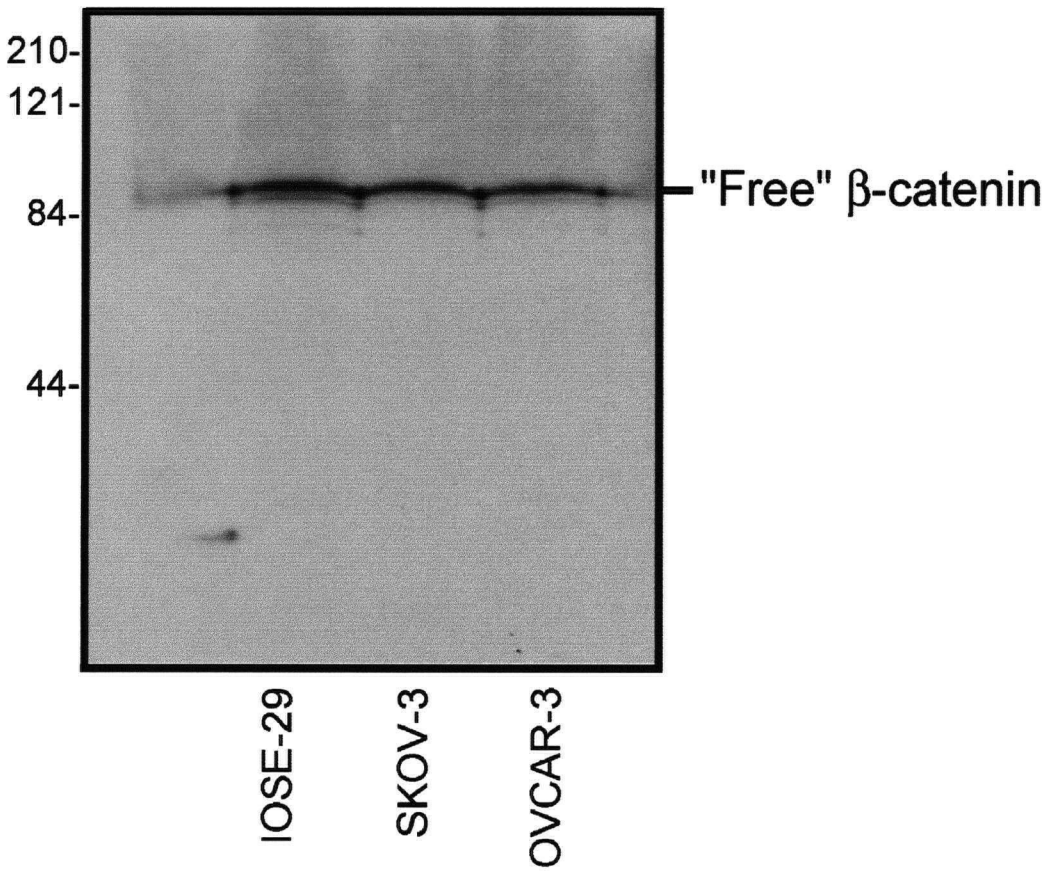
Examination of whole cell lysates by Western Blotting demonstrated that there were no significant differences in total steady state levels of β-catenin between the IOSE-29, OVCAR-3, and SKOV-3 cell lines. However, in agreement with the immunofluoresent staining, there were differences in cytoskeletal associated β-catenin amongst the cell lines. Therefore, we wanted to determine if differences in cytoskeletal associated β-catenin would result concurrent changes in the amount of "free" cytosolic β-catenin. Published data using other cell lines has shown that an increase in the amount of cytoskeletal, junctional β-catenin results in a concurrent decrease in the amount of "free" cytosolic β-catenin which is capable of signaling to the nucleus (Fagotto et al., 1996, Miller and Moon 1997).

Direct assessment of "free" cytosolic β-catenin was carried out by using a fusion protein consisting of the E-cadherin cytoplasmic domain, which contains intact β-catenin binding sites, fused to glutathione S-transferarse (GST) (Aberle et al., 1994). Cell extracts were incubated with the GST-E-cadherin fusion protein and GST protein complexes were precipitated with glutathione-sepharose beads and were then subjected to Western blot analysis. The IOSE-29 cells, which had less cytoskeletal associated β-catenin compared to the OVCAR-3 cells, also had more "free" cytosolic β-catenin when compared to the OVCAR-3 cells. The SKOV-3 cells also had more "free" cytosolic β-catenin than the

OVCAR-3 cells (Fig 5). These data suggest that in the absence of E-cadherin-mediated adherens junctions, there is more β-catenin available in the IOSE-29 and SKOV-3 cells to translocate into the nucleus and transactivate target genes compared to the situation in the OVCAR-3 cells where functional adherens junctions are present.

Figure 5. "Free" Cytosolic β-catenin in IOSE-29 ,OVCAR-3 and SKOV-3 Cells

IOSE-29, OVCAR-3 and SKOV-3 whole cell lysates (1000ug of protein) were incubated with an E-cadherin-GST fusion protein. Protein complexes were precipitated with glutathione-sepharose beads and levels of "free" cytosolic β-catenin were resolved by SDS-PAGE analysis and Western Blotting with an anti-β-catenin antibody. OVCAR-3 cells had a decreased amount of "free" cytosolic β-catenin when compared to that observed in both the IOSE-29 and SKOV-3 cells. Data shown is representative of two independent experiments


210-
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"Free" β-catenin
IOSE-29
SKOV-3
OVCAR-3

LEF-1 Promoter Activity In IOSE-29, OVCAR-3, and SKOV-3 Cell Lines

Free cytoplasmic β-catenin can translocate into the nucleus where it can bind to the LEF-1 family of transcription factors and activate the transcription of specific target genes. Thus, we reasoned that differences in the localization and free cytoplasmic pools of β-catenin between the ISOE-29, OVCAR-3, and SKOV-3 cell lines may result in different levels of LEF-1 signaling. To test this tentative hypothesis the three cell lines were trasiently transfected with a construct containing a promoter driven by four concantomerized TCF/LEF-1 binding sites fused to firefly luciferase reporter gene (TOPFLASH) (van de Wetering et al., 1997). A second construct containing either a nonspecific CMV or HSV promoter fused to *Renilla* luciferase was used to control for transfection efficiencies.

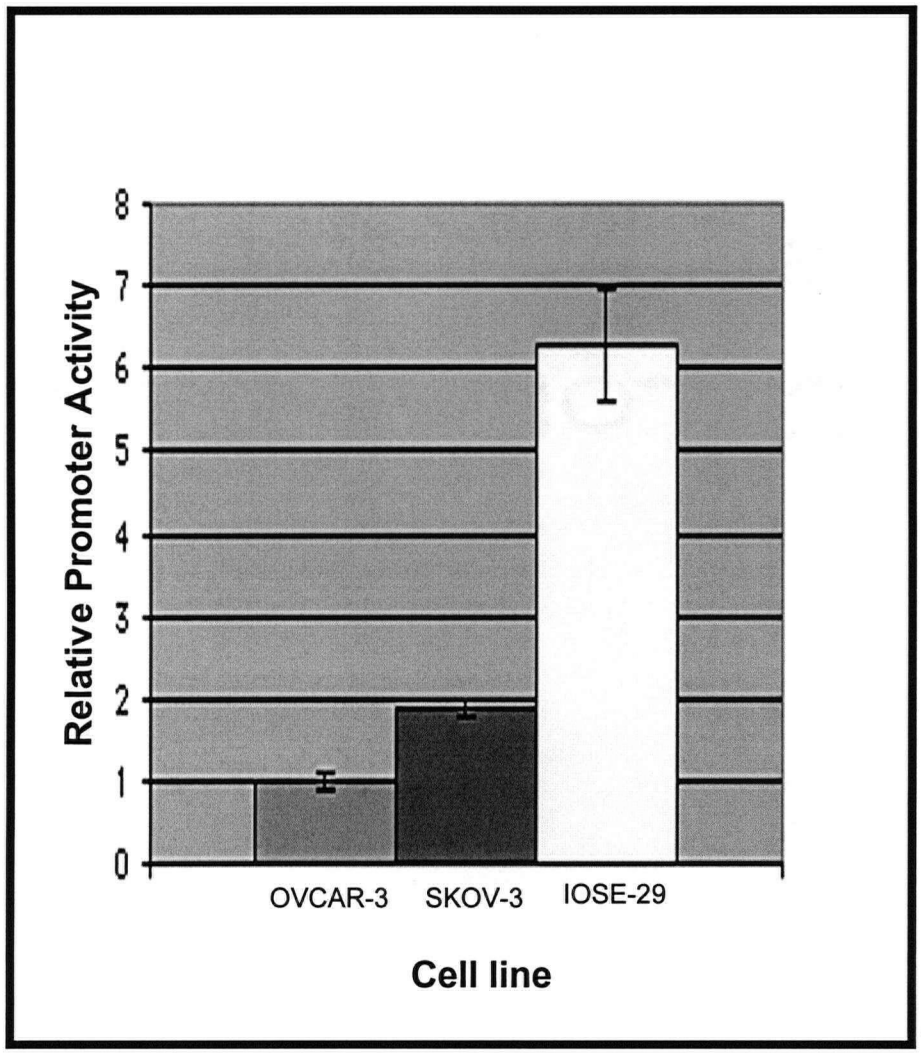
The IOSE-29 cells, which do not form adherens junctions and have more "free" cytoplasmic β-catenin compared to the OVCAR-3 cells showed a three-fold higher LEF-1 reporter activity when compared to the OVCAR-3 cells (Fig 6). The SKOV-3 cells also showed an increase in the amount of LEF-1 reporter activity when compared to that observed in the OVCAR-3 cells (Fig 6). As one of the target genes for LEF-1 activation is the mesenchyme-inducing siamosis gene (Nelson and Gumbiner 1998), this suggests that the absence of adherens junctions and associated high levels of free β-catenin may contribute to the epithelial to mesenchymal transformation that occurs when OSE cells

are maintained in monolayer culture. As a corollary, the presence of E-cadherin-mediated adherens junctions and the associated decrease in free β-catenin observed in OVCAR-3 cells suggests that the resulting decrease in LEF-1 activity may contribute to the mesenchymal to epithelial transformation that is a prominent feature of well differentiated ovarian carcinoma cells.

Figure 6. LEF-1 Promoter Activity in IOSE-29, OVCAR-3 and SKOV-3 Cell Lines.

IOSE-29, OVCAR-3, and SKOV-3 cell lines were transiently transfected with a construct containing a promoter driven by four concantomerized TCF/LEF-1 binding sites fused to the firefly luciferase reporter gene (TOPFLASH). Transfection efficiencies were normalized by assessing the activity of a second reporter *Renilla* luciferease, which is under the control of the constituatively active HSV reporter or CMV promoter. Numbers are given as a relative activation compared to cells transfected with a FOPFLASH-firefly luciferase reporter which contains mutated TCF/LEF-1 binding sites. Luciferase activity in the OVCAR-3 cells were arbitrarily defined as 1. OVCAR-3 cells exhibited decreased LEF-1 promoter activity when compared to the both the IOSE-29 cells and SKOV-3 cells. The data are representative of four independent transfections performed in triplicate for each sample.

LEF-1 Promoter Activity

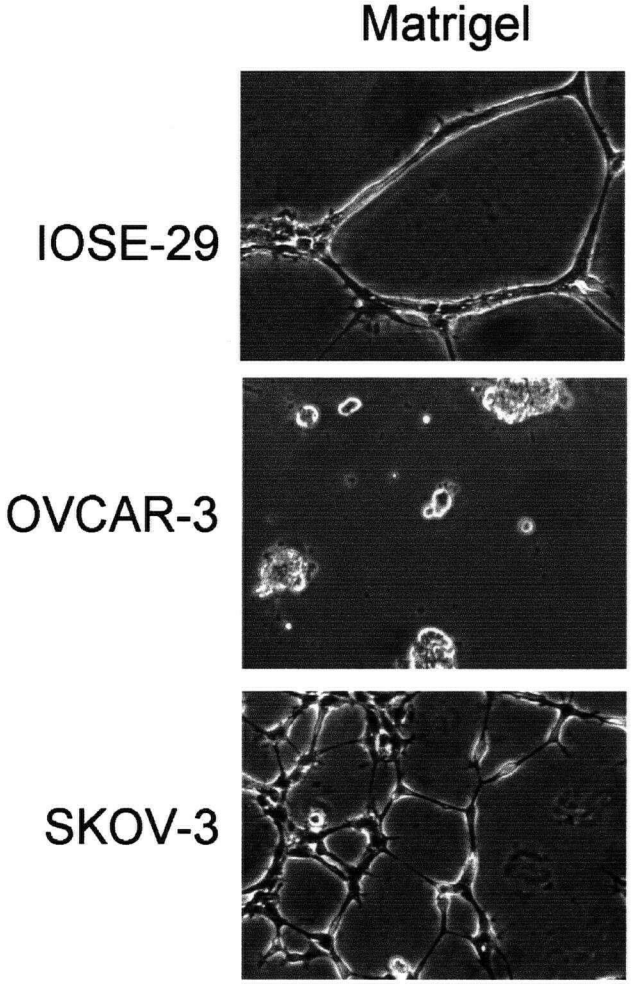


IOSE-29, OVCAR-3 and SKOV-3 Cells Grown on Three Dimensional Basement Membrane Gels

Matrigel mimics the environment of the basement membrane. When grown on matrigel, the IOSE-29 and SKOV-3 cells made long spindle like projections that migrated on the gel. This is a characteristic of wandering mesenchyme like cells. In contrast, OVCAR-3 cells did not migrate through the gel; rather, they sat on top of the gel and aggregated to form spheroids (Fig. 7).

Figure 7. IOSE-29, OVCAR-3 and SKOV-3 Cells Grown on Three Dimensional Basement Membrane Gels

Cells were maintained on Matrigel for 24 hours. OVCAR-3 cells sat on top of the gel and formed spheriods. In contrast both IOSE-29 and SKOV-3 cells migrated on the gel. Data shown is representative of 2 independent experiments.


Matrigel
IOSE-29
OVCAR-3
SKOV-3

3.2 Effects of Modulation of E-cadherin Expression in IOSE-80pcEC Clones

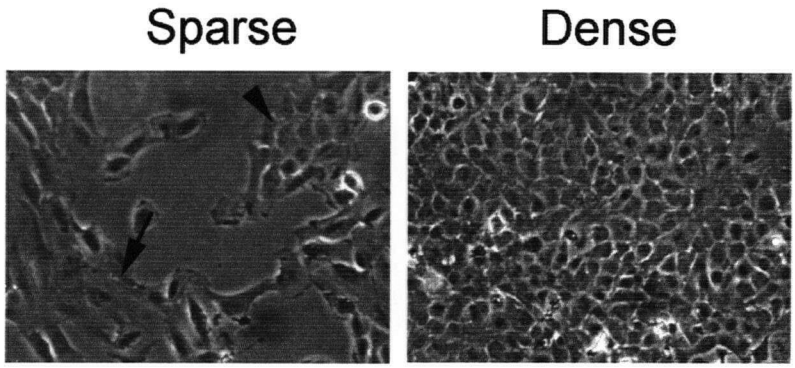
Characterization of the IOSE-80pc Cell Line

The culture life span of the OSE-80 cells was achieved through transfection with simian virus-40 derived large T antigen to produce the IOSE-80 strain (Auersperg). A small proportion of the strain then went through crisis generating the immortal IOSE-80 post crisis cell line (IOSE-80pc A. Godwin unpublished data). The IOSE-80pc cell line consists of a morphologically heterogeneous population of cells. When subconfluent, the majority of the cells exhibit the typical bipolar fibroblastic appearance of other cultured normal ovarian surface epithelial cells that have undergone EMT; however, some cells within this population do display an epithelial cobblestone appearance especially at high density (Fig. 8a). While some cells within the IOSE-80pc population do appear to be phenotypically epithelial, the IOSE-80pc cells, like OSE cells *in vivo*, do not express E-cadherin as shown by both immunofluoresence staining and Western blot analysis of whole cell lysates (Fig. 10 and Fig. 11a). To demonstrate the IOSE-80pc line was truly derived from the initial large T antigen transfected population, immunofluorescence staining for large T was carried out (Fig. 8b). All nuclei identified by DNA-binding DAPI staining were also large T positive.

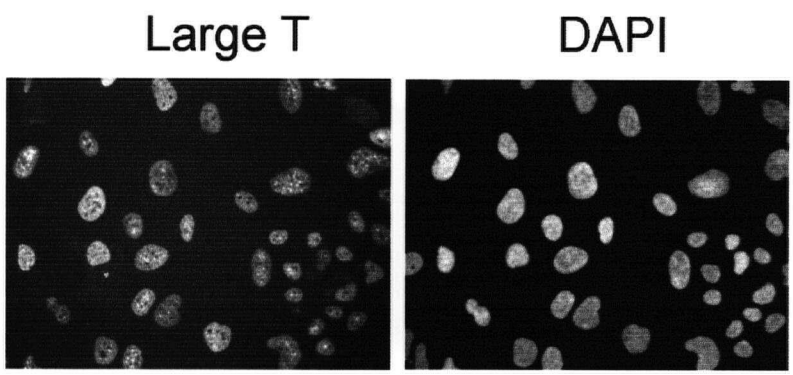
Figure 8. Morphology of the IOSE-80pc Cell Line

IOSE-80pc cells were maintained monolayer culture. a) Morphology was assessed by live phase microscopy. The majority of IOSE-80pc cells exhibited bipolar fibroblastic phenotype (arrow); however, some cells within the population do grow in a typical cobblestone pattern characteristic of epithelial cells when they are maintained at high density. 8b) Immunofluorescence double-staining for large T antigen and DAPI demonstrated that all cells expressed large T antigen and that the protein was localized to the nucleus (phase X 125; immunofluorescence X 250).

a)



b)



Morphology of the IOSE-80pcEC Clones

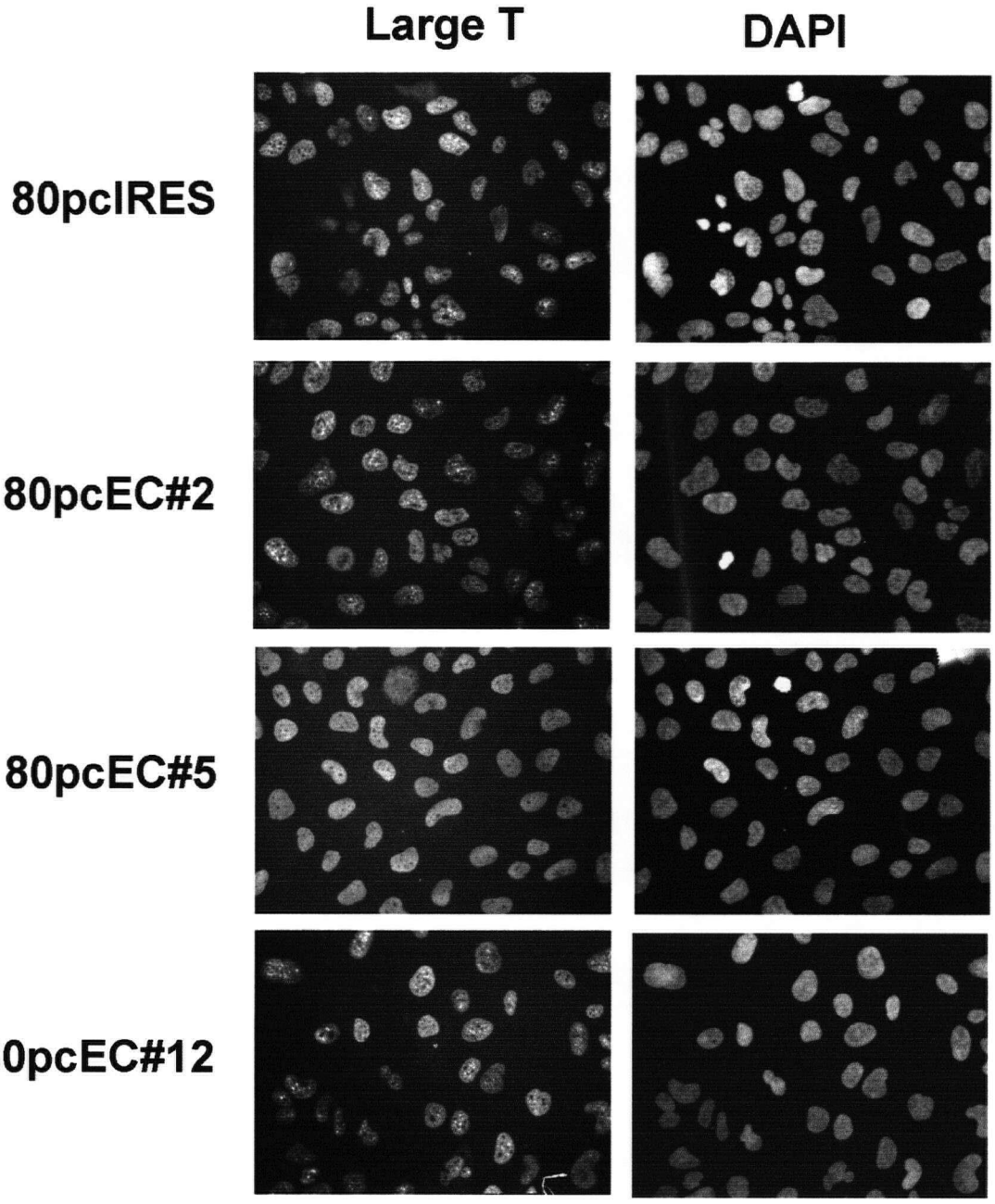
Normal OSE cells do not contain E-cadherin and they undergo a migratory EMT in monolayer culture. In contrast, early stage well-differentiated ovarian carcinoma cells express E-cadherin and they exhibit a stationary, committed epithelial phenotype. In late stage metastatic ovarian carcinomas E-cadherin is once again lost and the cells become migratory and mesenchymal (Davies et al., 1998). One factor that may account for these differences in phenotype are E-cadherin-mediated adherens junctions. I have shown above that adherens junctions are present in well differentiated OVCAR-3 cells and that these cells are stationary. I have also shown that adherens junctions are not present in IOSE-29 and poorly differentiated SKOV-3 ovarian carcinoma cells and I have shown that these cells are migratory. Therefore, to determine if adherens junctions are capable of inducing a switch between migratory and stationary phenotypes I characterized clones of IOSE-80pc cells in which E-cadherin was ectopically expressed by stable transfection (IOSE-80pcEC; S. Maines-Bandiera and N. Auersperg, unpublished data, see Materials and Methods for details).

In monolayer culture the IOSE-80pc line (Fig 8), the mock-transfected IOSE-80pcIRES line and the three E-cadherin expressing clones (Fig 9b) all had similar morphologies, particularly in dense culture. When the cultures were sparse the E-cadherin expressing clones appeared to be slightly more cobblestoned and epithelial.

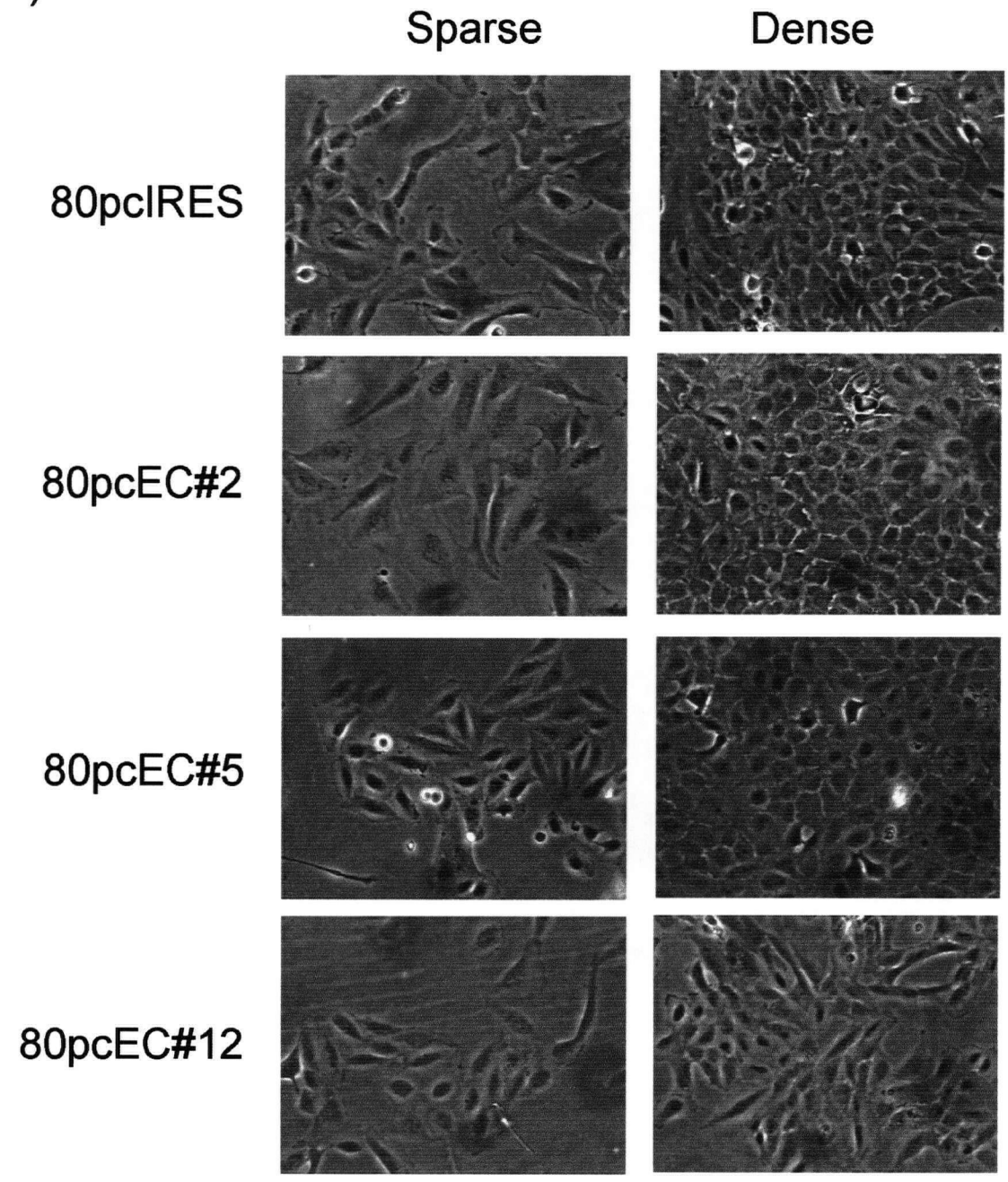
Figure 9. IOSE-80pcEC Clones in Monolayer Culture

a) Mock transfected IOSE-80pcIRES cells and the three IOSE-80pcEC clones maintained in subconfluent monolayer culture were dual-stained for DNA by DAPI staining and large T antigen by immunofluorescence. Note that all visible nuclei were positive for large T antigen (X250). b) IOSE-80IRES controls and IOSE-80pcEC clones were maintained as sparse or dense monolayer cultures and examined by live phase microscopy (X125).

a)



a)



Localization of Adherens Junction Proteins in the IOSE-80pc Cells and the IOSE 80pcEC Clones

I next wanted to assess the adherens junction status in the IOSE-80pcEC clones. To determine E-cadherin localization, immunofluorescence staining for the protein was performed (Fig. 10). Immunofluorescence staining of parental IOSE-80pc cells and mock transfected, IOSE-80pcIRES, cells indicated that both cell lines completely lacked E-cadherin. Although all three clones expressed E-cadherin, the localization of the protein differed between the three. In the IOSE-80pcEC#2 clone, E-cadherin was localized to sites of cell-cell interaction and was present in a continuous uniform pattern at these sites. While E-cadherin was also present at sites of cellular interaction in the IOSE-80pc#5 clone, localization of the protein was not uniform. In the IOSE-80pcEC#12 clone E-cadherin appeared to stain heterogeneously throughout the cytoplasm. This suggests that E-cadherin is present in all three IOSE-80pcEC clones; however, based on the localization of the protein, functional adherens junctions may not be present in all three clones.

We next stained for the adherens junction linker protein β-catenin (Fig. 10). Because β-catenin binds to the cytoplasmic tail of E-cadherin we expected that differences in the localization of E-cadherin would also result in differences in β-catenin localization. In both parental and mock transfected cells some β-catenin was present at

sites of cell-cell interaction; however, β-catenin located at these sites was organized in a discontinuous loose zipper-like pattern and a number of cells had diffuse cytoplasmic staining. In both IOSE-80pcEC#2 and IOSE-80pcEC #5 clones, β-catenin was also found at sites of cell-cell contact; however, unlike the parental and vector control cell lines, β-catenin stained in a more continuous uniform line. In addition, in the IOSE-80pcEC #5 clone, there were occasional cells with bright β-catenin staining over the nucleus. In IOSE-80pcEC#12 clone β-catenin staining was similar to that of the parental cell lines, staining discontinuously at sites of cell-cell interaction.

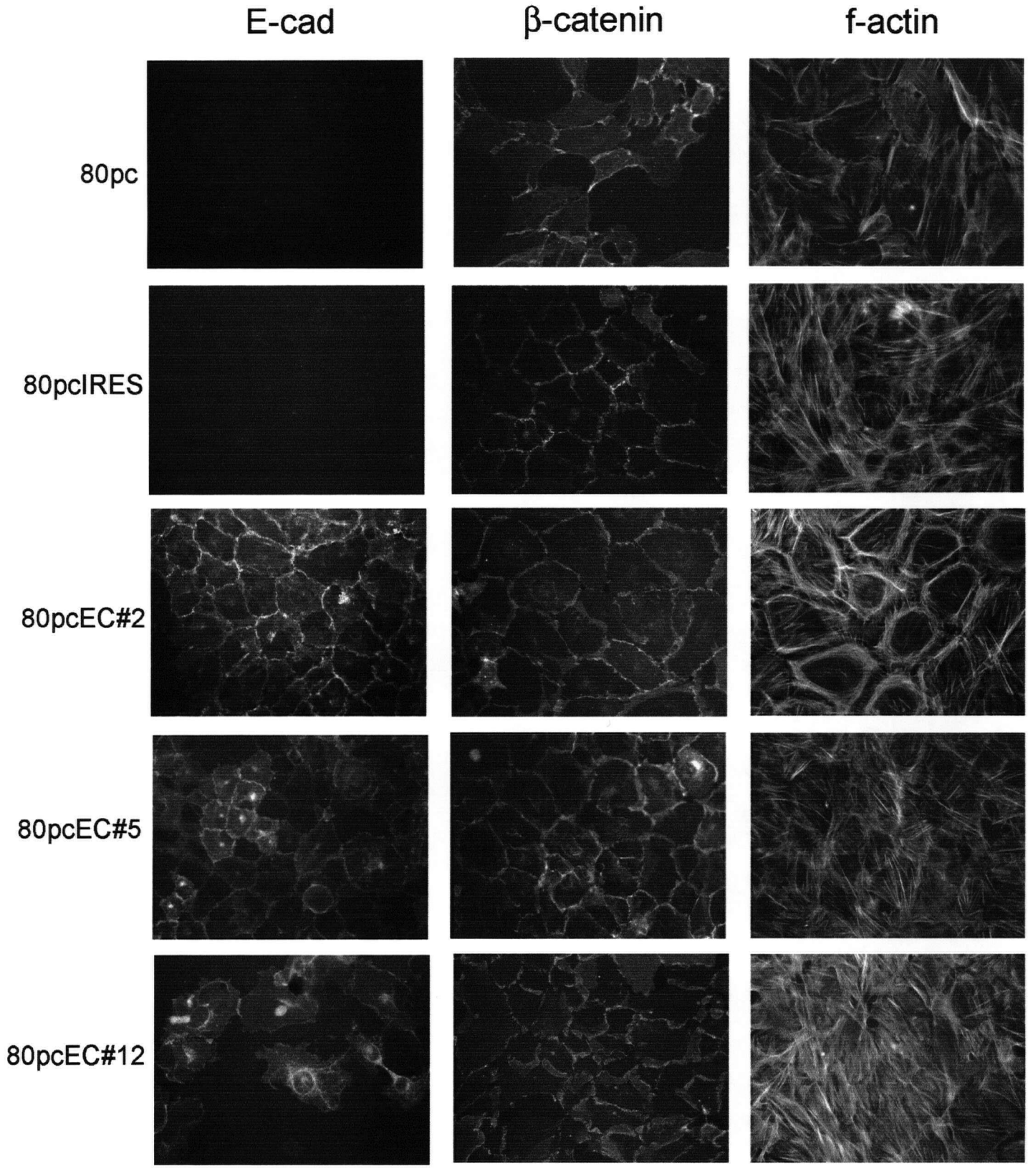
β-catenin becomes physically linked to f-actin when it is associated with E-cadherin in the adherens junction; therefore, our last criteria to determine if functional adherens junctions were present in the IOSE-80pcEC clones, and not in the IOSE-80pc parental cell lines, was to examine the localization of f-actin within the cells (Fig. 10). Perhaps the most striking differences between parental cell lines and the three clones was in the localization of f-actin. In the IOSE-80pc and IOSE-80pcIRES cells f-actin was present in cytoplasmic stress fibers which is a characteristic of migratory mesenchymal cells. In both clone #5 and clone #12 f-actin staining was similar to that of the parental cell lines. In contrast, in the IOSE-80pcEC#2 clone, considerable f-actin was cortical and associated with sites of cell contact. The presence of cortical actin, is an indication that

functional adherens junctions may have been formed in the IOSE-80pcEC #2 clone, but not in the parental and vector control cell lines or in IOSE-80pcEC clones #5 and #12.

In both the IOSE-80pcEC#2 and IOSE-80pcEC#5 clones E-cadherin was localized at sites of cell-cell interaction. Not surprisingly these clones also showed strong junctional β-catenin staining, however, only in the IOSE-80pcEC #2 clone was there the presence of junctional f-actin. Taken together, these data suggest that functional adherens junctions formed only in IOSE-80pcEC clone #2, which was the population of cells that expressed the highest levels of E-cadherin (Fig. 11).

Figure 10. Immunofluorescence Staining of Adherens Junctions Proteins in the IOSE-80pcEC Clones

Immunofluorescence staining for adherens junctions proteins E-cadherin, β-catenin, and f-actin was carried out on parental IOSE-80pc, mock-transfected IOSE-80pcIRES, and IOSE-80pcEC Clones #2, #5, and #12 (X250; different fields).


E-cad
β-catenin
f-actin
80pc
80pcIRES
80pcEC#2
80pcEC#5
80pcEC#12

Steady-State Levels of E-cadherin and β-catenin in the IOSE-80pc Cells and the IOSE-80pcEC Clones

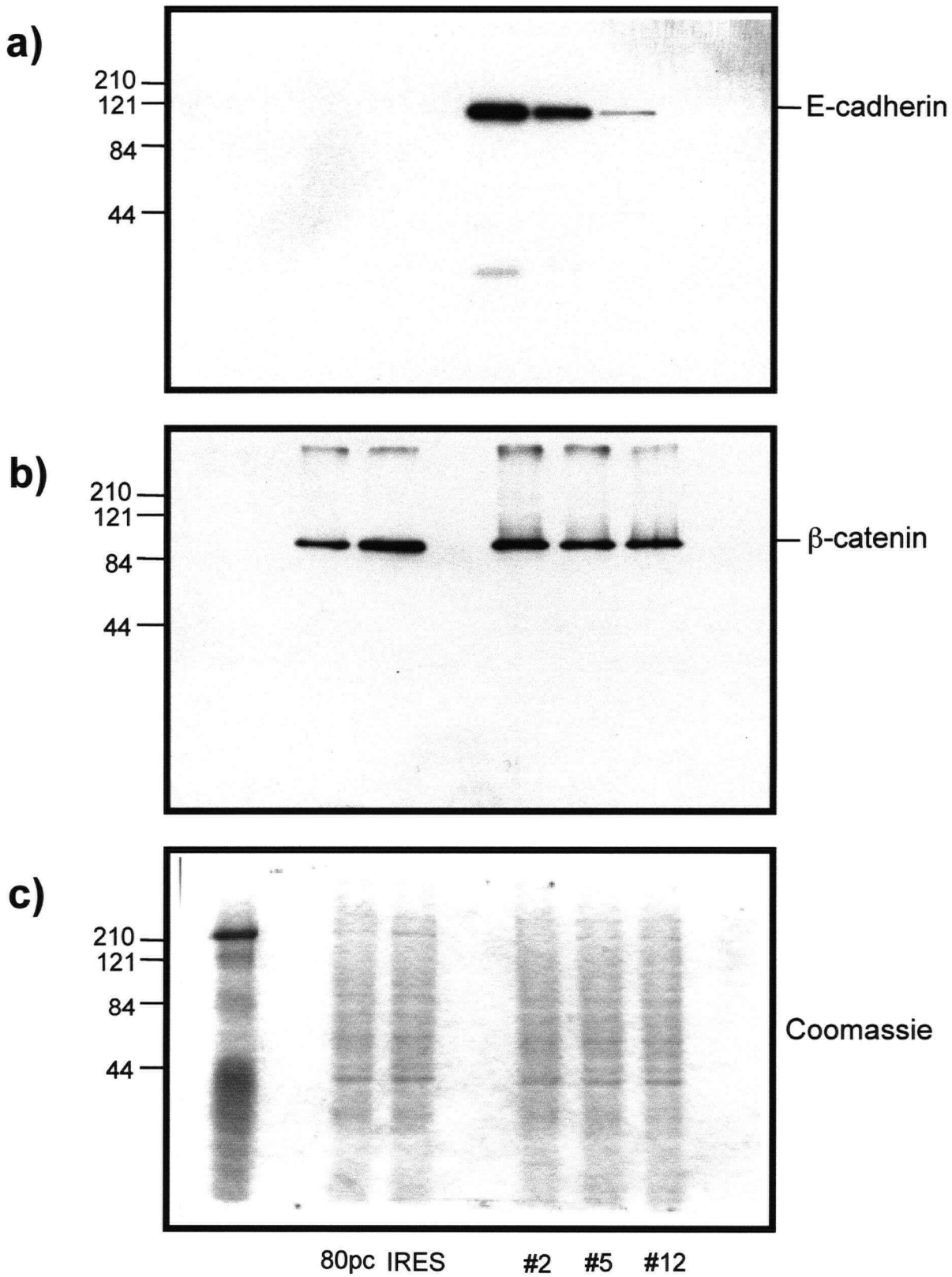
To confirm the presence of adherens junction proteins implied by immunofluorescence staining, Western blot analysis was performed on the whole cell lysates of the parental IOSE-80pc cells, mock transfected IOSE-80pcIRES cells, and the IOSE-80pcEC clones. As expected, based on the immunofluorescence staining, both parental and vector control cell lines completely lacked E-cadherin while all three of the IOSE-80pcEC clones did express the 120kDa E-cadherin protein (Fig. 11a). However, the amount of total E-cadherin did vary between the three clones. The IOSE-80pcEC#2 clone expressed the most E-cadherin; IOSE-80pcEC#5 clone expressed an intermediate amount of E-cadherin; and IOSE-80pcEC#12 clone expressed the least amount of E-cadherin.

Total cell lysates were also probed for β-catenin (Fig. 11b). No differences were observed in the amount of steady state levels of β-catenin between parental and vector control cell lines and that of the IOSE-80pcEC clones. However, this observation gave no indication as to whether or not the differences in E-cadherin levels altered the levels of junctional β-catenin as suggested by immunofluorescence microscopy. Therefore, I next examined the cytoskeletal-associated β-catenin in the three clones.

Figure 11. Western Blot Analysis of Whole Cell Lysates from the IOSE-80pc Cells and the IOSE-80pcEC Clones

Western blot analysis was performed on whole cell lysates from parental IOSE-80pc cells, mock-transfected IOSE-80pcIRES cells and the IOSE-80pcEC clones. a) 15μg of whole cell lysates was resolved by SDS-PAGE, transferred to PVDF membrane and probed for E-cadherin. Note that IOSE-80pcEC #2 clone had the highest E-cadherin levels. b) 2μg of whole cell lysates was resolved by SDS-PAGE, transferred to PVDF membrane, and probed for β-catenin. Note that steady-state levels were very similar in all three lines. c) 20μg of whole cell lysates where resolved by SDS-PAGE and stained for Coomassie Blue to demonstrate equal loading.

Data shown is representative of three independent experiments.


a)
210
121
84
44
E-cadherin
b)
210
121
84
44
β-catenin
c)
210
121
84
44
Coomassie
80pc IRES
#2
#5
#12

Comparison of Cytoskeletal Fractions of the IOSE-80pc Cells and the IOSE-80pcEC Clones

Immunofluorescence staining suggested there might be differences in the efficiency of adherens junction formation between the IOSE-80pcEC clones. When they are complexed in the adherens junction, both E-cadherin and β-catenin are complexed to the cytoskeleton. Therefore, I assessed the levels of cytoskeletal-associated E-cadherin and β-catenin in the three clones and compared them to parental and mock transfected cells.

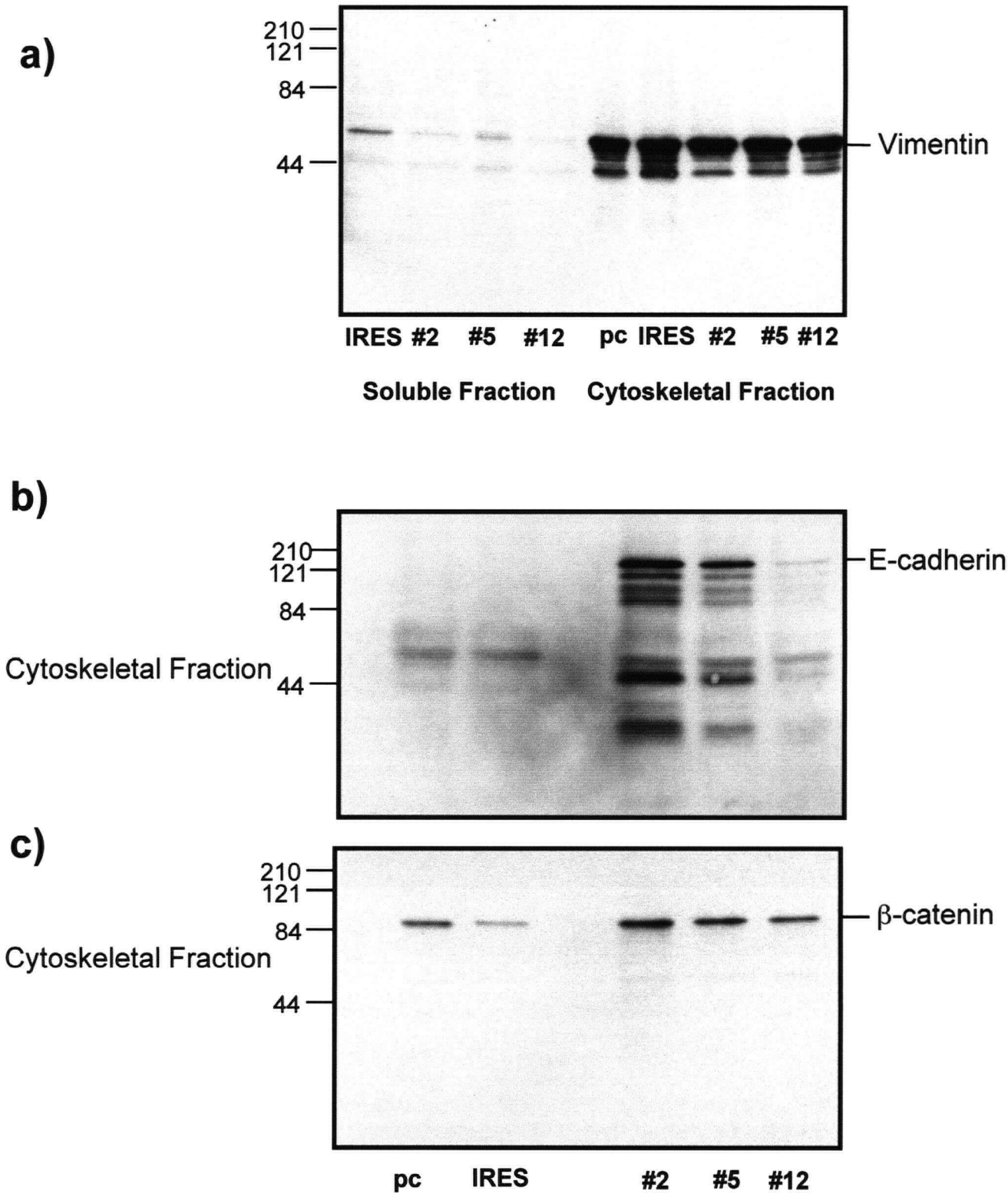
Cytoskeletal fractionation was achieved by lysing the cells in 0.5% Triton-X-100 of which the insoluble portion of the lysate contained the cytoskeleton. This was confirmed by probing the two fractions for the cytoskeletal intermediate filament protein vimentin (Fig. 12a). No E-cadherin was present in the cytoskeletal Triton-X100 insoluble fraction of the IOSE-80pc cells or IOSE-80pcIRES mock transfected cells. In contrast, E-cadherin was present in the cytoskeletal fraction of the IOSE-80pcEC clones. As expected, based on immunofluorescence staining and Western blot analysis of whole cell lysates, the greatest amount of cytoskeletal-associated E-cadherin was present in IOSE-80pcEC clone #2 an intermediate amount was present in IOSE-80pcEC #5 clone, and the lowest amount was present in IOSE-80pcEC clone#12.

As predicted, differences in E-cadherin localization seen in immunofluorescence staining and confirmed by Western blot analysis of the cytoskeletal fraction of the cell lines resulted in differences in the localization of β-catenin. The IOSE-80pc parental and vector control cell lines, which completely lacked E-cadherin expression, had low levels of cytoskeletal-associated β-catenin. In contrast, the IOSE-80pcEC#2 clone, which had the greatest amount of cytoskeletal associated E-cadherin, also showed the greatest amount of cytoskeletal-associated β-catenin. The IOSE-80pcEC clone #5, which intermediate amounts of cytoskeletal-associated E-cadherin also showed intermediate amounts of cytoskeletal associated β-catenin. Not suprisingly, the IOSE-80pcEC#12 clone, which had very little cytoskeletal-associated E-cadherin, also had low levels cytoskeletal-associated β-catenin which were comparable to the parental and control lines (Fig. 12b and Fig. 12c). These data fully support the immunofluorescence staining in which: i) no junctional E-cadherin and little junctional β-catenin was present in the IOSE-80pc parental and mock control cells lines, ii) the most junctional E-cadherin and β-catenin was present in the IOSE-80pcEC clone #2, iii) intermediate amounts of junctional E-cadherin and β-catenin was present in IOSE-80pcEC clone #5 and, iv) the least amount of junctional E-cadherin and β-catenin was present in IOSE-80pcEC clone #12.

Therefore, it appears that the presence of the adherens junctions in the E-cadherin clones can be summarized as follows: Clone #2 > Clone#5 > Clone#12. This suggests that a threshold of E-cadherin must be reached prior to adherens junction formation as the levels of expression of the cell adhesion molecule can be summarized in a similar manner.

Figure 12. Western Blot Analysis of Cytoskeletal Fractions in the IOSE-80pc Cells and the IOSE-80pcEC Clones

Cytoskeletal fractionation was achieved by lysing cell lines with 0.5% Triton-X 100. Soluble and insoluble cytoskeletal portions of the lysates were resolved by SDS-PAGE and analyzed by Western blotting using a) anti-vimentin, b) anti-E-cadherin and c) anti-β-catenin antibodies. Note that the great majority of vimentin was located in the insoluble cytoskeletal portion of cell lysates which indicates that fractionation was successful. Data shown is representative to two independent experiments.


a)
210
121
84
44
Vimentin
IRES #2 #5 #12 pc IRES #2 #5 #12
Soluble Fraction
Cytoskeletal Fraction
b)
210
121
84
Cytoskeletal Fraction
44
E-cadherin
c)
210
121
84
Cytoskeletal Fraction
44
β-catenin
pc IRES #2 #5 #12

LEF-1 Promoter Activity in the IOSE-80pc Cells and the IOSE-80pcEC Clones

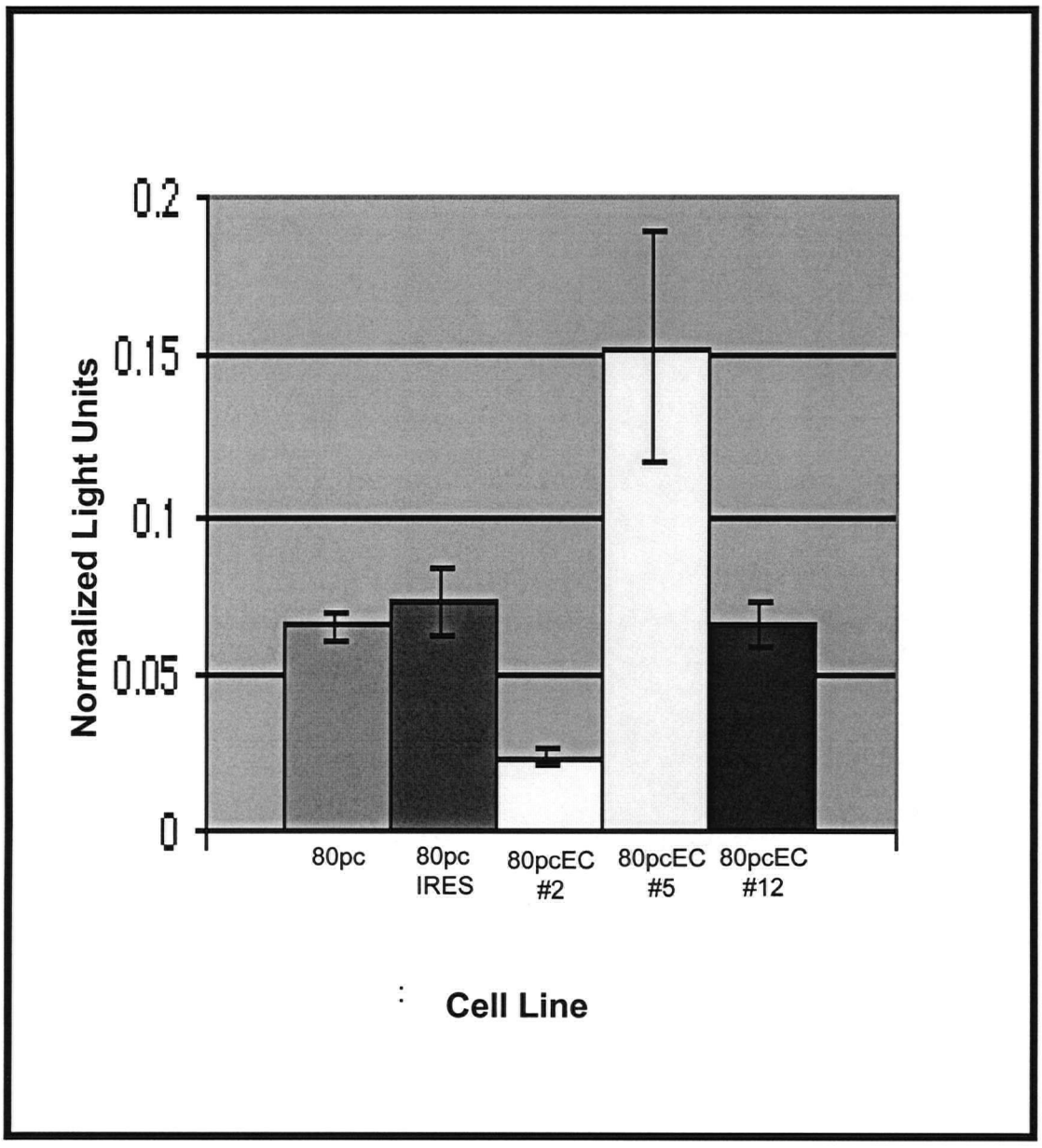
When it is not complexed to the cytoskeleton in the adherens junction, "free" β-catenin can also act as a signaling molecule by binding to the LEF-1 family of transcription factors and transactivating target genes in the nucleus. I have demonstrated that modulating E-cadherin expression in immortalized OSE cells also modulates cytoskeletal-associated β-catenin. Therefore, I next asked if the differences in cytoskeletal β-catenin localization would translate to differences in the transcriptional activity of β-catenin. To test this hypothesis, the cell lines were transiently transfected with a construct containing a promoter driven by four concantomerized LEF-1 binding sites fused to a firefly luciferase reporter gene (TOPFLASH). A second construct containing a nonspecific CMV promoter fused to *Renilla* luciferase was used to control for transfection efficiencies. IOSE-80pc and IOSE-80pcIRES control cell lines had comparable levels of LEF-1 promoter activity (Fig. 13). IOSE-80pcEC clone #2 which had the greatest amount of cytoskeletal-associated β-catenin was the only clone which showed a decrease in LEF-1 promoter activity when compared to parental and mock transfected cell lines. This result fit my prediction that adherens junction formation can decrease LEF-1 signaling, presumably by decreasing the pool of free cytoplasmic β-catenin. The fact that LEF-1 activity was unchanged in IOSE-80pcEC Clone #12 compared to controls also fit the prediction, as these cells do not form adherens junctions

with increased cytoskeletal β-catenin. The one clone that did not fit the prediction was IOSE-80EC clone #5. These cells had a moderate increase in cytoskeletal ß-catenin which did not translate into a moderate decrease in LEF-1 activity. Instead the reverse occurred and LEF-1 activity was increased in these cells.

Figure 13. LEF-1 Promoter Activity in the IOSE-80pc Cells and the IOSE-80pcEC Clones

Cells were transiently transfected with a construct containing a promoter driven by four concantomerized LEF-1 binding sites fused to the firefly luciferase reporter gene (TOPFLASH). Transfection efficiencies were normalized by assessing the activity of a second reporter, *Renilla* luciferase, which is under the control of the constituatively active CMV promoter. Numbers are given as a relative activation compared to cells transfected with FOPFLASH firefly luciferase reporter which contains mutated, non-responsive LEF-1 binding sites. The data are representative of three independent transfections performed in triplicate for each sample.

LEF-1 Promoter Activity



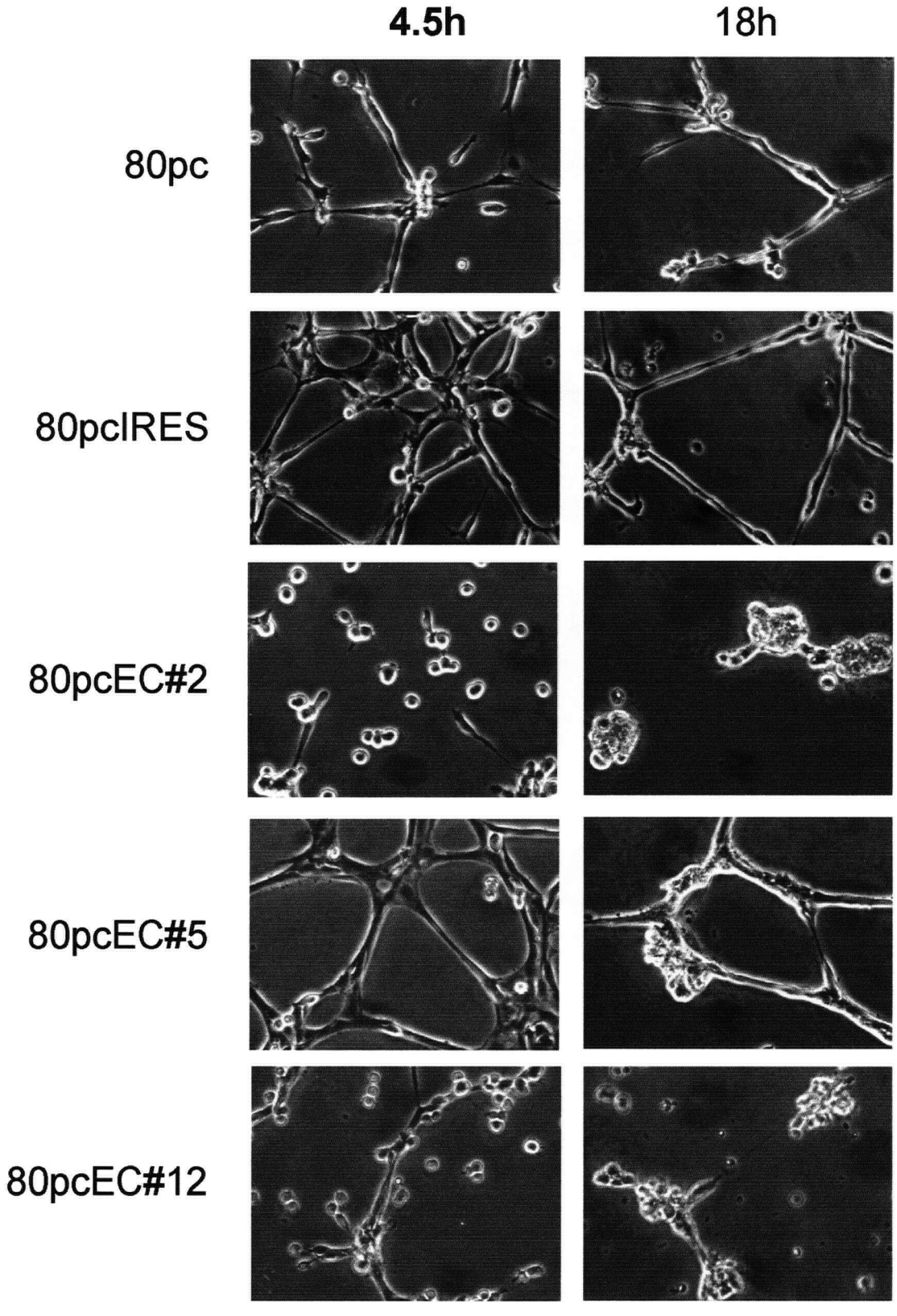
The IOSE-80pc Cells and the IOSE-80pcEC Clones Cultured on Three Dimensional Basement Membrane Gels

I have already demonstrated that mesenchymal IOSE-29 cells and late stage de-differentiated SKOV-3 ovarian carcinoma cells, which do not have adherens junctions, migrated on Matrigel. In contrast, well differentiated OVCAR-3 cells, which have adherens junctions, did not migrate on Matrigel. Therefore, to determine if E-cadherin-mediated adherens junctions were responsible for this different migratory phenotype, I cultured IOSE-80EC clones on Matrigel.

Both parental IOSE-80pc and mock transfected IOSE-80pcIRES controls migrated on Matrigel. In contrast, IOSE-80pcEC clone#2, which formed functional adherens junctions (most cytoskeletal-associated E-cadherin and β-catenin, low LEF-1 promoter activity) sat on top of the gel and over time the cells formed tight non migratory spheriods. IOSE-80pcEC clone #5 (intermediate cytoskeletal-associated E-cadherin and β-catenin, high LEF-1 promoter activity) made long spindle like projections on the gel and appeared to be the most migratory amongst the three E-cadherin clones. IOSE-80pcEC clone #12 (low cytoskeletal-associated E-cadherin and β-catenin, moderate LEF-1 promoter activity) did not form tight clusters, but rather the cells made short projections on the gel. These data indicate that ectopic adherens junction formation decreases OSE cell migratory activity, which is a hallmark of early stage ovarian carcinoma formation.

Figure 14. The IOSE-80pc Cells and the IOSE-80pcEC Clones Cultured on Three Dimensional Basement Membrane Gels

IOSE-80pc and IOSE-80pcEC clones were cultured on Matrigel. Both parental IOSE-80pc and mock transfected IOSE-80pcIRES made spindle like projections and migrated on the gel. In contrast, the IOSE-80pcEC clone #2 sat on top of the gel and clustered to form non migratory spheriods. IOSE-80pc clone #5 migrated on the gel forming long spindle like projections. IOSE-80pcEC clone #12 did not form tight spheroids and the cells made short projections migrating on the gel. Data shown is representative of two independent experiments.


4.5h
18h
80pc
80pcIRES
80pcEC#2
80pcEC#5
80pcEC#12

Discussion

The cells of the normal human OSE have a plastic, uncommitted phenotype and they do not express E-cadherin. Thus, in the resting OSE the cells are epithelial while in post-ovulatory wounds the cells become migratory and mesenchyme-like. In contrast, OSE cells lining inclusion clefts and inclusion cysts have a more restricted cohesive epithelial phenotype, loose their mesenchyme-like characteristics, and they express E-cadherin. It has been proposed that OSE cells within these inclusion clefts and cysts may be weakly pre-neoplastic because they display the more committed phenotype of differentiated Mullerian duct-derived epithelia that are a hallmark of ovarian carcinomas. In addition, differentiated ovarian carcinomas, which can form de novo in a small proportion of inclusion cysts, also have a restricted cohesive epithelial phenotype and they express E-cadherin (Salazar et al., 1996). Taken together, these observational data strongly suggest that E-cadherin may help initiate cohesive preneoplastic changes in the OSE. If this is indeed the case, it is not unreasonable to propose that such changes would be mediated by cohesive E-cadherin-dependent adherens junctions. Therefore, I first determined if an ectopic, forced expression of E-cadherin was capable of initiating adherens junction formation in cultured OSE cells that exhibit both epithelial and mesenchyme-like characteristics. I then determined if the ability of the cells to form adherens junctions was associated with a more restricted, cohesive epithelial phenotype.

Adherens Junction Formation in E-Cadherin Expressing OSE Cells

The IOSE-80pc cell line, which is E-cadherin negative, has gone through crisis in culture and it is truly immortal. It also exhibits the dual epithelial and mesenchymal characteristics of the uncommitted OSE phenotype. For example, IOSE-80PC cells express epithelial cytokeratin (Auersperg pers. communication) as well as mesenchymal vimentin (see below) intermediate filament proteins. They are also migratory in basement membrane gels, a characteristic which is not exhibited by cohesive, epithelial restricted ovarian carcinoma cells. ß-catenin is somewhat diffuse and the f-actin is arranged in cytoplasmic stress fibers, both of which indicated that significant cell-cell junctions do not form in the IOSE-80pc line. After stable transfection and selection with an E-cadherin expression vector the resulting polyclonal population of IOSE-80pcEC cells was extremely heterogeneous with respect to E-cadherin levels and the ability to form adherens junctions. Therefore, clones were isolated by limiting dilution. Three clones were chosen for further study based on the differences in E-cadherin expression in each.

Of the three clones chosen for further study the IOSE-80pcEC Clone #2 expressed the highest level of E-cadherin. In this clone E-cadherin and β-catenin were both localized at continuous lines of contact between cells. There was also a significantly higher proportion of ß-catenin complexed with the cytoskeleton in clone #2 compared to

that the of parental or mock-transfected control cells. Therefore, it appears that functional, cytoskeleton-complexed adherens junctions did form in clone #2.

The IOSE-80pcEC Clone #5 expressed an intermediate level of E-cadherin. While most of the E-cadherin was localized to sites of cell-cell contact in this clone, ß-catenin localization was discontinuous and serrated at these sites. In addition, there was some diffuse, cytoplasmic ß-catenin staining in this clone. While there was some increase in ß-catenin complexed with the cytoskeleton in Clone #5 it was not as dramatic as that seen in Clone #2. Therefore, it does appear that only partial adherens junction formation occurred in clone #5.

IOSE-80pcEC Clone #12 expressed the lowest level of E-cadherin. Both E-cadherin and ß-catenin staining were heterogenously distributed in these cells. In addition, there was little or no increase in the amount of ß-catenin complexed to the cytoskeleton. Therefore, it does not appear that functional adherens junctions formed in these cells.

Role of β-catenin/LEF-1 Signaling in EMT

ß-catenin-dependent LEF-1-mediated transcriptional activation, hereafter described as LEF-1 activity, is a downstream effector of the canonical Wnt signalling pathway. This pathway is essential for developmental events that require tissue rearrangements. For example, it regulates cellular movements during gastrulation, it is

required for body axis formation, and it is involved in the formation of paraxial mesoderm (Moon et al., 1998, Yamaguchi et al., 1997). In cell culture, inappropriate inductions of an epithelial to mesenchymal transformation (EMT), all of which are associated with a downregulation of E-cadherin expression, result in an upregulation of LEF-1 activity, presumably because there is an increase in non-junctional, non-proteosome-targetted β-catenin available for translocation to the nucleus (Eger et al., 2000, Gradl et al., 1999, Novak et al., 1998). Recent evidence also suggests that LEF-1 activity itself contributes directly to EMT. Specifically, forced overexpression of LEF-1 causes a translocation of β-catenin into the nucleus, increased LEF-1 activity and a dramatic induction of EMT in epithelial tumor cells (Kim et al 2002). Thus, it is not unreasonable to suggest that a decrease in LEF-1/ß-catenin signaling could contribute to the opposite phenomenom, namely the mesenchymal to epithelial transformation that is a hallmark of the early stages of ovarian carcinoma formation.

Initially, I compared the LEF-1 activities in mesenchymal, IOSE-29 cells and in two ovarian carcinonoma cell lines. One of the carcinoma lines, OVCAR-3, is well differentiated and epithelial. Thus, we consider OVCAR-3 cells to be at an early to intermediate stage of ovarian carcinoma progression. The other carcinoma line, SKOV-3, is poorly differentiated and mesenchymal. Thus, we consider SKOV-3 cells to be at a late stage of ovarian carcinoma progression. The mesenchymal IOSE-29 and mesenchymal SKOV-3 lines had the highest LEF-1 activity. These cells also had no (IOSE-29) or low

E-cadherin (SKOV-3), did not form adherens junctions, and they had high levels of free β-catenin. In contrast, the low LEF-1 activity OVCAR-3 cell line expressed considerable E-cadherin, formed adherens junctions and had low levels of free β-catenin. While correlative, these findings agree with the notion that the degree of LEF-1 activity regulates the degree phenotypic switching between mesenchymal and epithelial phenotypes in ovarian carcinoma. These data also led me to hypothesize that E-cadherin-mediated adherens junction formation itself regulates LEF-1 signaling in OSE cells by altering the pool of free ß-catenin.

To directly test whether or not E-cadherin-mediated adherens junction formation regulates LEF-1 activity in OSE cells I examined the IOSE-80pcEC clones. Clone #2, which had the highest E-cadherin expression and formed functional adherens junctions had the lowest LEF-1 activity. The concept that E-cadherin can depress LEF-1 activity by sequestering free ß-catenin has been proposed previously (Gottardi et al., 2001, Moon, Fagotto et al., 1996). However, I would like to go one step further and suggest that this sequestration is most stable when it is linked to the cytoskeleton in the adherens junction complex. For example, while clone #5 expressed moderate levels of E-cadherin it did not form adherens junctions and β-catenin was not linked to the cytoskeleton, perhaps because of low stability E-cadherin/β-catenin interactions that prevented proteosomal degradation. As a result there was an increase in LEF-1 activity in clone #5 compared to

the control lines. The latter result lead me to consider an intriguing possibilty: are there situations where increased E-cadherin expression (ie. an initiating event in OSE carcinogenesis) can lead to an increase in LEF-1 activity? If this is indeed the case the ectopic E-cadherin expression that occurs early in OSE cell carcinogenesis could be directly oncogenic given that LEF-1 activity can act to induce the expression of proliferative genes such as myc and cyclinD1 (Shtutman et al., 1999, He et al., 1998)

Given the fact that the differences in LEF-1 activity correlated with changes in cytoskeletal-associated ß-catenin I examined the distribution of f-actin which is the cytoskeletal compartment of adherens junctions. f-actin predominantly formed cytoplasmic stress fibers which is a characteristic of mesenchymal cells in the parental IOSE-80pc cells and in clone 12 not expressed E-cadherin at low levels. Much of the f-actin was re-localized to regions of cell-cell contact in the adherens junction forming clone #2 which expressed high levels of E-cadherin. This did not occur in the mid-level E-cadherin expressing clone #5. Therefore, in situations where the levels of ectopic E-cadherin expression are not sufficient to fully recruit f-actin to the adherens junction there may be to an increase in β-catenin that is sequestered from proteosomal degradation but free to enter the LEF-1 signaling pathway.

Increased LEF-1 activity is correlated with mesenchyme-like properties. Given the fact that mesenchymal cells have the ability to migrate through extracellular matrices,

I cultured the IOSE-80pcEC clones on three dimensional basement membrane gels to assess changes in these abilities. Clone #2 which had the lowest LEF-1 promoter activity and the highest amounts of cytoskeletal associated E-cadherin and β-catenin, did not migrate on the gel but rather sat on top of the gels and the cells formed epithelial spheriods over time. In contrast, although clone #5 had moderate amounts of cytoskeletal associated E-cadherin, this clone was the most migratory when cultured on three dimensional basement membrane gels. Clone #12 which had the least amount of cytoskeletal associated E-cadherin and β-catenin, but lower LEF-1 activity, appeared to be less migratory than clone #5. Therefore, high LEF-1 activity may result in the mesenchymal property to migrate through extracellular matrices as seen in IOSE-80pcEC clone #5. Conversely, successful recruitment of β-catenin to the cytoskeleton may result in a true mesenchymal to epithelial transition by decreasing the amount of β-catenin available to translocate into the nucleus and activate LEF-1 activity as was seen in the IOSE-80pcEC clone #2.

Conclusions

Forced expression of E-cadherin in normal OSE cells resulted in the formation of functional adherens junctions only when E-cadherin, β-catenin, and f-actin were all localized to sites of cell-cell interaction. Proper localization of E-cadherin to sites of cell cell contact resulted in an increase in the amount of cytoskeletal associated β-catenin and

a decrease in LEF-1 activity. It is possible that this decrease in LEF-1 activity is due to a decrease in the amount of "free" cytosolic β-catenin available to translocate into the nucleus as was seen in between differentiated (OVCAR-3) and dedifferentiated (SKOV-3) cell lines. Decreased LEF-1 activity was also correlated with an increase in non-migratory spheroids in basement-membrane culture. Thus, the formation of functional adherens junctions in ovarian surface epithelial cell lines results in the acquisition of a non-mesenchymal epithelial phenotype. This phenotype is a hallmark of early stage ovarian carcinomas. Therefore E-cadherin may play an initiating role in the formation of epithelial ovarian carcinoma,

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