E-CADHERIN PROMOTES TUMORIGENICITY IN HUMAN OVARIAN SURFACE EPITHELIUM

by

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ABSTRACT

Ovarian cancer is the most lethal gynecological cancer in the Western world, but comparatively little is known about its development. Epithelial ovarian carcinomas are thought to originate in the ovarian surface epithelium (OSE), i.e. the mesothelium covering the ovary, but experimental evidence for this origin has been lacking. Contrary to most epithelia where neoplastic progression is associated with loss of E-cadherin, this cell-cell adhesion molecule is sparse in normal human OSE but its expression increases in ovarian epithelial metaplasia and neoplasia. Concurrently, the tumors tend to acquire characteristics of the complex epithelia of the oviduct and uterus. The high proportion of ovarian cancers where such aberrant Mullerian differentiation occurs suggests that this change may confer a selective advantage on the transforming cells. Previous studies in our laboratory showed that increased E-cadherin expression may be a cause of such Mullerian differentiation. E-cadherin was transfected into SV40 large T antigen-immortalized, E-cadherin-negative cells derived from normal OSE. Constitutive expression of E-cadherin re-established normal epithelial markers that had been lost in culture, such as keratin, and induced markers of metaplasia and neoplasia, such as CA125. In the present study, SV40-immortalized, E-cadherin-transfected cells, but not the E-cadherin-negative controls, were found to be anchorage independent and formed transplantable, invasive subcutaneous and intraperitoneal adenocarcinomas in 100% of injected SCID mice. Intraperitoneally injected tumor cells seeded the mesenteries and omentum, invaded the liver and thigh musculature, and produced ascites. The presence of SV40 large T antigen in the tumor cell nuclei confirmed their origin in the transfected OSE cells. This is the first experimental model of ovarian adenocarcinomas derived by genetic manipulations of normal human OSE. The results confirm the potential of OSE to give rise to ovarian epithelial carcinomas and suggest that upregulation of E-cadherin may play a pivotal role in their progression.

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LIST OF ABBREVIATIONS

BRCA1	- breast cancer susceptibility gene 1
BSA	- bovine serum albumin
BSS	- balanced salt solution
CA-125	- cancer antigen 125
EC	- E-cadherin
EDTA	- ethylenediamine tetra-acetate
FBS	- fetal bovine serum
HMF	- Hepes-buffered Mg-free saline
IOSE	- immortalized ovarian surface epithelium
Lef	- leukocyte enhancing factor
NCS	- newborn calf serum
NK	- natural killer cells
OCT	- optimal cutting temperature
OSE	- ovarian surface epithelium
PBS	- phosphate buffered saline
PMSF	- phenylmethane sulfonyl fluoride
RB	- retinoblastoma
SCID	- severe combined immunodeficiency
SDS-PAGE	- sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SV40	- simian virus 40
Tag	- large T-antigen
Tcf	- T-cell factor
TsIOSE	- temperature sensitive immortalized ovarian surface epithelium

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"If I have seen further, it is by standing on the shoulders of giants." – Isaac Newton

Thank you everyone.

1. INTRODUCTION

1.1 Ovarian Cancer

Ovarian cancer is a highly lethal disease with an insidious onset. It is the fifth leading cause of cancer deaths in women in the Western world. Although it only accounts for 4% of all cancers among women (Daly and Obrams, 1998), it ranks first in overall mortality from gynecologic cancers and ranks second among gynecologic cancers in incidence in the United States (Landis *et al.*, 1998). Due to the lack of early symptoms, approximately 76% of ovarian carcinoma patients are diagnosed at the advanced stage, with regional or distant metastasis already occurring, and respond poorly to therapy (Qazi and McGuire, 1995). Because of our inability to detect the disease before it becomes metastatic, 5-year survival rates have not changed significantly in over 25 years and remain a low and discouraging 38% (Chen and Berek, 1998).

The most common ovarian cancer is the epithelial type, with approximately 90% of malignant ovarian tumors originating from the ovarian surface epithelium (OSE) which is the mesothelial cell layer that surrounds the ovary (Nicosia *et al.*, 1991). The nonepithelial types of ovarian cancer comprise the other 10% and include the sex cord-stromal tumors (6% of malignant ovarian tumors), germ cell tumors (3%), and indeterminate tumors (1%) (Daly and Obrams, 1998).

While the exact cause of ovarian cancer is unknown, ovulation may play an important role in ovarian carcinogenesis since epidemiological studies have shown that the risk of ovarian epithelial cancer is decreased by factors that suppress ovulation like pregnancy, breast-feeding, and the use of oral contraceptives, whereas uninterrupted ovulation has been associated with increased risk (Berchuck 999and Carney, 1997). Several theories to explain the relationship

between uninterrupted ovulation and the development of ovarian cancer have been suggested: (1) the ovulatory defect produced on the ovarian surface during ovulation likely results in the increased proliferation and regeneration of the epithelial cells which provides an opportunity for mutation and malignant transformation; (2) the exposure of the epithelium to high levels of steroid hormones and gonadotropins during ovulation may increase the risk of ovarian cancer; and (3) the inclusion cysts formed due to the entrapment of epithelial cells during ovulation may represent precursor lesions in which transformation is facilitated by the presence of hormones and/or growth factors in the cyst fluid (Berchuck and Carney, 1997). However, in spite of the physiologic and clinical significance of the ovarian surface epithelium, it has been the least understood component of the ovary. It is important to study how the ovarian surface epithelial cells become transformed, escape growth regulation and metastasize in order to develop effective methods of disease prevention, detection, and treatment.

1.2 Ovarian Surface Epithelium

The ovarian surface epithelium is the simple pelvic mesothelium covering the ovary. Embryologically, the OSE is of mesodermal origin, having originated from the coelomic epithelium which overlies the gonadal ridge and therefore is closely related to the underlying ovarian stromal fibroblasts (Nicosia, 1983). In the embryo, the coelomic epithelium in the gonadal region is competent to develop along many different pathways. Coelomic epithelial cells penetrate into the fetal ovary and contribute to the development of granulosa cells. In addition, invagination of the coelomic epithelium gives rise to the Mullerian (paramesonephric) ducts, which are the primordia for the epithelia of the oviduct, uterus and upper vagina. Therefore, these epithelia and the OSE are closely related. As a matter of fact, the adult OSE is believed to be relatively uncommitted and closer to a stem cell form than the other coelomic

epithelial derivatives such as the endometrium and the oviductal epithelium because of the relatively simple phenotype of the adult OSE and its capacity to differentiate into a striking variety of ovarian carcinomas, frequently along the lines of Mullerian duct derivatives.

The OSE is a simple squamous to cuboidal epithelium, and like other epithelia, is characterized by keratin, mucin, desmosomes, apical microvilli and a basal lamina (Blaustein and Lee, 1979). However, as mentioned previously, in spite of these classical epithelial features, there are indications that OSE differentiation is not as firmly determined as in many other epithelia. *In vivo*, OSE cells coexpress keratin with vimentin which is a mesenchymal marker (Czernobilsky *et al.*, 1985). In addition, the epithelial differentiation markers E-cadherin and CA-125 are expressed weakly and inconsistently in human OSE, in contrast to oviductal and endometrial epithelium and the extraovarian mesothelium, in which both markers are uniformly and prominently expressed (Maines-Bandiera and Auersperg, 1997; Jacobs and Bast, 1989; Nouwen *et al.*, 1987; Kabawat *et al.*, 1983). CA-125 is an antigenic determinant on a high-molecular-weight glycoprotein recognized by a monoclonal antibody which was raised using an ovarian cancer cell line as an immunogen. Although also present in normal and other pathological states, serum CA-125 estimation is of clinical value in the pre-operative diagnosis and monitoring of ovarian malignancy and is considered the best prognostic indicator of this disease so far (De Bruijn *et al.*, 1997).

In culture, OSE cells produce extracellular matrix components characteristic of epithelial basement membrane like laminin and collagen type IV, as well as stromal components like collagen types I and III (Kruk *et al.*, 1994). In addition, OSE cells respond to a culture environment by modulating from an epithelial to a more mesenchymal phenotype as can be seen by the acquisition of anterior-posterior polarity, secretion of collagen types I and III, and loss of epithelial markers keratin and desmoplakin (Auersperg *et al.*, 1994). Cells generally respond to explantation into culture as they would to wounding and so the response of OSE cells to

explantation into culture most likely imitates their normal response to ovulatory rupture. The acquisition of an atypical and fibroblast-like phenotype may correspond to OSE cells that separate from one another and migrate during ovulatory repair *in vivo* (Kruk *et al.*, 1994).

In most tissues, the loss of normal differentiation and cellular organization are among the earliest histologically observable changes in carcinogenesis. This loss tends to increase with malignant progression. In contrast, epithelial ovarian carcinomas have more specialized epithelial markers and are frequently more highly differentiated than their tissue of origin (Van Niekerk *et al.*, 1993). As a matter of fact, ovarian carcinomas are classified according to their structural and functional resemblance to the highly specialized epithelia of the oviduct, endometrium, and endocervix. With neoplastic progression, the OSE loses mesenchymal characteristics and becomes more committed to an epithelial phenotype.

1.3 Immortalized OSE (IOSE)

Normal OSE constitutes only a very small part of the ovary and does not survive in culture for many passages; therefore, it has been difficult to obtain OSE cells in sufficient numbers for large-scale experiments. To address this problem, the growth potential of cultured OSE was increased through transformation with simian virus 40 (SV40) large-T antigen (Maines-Bandiera *et al.*, 1992). Large T-antigen (Tag) localizes predominantly in the nucleus and binds to several cellular proteins including the products of the tumor suppressor genes p53 and p105 RB. This binding results in interference with the growth regulatory activities of the p53 and p105 RB proteins (Lane, 1989). p53 is a tumor suppressor gene product that stops growth in DNA damaged cells and regulates apoptosis. Rearrangements, deletions and missense mutations of the p53 gene were found to be the most common genetic alterations found in human cancers (Holstein *et al.*, 1991). Tag binds to and either forms a stable complex with the p53

protein or phosphorylates it, rendering it inactive. p105 RB is a nuclear phosphoprotein which in its hypophosphorylated form is suggested to be active in the G1 phase of the cell cycle, exerting a negative growth effect on the cells. SV40 Tag binds to the hypophosphorylated form of p105 RB (Ludlow *et al.*, 1989) and stimulates its phosphorylation therefore removing its negative growth effect (Hu *et al.*, 1992). As a result, phosphorylation allows progression of the cells into the S phase. The Tag transformed OSE cells share characteristics with cells in early stages of neoplastic progression. They have a reduced dependence on exogenous growth factors as well as an extended life span and increased growth rate and saturation density compared to normal OSE. However, they remained non-tumorigenic in nude mice and lacked other characteristics of ovarian epithelial neoplasms like CA-125 and E-cadherin expression (Maines-Bandiera *et al.*, 1992). SV40-immortalized cell lines tend to retain the differentiated characteristics of the cells at the time of immortalization (Lemoine *et al.*, 1989). In keeping with this phenomenon in other cell types, IOSE cultures that had already undergone epithelio-mesenchymal conversion at the time of immortalization demonstrated an atypical, fibroblastic-like morphology.

1.4 E-cadherin

E-cadherin is an epithelial differentiation marker that may play an important role in OSE metaplasia and neoplastic progression. E-cadherin is a 120 kDa transmembrane glycoprotein and is a member of the cadherin superfamily whose main functions are to maintain tissue integrity and differentiation through homotypic calcium-dependent intercellular adhesion (Geiger and Ayalon, 1992; Takeichi, 1991). Cadherins are differentially expressed in development, and expression is often restricted to specific cell types within a tissue (Takeichi, 1991). One proposed function for the differential cadherin expression is to mediate cell sorting wherein

expression of different cadherins in a mixture of cells results in sorting of cells into populations that express the same cadherin (Steinberg and Takeichi, 1993). The restricted expression of different cadherins to specific cell types also indicates that cadherins may also be involved in the differentiation of specialized cell phenotypes (Takeichi *et al.*, 1990). The E-cadherin molecule is divided by its single-pass transmembrane region into a highly conserved intracellular domain and a glycosylated extracellular domain (Takeichi, 1991; Grunwald, 1993). The carboxy-terminal cytoplasmic part is known to interact via catenins with the actin cytoskeleton of the cell (Kemler, 1993) while the amino-terminal extracellular region with its repeated domains is important for intercellular adhesion and homotypic binding specificity.

E-cadherin is the prime mediator of cell-to-cell adhesion in epithelial cells and is a characteristic component of most normal epithelia, including those of the oviduct, endometrium, and uterine cervix. E-cadherin has also been proposed as a master gene that activates and regulates gene expression that is required for epithelial differentiation (Hay, 1995). Cells transfected with the E-cadherin gene develop an epithelial morphology (Nagafuchi *et al.*, 1987) and characteristic epithelial intercellular junctions (Marrs *et al.*, 1995; Gumbiner *et al.*, 1988). On the other hand, disruption of E-cadherin mediated cell adhesion between neighboring epithelial cells with the use of specific antibodies results in cell dissociation and the acquisition of a fibroblastic morphology (Behrens *et al.*, 1985). Furthermore, E-cadherin functions as a suppressor of invasion and metastasis in carcinogenesis by the maintenance of intercellular adhesion among differentiated malignant cells (Birchmeier and Behrens, 1994). Thus, E-cadherin expression levels tend to be highest in normal epithelia, persist to varying degrees in differentiated carcinomas, and become downregulated as malignant tumors become undifferentiated and invasive (Birchmeier, 1995; Pierceall *et al.*, 1995; Verneulen *et al.*, 1995).

1.5 E-cadherin in OSE

In contrast to the general situation, E-cadherin expression is more limited in normal OSE than in epithelial ovarian carcinomas (Sundfeldt *et al.*, 1997). E-cadherin is present rarely on the ovarian surface but is increased in surface invaginations and particularly in epithelial inclusion cysts. More importantly, its expression is absent in flat OSE, variable in cuboidal and most prominent in columnar OSE regardless of location (Maines-Bandiera and Auersperg, 1997). Columnar OSE with its increased E-cadherin expression is frequently found in inclusion cysts which are sites of frequent metaplastic and dysplastic changes. Therefore, the appearance of E-cadherin in columnar OSE may represent an early step in the increased commitment to epithelial phenotypes that accompanies metaplasia and neoplastic progression. E-cadherin expression increases in metaplastic and dysplastic lesions and in ovarian carcinomas and is diminished only in some invasive lesions and in the ascites form (Veatch *et al.*, 1994).

1.6 E-cadherin in IOSE

The ability of E-cadherin to induce epithelial differentiation in cells and its expression in ovarian carcinomas while being absent or rare in normal OSE raises questions regarding its role in the aberrant differentiation, i.e. the acquisition of oviductal and endometrial characteristics, of OSE cells undergoing neoplastic progression. To examine its role, E-cadherin was constitutively-expressed in SV40 Tag immortalized, E-cadherin-negative OSE cells by transfecting them with a full length mouse E-cadherin cDNA under the control of the β-actin promoter (pBATEM2) (Auersperg *et al.*, 1999). Epithelial morphology was restored to the cells, the catenins were increased in quantity and colocalized with E-cadherin at the intercellular junctions and keratin was reexpressed. Like neoplastic OSE but unlike normal OSE, the E-cadherin-transfected cells secreted the tumor marker CA-125 at levels comparable to ovarian

cancer lines, and their growth pattern in 3-dimensional sponge cultures is similar to that of the ovarian cancer cell line OVCAR-3 (Auersperg *et al.*, 1999). Therefore, E-cadherin induced phenotypic and genetic changes that are associated with metaplasia and neoplastic progression in the precursor cells of epithelial ovarian carcinomas.

1.7 Immunocompromised mouse models in tumorigenicity assays

The use of immunocompromised mice in medical research has increased tremendously in recent years. The lack of suitable animal models that mimic the normal functions of human tissues has seriously hampered progress in understanding human disease mechanisms and the development of new therapies. The inability to manipulate physiological conditions and obtain tissue samples at various times during the disease process from human patients necessitates the establishment of a small animal model transplanted with functional human cells and/or tissues that would allow investigation of the mechanisms involved in human disease. The major problem in the transplantation of human tissue into animals has been the destruction of the engrafted tissue by the host immune system. To overcome this problem, immunodeficient animals such as nude mice have been used as hosts for human tissues and cells.

Nude mice are athymic and lack a functional T-cell system. T-cell maturation cannot occur due to thymic hypoplasia and this results in the failure of cell-mediated immune reactions such as allograft rejection, delayed type hypersensitivity, and antibody responses to T-cell dependent protein antigens. Athymic nude mice still possess appreciable numbers of immune effector cells with anti-tumor activities like B cells, natural killer (NK) cells and macrophages (reviewed in Oakley *et al.*, 1993). Nude mice also have residual T cells with potential anti-tumor activities (Silobrcic *et al.*, 1990).

Another animal model to use is the severe combined immunodeficient (SCID) mouse model. SCID mice have an autosomal recessive mutation (*scid*) that interferes with the rearrangement of immunoglobulin and T-cell receptor genes in lymphoid progenitors, resulting in the lack of both T and B lymphocytes (Schuler *et al.*, 1986). This results in low serum immunoglobulin levels and lack of functional T cells (reviewed in Hendrickson, 1993 and Sandhu *et al.*, 1996). The state of immunological deficiency of the SCID mouse is more severe that that observed in the athymic nude mouse. However, these animals do show leakiness in later stages of life with virtually all SCID mice having detectable B- and T-cells at one year of age (Nonoyama *et al.*, 1993; Mosier *et al.*, 1993). In addition, these mice are profoundly sensitive to x-ray radiation due to defects in their DNA repair mechanisms (Fulop and Phillips, 1990).

Since SCID mice suffer from a broader immunodeficiency than nude mice, it seems reasonable to assume that they might be more susceptible recipients for human tumor growth and metastatic spread. There are some studies which indicate that human tumor xenografts grow equally well in nude and SCID mice (Oakley *et al.*, 1993, Xie *et al.*, 1992). But there are also several studies that have shown that SCID mice have a better take for some of the human tumor types than nude mice (Phillips *et al.*, 1989; Nomura *et al.*, 1991; Shibayama *et al.*, 1991; Wang and Stearns, 1991). Furthermore, the metastatic capacity of human tumor cells is better expressed in SCID mice than in nude mice. (Furukawa *et al.*, 1993; Xie *et al.*, 1992). Therefore, it would seem that SCID mice are better models for the study of the mechanisms of human cancers, especially the study of human cancer metastasis.

2. OBJECTIVE

1) To test the hypothesis that IOSE-29EC, a SV40-Tag-immortalized and E-cadherin transfected OSE line is tumorigenic.

3. MATERIALS AND METHODS

3.1 Cell lines and cell culture

The IOSE-29 cell line (referred to as IOSE-Mar in some previous publications) was generated by transfecting normal human OSE cells in passage 5 with the immortalizing SV40 virus early genes (Maines-Bandiera *et al.*, 1992). As described previously (Auersperg *et al.*, 1999) IOSE-29 cells at passage 11 were co-transfected by lipofectamine (Life Technologies, Gaithersburg MD) with pSV2neo and an expression vector containing a full length mouse E-cadherin cDNA under the control of the ß-actin promoter (pBATEM2) (Southern *et al.*, 1982; Nose *et al.*, 1988). The cells were selected with G418, and reselected by differential adhesion. This procedure produced line IOSE-29EC, which expresses E-cadherin at levels similar to those observed in the human ovarian carcinoma-derived positive control cell line OVCAR-3 (Hamilton *et al.*, 1983). The IOSE-29neo cell line, generated from IOSE-29 cells transfected with pSV2neo alone, does not express E-cadherin (Auersperg *et al.*, 1999). In the course of the present study, two additional cell lines, designated IOSE-29EC/T4 and IOSE-29EC/T5, were established from tumors that arose in IOSE-29EC-inoculated SCID mice.

The cells were maintained in medium 199/MCDB 105 (1:1) (Sigma, St. Louis MO) with either 5% FBS or 10% NCS (HyClone Laboratories, Logan UT), at 37°C in a 5% CO_2 atmosphere. They were subcultured with 0.06% trypsin (250:1)/ 0.01% EDTA in Ca, Mg-free

Hanks'BSS. To define the growth potential of line IOSE-29EC, these cells were serially propagated at 1:10 split ratios, with cell counts at each passage.

3.2 Immunofluorescence

Cells grown on glass coverslips and 5 μm sections cut from frozen O.C.T tissue blocks were either fixed in cold (-20°C) methanol and permeabilized in cold methanol-acetone (1:1) for keratin and SV40 Tag, or fixed with 10% formalin in Hepes-buffered Mg-free saline supplemented with $CaCl_2$ (HMF) (Hirano *et al.*, 1987) and permeabilized in cold methanol for 5 min. for E-cadherin. The cells and sections were washed with the appropriate buffer (HMF for E-cadherin, PBS for the rest) and incubated with 5% normal goat serum (NGS) in 1% BSA/buffer to block non-specific sites, followed by antibodies against SV40 Tag (mouse monoclonal, Oncogene Research, Cambridge, MA), E-cadherin (mouse monoclonal, Transduction Labs, Lexington, KY; rat anti-mouse E-cadherin ECCD-2, ZyMed Laboratories, San Francisco, CA), keratin (rabbit polyclonal, Dako, Carpenteria, CA; AE1/AE3, generously provided by Dr. T.T. Sun), or β-catenin (mouse monoclonal, Bio-Can). After incubations with the appropriate secondary antibodies, fluorescence was visualized by epifluorescence microscopy.

3.3 Immunocytochemistry

Cells on glass coverslips and thawed sections were fixed in 10% PBS- buffered formalin for 30 min. Endogenous peroxidase was blocked using 3.0% (v/v) hydrogen peroxide in methanol for 20 min., and non-specific sites with 5% NGS for 30 min. Monoclonal anti-CA-125 (OC125, generously provided by Dr. R. Bast) at a 1:2000 dilution was applied for 1 hr, followed sequentially by biotinylated goat anti-mouse antibody and horseradish peroxidase-

strepavidin conjugated IgG (Kirkegaard & Perry Labs, Gaithersburg, MD) for 1 hr and 30 min respectively. The signal was developed using 0.025% diaminobenzidine /0.01% H_2O_2/0.04% $NiCl_2$. The same procedure was carried out for E-cadherin using the mouse monoclonal anti-E-cadherin antibody (Transduction Laboratories) and HMF buffer.

3.4 SDS-PAGE electrophoresis and Western blot analysis

For E-cadherin immunoblotting, whole cell lysates were prepared by scraping confluent cultures into RIPA buffer (0.1% sodium dodecyl sulphate, 1% deoxycholate, 1% NP-40) containing protease inhibitors (EDTA, leupeptin, pepstatin A, PMSF, and aprotinin). After brief vortexing the solution was centrifuged at 14,000 rpm for 15 minutes and the supernatant was stored at –70°C. Protein determinations were done with the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Richmond, CA). Twenty μg of total soluble proteins were resolved by SDS-polyacrylamide gel electrophoresis (5% stacking and 7.5% resolving gels). Proteins were electroblotted onto Hybond ECL membrane (Amersham, Amersham Bucks, UK) using a Mini Trans-Blot cell apparatus (Bio-Rad) and probed with the mouse monoclonal anti-E-cadherin antibody (1:1000) from Transduction Laboratories. Primary antibody binding was assessed using horseradish peroxidase-labelled goat anti-mouse IgG (1:5000) (Jackson ImmunoResearch Laboratories, West Grove, PA) that was visualized by the enhanced chemiluminescence detection system (Amersham, Arlington Heights IL).

3.5 Assays for anchorage independence

Colony formation in semi-solid agar was assayed by suspending 1.3 x 10^4 cells in 2 ml of medium 199/MCDB105/10% NCS with 0.33% agarose (Life Technologies), and placing this suspension on top of 5 ml of solidified 0.5% agarose in the above medium. 60 ml tissue culture

dishes with 2 mm grids (Nunclon) were used for this experiment. Triplicate cultures for each cell type were maintained for 14 days at 37°C in a 5% CO_2 atmosphere with fresh medium added after one week. Colonies equal to or greater than 50 μm in diameter were counted after two weeks. The experiment was repeated 3 times.

3.6 Tumorigenicity assays

Female BALB/c SCID mice, 4 to 6 weeks old, were obtained from the Terry Fox Laboratory (Vancouver B.C., Canada). The animals received proper care according to the rules of the University of B.C. Committee on Animal Care, and received food and water *ad libitum*. In the first tumorigenicity assay, they were injected with IOSE-29 cells or IOSE-29EC cells, either subcutaneously in the thigh regions or intraperitoneally. Approximately 5 x 10^6 cells were injected in 0.2 ml of CO_2-independent medium (Life Technologies), with or without Matrigel, which has been reported to stimulate tumor formation by cultured cells (Mullen *et al.*, 1996). The date of the first indication of tumor growth was noted, and the animals were killed when they appeared acutely uncomfortable or at the termination of the experiment after 4 months. The extent of the tumor burden was determined by gross examination of the peritoneal and thoracic cavities and of the subcutaneous tissues in the region of the injection sites, and by microscopic examination of the abdominal organs and of extra-abdominal regions suspected of tumor formation or invasion. Resected tissues were either embedded in O.C.T. and stored at –70°C for cryostat sections, or fixed in buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. To ascertain that the tumors were derived from the injected cells, parts of two tumors were minced with scalpels, cultured in medium 199/MCDB105/5% FBS, and stained for SV40 Tag by the methods described above. Additional cells from these two tumors gave rise to the lines IOSE-29EC/T4 and IOSE-29EC/T5. In the second tumorigenicity assay, all cells

were injected intraperitoneally without Matrigel. Otherwise, the protocol was identical to the one in the first assay. The following cell lines were used in the second assay: IOSE-29, IOSE-29neo, IOSE-29EC, IOSE-29EC/T4 and IOSE-29EC/T5.

4. RESULTS

4.1 Characterization of the IOSE-29 and IOSE-29EC cell lines

IOSE-29 cells grew as fibroblastic monolayers which was consistent with the epithelio-mesenchymal conversion that these cells had previously undergone (Fig 1a) (Maines-Bandiera *et al.*, 1992). In contrast, the IOSE-29 cells that had been transfected with the mouse E-cadherin cDNA, designated IOSE-29EC, grew as epithelial monolayers which formed polarized epithelial domes (hemicysts) at high cell densities (Fig. 1b). Such domes were never observed in cultures of normal, metaplastic or immortalized OSE but did occur in the ovarian carcinoma line OVCAR-3 (data not shown). Like normal OSE, IOSE-29 cells lacked E-cadherin (Figs. 1c), while IOSE-29EC cells produced E-cadherin which accumulated at the cell-cell junctions (Figs. 1d, 2). In contrast to the keratin-negative parental IOSE-29, IOSE-29EC cells contained abundant keratin filaments (Figs 1e,f). In agreement with previous observations, CA-125 was absent in line IOSE-29 (Maines-Bandiera *et al.*, 1992), but it was present in substantial amounts in IOSE-29EC (Figs. 1g,h).

The IOSE-29EC cells were propagated over more than 200 population doublings, indicating that they represent a continuous cell line.

Taken together, the morphology, differentiation markers and growth characteristics of the E-cadherin-transfected IOSE-29EC cells closely resembled those of the ovarian carcinoma line OVCAR-3. Importantly, however, these two lines could be distinguished because OVCAR-3 cells lacked the immortalizing SV40 Tag that was consistently found in all cells of lines IOSE-29 and IOSE-29EC (data not shown).

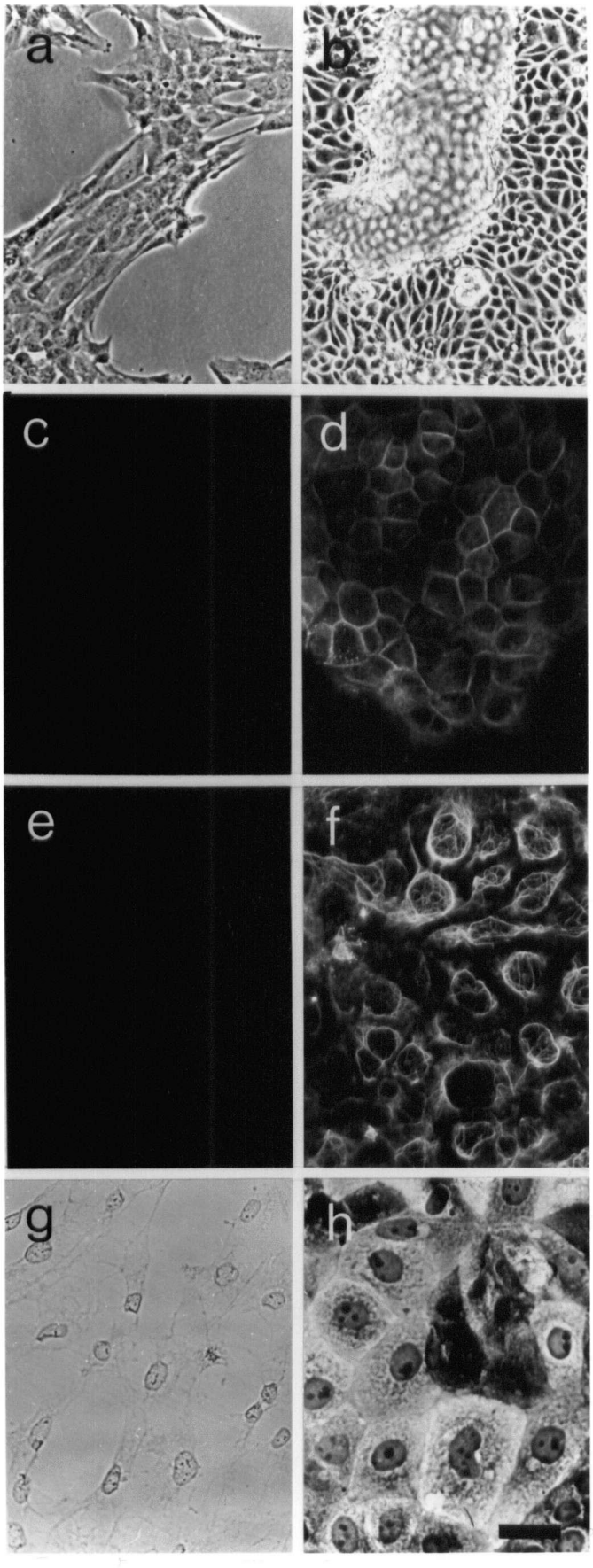

Figure 1. Characteristics of IOSE-29 and IOSE-29EC lines.

Cultures of IOSE-29 cells (a,c,e,g) and IOSE-29EC cells (b,d,f,h) were photographed by phase microscopy (a,b) or fixed and stained by immunofluorescence for E-cadherin (c,d) and keratin (e,f) or by immunocytochemistry for CA-125 (g,h). *Note* dome formation in the upper part of (b). Bar = 120 μm for a,b; bar = 20 μm for c,d,e,f,g,h.

4.2 First tumorigenicity assay

In this assay, twelve SCID mice were injected with IOSE-29 cells, and 11 mice with IOSE-29EC cells. The injection sites were either subcutaneous or intraperitoneal, and each site was inoculated with cells in either plain culture medium or medium with Matrigel (Table 1). Over 4 months, tumors developed in all mice injected with IOSE-29EC cells, independently of the site or use of Matrigel. In contrast, no tumors were found in any of the mice injected with IOSE-29 cells. Among the animals with intraperitoneal injections, one was killed within the first month after injection to examine early stages of tumor formation, and a microtumor, approximately 10-15 cells in diameter, was detected attached to the omentum. Another animal died with widespread abdominal/pelvic tumor masses after 9 weeks. Of the remaining mice in the intraperitoneal group, 6 animals became lethargic and or limped 10 – 14 weeks after injection and were sacrificed 2-3 weeks later. The limping was subsequently found to be due to tumor invasion of the thigh musculature. Most animals with subcutaneous inoculations developed palpable or visible masses, which sometimes ulcerated the skin at the sites of injection, after 13-14 weeks. The experiment was terminated after 4 months.

4.2.1 Characterization of tumor sections

Histologically, all tumors were classified as poorly differentiated adenocarcinomas. The tumors were composed mostly of solid cell masses, but in some areas, the neoplastic cells formed papillae and gland-like structures (Fig. 2a,c). The intraperitoneal injections resulted in tumor implants on the abdominal/pelvic wall, omentum and mesenteries in all animals (Fig 2,a,b,h). In addition, neoplastic cells were found on the diaphragm and uterine fundus, within the liver parenchyma (Fig. 2e), the mammary fat pads, lymphatic vessels (Fig.2b) and in the bursa

Table 1. ***Tumor formation in SCID mice injected with IOSE-29EC and IOSE-29 cells.***

Cell Type	Conditions		Tumor Incidence	Time from Injection (weeks)		Tumor Size[b]
	Site	Matrigel		Onset of symptoms	Termination	
IOSE-29EC	s.c.	+	2/2	1, 14	2, 15	0.8
		-	3/3	13, 13, 13	13, 15, 18	1.2
	i.p.	+	3/3	N/S, 10, N/S	4[a], 11, 13	1.0
		-	3/3	10, 10, 10	11, 13, 12	0.8
IOSE-29	Same as for IOSE-29EC		0/12	N/S	18	N/A

N/S = no symptoms; N/A = not applicable

[a] This mouse was terminated to examine an early stage of tumorigenesis and was found to have microtumors in the mesentery.

[b] Maximum diameter of the largest tumor in cm.

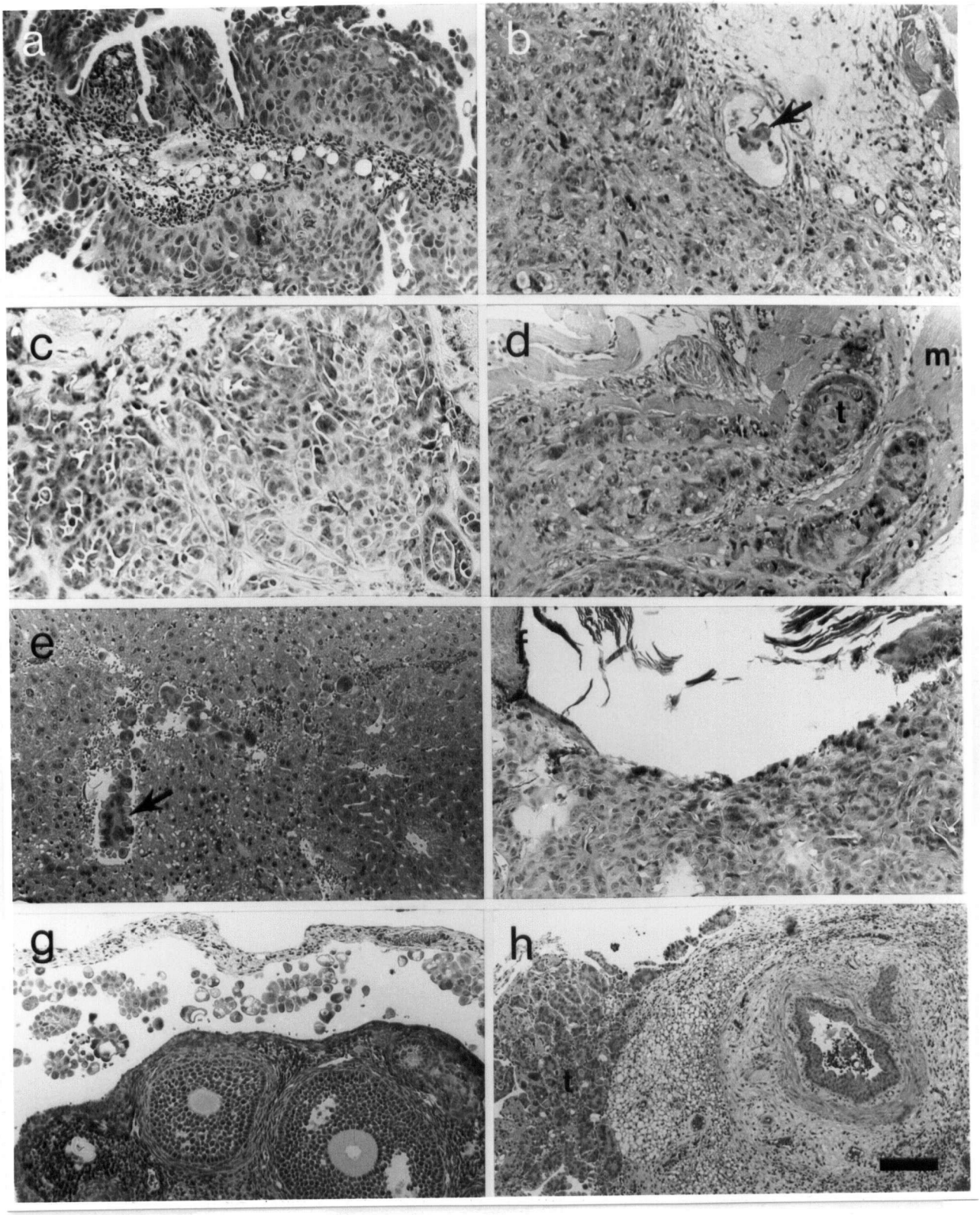

Figure 2. Histological sections of tumors.

Intraperitoneal (a,b,e,g,h) and subcutaneous (c,d,f) adenocarcinomas formed by IOSE-29EC cells in SCID mice were paraffin embedded, sectioned and stained with hematoxylin and eosin. a, tumor deposits with papillary projections coat mesentery; b, poorly differentiated tumor *(on left)* and tumor cells within a lymphatic vessel *(arrow)* ; c, formation of glandular structures; d, muscle invasion *(m, muscle; t, tumor);* e, metastasis to the liver *(arrow)*; f, ulceration of the skin. Normal skin is visible at the extreme left; g, clusters of tumor cells free within the periovarian space; h, large deposits of tumor cells on the pelvic wall overlying the ureter. *Note* lack of any desmoplastic reaction. Bar = 100 μm.

surrounding the ovaries (Fig. 2g). In all animals, the neoplastic cells had penetrated the pelvic peritoneum and invaded the adjacent thigh muscles. None of the animals showed evidence of ascites. The subcutaneous tumors were composed of multiple firm nodules up to 1.2 cm in diameter. Conspicuous blood vessels which converged on these nodules suggested an angiogenic response. The tumor cells invaded adjacent muscle (Fig. 2d) and ulcerated the overlying skin (Fig. 2f). The addition of Matrigel at the time of injection did not appear to influence the rate of tumor progression either subcutaneously or intraperitoneally. Examination of several tumors for differentiation markers revealed E-cadherin and keratin, though both of these markers were irregularly distributed and stained more weakly than adjacent normal tissues such as intestinal epithelium. CA-125 was abundant and present in most areas within the tumors (Fig. 3).

4.2.2 Characterization of tumor-derived lines

Parts of two intraperitoneal tumors (Nos. 4 and 5) were used to establish the cultured cell lines IOSE-29EC/T4 and IOSE-29EC/T5. To date, these lines have been passaged 8 and 7 times respectively. Both lines exhibited an epithelial morphology and formed domes, but differed from the original IOSE-29EC line by increased variation in cell size, multinucleation and numerous atypical mitotic figures, and a tendency to pile up and form ridges when crowded. The tumor-derived lines maintained the expression of E-cadherin, keratin and CA-125, but in reduced overall intensity and with more intercellular variation than the original IOSE-29EC line (Fig. 4). They were also positive for Tag (Fig. 5) confirming their exogenous origin. Immunoblot analysis (Fig. 6) showed E-cadherin protein in both tumor-derived lines. Although relatively little E-cadherin was detectable by immunofluorescence, immunoblot analysis suggested that the tumor-derived cells contained similar amounts (IOSE-29EC/T4) or more (IOSE-29EC/T5)

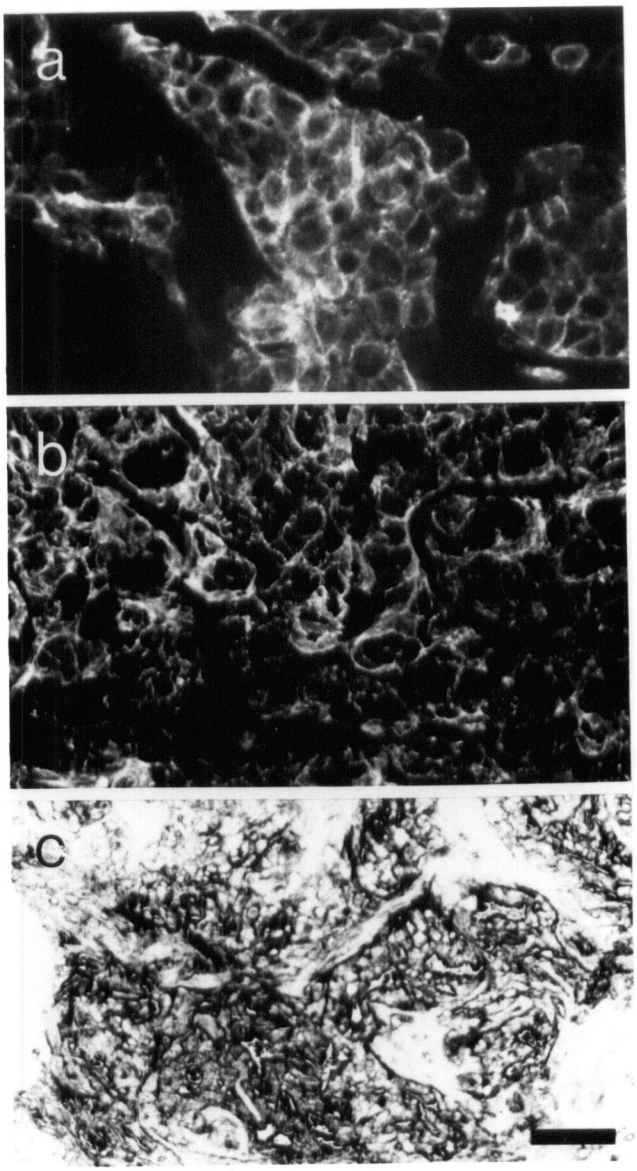

Figure 3. Expression of epithelial markers E-cadherin, keratin, and CA-125 in tumor sections.

Differentiation *in vivo* of tumor No. 4 (a,c) and tumor No. 5 (c), stained by immunofluorescence for E-cadherin (a) and keratin (b) and by immunocytochemistry for CA-125 (c). Bar = 30 μm (a,b); bar = 120 μm (c).

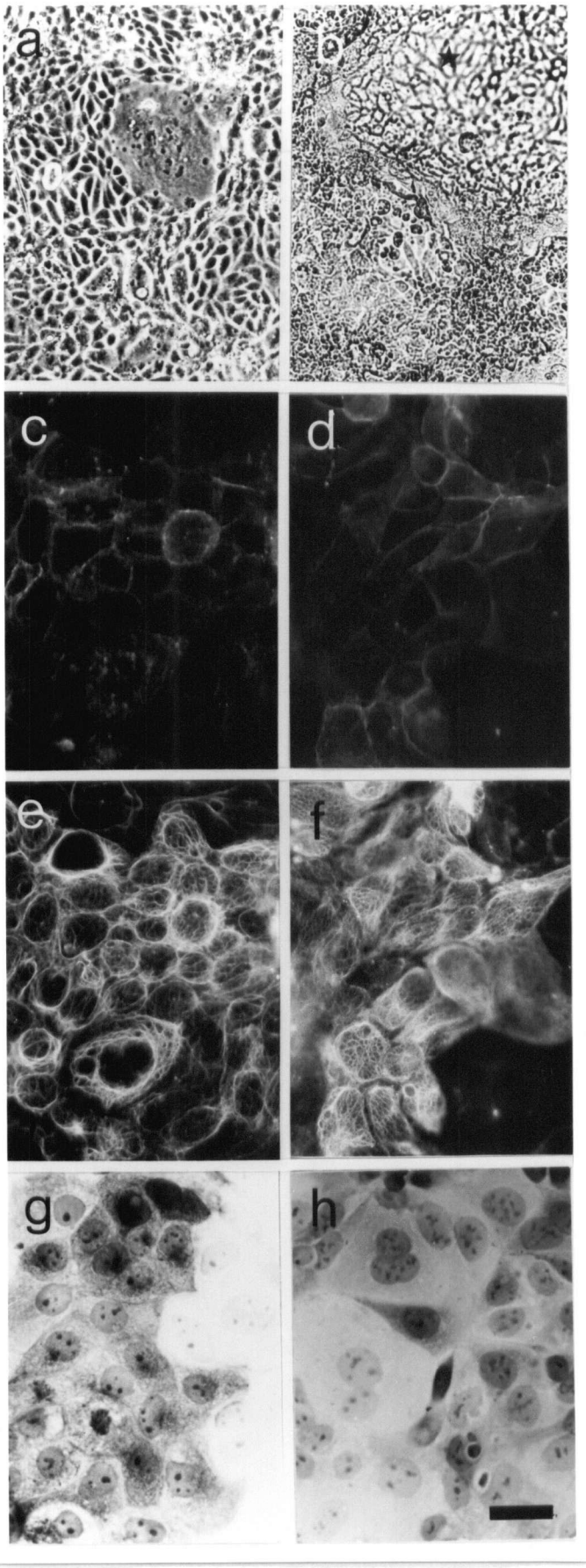

Figure 4. Expression of epithelial markers E-cadherin, keratin, and CA-125 in tumor-derived cell lines.

Differentiation of the tumor-derived cultured cell lines IOSE-29EC/T4 (a,c,e) and IOSE-29EC/T5 (b,d,f), photographed live by phase microscopy (a,b) or fixed and stained by immunofluorescence for E-cadherin (c,d) and keratin (e,f) and by immunochemistry for CA-125 (g,h). *Note* dome formation in (b) and *note* that, in contrast to line IOSE-29EC in Fig. 1, the expression of keratin, E-cadherin and CA-125 is weaker and, in parts of the cultures, undetectable. Bar = 120 μm for a,b; bar = 20 μm for c,d,e,f,g,h.

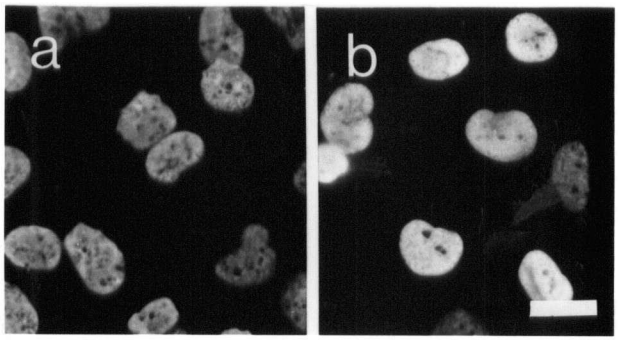

Figure 5. SV40 Tag expression in the tumor-derived cell lines.

Expression of SV40 large T antigen in the nuclei of cultured IOSE-29EC/T4 (a) and IOSE-29EC/T5 (b) cells confirms their origin from line IOSE-29. Bar = 20 μm.

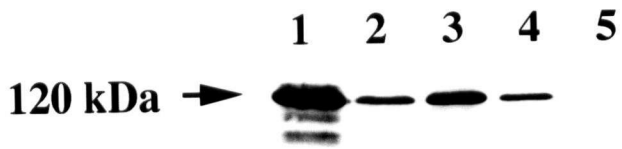

Figure 6. Western blot for E-cadherin expression.

Whole cell lysates were probed by immunoblotting for E-cadherin (120kd). Twenty μg of total protein were loaded in each lane. Lanes: 1) OVCAR-3 (positive control); 2) IOSE-29EC/T4; 3) IOSE-29EC/T5; 4) IOSE-29EC; 5) IOSE-29.

protein than their parent line IOSE-29EC. This discrepancy suggests that, in the tumor-derived lines, E-cadherin was produced but was not localized at the cell-cell junctions. A look at β-catenin in these cell lines showed that β-catenin was still localized at the cell-cell junctions in these cells (Fig. 7).

4.3 Second tumorigenicity assay

The procedures were similar to the first assay except that all mice were injected intraperitoneally, without Matrigel. IOSE-29, IOSE-29neo, IOSE-29EC, IOSE-29EC/T4 and IOSE-29EC/T5 were compared. Over 4 months, there were no tumors in the control groups IOSE-29 and IOSE-29neo, while all animals injected with the E-cadherin-expressing lines developed tumors (Table 2). The tumors formed by lines IOSE-29EC/T4 and IOSE-29EC/T5 were larger that those produced by IOSE-29EC (Table 2). In all animals there were tumor masses in the omentum and mesentery and many small (1-2mm diameter) tumor nodules seeding the peritoneum, diaphragm and even pleura, and in all but one mouse a large (0.5-1.5 cm diameter) tumor occupied most of the pelvis. Grossly visible metastases were observed in the liver of some animals. In addition, all animals inoculated with IOSE-29EC/T4 produced several ml of bloody ascites fluid which contained clusters of epithelial cells (Fig. 8). In spite of their considerable tumor burden, the mice showed surprisingly little discomfort or symptoms up to near the end of the experiments.

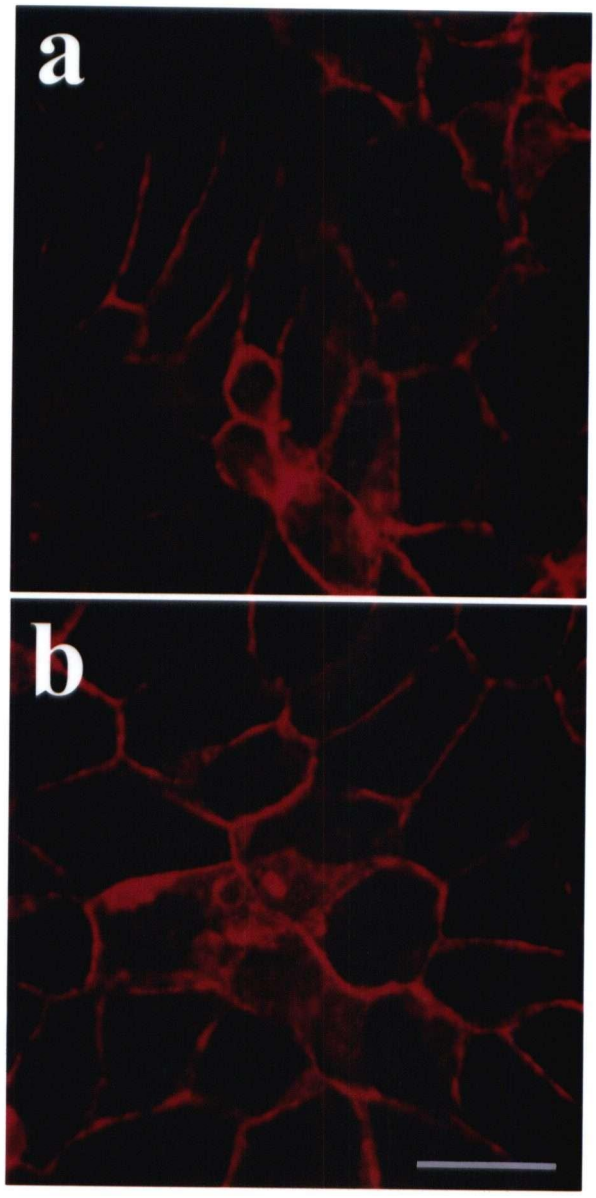

Figure 7. β-catenin expression in the tumor-derived lines.

Immunofluorescence staining of β-catenin in tumor-derived lines IOSE-29EC/T4 (a) and IOSE-29EC/T5 (b) show that β-catenin is predominantly localized at the cell-to-cell borders. Bar = 20 μm.

Table 2. ***Tumor formation in SCID mice injected intraperitoneally with IOSE-29 derived cells.***

Cell Type	Tumor Incidence	Time from Injection (weeks)		Tumor Characteristics	
		Onset of Symptoms	Termination	Size[a]	Presence of Ascites
IOSE-29	0/3	N/S	17	N/A	no
IOSE-29neo	0/3	N/S	17	N/A	no
IOSE-29EC	3/3	N/S	17	1.0	no
IOSE-29EC/T4	4/4	6, 15, 15, 15	10, 17, 17, 17	2.0	yes
IOSE-29EC/T5	3/3	7, 15, 15, 15	10, 17, 17	1.5	no

N/S = no symptoms; N/A = not applicable

[a] Maximum diameter of the largest tumor in cm.

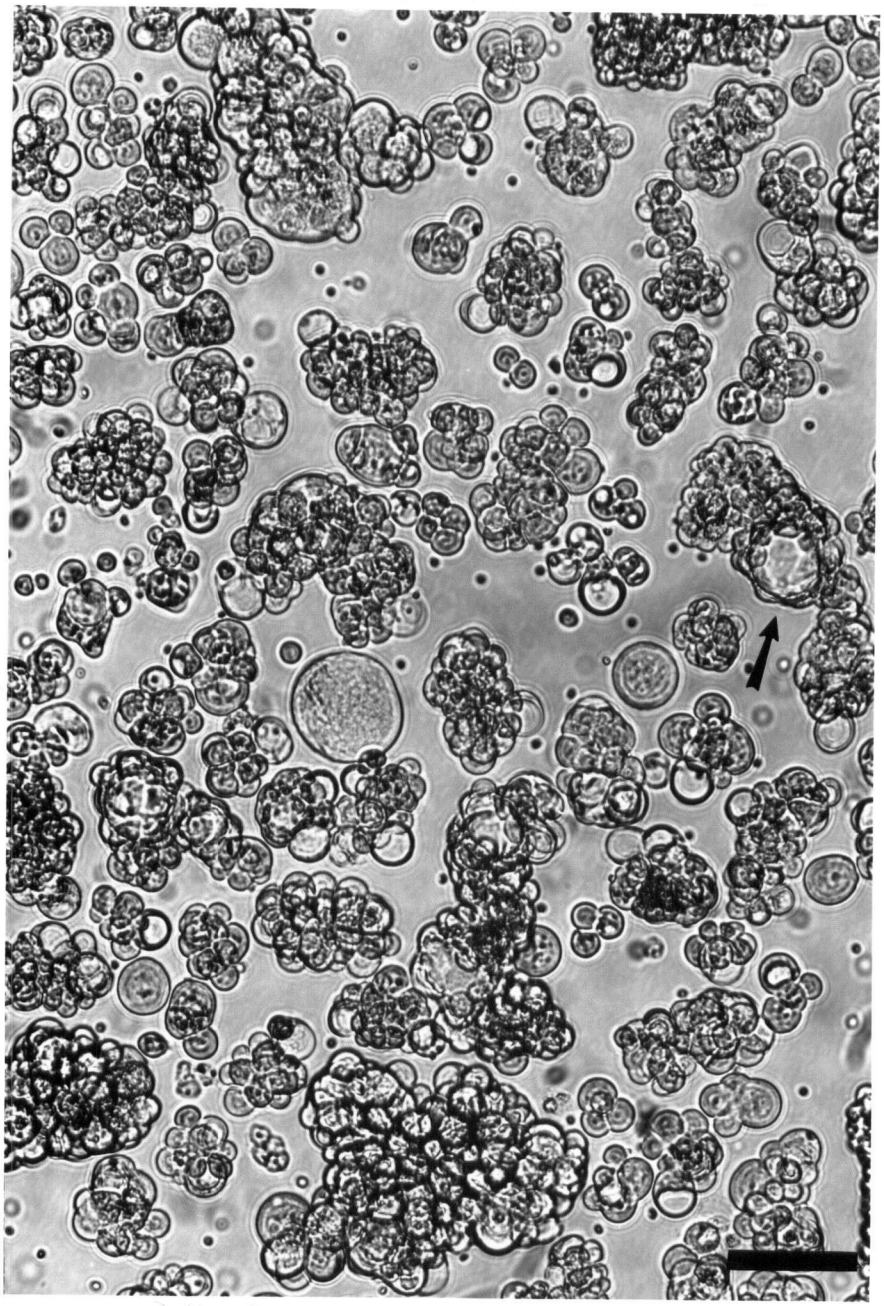

Figure 8. Ascites obtained from an IOSE-29EC/T4 injected SCID mouse.

Phase microscopy picture of freshly obtained ascites show clustering of tumor cells and the presence of cysts (*arrow*). Bar = 120 μm.

4.4 Anchorage independence as an *in vitro* tumorigenicity assay

As shown in Fig. 9, IOSE-29 cells formed very few, small colonies in soft agar, while the IOSE-29EC colonies, were more numerous and larger, indicating that these cells had acquired a significant degree of anchorage independence. As can be expected given their nature and origin, the number and size of anchorage independent colonies was significantly greater in cultures of cells obtained from the SCID mouse-derived tumors than in cultures of the original IOSE-29EC line (Fig. 9).

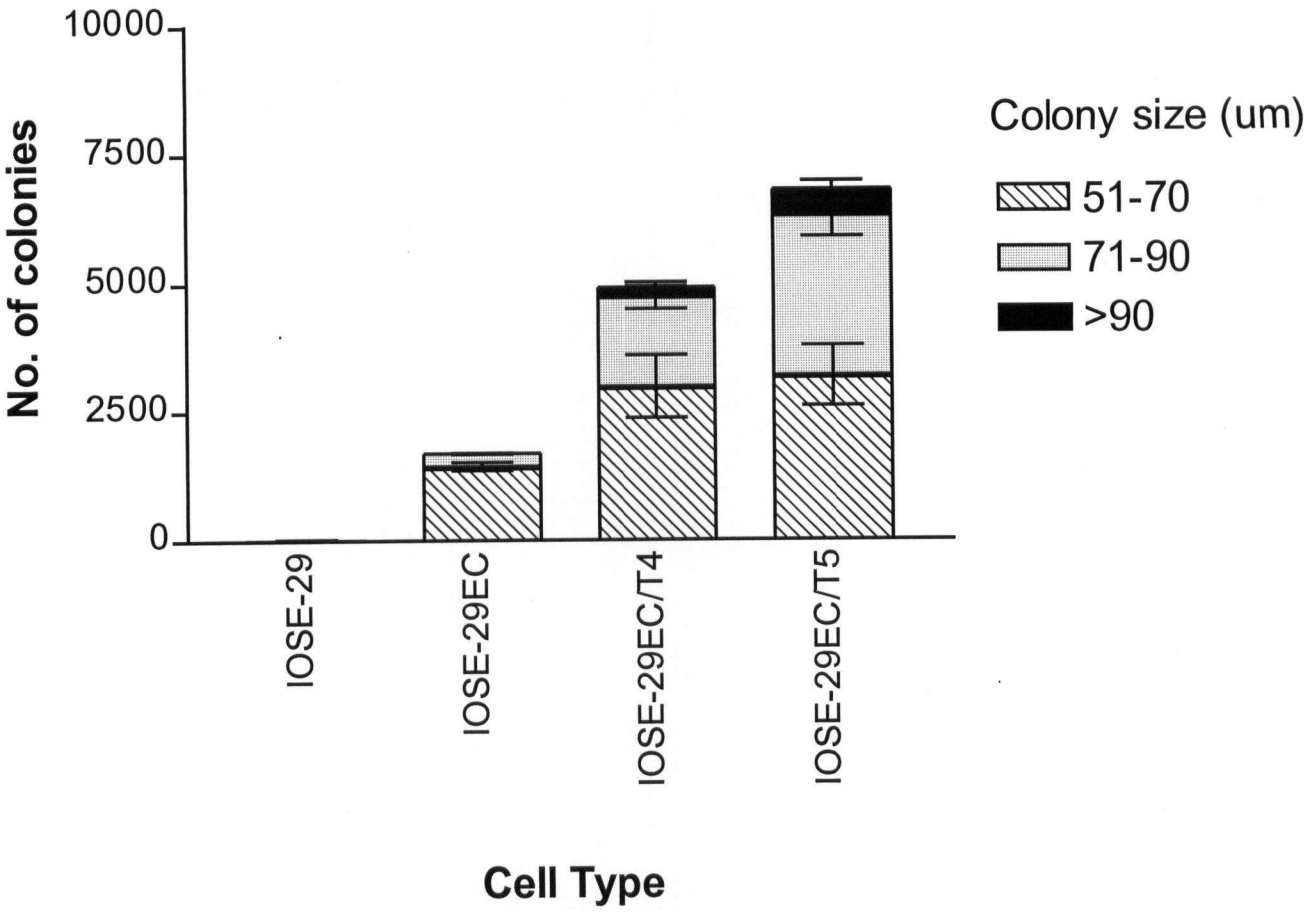

Figure 9. Anchorage independent growth of the IOSE-29 derived cell lines.

1.3 x 10^4 cells were suspended in 0.33% agarose in medium 199:MCDB (1:1) plus 10% NCS. After 2 weeks, the number of colonies equal to or greater than 50 μm in diameter were counted. The results are means of triplicate counts with standard deviations, and are representative of 3 separate experiments.

5. DISCUSSION

The ovarian surface epithelium is the least understood component of the ovary despite its important physiologic roles and clinical significance in ovarian carcinogenesis. In addition, in spite being the most lethal gynecologic cancer in the Western world (Landis *et al.*, 1998), comparatively little is known about the development and biology of these neoplasms. As a matter of fact, evidence supporting the widely accepted theory that ovarian epithelial carcinomas arise from the OSE, while strong, is based mainly on correlative studies and histopathological examination of clinical lesions (Dubeau, 1999). The present report demonstrates, for the first time, the conversion of normal human OSE to a phenotype resembling ovarian epithelial adenocarcinomas, through the introduction of defined genes.

The introduction of SV40 large T antigen (Tag) into normal human OSE cells produced the cell line IOSE-29. This p53- and p105 RB-defective OSE cell line demonstrated an extended life span, increased growth rate and saturation density, and reduced serum requirements. However, in spite of these Tag-induced neoplastic properties, IOSE-29 remained nontumorigenic in nude mice and lacked other characteristics of ovarian epithelial neoplasms such as CA-125 expression (Maines-Bandiera *et al.*, 1992). IOSE-29 was also nontumorigenic in the more immunocompromised SCID mice as shown in this report. Co-transfection of E-cadherin and neomycin resistance genes into these immortalized cells produced line IOSE-29EC (Auersperg *et al.*, 1999). IOSE-29neo, the sham control line generated by transfection of IOSE-29 cells with only the neomycin resistance gene, retained the characteristics of IOSE-29. In contrast, the constitutive E-cadherin expression in IOSE-29EC induced mesenchymal-epithelial conversion which could readily be observed by the reversion from an atypical morphology to a more epithelial phenotype. Furthermore, normal epithelial differentiation markers that had been lost in culture like keratin, were restored. This phenomenon is not surprising since transfection with E-

cadherin has been shown to cause various degrees of epithelialization of mesenchymal cells (Hay, 1995; Marrs and Nelson, 1996). The induction of mesenchymal-epithelial conversion by E-cadherin in the IOSE-29 cells illustrates the capacity of this adhesion molecule to initiate epithelial differentiation by remodeling the distribution of preexisting proteins and by the induction of new proteins (Marrs and Nelson, 1996). One consequence of E-cadherin transfection was the secretion of CA-125, which was produced at levels comparable to ovarian carcinoma cell lines, while secretion was negligible in the SV40-immortalized cells lacking E-cadherin (Auersperg *et al.*, 1999). This glycoprotein, which is essentially absent in normal OSE but is a normal secretory product of the oviductal and endometrial epithelia, is elevated in a high proportion of ovarian carcinomas and is currently the most important marker to monitor the clinical course of ovarian cancer (But and Goreisek, 1996). Another striking change induced by E-cadherin was dome (hemicyst) formation which is indicative of tight junction formation and vectorial ion transport in highly polarized epithelia. Similar polarization has been induced in other cell types, including fibroblasts, by transfection with E-cadherin (Marrs and Nelson, 1996). Dome formation in culture is one of many differentiated characteristics that are acquired by ovarian carcinoma cells (e.g. OVCAR-3), but not by normal, SV40-immortalized and metaplastic OSE cells. Therefore, forced E-cadherin expression in immortalized OSE induced characteristics which signify progression to a neoplastic form. It was the presence of these characteristics in IOSE-29EC and its similarity with the ovarian carcinoma line OVCAR-3 that prompted us to test for the line's tumorigenicity and anchorage independence.

As shown in the results, IOSE-29EC is tumorigenic in SCID mice while the E-cadherin-negative lines IOSE-29 and IOSE-29neo were not. These data are supported by the anchorage independence data. The induction of anchorage independent growth in human cells *in vitro* has been shown to be a relatively early event in the tumorigenic process (Zimmerman and Little,

1983). Anchorage independent growth *in vitro* by IOSE-29EC supports the idea that neoplastic progression has already occurred in this line even before inoculation into SCID mice. Our results do not contravene the generally held view that neoplastic progression and E-cadherin expression are inversely related. This is because our results pertain specifically to ovarian carcinogenesis, which differs from carcinogenesis in other tissues by the tumors' propensity to acquire increasingly complex epithelial characteristics early in neoplastic progression. These characteristics, which mimic the differentiation of the Mullerian duct-derived epithelia of the oviduct and endometrium, reflect the noncommitted pleuripotential nature of adult human OSE and the common embryonic origin of OSE and the Mullerian ducts in the urogenital coelomic epithelium. The aberrant Mullerian differentiation of neoplastic OSE includes the appearance of the classical epithelial differentiation marker E-cadherin, which is rare in normal OSE (Wong *et al.*, 1999; Maines-Bandiera and Auersperg, 1997; Sundfeldt *et al.*, 1997) but abundant in benign ovarian epithelial tumors and in early stage ovarian epithelial carcinomas (Sundfeldt *et al.*, 1997; Inoue *et al.*, 1992; Darai *et al.*, 1997; Peralta Soler *et al.*, 1997; Davies *et al.*, 1998). In addition, in spite of the demonstrated *in vivo* invasiveness of IOSE-29EC tumors, our data do not conflict with a role of E-cadherin as a suppressor of invasiveness and its loss in advanced ovarian carcinomas (Veatch *et al.*, 1994).). The observed invasive regions may well represent a subpopulation of cells at a more advanced stage of carcinogenesis in which E-cadherin expression is reduced or absent. The reduced expression of E-cadherin in the more tumorigenic and anchorage independent tumor-derived lines IOSE-29EC/T4 and IOSE-29EC/T5 supports this idea. Morever, there are some reports which describe persistent E-cadherin even in metastatic lesions (Sundfeldt *et al.*, 1997; Inoue *et al.*, 1992). It should be noted that the exact mechanism for the suppressive role of E-cadherin in tumor development is not known.

The precise role of the transfected, constitutively expressed E-cadherin in the development of the neoplastic phenotype of line IOSE-29EC has not yet been defined. It is well known that tissue morphology and adhesive interactions of normal and neoplastic cells profoundly influence their growth and differentiation (Ingber, 1997; Redfield *et al.*, 1997; Roskelley *et al.*, 1995). The morphologic changes and dome formation in IOSE-29EC may have been influenced directly by cadherin-mediated intercellular junction formation, but the induction of keratin and CA-125 suggests that E-cadherin also altered gene expression. E-cadherin can be found at the cell-cell adherens junctions which are specialized regions of the plasma membrane connected with cytoskeletal actin filaments. The cytoplasmic domain of E-cadherin interacts with the α-, β- and γ-catenins. (Takeichi, 1991; Kemler, 1993; Tsukita *et al.*, 1993; Troyanovsky *et al.*, 1994). It is these catenins that mediate the connection between E-cadherin and the cytoskeletal actin network. One of the catenins, β-catenin, which binds directly to the intracellular domain of E-cadherin, is also a pivotal transcriptional regulator in signal transduction (Bullions and Levine, 1998). β-catenin binds to and activates the leukocyte enhancing factor (Lef) or T-cell factor (Tcf) family of transcription factors. This is important since the Tcf/Lef-β-catenin complex is activated in human tumors. The cytoplasmic pool of β-catenin, which participates in signal transduction, is highly regulated by several mechanisms, and it has been shown that in colon carcinoma cells, E-cadherin has the ability to recruit β-catenin to the cell membrane and prevent its nuclear localization and transactivation (Orsulic *et al.*, 1999). In IOSE-29EC cells, the steady state levels of β-catenin were increased over those in IOSE-29, though most of this protein seemed to be associated with cell-cell junctions (Auersperg *et al.*, 1999). It will be important to determine whether Tcf/Lef-β-catenin activity is altered in line IOSE-29EC as compared to IOSE-29, and if so, what the specific contributions of such changes are to the altered expression of the epithelial differentiation genes.

Another interesting thing to investigate in the cell lines would be BRCA1. Mutations of the breast cancer susceptibility gene BRCA1 confer increased risk for breast, ovarian, and prostatic cancers (Ford *et al.*, 1994). The product of the BRCA1 gene has been implicated in regulation of cell proliferation, cell cycle progression, apoptosis induction and DNA repair and recombination (Irminger-Finger *et al.*, 1999). Just recently, Fan *et al.* (1999) have shown that wild-type BRCA1 inhibits estrogen receptor signalling in several cancer lines. This is relevant because it has also been shown that estrogen has the ability to upregulate E-cadherin expression in the mouse OSE (MacCalman *et al.*, 1994). Thus, it can be imagined that in OSE cells where BRCA1 is mutated and non-functional, estrogen-dependent transcriptional pathways that are involved in E-cadherin expression are not suppressed, leading to increased E-cadherin expression. This phenomenon possibly provides a link between BRCA1 mutations and E-cadherin expression in the promotion of ovarian carcinogenesis.

The tumorigenic potential of the IOSE-29EC cells which was revealed in this study was unexpected. Admittedly, the similar characteristics demonstrated by IOSE-29EC to ovarian carcinoma line OVCAR-3, especially CA-125 expression and growth in 3-dimensional sponge matrix culture (Auersperg *et al.*, 1999), support the possibility of tumorigenicity. Intuitively however, it would be anticipated that the expression of E-cadherin would prevent, rather than enhance the development of neoplastic characteristics. It is important to note in this regard that the IOSE-29EC tumors, though invasive and histologically poorly differentiated, exhibited some characteristics of low grade neoplasms. The long latent periods from inoculation to evidence of tumor formation suggest that the IOSE-29EC cells were *potentially* tumorigenic, but that they initially lacked properties required for progressive growth *in vivo* and remained dormant until such properties were acquired. Furthermore, they elicited no desmoplastic reaction and no ascites, which are two of the most common causes of clinical symptoms in advanced ovarian

cancer. It is also reminiscent of the clinical picture of ovarian cancer that, in the present study, the animals appeared healthy and symptom-free even after the tumors were widespread.

When the tumor-derived lines IOSE-29EC/T4 and IOSE-29EC/T5 were reinjected into SCID mice, both lines produced ascites, although IOSE-29EC/T4 produced significantly more ascites than IOSE-29EC/T5. In addition, tumors observed in IOSE-29EC/T4 and IOSE-29EC/T5 injected mice were more widespread and larger than that in IOSE-29EC injected mice. This suggests that passage in vivo may have resulted in further neoplastic progression. It is also interesting to note that these tumor-derived OSE lines have less E-cadherin by immunofluorescence compared to line IOSE-29EC although by Western blot, there seemed to be more (IOSE-29EC/T5) or equal amounts (IOSE-29EC/T4) of E-cadherin protein compared to IOSE-29EC. E-cadherin in the tumor-derived lines may be localized differently and maybe in the cytoplasm instead of being predominantly membrane-bound as in line IOSE-29EC. However, immunofluorescence and immunohistochemical analysis of these tumor-derived lines failed to reveal cytoplasmic E-cadherin staining (data not shown). This may just be due to the limitations of the detection techniques utilized. A way to determine whether E-cadherin is localized in the cytoplasm in these cells would be to do a cell fractionation of the lysates and then run both in Western blots. The downregulation of membrane-bound E-cadherin and production of ascites in the tumor-derived lines indicate that these OSE lines and IOSE-29EC may represent different stages of ovarian carcinogenesis.

Though IOSE-29EC cells and the tumor-derived lines did not form tumors rapidly, most of them clearly remained viable *in vivo* for many weeks before their growth began to cause symptoms. Recent studies have shown that cell-cell contact increases survival in several cell types (Howell and Doane, 1998; Trolice *et al.*, 1997). Kantak and Kramer (1998) demonstrated

that E-cadherin-mediated intercellular adhesion prevents apoptosis and promotes growth of carcinoma cells that are maintained in suspension as aggregates, while single suspended cells die. The authors propose that such intercellular adhesion substitutes for anchorage to substrata, which is required for cell survival and growth. Our experiments did indeed show that the conversion of IOSE-29 cells to IOSE-29EC cells conferred anchorage independence. These results suggest that intercellular adhesion within cell clusters may have contributed to their survival *in vivo*, and that E-cadherin here may have functioned as a survival factor. Recent data by Pece *et al.* (1999) supports this theory by showing that E-cadherin-mediated adherens junctions can activate phosphotidylinositol (PI) 3-kinase, which is known to play a central role in a number of cellular processes, including cell survival (Wymann and Pirola, 1998). In addition, recent data by Schiffenbauer *et al.* (1997) demonstrated that neoplastic ovarian epithelial cells persist *in vivo* but remain as dormant microtumors unless they are induced to produce angiogenic factors, which can be accomplished by increasing circulating gonadotrophin levels. Since OSE cells express gonadotrophin receptors (Zheng *et al.*, 1996), an analogous situation could be envisioned where prolonged cyclical hormonal stimulation in the female hosts of the IOSE-29/EC-derived microtumors may have initiated angiogenesis and, eventually, tumor growth. In this scenario, the nontumorigenic IOSE-29 cells, being anchorage dependent, may have died *in vivo* before further, hormonally-induced, promoting changes took place whereas the prolonged survival of IOSE-29EC cells provided enough opportunity for these changes to occur. It has also been shown in many systems, including rat OSE (Howell and Doane, 1998), that cadherins alter the response of cells to growth factors and other external influences that promote neoplastic progression. It remains to be determined whether IOSE-29 and its E-cadherin-expressing derivatives differ in their responsiveness to hormonal influences, including gonadotrophins.

It is particularly interesting, in relation to the above, that in the clinical situation the E-cadherin-positive serous papillary adenocarcinomas, which make up the majority of ovarian epithelial cancers, disseminate as cohesive papillary cell clusters which detach from the primary ovarian tumors and seed the pelvic cavity. Furthermore, these neoplasms, like the IOSE-29EC-derived tumors, initially may show little invasiveness and remain asymptomatic until they are widespread. In contrast, a less common subset of ovarian epithelial cancers, the endometrioid variety, also express E-cadherin but tend to spread by local invasion within the ovary. Importantly, a high proportion of these endometrioid ovarian carcinomas, but not the other subsets, have mutations in the β-catenin gene, which would be expected to interfere with E-cadherin function and thus to initiate cell dispersion and tumor spread (Palacios and Gamallo, 1998). These clinical features support the concept of a role for E-cadherin in regulating both survival and spread of epithelial ovarian carcinomas in the early stages.

The IOSE-29EC cells express both SV40-Tag-induced and E-cadherin-induced neoplastic properties. Tag inactivates the tumor suppressor proteins Rb and p53 and thereby disregulates the normal controls of proliferation and apoptosis (Wang and Harris, 1997). Abnormal expression of Rb has not been reported in benign and borderline ovarian neoplastic lesions and only rarely in ovarian carcinomas (Taylor *et al.*, 1995). However, p53 is inactivated in a high proportion of epithelial ovarian carcinomas and this defect tends to occur early in ovarian carcinogenesis (Runnebaum *et al.*, 1996). Since E-cadherin expression increases in the course of ovarian carcinogenesis, the unusual combination of inactivated p53 and overexpressed E-cadherin, present in IOSE-29EC and its tumor-derived sublines, is a common occurrence in clinical ovarian epithelial neoplasms.

Our data demonstrate that E-cadherin may be responsible for the initiation of epithelial differentiation, acquisition of anchorage independence, and the promotion of tumorigenicity of OSE, suggesting a causal relationship between E-cadherin expression and neoplastic progression. However, the data do not absolutely resolve whether E-cadherin is the sole mediator of these neoplastic changes. As a matter of fact, it is unknown whether E-cadherin acts like an embryonic inducer in these cells or whether constitutive E-cadherin expression is necessary to maintain these changes. Immortalization with SV40 introduces genetic changes and instabilities that are not present in normal OSE. The presence of these changes makes it unclear whether E-cadherin was solely responsible for the induction of neoplastic properties in OSE or, more likely, if it accomplishes this in conjunction with p53 and RB deactivation. However, our laboratory has now produced a temperature sensitive immortalized OSE cell line (tsIOSE) that can potentially address this problem. The tsIOSE line is an OSE line immortalized with a temperature sensitive Tag protein. Tag is expressed at the permissive temperature of 34 °C, inactivating p53 and p105 RB, but it is not expressed at 37 °C (Leung, 1997). This provides an opportunity to generate an E-cadherin expressing IOSE line that has normal p53 and p105 RB function, thus making it possible to investigate more closely the role of E-cadherin expression in OSE.

There is a remote possibility that the neoplastic properties observed in IOSE-29EC are unique to that cell line and may not be applicable to OSE in general. To address this possibility, our laboratory has recently transfected a second non-tumorigenic SV40-immortalized OSE line, derived from a different woman, with E-cadherin. The anchorage independence and tumorigenicity assays are currently being done and are not yet completed. However, the resulting changes in differentiation markers and morphology in the new line are identical to those of line IOSE-29EC (data not shown). This reproducibility of the results with regards to markers and morphology strongly supports the theory that there is a causal relationship between the

expression of E-cadherin in OSE and its neoplastic progression. To further investigate the relationship between E-cadherin expression and neoplastic progression in OSE, E-cadherin downregulation experiments are currently being designed. The use of antisense oligonucleotides is a good method of achieving specific inhibition of a targeted gene expression (Stein *et al.*, 1991). They are useful in investigating the role of specific molecules in a process and have been used successfully in studying the role of E-cadherin in embryo development (Ao and Erickson, 1992; Chen and Hales, 1995). In this case, administration of oligonucleotides antisense to the E-cadherin cDNA sequence will inhibit gene expression resulting in functional down-regulation of E-cadherin in our transfected and tumor-derived OSE cell lines. This will enable us to better investigate the apparent role of E-cadherin in promoting neoplastic progression and tumorigenicity in human OSE cells.

6. CONCLUSIONS

Similar results in two separate tumorigenicity assays in the present report demonstrate conclusively that the E-cadherin-transfected IOSE-29EC line is tumorigenic. Even prior to inoculation into SCID mice, this line has also shown anchorage independent growth indicating that it is unlikely that this line's increased tumorigenic potential was acquired *in vivo*. In addition, experiments in this report have generated two tumor-derived cell lines (IOSE-29EC/T4 and IOSE-29EC/T5) which, as shown by their increased anchorage independent growth and tumorigenicity, represent later, more advanced stages of ovarian carcinogenesis. This idea is supported by the fact that these lines seem to have a more heterogenous expression of E-cadherin and keratin. Finally, the similarity of our experimentally derived tumors to clinical ovarian tumors leads us to believe that this is the first experimental model of ovarian adenocarcinomas derived by genetic manipulations of normal human OSE. The results provide direct evidence that ovarian carcinomas arise from the OSE and also suggest that E-cadherin expression may play a causal role in ovarian neoplastic progression. The capacity of the E-cadherin-transfected cells to form adenocarcinomas indicates that this adhesion molecule, directly or through other components of the Mullerian phenotype, contributes to the neoplastic conversion of OSE.

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