THE ROLES OF E-CADHERIN AND HEPATOCYTE GROWTH FACTOR IN HUMAN

OVARIAN SURFACE EPITHELIAL PHYSIOLOGY AND PATHOLOGY

By

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ABSTRACT

Epithelial ovarian cancer is the most lethal gynecologic malignancy in the Western world. This neoplasm arises *de novo* from the ovarian surface epithelium (OSE). The OSE is a simple mesothelium which, paradoxically, acquires complex epithelial characteristics in the course of neoplastic progression. The present study investigated the roles of cellular constituents that are known to contribute to epithelial differentiation: E-cadherin and hepatocyte growth factor (HGF). Two experimental culture models were used: (1) OSE from women without (NFH-OSE) and with (FH-OSE) familial histories of ovarian cancer; and (2) OSE at progressive stages of neoplastic transformation, created by sequentially introducing SV40 large T antigen and E-cadherin into normal OSE, which produced lines IOSE-29 and IOSE-29EC. IOSE-29EC was tumorigenic and from the tumor we generated line IOSE-29EC/T4. The data indicate that E-cadherin and HGF receptor Met expression was enhanced and stabilized in FH-OSE and neoplastic OSE, but not in NFH-OSE. Importantly, coexpression of HGF and Met, resulting in constitutive activation of Met, was demonstrated in FH-OSE and ovarian cancer cell lines, suggesting an autocrine HGF-Met activity in these cells. Such an increased autonomy in FH-OSE may enhance the susceptibility of these cells to neoplastic transformation. HGF stimulated growth in normal OSE, IOSE-29 and the ovarian cancer cell line OVCAR-3, but inhibited the growth of IOSE-29EC and IOSE-29EC/T4. HGF induced scattering and branching tubulogenesis in IOSE-29EC and IOSE-29EC/T4 but not in IOSE-29 or OVCAR-3. These different responses to HGF could be related to different levels and kinetics of ERK activation. Increased expression of CK2 and PI3K effectors (Akt2, GSK3β and p70 S6K), downregulation of PKG and upregulation of MEK6 accompanied the neoplastic transformation of OSE. Akt2 and p70 S6K were constitutively phosphorylated in neoplastic OSE, and to a lesser degree in

FH-OSE, but rarely in NFH-OSE. The inhibition of PI3K activity induced apoptosis in OVCAR-3 and metaphase arrest in IOSE-29EC, but had less effect on IOSE-29. These observations, in conjunction with the demonstrated capacity of E-cadherin and HGF to contribute to epithelial differentiation, support the hypothesis that these molecules play a role in the initiation of the aberrant differentiation which characterizes ovarian carcinogenesis.

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LIST OF ABBREVIATIONS

APC   Adenomatous polyposis coli

BCIP   5-bromo-4-chloro-3-indolyl phosphosphate

bFGF   Basic fibroblast growth factor

BSA   Bovine serum albumin

CK2   Casein kinase II

CREB   cAMP-responsive element binding

DIG   Digoxigenin

DMSO   Dimethylsulfoxide

DTT   Dithiothreitol

ECL   Enhanced chemiluminescence

ECM   Extracellular matrix

EGF   Epidermal growth factor

ER   Estrogen receptor

ERK   Extracellular regulated kinase

FBS   Fetal bovine serum

HOG	High osmolarity glycerol
ILK	Integrin-linked kinase
IOSE	SV40-immortalized OSE
ISH	*In situ* hybridization
JNK	Jun N-terminal kinase
JNKK	Jun N-terminal kinase kinase
LEF-1	Lymphoid enhancer factor-1
LOH	Loss of heterozygosity
MAPK	Mitogen activated protein kinase
MAPKAPK2	Mitogen activated protein kinase -activated protein kinase-2
M-CSF	Macrophage-colony stimulating factor
MEK	Mitogen activated protein kinase kinase /ERK kinase
MEKK	MEK kinase
MKK	Mitogen activated protein kinase kinase, MEK
MLK	Mixed lineage kinase
mTOR	Mammalian target of rapamycin
NBT	Nitroblue tetrazolium

NCS   Newborn calf serum

NFH-OSE   OSE from women with no family histories of ovarian cancer

OSE   Ovarian surface epithelium

PAK   p21-activated kinase

PBS   Phosphate buffered saline

PDK   Phosphatidylinositol 3,4-biphosphate-dependent kinase

PHAS-1   pH- and acid-stable protein

PI3K   Phosphatidylinositol 3 -kinase

PKC Ca^{2+}/phospholipid-dependent protein kinase

PKG   cGMP dependent protein kinase

PMSF   Phenylmethylsulfonyl fluoride

Rsk   Ribosomal S6 kinase

RT-PCR   Reverse-transcription polymerase chain reaction

S6K   S6 kinase

S.D.   Standard deviation

SDS-PAGE   Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SF   Scatter factor

SH2   Src homology region 2

STAT   Signal transducers and activators of transcription

SV   Simian virus

TBS   Tris-buffered saline

TCF   T-cell factor

TGFβ   Transforming growth factor β

u-PA   Urokinase type plasminogen activator

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1. INTRODUCTION

1.1. Rationale/Objective

Ovarian cancer is the most lethal gynecologic malignancy in North America. Over 90% of ovarian cancers arise from the ovarian surface epithelium (OSE), which is the pelvic mesothelium that overlies the surface of the ovary. In contrast to carcinomas derived from most epithelia that have lost their capacity to differentiate during neoplastic transformation, OSE-derived carcinomas express Mullerian phenotypes (i.e. the phenotypes of oviductal, endometrial and endocervical epithelia) concomitant with more complex epithelial markers as compared to normal OSE. The high frequency of Mullerian epithelial differentiation in ovarian neoplasia suggests that these changes confer a selective advantage to the transforming OSE. Interestingly, characteristics of Mullerian epithelia are already found in apparently normal OSE from women with histories of familial ovarian cancer, indicating that such aberrant epithelial differentiation may be a very early or predisposing step in ovarian carcinogenesis. These observations motivated the objective of the present study, to define more precisely the molecular basis for increased epithelial differentiation in the neoplastic progression of OSE. Specifically, we tested the hypothesis that E-cadherin and HGF-Met contribute in an important way to Mullerian differentiation in the course of ovarian carcinogenesis.

The specific aims of this study were:

1. Compare the expression of cadherins, HGF and its receptor Met in normal and malignant OSE.

2. Investigate the roles of HGF in normal and (pre)neoplastic OSE and the mechanisms that underlie its activities in these cells.

3. Examine the regulation of a variety of intracellular protein kinases in the neoplastic transformation of OSE.

4. Characterize the contribution of PI3K on OSE cell cycle progression.

1.2. Ovarian surface epithelium (OSE)

1.2.1. Biology of OSE *in vivo*

In the embryo, ovarian surface epithelium (OSE) originates from the coelomic epithelium, which in turn arises from the underlying mesenchyme. During and just before the arrival of primitive germ cells in the gonadal ridge, the coelomic epithelial cells undergo marked proliferation and some of the cells penetrate the underlying stroma. Continued growth of these cells in the cortex gives rise to cortical cords, which subsequently break up into isolated cell clusters, each surrounding one or more primitive germ cells. These surrounding epithelial cells are the pregranulosa cells. Later in the embryonic life, the coelomic epithelium on the lateral surface of the urogenital ridge proliferates and forms several invaginations, which coalesce to form the Mullerian ducts. The paired Mullerian ducts subsequently give rise to the epithelia of the upper genital organs such as the fallopian tube, uterus and endocervix (Parmley, 1987; Barber, 1988). Thus, the embryonic coelomic epithelium in the genital ridge area is competent to differentiate along many different pathways. Adult OSE retains the uncommitted and pleuripotential properties of its embryonic precursor, the coelomic epithelium. The phenotypic plasticity of adult OSE is particularly emphasized by its capacity to undergo epithelio-mesenchymal conversion and to develop along the lines of Mullerian duct derivatives in response to physiological and pathological influences.

Though OSE has an inconspicuous appearance and represents only a diminutive fraction of the diverse cell types that comprise the ovary, this epithelium accounts for 80-90% of all ovarian cancers and plays a key role in normal ovarian physiology. In adult women, the OSE transports materials to and from the peritoneal cavity, and may actively participate in the mechanics of follicular rupture (Motta *et al.*, 1980). OSE produces proteolytic enzymes which are thought to degrade the tunica albuginea and the underlying follicular theca, thereby weakening the ovarian surface to the point of rupture (Bjersing and Cajander, 1975; Kruk *et al.*, 1994). This process is also in part facilitated by apoptosis of OSE at the impending site of follicular rupture (Murdoch, 1995). Ovulation creates a wound at the ovarian surface, and this defect is repaired by proliferation of OSE. These observations suggest that OSE has secretory, regenerative and transport functions, which vary with the reproductive cycle and are likely hormone-dependent (Murdoch, 1995).

OSE is a modified mesothelium that varies from squamous to cuboidal as it follows the contours of the ovary. This mesodermally derived epithelium is characterized by keratin types 7, 8, 18, 19, mucin and apical microvilli and is separated from the underlying stroma by a basal lamina upon the tunica albuginea (van Niekerk *et al.*, 1991). The cells are held together laterally by desmosomes and communicate via gap junctions. The lack of the epithelial differentiation markers E-cadherin and CA125 and the expression of mesenchymal (vimentin) concurrently with epithelial (keratin) intermediate filaments suggest that OSE is not as firmly determined as most other adult epithelia, including epithelia of the oviduct and endometrium (Kabawat *et al.*, 1983; Czernobilsky *et al.*, 1985; Sundfelt *et al.*, 1997; Maines-Bandiera and Auersperg, 1997; Davies *et al.*, 1998). These findings also provide evidence that the close developmental proximity of normal OSE to ovarian stromal fibroblast persist in the adults. During the reproductive life of a woman, invagination of the

surface epithelium into the cortical stroma gives rise to epithelial crypts and inclusion cysts. These structures are considered weakly preneoplastic and may facilitate malignant transformation of entrapped OSE by generating a microenvironment enriched of growth factors, cytokines and hormones (Scully, 1995; Berchuck and Carney, 1997).

1.2.2. Biology of OSE *in vitro*

In spite of the clinical importance of OSE, the study of this epithelium has been limited by the lack of appropriate models. In the past decade, several methods reported permit culture of the OSE of the rat and rabbit. However, their usefulness as models for human ovarian cancer is limited because epithelial ovarian cancer rarely arise in OSE of these animal species (Adams and Auersperg, 1981; Nicosia *et al.*, 1984, 1985). This may well be because the biology of the surface epithelium of these animals is different from that of human. For example, rodent OSE expresses the epithelial differentiation marker E-cadherin (Hoffman *et al.*, 1993), and shows spontaneous malignant transformation in long-term cultures (Godwin *et al.*, 1992, 1993), which never happens in human OSE. Such differences may alter the response of cells to their environment and the sequence of molecular events in the pathogenesis of ovarian cancer.

In 1984, Auersperg *et al.* developed the culture method for human OSE (Auersperg *et al.*, 1984). Human OSE cells typically form cobblestone epithelial monolayers in culture and assume flattened epithelial or atypical, fibroblast-like forms with time and passages (Auersperg *et al.*, 1984; Kruk *et al.*, 1990; Nakamura *et al.*, 1994). By analogy, this epithelio-mesenchymal conversion of OSE *in vitro* may mimic the regenerative responses that occur *in vivo* following ovulatory ruptures during which OSE cells disperse, migrate, and proliferate. Cultured OSE lacks desmoplakin I and II that are found in OSE *in vivo*. OSE

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cells express both epithelial and mesenchymal intermediate filament proteins, which are keratin and vimentin (Auersperg et al., 1994). OSE is capable of depositing and reconstructing extracellular matrix (ECM). It produces not only epithelial (laminin and collagen IV) but also mesenchymal (collagen I and III) components of ECM. The production of mesenchymal ECMs is acquired by OSE in culture, which may facilitate the cells to modulate from an epithelial to a mesenchymal morphology in response to environmental cues (Auersperg et al., 1994). OSE cells also secrete chymotrypsin-like and elastase-like peptidases, metalloproteases and plasminogen activator inhibitor, which may facilitate ovulatory rupture in vivo (Kruk et al., 1994). OSE expresses integrins which bind to laminin, collagens, fibronectin and vitronectin, and these interactions may play a role in the adhesion, spreading, proliferation and matrix reconstitution of normal OSE following postovulatory ruptures (Kruk et al., 1994; Cruet et al., 1999). c-jun is present in both dividing OSE in culture and resting OSE in vivo. Given that c-jun is an immediate early gene associated with cell proliferation, its presence in resting OSE suggests that this protein may be post-translationally activated upon mitogenic stimulation and play a role in the post-ovulation wound repair of the ovarian surface (Neyns et al., 1996).

1.2.3. Endocrine, paracrine and autocrine factors

Human OSE has only a limited life span in culture (Kruk et al., 1990; Nakamura et al., 1994). In vitro studies indicate that growth factors, cytokines and gonadotropins can modulate growth of human OSE, and these factors may be involved in the growth regulation of normal OSE in vivo. Early passage OSE has been shown to proliferate in response to epidermal growth factor (EGF), suggesting that EGF may play a role in the rapid repair of the ovulatory defect (Siemens and Auersperg, 1988). In SV40-immortalized OSE (IOSE),

EGF promotes survival but not proliferation of these cells, and that is mediated through a mitogen-activated protein (MAP) kinase pathway (McClellan *et al.*, 1999). Normal OSE cells also produce amphiregulin, which has homology with EGF and appears to control cell proliferation under normal conditions. Consistent with responsiveness to EGF, normal OSE cells express EGF receptor but little or no of the related HER-2/neu receptor (Rodriguez *et al.*, 1991). Hepatocyte growth factor (HGF) stimulates proliferation of cultured bovine and human OSE (Parrott and Skinner, 2000). Basic fibroblast growth factor (bFGF) stimulates the proliferation of rabbit OSE and maintains the viability in cultured rat OSE (Pierro *et al.*, 1996; Gulati and Peluso, 1997; Trolice *et al.*, 1997). The latter function involves alterations in intracellular calcium levels, and can be mimicked by N-cadherin-mediated intercellular adhesion, or counteracted by HGF (Gulati and Peluso, 1997; Trolice *et al.*, 1997). In contrast to most other growth factors, TGFβ acts as an autocrine growth inhibitory factor in normal OSE and also counteracts the growth-stimulatory effect of EGF (Berchuck *et al.*, 1992; Vigne *et al.*, 1994). This may account for the stationary nature of OSE *in vivo*. OSE has the capability to secrete bioactive cytokines, including interleukin-1 (IL-1) and IL-6, macrophage-colony stimulating factor (M-CSF), granulocyte CSF (G-CSF), and granulocyte-macrophage CSF (GM-CSF) (Ziltener *et al.*, 1993). These cytokines stimulate proliferation of normal OSE (Berchuck *et al.*, 1993). Studies of human ovaries have identified both estrogen receptor (ER)-α and ER-β in normal OSE (Brandenberger *et al.*, 1998; Hillier *et al.*, 1998). Though normal OSE expresses estrogen and progesterone receptors, neither 17β-estrogen nor progesterone has any proliferative effect on these cells (Karlan *et al.*, 1995). Glucocorticoids are mitogenic in normal OSE (Siemens and Auersperg, 1988; Karlan *et al.*, 1995). OSE cells also have receptors for gonadotropins and gonadotropin-releasing hormone (GnRH). While gonadotropins, such as human chorionic

gonadotropin (hCG), luteinizing hormone (LH) and follicle-stimulating hormone (FSH), stimulate growth in OSE, GnRH has a growth-inhibitory effect (Kang *et al.*, 2000).

1.3. OSE and familial ovarian cancer

1.3.1. Familial ovarian cancer

The hereditary ovarian cancer syndromes are the most well acknowledged risk factor for the development of ovarian cancer. The lifetime risk triples to 5% for women with one affected first-degree relative (mother, sister or daughter), and the risk may be as high as 40% for those with two or more affected relatives. These women also tend to develop the disease at a younger age than the general population. There are three main hereditary ovarian cancer syndromes, which are consistent with the inheritance of a mutant copy of one of the tumor suppressor genes. The most common form (65-75%) is the hereditary breast/ovarian cancer syndrome, which includes families whose female members have had ovarian and breast cancer over multiple generations (Miki *et al.*, 1994), which appears to be linked to the BRCA1 locus on chromosome 17q. Germline mutations in the BRCA1 gene have been described in the majority of families predisposed to develop familial breast/ovarian cancers, implicating that BRCA1 is a bonafide tumor suppressor gene. Most of the mutations are loss-of-function nonsense or frameshift alterations, which result in the loss of protein synthesis or production of a truncated protein. Though the biological functions of the protein are yet unclear, the expression pattern of BRCA1 during mouse development, and the cell cycle- and hormone-regulated expression of BRCA1 suggest a relationship with differentiation and cell proliferation (Marquis *et al.*, 1995; Gudas *et al.*, 1995, 96). Mutational inactivation of BRCA1 might thus be expected to affect the expression of other genes, involved presumably in the regulation and growth or differentiation in breast

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and ovarian epithelium. Moreover, a role for BRCA1 in conjunction with the RAD51 protein has been implicated in the repair of double-strand DNA breaks, suggesting that an additional function may lie in the maintenance of genomic stability (Scully *et al.*, 1997). Most of the breast/ovarian cancer families that are not linked to BRCA1 are linked to the newly described BRCA2 (Wooster *et al.*, 1994; Takahashi *et al.*, 1996). Unlike BRCA1, inherited mutations in BRCA2 appear to contribute to ovarian cancer with a lower penetrance. Less frequently (10-15%), ovarian cancer occurs as part of the hereditary non-polyposis colorectal cancer (HNPCC) or Lynch II syndrome, which arises from an inherited defect in MSH2 (chromosome 2p), MLH1 (chromosome 3p), PMS1 (chromosome 2q) or PMS2 (chromosome 7p). The proteins encoded by these genes participate in the same DNA mismatch repair pathway, and loss-of-function mutations are associated with genetic instability of affected family members. HNPCC kindred are also at increased risk for cancers other than of the ovary, such as colon and endometrium (Lynch *et al.*, 1993). Hereditary site-specific ovarian cancer is the least common familial syndrome. Our knowledge of this syndrome is relatively limited. Prophylactic oophorectomy is currently the main method to prevent ovarian cancer. In addition, oral contraceptives, even if taken for 5 years, reduce the incidence of ovarian cancer.

1.3.2. OSE from women with familial ovarian cancer (FH-OSE)

Characteristics of preneoplastic phenotypes were initially demonstrated by studies of ovaries from identical twins where one of each pair had an invasive carcinoma of the ovary (Gusberg and Deligdisch, 1984) and contralateral ovaries from women with unilateral ovarian cancer (Bell and Scully, 1994; Mittal *et al.*, 1993). In those ovaries, tubal metaplasia with severe dysplasia was present on the surface epithelium or its inclusion cysts. A non-

blind study further indicated that ovaries from women with hereditary ovarian cancer syndromes demonstrate increased papillomatosis and pseudostratification of the OSE, as well as an increase in inclusion cysts and invaginations than in ovaries removed for other indications (Salazar et al., 1996). In another, blind, study, nuclear changes were observed in the OSE of such women (Werness et al., 1999). On the other hand, two other blind comparisons between carriers and non-carriers reported no recognizable difference of histologic features of the ovarian epithelium (Sherman et al., 1999; Stratton et al., 1999). Thus, there is still conflicting evidence as to whether there are histologically recognizable precancerous lesions in ovarian specimens from women who have inherited the predisposing mutations in BRCA1 or BRCA2 genes.

Our laboratory compared cultured normal OSE of the general population (NFH-OSE) to overtly normal OSE from women with family histories of ovarian cancer (FH-OSE) obtained at prophylactic oophorectomies. Our data showed that some epithelial characteristics that are present in Mullerian epithelia and ovarian cancer cells but are often lacking or weakly expressed in normal OSE are already found in cultures of FH-OSE. These phenotypic alterations include enhanced and a more stable CA125 production, a higher proportion of keratin-positive cells concomitant with a significantly reduced production of the stromal marker collagen III in late passages (Auersperg et al., 1995; Dyck et al., 1996). In addition, an increased commitment to an epithelial morphology on plastic and in three-dimensional sponge matrix is demonstrated in FH-OSE, but not in OSE from women with no such family histories (NFH-OSE) (Dyck et al., 1996). Furthermore, an increased telomeric instability and a shorter telomere length are observed in FH-OSE (Kruk et al., 1999), suggesting an increased genetic instability in these cells which may enhance the susceptibility of FH-OSE to neoplastic transformation. Together, the identification of

phenotypic differences of FH-OSE secondary to genetic predisposition suggests that other changes precede the loss of both copies of BRCA1 gene. These findings are not only of great importance for women in these affected families who are at high risk of developing the malignancy, but also have wider implications for the understanding of the epigenetic mechanisms underlying sporadic ovarian cancer.

1.4. Epithelial ovarian cancer

1.4.1. Overview

Ovarian cancer causes more deaths than any other cancers of female reproductive organs. It ranks the fifth most common cause of cancer deaths in North America behind lung, breast, pancreas and colorectal cancers. Ovarian cancer affects 28,000 women every year in Canada and the United States, and approximately 15,000 women will die of this neoplasm annually (Landis *et al.*, 1999). The main reasons for this poor prognosis are (1) the notorious subtle and non-specific early symptoms and (2) the absence of an effective molecular marker for early detection. Currently, the most widely used ovarian tumor marker is CA125, which is elevated in 80% of cases of ovarian cancer, but unfortunately, in only 30-50% of stage I cases and often in the presence of other neoplasms and non-neoplastic disorders. It is therefore not surprising that over two thirds of patients have cancers spread beyond the ovary at the time of diagnosis. Among these patients, only 10-20% can be cured with currently available therapy. However, if the tumor is still confined to the ovaries, the chances of survival increase to 80-90%. In spite of its clinical importance, the early events in the development of ovarian cancer are poorly understood.

The etiology of ovarian cancer, as in other human cancers, is multifactorial and not completely understood. Environmental agents including high fat diet, talc, asbestos,

smoking and infectious agents have been implicated. Moreover, social and geographic factors have also been emphasized. "Incessant ovulation" has been postulated to be etiologically related to the development of ovarian cancer (Fathalla, 1971) because the postovulatory proliferation of OSE may increase the frequency and accumulation of spontaneous mutations which are major factors *en route* to transformation. It has also been suggested that ovulation may increase the risk of ovarian cancer because of exposure of the epithelium to high levels of steroid hormones and gonadotropins. Thus, the use of oral contraceptives, along with pregnancy and breast-feeding, have protective effects on ovarian cancer, while nulliparous women, infertile women and women who have early menarche and late menopause are at higher risk of developing the disease. Concomitantly, ovarian cancer is rare in women with Turner's syndrome, who are anovulatory (Berchuck and Carney, 1997).

Epithelial ovarian cancers account for over 90% of all ovarian neoplasms. Of these, about 80% are serous adenocarcinomas and are thought to arise *de novo* from the OSE and its epithelial inclusion cysts (Scully, 1995). The implication of OSE as the source of epithelial ovarian cancer was based mainly on the histo-pathological examination of clinical lesions. Recently, our laboratory provided the first direct experimental proof that normal human OSE has the capacity to give rise to ovarian epithelial adenocarcinomas (Auersperg *et al.*, 1999; Ong *et al.*, 2000). It has also been proposed that ovarian epithelial cancer may derive from cell lineages other than the OSE. Common molecular markers and the presence of synchronous ovarian and gastric intestinal mucinous tumors suggests that mucinous tumors of the ovary may represent metastases of gastric intestinal tumors to the ovary (Young *et al.*, 1991; Peralta Soler *et al.*, 1997). It has also been suggested epithelial ovarian carcinomas may arise in the rete ovarii (Dubeau, 1999). Endometriosis, which is a

common pathological condition characterized by the migration of endometrial-like tissues outside the uterus, is thought to be the precursor of endometrioid and clear cell ovarian carcinomas. The arguments in favor of this idea are 1) tubal ligation reduces the risk of ovarian cancer and 2) common genetic alterations found in endometrioid ovarian carcinomas and the adjacent endometriotic lesions (Jiang *et al.*, 1996, 1998). An unusual aspect of ovarian carcinogenesis is the progression from a simple, rather primitive epithelium to a more highly differentiated phenotype that acquires characteristics of oviductal and endometrial epithelia, which is lost again only in late stage carcinomas. Histologically, depending on their resemblance to Mullerian duct derived epithelium, the epithelial ovarian carcinomas are classified as serous (40%) (oviduct-like), endometrioid (20%) (uterine-like), and mucinous (10%) (endocervix-like) adenocarcinomas, as well as several less common types. Similar to these epithelia, differentiated ovarian carcinomas express high molecular weight keratins, CA125 and E-cadherin, possess cilia and form glands and papillae but lack the mesenchymal markers that are present in normal OSE (reviewed in Auersperg *et al.*, 1998). In culture, ovarian carcinoma cells retain an epithelial morphology and express CA125 indefinitely, which signifies a determined epithelial phenotype (Auersperg *et al.*, 1995). This is in contrast to carcinomas arising from epithelia of most other tissues where neoplastic progression is accompanied by a progressive loss of differentiation.

1.4.2. Endocrine, paracrine and autocrine factors

While the secretion of growth factors and cytokines is a function of normal OSE, their escape into dysregulated autocrine loops may be important to the development and progression of ovarian cancer. Autocrine growth stimulation of CSF-1 through the *fms*

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receptor has been found in some ovarian cancers (Bast *et al.*, 1995). The EGF receptor has been retained in some malignant OSE, and overexpression of the EGF receptor and the related HER-2/neu receptor in ovarian tumor tissues are often associated with poor prognosis (Slamon *et al.*, 1989; Rodriguez et al., 1991). Most ovarian cancer cell lines have also lost the ability to produce, activate or respond to TGFβ (Berchuck *et al.*, 1992), which normally act as an autocrine growth inhibitory factor in normal OSE. Estrogen appears to be mitogenic in some ovarian cancer cell lines (Nash *et al.*, 1989). These ovarian carcinoma cells that retain estrogen receptor expression are exquisitely sensitive to anti-estrogen (tamoxifen) treatment (Nash *et al.*, 1989). Development of ovarian cancers has been related to excessive gonadotropin production associated with the onset of the menopause or the use of fertility drugs during assisted fertilization. Receptors for gonadotropic hormones have been localized to ovarian cancer cell lines. These findings provide a possible explanation for the increased risk of developing ovarian cancer in postmenopausal women who are known to have high circulating levels of gonadotropins. GnRH has growth inhibitory effect on ovarian cancer cells, suggesting that GnRH analogs may provide a possible therapeutic approach in this neoplasm (Kang *et al.*, 2000).

1.4.3. Altered gene functions

Aberrant gene expression and signal transduction are observed in a variety of human cancers. For example, inappropriate expression of HER2/neu receptor and the macrophage colony stimulating factor receptor (*c-fms*) has been implicated in the altered cellular signaling found among ovarian cancers (reviewed in Bast *et al.*, 1998). Signaling molecules such as the *ras* oncogene are mutated in approximately half of all tumors and in over 30% of epithelial ovarian cancer cells. Mutated *ras* genes may lead to alterations in the

control of cell growth. While the pp60$^{c\text{-}Src}$ protein kinase is expressed in normal OSE, both the Src protein level and its kinase activity are significantly increased in some late-stage ovarian cancer cells. Studies with an antisense *c-src* construct suggest that activated Src protein kinase is involved in ovarian tumor growth and maintenance (Wiener *et al.*, 1999). The immediate early genes *c-jun* and *jun-B* genes are highly expressed in a subset of human ovarian tumors that have a more undifferentiated, aggressive phenotype and in most ovarian cancer cell lines. It has been suggested that tumors with a high *c-jun* content tend to be diagnosed at an earlier stage than their *c-jun* negative counterparts and respond better to platinum-based chemotherapy (Neyns *et al.*, 1996). Amplification and overexpression of the *c-myc* gene in ovarian cancer cell lines and tumor biopsy specimens have also been shown. This amplification is more common in serous adenocarcinoma and associated with advanced ovarian cancer, suggesting a role in the neoplastic progression. Activated PI3K has been implicated in ovarian cancers. The *PIK3CA* gene, which encodes the p110α catalytic subunit of PI3K, is amplified in approximately 40% of ovarian cancers (Shayesteh *et al.*, 1999). The overexpression of p110α is associated with increased p110-p85 heterodimer formation, and a resulting increase in PI3K activity. A downstream effector of PI3K, Akt2, is also overexpressed in 10-20% ovarian carcinomas. This appears to be especially frequent in undifferentiated ovarian tumors and is associated with a poor prognosis (Cheng *et al.*, 1992; Bellacosa *et al.*, 1995). Mutations in the PTEN/MMAC1 gene, a negative regulator of PI3K, occur predominantly, if not exclusively, in ovarian tumors of endometrioid origin with a 26% frequency (Ali *et al.*, 1999). Inactivation of this protein may contribute to PI3K-mediated proliferation and invasiveness in some human ovarian cancers. Treatment with the PI3K inhibitor LY294002 decreases proliferation and increases apoptosis of many ovarian cancer cell lines *in vitro* and inhibits dissemination of

ovarian cancer cells to peritoneal surfaces and the development of ascites *in vivo* (Shayesteh *et al.*, 1999; Hu *et al.*, 2000). Thus, the increased PI3K activity may allow ovarian epithelial cells to avoid apoptosis and to survive to evolve into a more malignant phenotype.

Mutations in cell cycle regulators are common in human cancers. p53 functions as a surveillance mechanism in which cells that have undergone genetic damage are arrested in the G1 phase of the cell cycle to allow for DNA repair. Loss of p53 function, including allelic losses and mutations of the p53 gene, has been described in 10-15% of early-stage and 40-50% of advanced stage ovarian cancers (Berchuck *et al.*, 1994). Cyclin D1 and cyclin E are frequently overexpressed in ovarian carcinomas (Masciullo *et al.*, 1997; Marone *et al.*, 1998). Cyclin-dependent kinase inhibitors (CKI) are also altered in ovarian cancer; loss of p21 (Barboule *et al.*, 1995), p16 (Milde-Langosch *et al.*, 1998) and p27 (Masciullo *et al.*, 1999) expression has been reported in 20-25% of ovarian carcinomas.

1.5. Cadherins in OSE and epithelial ovarian cancer

1.5.1. E-cadherin

Cadherins are a family of calcium-dependent intercellular adhesion molecules that mediate selective cell-cell adhesion through their association with catenins. These changes in adhesive function accompany development and remodeling of a variety of tissues and organs, including the ovary. E-cadherin is expressed by almost all epithelia and plays crucial roles in maintaining the morphological and functional integrity of these tissues. In addition, E-cadherin has the capacity to induce epithelial differentiation in embryonic and adult mesenchymal cells (Hay, 1995). Immunoreactive E-cadherin was observed in isolated rat and porcine OSE cells (Hoffman *et al.*, 1993; Ryan *et al.*, 1996), and in human oviductal,

endometrial and endocervical epithelia (van der Linden *et al.*, 1994, 1995), but in OSE of human ovaries only when cell shapes approach those of metaplastic epithelium (Sundfeldt *et al.*, 1997; Davies *et al.*, 1998; Maines-Bandiera and Auersperg, 1997). This suggests that conditional expression of E-cadherin in human OSE may be a trigger for neoplastic transformation or an indicator of other underlying preneoplastic changes. E-cadherin is present at high levels in dysplastic lesions and in well-differentiated ovarian carcinomas, and diminishes in only some, but not all, highly metastatic ovarian tumor cells and ascites (Veatch *et al.*, 1994; Darai *et al.*, 1997; Maines-Bandiera and Auersperg, 1997; Sundfeldt *et al.*, 1997; Davies *et al.*, 1998). In parallel, β-catenin expression levels are decreased in metastatic lesions of ovarian carcinomas (Fujimoto *et al.*, 1997; Davies *et al.*, 1998). This is in contrast to most epithelia where E-cadherin is present at highest levels in normal epithelia, persists to lesser degrees in differentiated carcinomas, and becomes down-regulated or non-functional in undifferentiated, invasive tumors (Takeichi, 1993; Birchmeier, 1995). This paradoxical observation is related to the tendency of OSE-derived carcinomas to acquire more complex epithelial characteristics than those of normal OSE. This is in contrast to the neoplastic transformation of epithelia at most organ sites which is characterized by a loss of the capacity to differentiate. Recently, Auersperg *et al.* demonstrated that the constitutive expression of E-cadherin in SV40-immortalized OSE induces epithelial markers that are associated with ovarian neoplasia and tumorigenicity in SCID mice (Auersperg *et al.*, 1999; Ong *et al.*, 2000). Thus, E-cadherin may function as an inducer of Mullerian epithelial differentiation in the course of OSE transformation. In keeping with such a causative role of E-cadherin in ovarian cancers, mutations of the E-cadherin gene or LOH of chromosome 16q are rare in these neoplasms. Of a series of 63 ovarian

tumors, only one somatic missense mutation and one LOH were reported (Risinger *et al.*, 1994).

1.5.2. N-cadherin

Like other cells of mesodermal origin, including renal tubular cells and mesothelial cells, the coelomic epithelium-derived OSE and granulosa cells are primarily connected by N-cadherin. In the ovary, N-cadherin not only operates as an adhesion molecule but also regulates some physiological functions of ovarian tissues, including the control of apoptosis of rat OSE and granulosa cells (Gulati and Peluso, 1997). The expression of N-cadherin persists in most benign and borderline ovarian tumors, and is heterogenously coexpressed with E-cadherin or absent in ovarian carcinomas. Serous and endometrioid ovarian carcinomas express both N- and E-cadherin while mucinous ovarian tumors only express E-cadherin (Peralta Soler *et al.*, 1997). It has been proposed that the coexpression of E- and N-cadherin characterize epithelial differentiation of cells of mesodermal origin, such as in renal cell carcinomas (Shimazui *et al.*, 1996). Unlike ovarian carcinomas, mesotheliomas express only N-cadherin (Peralta Soler *et al.*, 1995). This difference illustrates that the propensity to Mullerian epithelial differentiation is a unique feature characteristic of tumors that arise from ovarian mesothelial cells, and is not seen in tumors that arise from extraovarian mesothelium. In contrast to E-cadherin, N-cadherin does not appear to act as a suppressor of tumor invasion. Instead, N-cadherin is often expressed in place of E-cadherin in development and tumor progression of squamous cell carcinoma, and is associated with a more motile and invasive phenotype (Islam *et al.*, 1995).

1.5.3. P-cadherin

P-cadherin was originally identified in mouse placental tissue as a molecule that appeared to act as a connector between the embryo and uterus. In the human, P-cadherin is expressed in stratified squamous, pseudo-stratified and transitional epithelia but not in simple epithelia (Shimoyama *et al.*, 1989). P-cadherin is present in the epithelia of Mullerian duct derivatives (van der Linden *et al.*, 1994, 1995). In some tissues such as gastric epithelium, P-cadherin diminishes with neoplastic progression, but appears in carcinomas of others, such as lung, where it is not expressed in the normal counterpart (Shimoyama *et al.*, 1989).

1.5.4. β-catenin

Cadherin has been implicated as an important indirect regulator of gene expression through its interaction with β-catenin which participates in Wnt signaling. In normal cells, adenomatous polyposis coli protein (APC) (Rubinfeld *et al.*, 1993; Su *et al.*, 1993) and the RGS domain protein axin (Zeng *et al.*, 1997; Behrens *et al.*, 1998) serve as scaffolds, binding both β-catenin and the glycogen synthase kinase (GSK3β) (Rubinfeld *et al.*, 1996). This facilitates the serine and threonine phosphorylation of β-catenin, which promotes ubiquination and the subsequent degradation in proteosomes. Mutation or deletion of serine and threonine residues of β-catenin results in highly stable forms of β-catenin that are constitutively active. In the nucleus, the binding of β-catenin to transcription factors of the lymphoid enhancer factor-1 (LEF-1)/T-cell factor (TCF) family may displace co-repressors from LEF/TCF (Morin *et al.*, 1997; Rubinfeld *et al.*, 1997). This activates regulators of cell growth and proliferation, c-myc and cyclin D1 (He *et al.*, 1998; Shtutman *et al.*, 1999), and the genes for metalloproteinase matrilysin and fibronectin (Gradl *et al.*, 1999; Crawford *et*

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al., 1999), which are regulators of cell migration and invasion. These findings suggest that β-catenin nuclear signaling may underlie growth regulation as well as the invasive phenotype in human tumors. The importance of these relationships is highlighted by recent findings that these residues are often mutated in a variety of human cancers, including that of the ovary. Palacios and Camallo (1998) have reported mutations of the β-catenin gene predominantly, if not exclusively, in endometrioid ovarian cancers. APC was originally identified as a tumor suppressor protein in colorectal cancer. However, mutation of APC is not an important event in ovarian carcinogenesis (Allan *et al.*, 1994).

1.6. Hepatocyte growth factor (HGF) and its receptor Met in OSE and epithelial ovarian cancer

1.6.1. Structure and function of HGF

Hepatocyte growth factor (HGF), identical to scatter factor (SF), is a secretory glycoprotein. It is encoded as an inactive single chain precursor, which is converted to the mature, heterodimer form by extracellular proteolytic processing, and yields two polypeptide chains, the 69 α-chain and 34 kDa β-chain (Nakamura *et al.*, 1987, 1989; Miyazawa *et al.*, 1989). This processing is activated by proteases in response to tissue damage and tumor progression (Miyazawa *et al.*, 1996). It is interesting to note that although HGF shares significant amino acid sequence identity with that of plasminogen, HGF does not possess serine protease activity. This is due to amino acid substitution within the catalytic domain of serine proteases (Nakamura *et al.*, 1989; Donate *et al.*, 1994).

HGF is a multipotent growth factor affecting growth, motility, morphogenesis and angiogenesis in a variety of cell types, but not fibroblasts. It is a strong mitogen for epithelial cells, melanocytes and endothelial cells, but is cytotoxic for several cancer cell lines. HGF is

also a potent stimulator of cell motility, which is an important process during development, wound healing, tumor invasion and metastasis. Such activity depends on changes in adhesive properties among cells, modification of ECM, and a concomitant induction of cell movement. To modulate cadherin-mediated adhesion at zonulae adherens, HGF may either inhibit the assembly of adherens junctions (Pasdar et al., 1997) or promote the dismantling of cadherin-catenin adhesion complexes (Watabe et al., 1993; Shibamoto et al., 1994; Tannapfel et al., 1994). Tyrosine phosphorylation of β-catenin, and perhaps cadherins, has been shown to be possible mechanisms in the disruption of cadherin-mediated adhesion at zonulae adherens and desmosomes (Watabe et al., 1993; Shibamoto et al., 1994; Tannapfel et al., 1994). Also, HGF alters intercellular communications mediated by gap junctions and inhibits association of tight junctions (Moorby et al., 1995; Ikejima et al., 1995). HGF is capable of modifying cell-ECM adhesion by stimulating the expression of integrins and the subsequent recruitment of integrins, cytoskeleton proteins, pp125 focal adhesion kinase (FAK) and paxillin to focal adhesions, and in addition downregulating fibronectin expression (Matsumoto et al., 1994; Jiang et al., 1996). Concomitant with these changes, there is an increased expression of motor proteins, such as myosin II, kinesin, myosin Iα and α-tubulin, which facilitate cell migration (Torok et al., 1996). HGF has also been shown to modulate genes in basement membrane invasion, including urokinase type plasminogen activator (u-PA) and matrix metalloproteinases, (Jeffers et al., 1996; Pepper et al., 1992; Grant et al., 1993). Importantly for this study, HGF has the capacity to induce epithelial morphogenesis. HGF induces the outgrowth of tubular structures of kidney and mammary epithelial cells in three-dimensional cell culture models, which are reminiscent of renal tubule and mammary gland differentiation in development (Montesano et al., 1991). Inappropriate expression of HGF-Met in NIH3T3 fibroblasts and certain cancer cell lines

induces the formation of cystic structures with enhanced epithelial differentiation, which mimic preneoplastic ovarian lesions (Tsarfaty et al., 1992, 1994).

1.6.2. HGF-Met in OSE and epithelial ovarian cancer

HGF and Met are principal mediators of paracrine epithelial-mesenchymal interactions. During mouse development, HGF is produced by the mesenchyme at the urogenital region in the vicinity of Met-expressing epithelia, suggesting that the development and morphogenesis of urogenital organs, including the ovary, depend on a paracrine regulation of HGF-Met (Sonnenberg et al., 1993). In the adult ovary, Met persists in the coelomic epithelium-derived OSE, granulosa cells and Mullerian epithelia (Prat et al., 1991; Di Renzo et al., 1994; Moghul et al., 1994; Parrott et al., 1994; Negami et al., 1995; Huntsman et al., 1999). Normal mesothelial cells, which share a common embryological origin and anatomical environment with OSE lack HGF and Met (Klominek et al., 1998). This suggests that Met expression might be a feature characteristic of coelomic epithelium derivatives at the urogenital ridge through local differentiation.

Immunohistochemical studies have localized expression of HGF to bovine, rat and human OSE (Wolf et al., 1991; Parrott and Skinner, 2000). The physiological influence of HGF on OSE depends on the presence or absence of a basement membrane component, fibronectin (Hess et al., 1999). For example, in rat OSE, HGF decreases N-cadherin-mediated cell contacts, increases intracellular calcium concentration, and ultimately induces apoptosis in vitro if these cells are cultured on plastic (Gulati and Peluso, 1997). On the other hand, HGF is mitogenic when rat OSE cells are plated on a fibronectin-like extracellular matrix (RGD peptide) (Hess et al., 1999). These modulations may facilitate rupture at the apex of the ovulatory follicle before ovulation, and the repair process at the

wound site following ovulation *in vivo*. These effects of HGF on OSE appear to be regulated by estrogen and gonadotropins *in vivo* (Liu *et al.*, 1994; Negami *et al.*, 1995).

While a paracrine interaction of HGF-Met is important in normal ovarian physiology, the generation of an autocrine HGF-Met loop has been implicated in tumorigenic transformation and progression (reviewed in Jeffers *et al.*, 1996). We and others have shown that Met is expressed at increased levels in differentiated ovarian carcinomas compared with normal OSE (Di Renzo *et al.*, 1994; Moghul *et al.*, 1994; Huntsman *et al.*, 1999). Recently, Sowter *et al.* (1999) demonstrated higher levels of HGF in malignant ovarian cystic and ascitic fluid from women with ovarian carcinomas than in peritoneal fluid of normal women, suggesting that HGF-Met may regulate in an autocrine manner in ovarian cancer cells. Recombinant HGF stimulates ovarian cancer cell proliferation and motility, and may contribute to the peritoneal dissemination of cancer cells during tumor progression (Corps *et al.*, 1997; Ueoka *et al.*, 2000).

1.6.3. Met signaling

HGF binds to heparin with low affinity and high capacity (Rosen *et al.*, 1989). These binding sites could probably act by retaining HGF in the pericellular space, and perhaps participate in Met activation. The biological activities of HGF are mediated through its receptor encoded by the *c-met* protooncogene, which is initially synthesized as a single-chain of 170-kDa precursor (Bottaro *et al.,* 1991). After glycosylation and proteolytic cleavage, the resulting dimer is composed of two chains of 50 kDa (α) and 145 kDa (β). There are three functionally distinct domains in the Met receptor: an extracellular ligand binding domain, comprising the 50 kDa α-chain and the N-terminal portion of the 145 kDa β-chain; a single transmembrane anchoring segment; a cytoplasmic domain contributed by

the C-terminal portion of the 145 kDa β-chain. The latter contains a tyrosine kinase domain, which groups Met as a member of the receptor tyrosine kinase family. Upon HGF binding, the Met receptor dimerizes and becomes phosphorylated on tyrosines located in the cytoplasmic region of the β-chain. Tyrosine 1349 and 1356 then act as specific docking sites for the binding and activation of intracellular signal transducers containing the Src homology region 2 (SH2) domains, such as phosphatidylinositol 3-kinase (PI3K), the Grb2/SOS complex, Ras GTPase activating protein (GAP), and phospholipase Cγ. Binding of different adaptor proteins mediates distinct biological activities of HGF.

<u>1.6.3.1. Signaling effectors</u>

a. The phosphatidylinositol 3-kinase (PI3K) pathway

Inositol phospholipids play a major role in signal transduction. As shown in Fig. 1, PI3K family lipid kinases phosphorylate the membrane lipid inositol at the D3 position, yielding PI 3-phosphate (PI(3)P), PI 3,4-biphosphate (PI(3,4)P_2) and PI 3,4,5-triphosphate (PI(3,4,5)P_3). These lipids can recruit cytoplasmic signaling proteins to the plasma membrane by binding to their PH domains and activate several protein kinases, such as several isoforms of PKC, in particular the ε, λ, δ and ζ. Although wortmannin can block receptor activation of PKC-ζ and PKC-λ in some cells, the connection between PI3K and PKC enzymes remains somewhat controversial. In 1995, Akt was identified as the first bonafide downstream target of PI3K. The PI3K/Akt pathway is important in the prevention of apoptosis by phosphorylating Bad, a death-promoting member of the Bcl-2 family. Phosphorylation of Bad by Akt creates a binding site for a member of the 14-3-3 protein family. Once bound to 14-3-3, Bad cannot dimerize with Bcl-2 or Bcl-x_L and neutralize their

FIGURE 1 - Schematic model for the signal transduction pathways regulated by growth factors and cytokines. Stimulation of a receptor activates the phosphatidylinositol 3-kinase (PI3K), Ras-Raf-mitogen activated protein kinase (MAPK), Jun N-terminal kinase (JNK) and p38 MAPK pathways as illustrated.

Signal Transduction Pathways
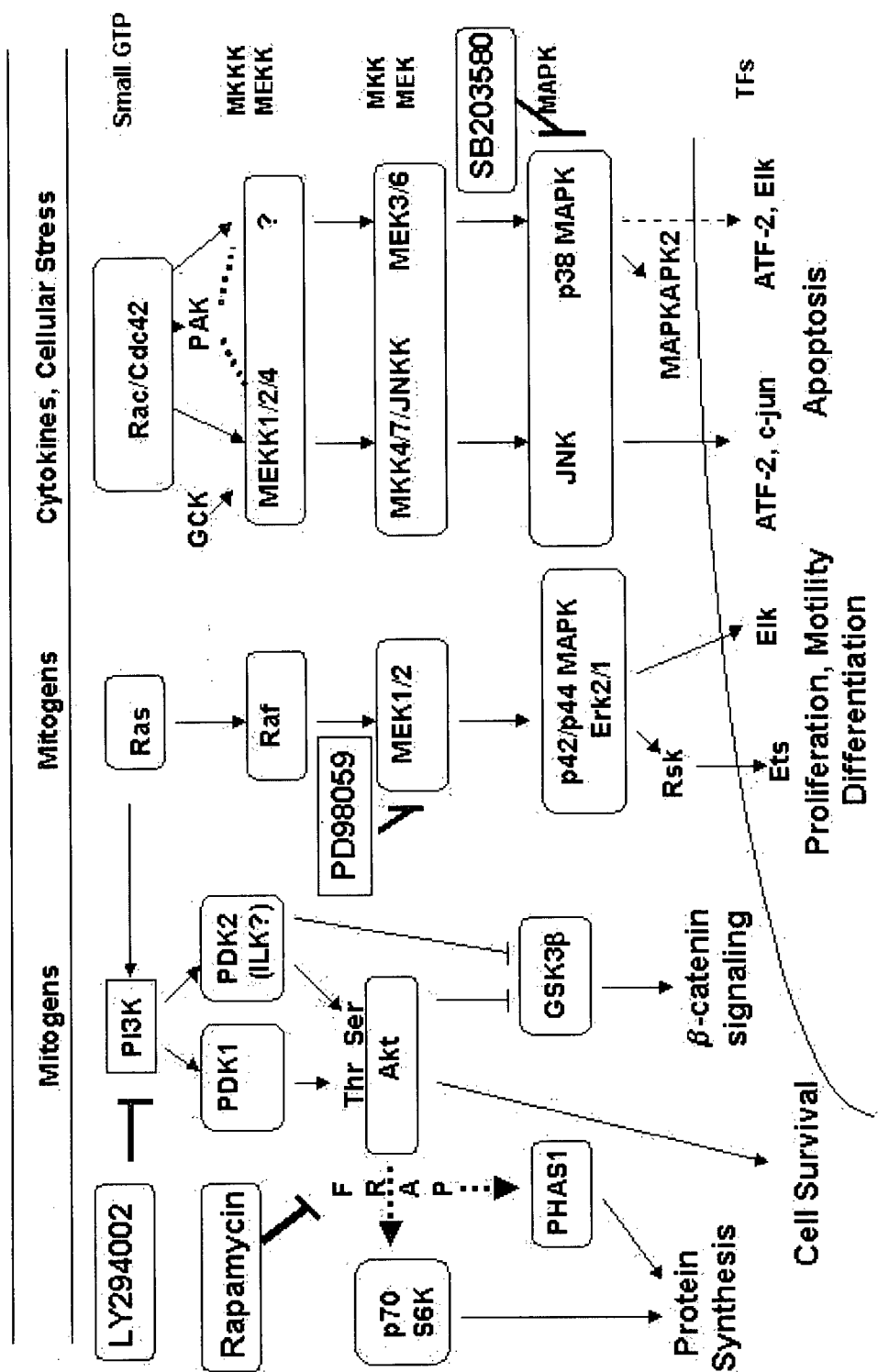
anti-apoptotic activities (Hemmings, 1997; reviewed in Toker and Cantley, 1997). There are several reports that Akt translocates to the nucleus after it is activated at the plasma membrane (Meier *et al.*, 1997). However, the nuclear targets of Akt remain to be identified. The intergrin-linked kinase (ILK) has been shown to phosphorylate Akt in a PI3K-dependent mechanism (Delcommenne *et al.*, 1998). Expression of ILK in mammalian cells downregulates GSK-3β activity and promotes the stabilization and nuclear accumulation of β-catenin (Delcommenne *et al.*, 1998; Novak *et al.*, 1998; Wu *et al.*, 1998). This results in anchorage-independent cell survival and cell cycle progression (Radeva *et al.*, 1997), as well as tumorigenesis in nude mice (Wu *et al.*, 1998). The PI3K/Akt pathway may also regulate the activity of the p70 S6 kinase (S6K). PDK1, the kinase that phosphorylates and activates Akt, can also phosphorylate and activate p70 S6K (Alessi *et al.*, 1998). In addition, the mammalian target of rapamycin (mTOR) or FKBP-12 rapamycin associated protein (FRAP), another kinase that is regulated by PI3K, may also play a role in activating p70 S6K (Brunn *et al.*, 1996). Thus, p70 S6K may be regulated by multiple PI3K-regulated protein kinases in a complicated manner. p70 S6K phosphorylates the ribosomal S6 protein, and this increases the translation of specific mRNAs and progression from G1 to S phase of the cell cycle (Andjelkovic *et al.*, 1997). Another downstream target of PI3K is the pH- and acid-stable protein (PHAS-1). Phosphorylation of PHAS-1 allows its dissociation from the eukaryotic initiation factor 4E, leading to mRNA translation. It was reported that PHAS-1 is a good substrate of mTOR *in vitro* (Beretta *et al.*, 1996; Fadden *et al.*, 1997). Thus, PI3K activation coordinates many cellular processes that include adhesion, survival, cytoskeletal rearrangement and protein synthesis.

b. Ras-Raf-MEK-MAPK cascade

Ras controls a kinase cascade that culminates in the activation of the ERK/MAPK. GTP-bound Ras binds to and activates Raf-1. Raf-1 then phosphorylates and activates two MKKs (MEK1 and MEK2), that in turn phosphorylate and activate the ERK1 and ERK2 MAPKs. Upon activation, the ERK kinases translocate to the nucleus where they phosphorylate and activate Ets-domain containing transcription factors including Ets-1 and Ets-2, and members of the ternary complex factor family, such as the Elk-1 transcription factor. In addition to directly activate transcription factors, the ERK kinases phosphorylate and activate the p90rsk kinase (Dalby *et al.*, 1998) which can phosphorylate and activate the cAMP-responsive element binding protein (CREB) transcription factor (Xing *et al.*, 1996). Ras is a potent inducer of cell cycle progression, proliferation and differentiation, presumably by controlling the expression of key cell-cycle regulators, such as cyclins D and E1 and p27Kip1. The Raf-MEK-ERK kinase cascade is probably not the only target for activated Ras. For example, Ras-GTP can bind to and activate PI3K (Rodriguez-Viciana *et al.*, 1994).

c. JNK and p38 MAPK cascades

Analogous to the Ras/Raf-1/MEK/ERK signaling module, JNK and p38 MAPK are regulated by multi-layered kinase cascades that are controlled by GTPases (reviewed in Leppa and Bohmann, 1999; Herlaar and Brown, 1999). Unlike the ERK kinases that are potently activated in response to growth factors, JNK and p38 MAPK are most potently activated by inflammatory cytokines and environmental stresses. The dual specificity kinases MKK4 and MKK7 phosphorylate and activate JNK, while MKK3 and MKK6 selectively activate p38 MAPK. Although this suggests that JNK and p38 MAPK can be

regulated independently, most stimuli that activate JNK also activate p38 MAPK and vice versa. Unlike Raf-1, which is directly activated by Ras, the MEKKs appear to be linked to the Rac1 and Cdc42 GTPases through kinases including the mixed lineage kinase (MLK), the germinal center kinase (GCK) and the p21-activated kinase (PAK). Activated forms of JNK and p38 MAPK translocate to the nucleus where they phosphorylate and activate different sets of transcription factors. In addition to directly phosphorylating nuclear transcription factors, p38 MAPK can phosphorylate and activate MAPKAPK-2, which in turn phosphorylates and activates the CREB transcription factor. Though the cellular functions of JNK and p38 MAPK in mammalian cells are not well documented, they have been implicated in apoptosis.

1.6.3.2. Signaling pathways to signal different responses

The mechanisms through which HGF exerts its pleiotropic effects have been only partially determined. However, a number of studies indicate that Ras is central to all Met-mediated responses. Ras can be activated either directly via Grb2/SOS recruitment to the activated HGF receptor or indirectly, through Shc phosphorylation (Pelicci *et al.*, 1995). Expression of a dominant-negative Ras or microinjection of Ras-neutralizing antibodies inhibit the motility signal of HGF in MDCK cells (Hartmann *et al.*, 1994; Ridley *et al.*, 1995). Mutation of Tyr1356 impairs Met association with Grb2 and drastically reduces the transforming potential of Tpr-Met, the oncogenic counterpart of the receptor. The same mutation impairs Met-mediated transformation, but not motility, in MDCK cells (Ponzetto *et al.*, 1996; Royal *et al.*, 1997). Treatment of intact cells with wortmannin blocks HGF-induced scattering and mitogenesis in epithelial cells, suggesting that Met-activation of PI3K is required for these activities (Royal and Park, 1995; Rahimi *et al.*, 1996). Cell scattering also

appears to require the activation of Ras (Hartmann *et al.*, 1994) and possibly the small GTP-binding proteins Rac and Rho (Takaishi *et al.*, 1994; Ridley *et al.*, 1995). The sensitivity of motile colon cancer cells to the MEK1 inhibitor PD184352 suggests that the mitogen activated protein kinase (MAPK) may also be important (Sebolt-Leopold *et al.*, 1999). Grb-2 mediated activation of the JNK pathway is required for Tpr-Met transformation (Rodrigues *et al.*, 1997). The Met receptor also binds and phosphorylates the signal transducers and activators of transcription (STAT)-3, and the ensuing nuclear signaling alone, albeit necessary, is not sufficient for promoting branching morphogenesis (Boccaccio *et al.*, 1998). Gab1 is an insulin receptor substrate-like protein that is phosphorylated by Met through direct interaction with Tyr1349, and Gab1-mediated PI3K activation seems sufficient to transduce a morphogenetic signal in various epithelial cell types (Weidner *et al.*, 1996; Nguyen *et.al.*, 1997; Niemann *et al.*, 1998).

1.7. Cell Cycle

The eukaryotic cell cycle is composed of five discrete stages: Go, the quiescent phase; G1, during which commitment to mitotic division takes place; S phase, when DNA is duplicated; G2, the second gap phase; and M (mitosis) phase, where chromosome segregation and cell division occurs. Several proteins have been implicated in mitosis. For instance, nucleolin is a 105-kDa protein that is associated with chromatin in the nucleus. The amount of nucleolin is cell cycle dependent. It is present at low levels in resting cells but is abundantly expressed and highly phosphorylated at mitosis (Peter *et al.*, 1990). The N-terminal region of nucleolin bears several Cdc2 phosphorylation consensus sequences and possibly other sites phosphorylated at mitosis by an unknown kinase (Peter *et al.*, 1990). Phosphorylation of nucleolin is important for chromosome condensation, nuclear

envelope breakdown and spindle formation. Core histones are integral components of nucleosomes. The N-tail of histone H3 plays a particularly important role in chromatin conformation changes. Removal of the tail prevents compaction of chromatin fibers *in vitro*. Although few H3 molecules are phosphorylated in interphase, almost all molecules become phosphorylated at the Ser10 residue in the N-tail during mitosis (Gurley *et al.*, 1978). The phosphorylation starts early in prophase, at the onset of chromosome condensation, reaches a maximum in metaphase, diminishes in anaphase and is absent in telophase (Sauve *et al.*, 1999). Chromosome segregation during mitosis occurs on a bipolar spindle that is constructed primarily from microtubules, hollow tubes of $\alpha\beta$-tubulin heterodimers. Chromosome-microtubule attachment is mediated by kinetochores that form on centromeres. The transition from metaphase to anaphase of mitosis is a key cell cycle event that depends on the regulation of spindle assembly, microtubule dynamics and microtubule-kinetochore interactions. Impairment of these control systems induces aberrant mitotic exit or apoptosis. Many of the chemotherapeutic drugs, including the vinca alkaloids and taxol that are commonly used in treating ovarian cancer patients, bind to tubulin or microtubules and inhibit the cell cycle at metaphase either by blocking microtubule polymerization or stabilizing microtubule dynamics (Rowinsky *et al.*, 1991).

2. MATERIALS AND METHODS

2.1. Materials

PCR primers were obtained from the oligonucleotide synthesis laboratory at the University of British Columbia. The probes used for E-cadherin and HGF/SF detection were derived from the cDNA plasmids pBATEM2 and pRS24 respectively, which were gifts kindly donated by Drs. Masatoshi Takeichi (Kyoto University, Japan) and George F. Vande Woude (National Health Institute, Bethesda). Rat tail type-I collagen stock solution was generously provided by Dr. Joanne Emerman (University of British Columbia). Inhibitors for specific kinases including the PI3K inhibitor LY294002, MEK1/2 inhibitor PD98059, p38 MAPK inhibitor SB203580 and mTOR/FRAP inhibitor rapamycin were bought from Calbiochem (San Diego, CA). Recombinant HGF and Quantikine human HGF immunoassay kit were purchased from R & D Systems (Minneapolis, MN). Digoxigenin (DIG)-DNA random primer labeling kit, RNA labeling kit and chemiluminescent detection kit were purchased from Boehringer Mannheim (Mannheim, Germany). Enhanced chemiluminescent (ECL) detection kit was obtained from Amersham (Oakville, Ont., Canada). DNA dye Hoechst 33258 was provided by Sigma (St. Louis, MO). Protein G agarose beads was bought from Gibco Life Technologies (Grand Island, NY). Other chemicals or reagents were purchased from either BDH (Vancouver, B. C., Canada) or Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

2.2. Antibodies

The mouse anti-E-cadherin (HECD1) and anti-Met (clone DQ13) antibodies were obtained from Zymed Laboratories Inc. (San Francisco, CA) and Upstate Biotechnology Inc. (Lake Placid, NY), respectively. The mouse anti-N-cadherin monoclonal antiobdy (13A9) was kindly provided by Dr. Margaret J. Wheelock (University of Toledo, OH). Primary antibody against human P-cadherin (NCC-CAD-299) was a gift from Dr. Masatoshi Takeichi (Kyoto University, Kyoto, Japan). TG-3 hybridoma cultures and H3P Ab monoclonal antibodies, which recognize nucleolin and histone H3 N-tails respectively, were generously provided by Dr. Michel Roberge (University of British Columbia). Primary antibodies for kinase detection were either purchased from Santa Cruz Biotechnology (Santa Cruz, CA), StressGen Biotechnologies Corp. (Vancouver, B. C., Canada) or Upstate Biotechnology Inc. (Lake Placid, NY), or kindly provided by Dr. Steven L. Pelech (University of British Columbia), as described in Table 1. Anti-phosphotyorosine (clone 4G10) antibody and neutralizing polyclonal anti-HGF antibody were purchased from Upstate Biotechnology Inc. (Lake Placid, NY) and R & D Systems (Minneapolis, MN), respectively. Species specific secondary antibodies conjugated to horseradish peroxidase and CY3-conjugated goat anti-mouse antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). As secondary antibodies, fluorescein isothiocyanate- (FITC) or Texas red-conjugated goat anti-mouse IgG was purchased from HyClone (Logan, UT).

Table 1. Antibodies used for kinase detection.

Antibodies were either purchased from Santa Cruz Biotechnology (Santa Cruz, CA), StressGen Biotechnologies Corp. (Vancouver, B. C., Canada) or Upstate Biotechnology Inc. (UBI) (Lake Placid, NY), or generously provided by Dr. Steven L. Pelech (University of British Columbia) (UBC). The corresponding immunogen peptide sequence or residues, the size of immunoproducts (kDa), the companies from which the antibody was purchased and the working concentration (μg/ml) are indicated.

TABLE 1 - Antibodies used for kinase detection

Antibodies	Kinase	Immunogen amino acid sequence or residues	Size (kDa)	Source	Working conc. (μg/ml)
Cdc2-CT	Cylin-dependent kinase (CDK1)	CT-CLSKMLVYDPAKRISKM ALKHPFDDLDNQIKKM	34	UBC	0.7
CK2α-III	Casein kinase II (CK2)	III-LKPVKKKKIKREIKILELR-GCC	38-43	UBI	0.6
ERK1-CT	Extracellular-regulated kinase (ERK)	CTCGGPFTFDMELDDLPKE LKELIFQETARFQPGAPEP	42-44	UBC	0.15
cGPK-CT	cGMP-dependent protein kinase (PKG)	CT-CDEPPPDDNSGWDIDF	75	UBC	1.3
GSK3β-XI	Glycogen synthase kinase (GSK) 3β	XI-CSHSFFDELRDDPNNK	48	UBC	1.0
p38 HOG-CT	p38 mitogen activated protein kinase (MAPK)	C20-residues 341-360	38-40	Santa Cruz	0.1
MEK6-SG	MEK6	C15-residues 320-334	38	Stress Gen	0.4
ILK1	Integrin linked kinase (ILK)	Recombinant human ILK-GST fusion protein	60	Stress Gen	1.0
PHAS-1	pH and acid stable protein (PHAS-1)	Residues 110-118	22-26	Stress Gen	1.0
RAC1-CT	Akt1	CT-CRRPHFPQFSYSASSTA	62	UBC	0.9

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Antibodies	Kinase	Immunogen amino acid sequence or residues	Size (kDa)	Source	Working conc. (μg/ml)
RAC2-CT	Akt2	CT-CRYDFLGLLEDQRT	62	UBC	0.9
mPKCβ	Protein kinase C (PKC)-α,β	M7-purified PKC, monoclonal	80	UBC	0.5
PI3K	Phosphatidylinositol 3-kinase (PI3K)	Recombinant p85 PI3K, monoclonal	85	UBC	1.0
Rsk1 (C21) SC	Ribosomal S6 kinase (Rsk)1	C21-residue 716-735	85-90	Santa Cruz	0.7
Rsk2-PCT	Ribosomal S6 kinase (Rsk)2	PCT-CNRNQSPVLEPVGRS	85-90	UBC	1.0
S6K-PNT	p70 S6 kinase (S6K)	NTAGVFDIDLDQPEDAGSE DELEEGGQLNESC	65-70	UBC	0.9

2.3. Cells and cell lines

Institutional approval for experimentation with human tissues was obtained prior to this study. OSE was obtained from ovarian biopsies and at laparoscopy from 62 women (age 27-60 years; mean age 45.9 years) with no family histories of breast/ovarian cancer (NFH-OSE), having surgery for non-malignant gynecologic diseases. Another group of 18 women (age 36-58 years; mean age 53.6 years) underwent prophylactic oophorectomies because of strong family histories of breast/ovarian cancer (FH-OSE). In this group, all women had at least two first-degree relatives with such cancer, and included 13 with known BRCA1 mutations (Table 2). The genetic analysis for the other 5 women is ongoing. One of these women had a pedigree indicative of Lynch II syndrome. Histopathological analysis confirmed the absence of neoplasms in all specimens. OSE fragments were scraped off the ovary and cultured intact without prior dissociation. They were characterized by their distinct morphology and keratin expression (Auersperg *et al.*, 1994).

IOSE-29 was obtained by immortalization of normal OSE with SV40 large T antigen (Maines-Bandiera *et al.*, 1992). Cotransfection of E-cadherin and neomycin-resistant (SV2neo) genes into IOSE-29 cells produced the IOSE-29EC cell line (Auersperg *et al.*, 1999) which was tumorigenic in SCID mice, and from that we generated the ovarian tumor cell line IOSE-29EC/T4 (Ong *et al.*, 2000) (Fig. 2). Parental cells sham-transfected with SV2neo were designated IOSE-29neo (Auersperg *et al.*, 1999). To ensure the genetic stability of these cultures, all experiments were performed within 3 passages for each cell line.

The human ovarian adenocarcinoma lines CaOV-3, OVCAR-3 and SKOV-3 were included in the study. OVCAR-3 and CaOV-3 served as positive controls while human dermal fibroblasts (HDF) and the human lung fibroblast cell line WI-38 as negative

TABLE 2 - Genetic information on women with strong family histories of breast/ovarian cancer*

Case number	BRCA1 mutation
HO-2775-154	+
OSE-153F	n.d.
OSE-154F	n.d.**
OSE-178F	n.d.
OSE-181F	+
OSE-187F	+
OSE-195F	+
OSE-201F	n.d.
OSE-204F	+
OSE-206F	+
OSE-214F	+
OSE-215F	+
OSE-233F	+
OSE-236F	+
OSE-269F	+
OSE-275F	+
OSE-286F	+
OSE-293F	+

n.d., not determined

*, two or more first-degree relatives with breast/ovarian cancer

**, pedigree suggestive of Lynch II syndrome

Normal OSE

|
SV40 Tag
↓

IOSE Decreased growth requirement
Increased growth rate
Extended life span
Genetic instability

E-cadherin

|
↓

IOSE-29EC Epithelial differentiation
Anchorage-independence
Infinite life span

|
↓

Tumorigenicity in SCID mice
IOSE-29EC/T4
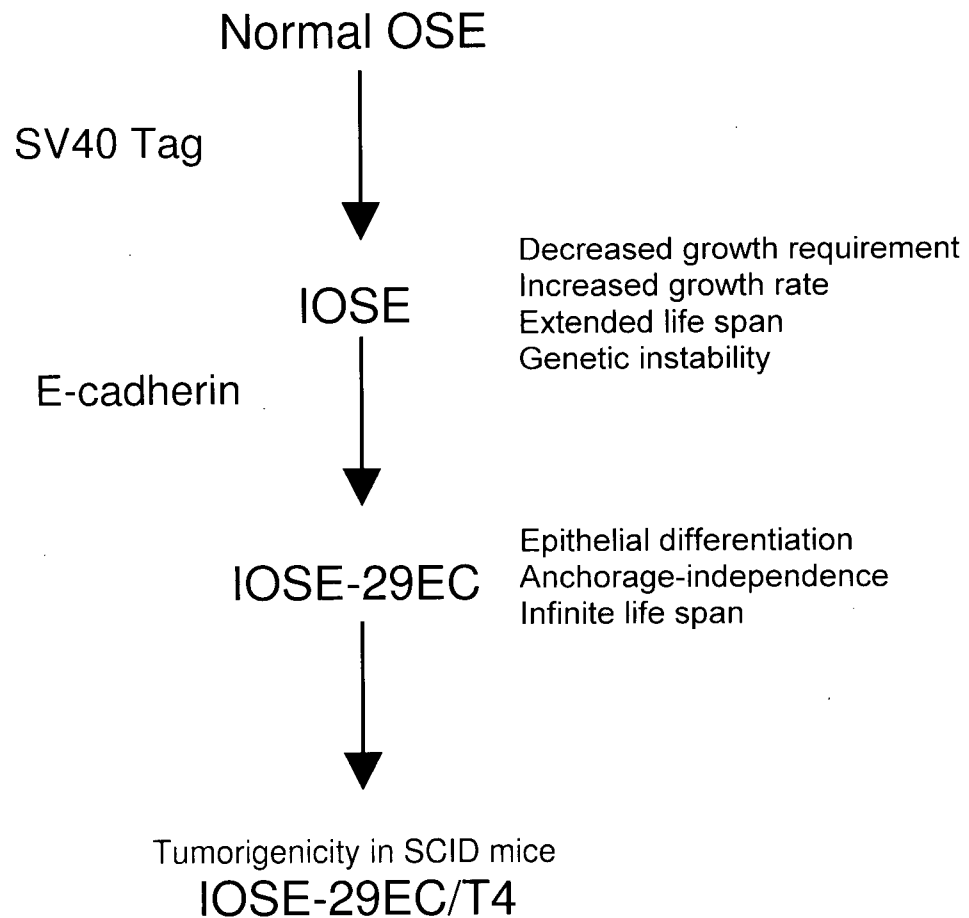
FIGURE 2 - Schematic diagram of the generation of SV40-immortalized OSE cell lines (IOSE) and their E-cadherin derivative (IOSE-29EC), which was tumorigenic in SCID mice (IOSE-29EC/T4). These lines possess properties that are representative of progressive stages in ovarian carcinogenesis.

controls for E-cadherin. The cervical cancer line C-4II (Auersperg et al., 1969; ATCC No. CRL1595) and human immortalized granulosa cells (SVOG) (Lie et al., 1996) were used as positive controls for P- and N-cadherin respectively. The human hepatoma line HepG2 was the negative control for HGF and positive control for Met, whereas WI-38 was the positive control for HGF.

2.4. Cell culture

Cells were routinely grown and maintained in medium 199/MCDB 105 (1:1) (Sigma, St. Louis, MO) supplemented with 10-15% fetal bovine serum (FBS) (Hyclone, Logan, UT) for OSE and fibroblasts, and with 5% newborn calf serum (NCS) for immortalized cell lines, E-cadherin-transfected IOSE and cancer cell lines, at 37°C in a humidified incubator with 5% CO_2:95% air. All were subcultured with 0.06% trypsin/0.01% EDTA in Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution. All cells were frozen in liquid nitrogen in medium 199/MCDB105 with 25% NCS and 10% dimethylsulfoxide (DMSO) while normal OSE was frozen under the same condition except with 45% FBS. OSE cultures in low passage were defined as senescent when the cells became large, flat and vacuolated and did not reach confluence over one month. In most cases, senescence occurred in passages 4-5. Because of the limited supply of these cells, not all OSE cases provided sufficient material to examine both mRNA and protein expression of E-cadherin and Met, and protein expression of all three types of cadherins.

For inhibitor studies, cells were pretreated with inhibitors for 30 min at 37°C prior to the addition of recombinant HGF at 20 ng/ml for 10 min in culture medium containing 2% NCS.

2.5. OSE cultures on various substrata

2.5.1. PolyHEMA

For culture on the non-adhesive substratum polyHEMA (poly-hydroxyethylmethacrylate, Sigma, St. Louis, MO), tissue culture dishes were coated by applying 125 μl/cm^2 of a 0.2-1.0 mg/ml solution of polyHEMA dissolved in ethanol onto the dish, and drying overnight in a tissue culture hood. Single cell suspensions of OSE and OVCAR-3 were plated at a cell density of 5x10^4/cm^2 onto the substratum and fixed after 4 days.

2.5.2. Matrigel

Growth factor-reduced Matrigel matrix, a reconstituted basement membrane gel derived from the Engelbreth-Holm-Swarm tumor (Collaborative Research, Bedford, MA), was thawed on ice, and 24-well plates previously coated with agar were coated with 0.4 ml/well of this solution on ice. After incubation for 30 min at 37°C to allow the matrix to gel, it was primed with medium containing 10% FBS for 1 h at 37°C. Cells were seeded at a density of 2x10^4/cm^2 in 10% FBS-containing medium with or without recombinant HGF at a concentration of 20 ng/ml. The cultures were maintained for 2 weeks and medium was changed every 2-3 days.

2.5.3. Collagen gel

The capacity of cells to form branching tubules in collagen gel was examined as described, with some modifications (Brinkmann *et al.*, 1995). In brief, collagen gel solution was polymerized by adding a mixture containing 7 parts of rat tail type-I collagen stock solution, 1 part each of 10x medium 199, 10% NCS and 22 mg/ml NaHCO$_3$ on ice. The

solution was then neutralized with 0.34 M NaOH. 300 µl/well of the cold neutralized collagen gel solution was plated onto 24-well plates and allowed to gel at 37°C. For each well, 2×10^4 cells were resuspended in 300 µl neutralized collagen solution and then seeded on top of the first layer. The collagen was allowed to gel before 1 ml culture medium containing 2% NCS was added. For specific experiments, the following inhibitors for specific kinases were added: PD98059 (50 µM), LY294002 (25 µM), rapamycin (20 nM) or SB203580 (10 µM). In addition, DMSO (control) or recombinant HGF (20 ng/ml) was added to the respective wells. The cultures were maintained for 21 days in an atmosphere of 5% CO_2/air with daily medium changes. For histological examination, cultures were fixed with 4% paraformaldehyde in phosphate buffered saline at the end of the experiment. The specimens were embedded in paraffin, sectioned and stained with hematoxylin and eosin. The experiments were repeated three times and representative fields were photographed.

2.6. Fluorescence microscopy

2.6.1. Cadherins

To demonstrate E-, P-, and N-cadherin, the cells were grown to confluence on glass coverslips, fixed in 3.7% paraformaldehyde in Hepes-buffered Mg-free saline supplemented with 2 mM $CaCl_2$, pH 7.4 (HMF), and extracted with $-20°C$ methanol for 20 min. The coverslips were blocked with 5% v/v normal goat serum/1% w/v bovine serum albumin (BSA) in HMF, incubated in primary antibody, mouse monoclonal antibody to human E-cadherin (HECD-1) (1:300) or human P-cadherin (NCC-CAD-299) (1:10) (Shimoyama *et al.*, 1989) or human N-cadherin (13A9) (undiluted) (Knudsen *et al.*, 1995). After rinsing, the secondary antibodies-FITC (1:100) or Texas red- (1:160) conjugated goat anti-mouse IgG were applied for 1 h. For negative controls, cells were incubated with normal goat serum

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instead of primary antibody. The number of positive cells was scored out of 1,000 cells per case. All incubations were carried out at room temperature.

2.6.2. Mitotic spread count

Cells were dissociated by 0.06% trypsin/0.01% EDTA, collected and centrifuged for 2-5 min at 200-400xg. The pellet was resuspended in 50 µl of the supernatant, and incubated with 1 ml 75 mM KCl for 8-10 min at room temperature. After centrifugation, 0.5 ml of Carnoy's fixative (1:3 v/v acetic acid:methanol) was added. After a 10-min incubation at room temperture, the cells were centrifuged, stained with the DNA dye Hoechst 33258 (0.5 µg/ml) and mounted onto the microscope plate. The number of cells at different mitotic stages was scored out of 1,000 cells counted.

2.6.3. Mitosis-specific antigens

Cells were grown to 70% confluence on coverslips. Following overnight treatment with LY294002, cells were fixed with 4% paraformaldehyde in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 40 min at 4°C. The coverslips were blocked with 0.1% w/v BSA in KB (TBS, 0.2% v/v Triton X-100) for 10 min, and incubated with primary antibody to human nucleolin (TG-3 hybridoma culture supernatant) (1:50) (Anderson *et al.*, 1998) or β-tubulin (E7) (1:15) or phosphorylated histone H3 N-tails (H3P Ab) (1:200) (Sauve *et al.*, 1999) for 90 min at ambient temperature. After three washes in TBS, the coverslips were incubated with CY3-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) (1:500) in KB for 90 min at room temperature. They were rinsed twice in PBS, stained with the DNA dye Hoechst 33258 (0.5

μg/ml), mounted on slides, and photographed. The experiment was performed in duplicates and the number of positive cells was scored out of 1,000 cells.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Total cytoplasmic RNA was isolated by the guanidium thiocyanate (GTC)-phenol-chloroform method (Chomcznski and Sacchi, 1987). In brief, cells were disrupted in GTC lysis buffer containing 4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% w/v N-lauroylsarcosine and 0.1 M β-mercaptoethanol. The lysate was purified by phenol-chloroform extraction. Total RNA was obtained by precipitation with an equal volume of isopropanol overnight at −20°C, and the pellet was resuspended in ribonuclease-free (treated with diethylpyrocarbonate, DEPC) water. The concentration and purity of RNA were determined based on absorbance at 260 nm measured by a spectrophotometer (DU-64, Beckman). cDNA was synthesized from total RNA using the First Strand cDNA synthesis kit (Pharmacia Biotech, Morgan, Canada) following the manufacturer's instructions. The reaction mixture (33 μl) containing 5 μg RNA, 11 μl bulk first-strand cDNA reaction mix, 20 pmole oligo-dT primer and 6 mM dithiothreitol (DTT) was incubated at 37°C for 60 min and terminated by heating at 90°C for 5 min.

To amplify the cDNA, 5 μl reverse-transcribed cDNA was subjected to PCR in a 100 μl reaction mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% w/v gelatin, 200 mM each of dATP, dCTP, dGTP, and dTTP, 2.5 units of Taq polymerase (GIBCO); and 0.5 pmole of each sense and antisense appropriate primers:

E-cadherin   5'-GGGTGACTACAAAATCAATC-3' and

                            5'-GGGGGCAGTAAGGGCTCTTT-3'.

HGF: 5'-AGTACTGTGCAATTAAAACATGCG-3' and

5'-TTGTTTGGGATAAGTTGCCCA-3';

Met: 5'-GGTGAAGTGTTAAAAGTTGGA-3' and

5'-ATGAGGAGTGTGTACTCTTG-3';

β-actin   5'- TGATCCACATCTGCTGGAAGGTGG-3' and

5'-GGACCTGACTGACTACCTCATGAA-3'.

All PCR primers span the introns to detect specific mRNA sequences. With these primers, the amplimers for E-cadherin, HGF, Met were 250-bp (nt. 2074-2325 Gene Bank No.Z13009), 378-bp (nt. 841-1219 GenBank M29145) and 324-bp (nt. 2667-2991 GenBank J02958), respectively. The amplification reaction was carried over 35 cycles. For E-cadherin, each cycle consisted of denaturation at 94°C for 60 s, primer annealing at 60°C for 90 s, extension at 72°C for 120 s. For HGF and Met, each cycle consisted of denaturation at 94°C for 30 s; primer annealing at 55°C for 45 s (HGF) or at 56°C for 30 s (Met); extension at 72°C for 30 s. This was followed by a final extension at 72°C for 15 min in a DNA thermal cycler (Perkin Elmer, Norwalk, CT). β-actin amplimer was included to normalize the relative amount of cDNA in each reaction, and a 510-bp fragment was amplified under similar condition, except the annealing temperature was 55°C. PCR controls were performed in the absence of cDNA to ensure that cross-contamination of samples did not occur. One tenth of the PCR products were then loaded on 2% agarose gels and stained with ethidium bromide.

2.8. Southern blot analysis

The PCR products in agarose gels were transferred onto nylon membranes (Hybond film, Amersham, United Kingdom) and hybridized with digoxigenin (DIG)-dUTP-labeled cDNA probes. Hybridization was performed at 68°C overnight in the presence of 5 x SSC

(75 mM sodium citrate, 0.75 M NaCl, pH 7.0), 0.1% w/v N-lauroylsarcosine, 0.02% w/v SDS and 1% w/v blocking reagent. The membranes were then washed twice with 2 x SSC and 0.1% SDS at room temperature, and twice with 0.1 x SSC and 0.1% SDS at 68°C. The hybridized products were visualized using the DIG chemiluminescent detection system. Briefly, membranes were rinsed in washing buffer and incubated with 1% blocking reagent in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl) (pH 7.5). After rinsing, membranes were incubated with anti-DIG conjugated alkaline phosphatase (1:10,000) in maleic acid buffer plus 0.3% Tween-20 (washing buffer). The membranes were washed twice with washing buffer and equilibrated for 2-5 min in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM MgCl2, pH 9.5). Chemiluminiscent reaction was started by the addition of CSPD (1:100), and hybridized signals were detected by exposure to X-OMAT AR film (Eastman Kodak Co., Rochester, NY).

The probe used for E-cadherin detection was a 0.7-kb Xhol-Smal fragment (nt. 1604-2320) excised from the mouse E-cadherin cDNA plasmid pBATEM2 (Nose et al., 1988). The probe used for HGF detection was a 0.8-kb EcoR1 fragment (nt. 255 – 1102) derived from the human HGF cDNA plasmid pRS24. The probes were labeled by the random primer method using the DIG labeling kit following the manufacturer's instructions. Briefly, 3 μg excised DNA template was denatured by heating for 10 min and quickly chilled on ice. The reaction mixture (20 μl) containing 2 μl hexanucleotide, 0.1 mM each of dATP, dCTP and dGTP, 65 nM dTTP, 35 nM DIG-11-dUTP and 2 units of Klenow enzyme was incubated overnight at 37°C. Labeled DNA was then precipitated with lithium chloride and ethanol. The yield of DIG-labeled, newly synthesized DNA was roughly determined in comparison to a DIG-labeled control DNA provided by the manufacturer in a dot blot followed by direct immunodetection with the chemiluminescent substrate.

2.9. *In situ* hybridization (ISH)

The probes for in situ hybridization of E-cadherin were prepared by recloning a XhoI-SmaI fragment (nt.1604-2320) from pBATEM2 into the pBlueScript II SK vector (Stratagene, La Jolla, CA). This fragment is homologous to the same fragment of the human gene. Antisense and sense riboprobes were generated with T7 and T3 RNA polymerases respectively, by *in vitro* transcription using digoxigenin-UTP following the manufacturer's instructions. In brief, linearlized DNA templates were purified by phenol-chloroform extraction followed by ethanol precipitation. The reaction mixture 1 µg linearized plasmid DNA, 1 mM each of ATP, CTP and GTP, 0.65 mM UTP, 0.35 mM DIG-UTP, 2 µl 10x DIG RNA labeling mix, 2 µl 10x transcription buffer, 20 units each of RNase inhibitor and RNA polymerase was incubated for 2 h at 37°C. RNase-free DNase 1 (20 units) were added to remove template DNA. To enhance penetration and avoid non-specific background due to the length of the cRNA, the labeled probes were alkali hydrolysed to an average length of 100-250 bp. Sense and antisense riboprobes were adjusted to the same concentration before use. RNase pre-treatment, hybridization with E-cadherin sense riboprobe, pBlueScript antisense riboprobe and water were used as controls.

The cells were grown to confluence on glass coverslips, rinsed in PBS and fixed in 4% paraformaldehyde with 5% acetic acid for 20 min at room temperature. After fixation, cells were rinsed in PBS, and treated with proteinase K (5 µg/ml in 100 mM Tris-HCl, pH 7.6, and 5 mM EDTA) for 30 min at 37°C. The cells were washed again in PBS, post-fixed in 4% paraformaldehyde, dehydrated in graded ethanol and air dried. The cells were covered with a hybridization buffer containing 50% v/v deionized formamide, 2x SSC (30 mM sodium citrate, 0.3 M NaCl, pH 7.0), 1x Denhardt's solution, 0.1% v/v SDS, 500 µg/ml Herring sperm DNA and 2 ng/µl DIG-UTP-labeled probe. Coverslips were sealed on slides with

rubber cement, denatured at 85°C for 3 min, and incubated in a humidified chamber overnight at 42°C. After hybridization, the cells were washed with 2 x SSC. Following the RNase treatment for 30 min at 37°C, the cells were rinsed in the same buffer, and then in 0.1 x SSC (1.5 mM sodium citrate, 0.15 NaCl, pH 7.0). The hybridization products were detected by incubating cells in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl and 50 mM $MgCl_2$ with 400 µg/ml nitro-blue tetrazolium (NBT) and 247.5 µg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Boehringer Mannheim, Germany) in the dark for 2-4 h. The reaction was stopped with water and the coverslips were mounted in aqueous mounting medium.

2.10. Western blotting

2.10.1. Determination of protein concentration

Protein concentration was determined by the dye binding method (Bradford, 1976) using the reagents and procedures for the microassay supplied by Bio-Rad laboratories (Bio-Rad, Richmond, CA). The standard curve was prepared by reading the absorbance of 0 to 2.5 µg/µl BSA at 630 nm by a microplate photoreader (Bio-Tek Instruments Inc.).

2.10.2. E-cadherin and Met Detection

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA) containing freshly added protease inhibitors (1 mM phenylmethyl sulfonyl fluoride (PMSF), 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A). Twenty µg protein in sample loading buffer (2.5% w/v SDS, 10% v/v glycerol, 50 mM HCl, pH 6.8, 0.5 M β-mercaptoethanol and 0.01% w/v bromophenol blue) were loaded and separated on 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 20-40 mA until the dye front reached the bottom of the gel. The proteins were

electrophorectically transferred onto nitrocellulose membrane at 30 mA at 4°C overnight. Membranes were blocked with 3% w/v non-fat dried milk in phosphate-buffered saline (pH 7.3) (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4) with 0.05% v/v Tween 20 and probed with primary antibody anti-human E-cadherin (HECD1) (1:2,000) or Met monoclonal antibodies (clone DQ13, 1 μg/ml) at 4°C overnight. After extensive washing, immune complexes were detected with goat anti-mouse secondary antibody conjugated with horseradish peroxidase (1:2,500) for 1 h followed by an ECL detection system.

2.10.3. Protein kinase detection

Cells were lysed in buffer (20 mM MOPS, pH 7.2, 5 mM EGTA, 2 mM EDTA, 0.1 mM sodium orthovanadate, 1 mM sodium fluoride, 24 mM β-glycerophosphate, 0.005 mM β-aspartic acid and 1% v/v Triton X-100) containing freshly added protease inhibitors (1 mM PMSF, 25 μg/ml leupeptin, 1.4 μg/ml pepstatin A, 2 μg/ml aprotinin and 0.5 mM DTT). Cell lysates containing 50 μg protein were separated on 11 to 12.5 % SDS-PAGE. Proteins were transferred onto nitrocellulose membranes. Membranes were stained in Ponceau S solution (0.1% w/v Ponceau S in 1% v/v acetic acid) for 30 s, followed by destaining in water. Blots were then blocked for 30 min with 5% w/v non-fat dried milk, and probed for 3 h with primary antibodies against specific kinases, as described in Table 1. After extensive washing, immune complexes were detected with either goat anti-mouse (1:3,000) or anti-rabbit (1:20,000) IgG conjugated with horseradish peroxidase for 1 h followed by an ECL detection system.

2.10.4. Immunoprecipitation for phosphoprotein detection

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA) containing freshly added phosphatase inhibitor (1 mM sodium fluoride, 1 mM sodium orthovanadate) and protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). Cell lysate (1 mg protein) was pre-cleared with protein G-Agarose at 4$^\text{o}$C for 2 h to reduce non-specific binding of proteins to the agarose. Clarified lysate was incubated with 1 μl mouse anti-Met IgG (clone DO-24) overnight at 4$^\text{o}$C. Immune complexes were collected using protein G-Agarose and then washed three times with RIPA buffer. The complexes were eluted in boiling SDS sample buffers. Immunoprecipitates were analyzed by 7.5% SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked with 5% BSA in low salt TBS (20 mM Tris-HCL, pH 7.5, 50 mM NaCl, 0.005% NP-40) (anti-phosphotyrosine) or 3% non-fat dried milk in PBS (Met). Blots were then probed with anti-phosphotyrosine (4G10, 1μg/ml) or anti-Met (clone DQ-13, 1μg/ml) overnight at 4$^\text{o}$C.

2.10.5. Stripping of Western blots

In certain circumstances, membranes were stripped in buffer containing 100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl, pH 6.7 for 30 min at 60°C with gentle agitation at every 10 min. The membranes were blocked with 5% w/v non-fat milk before reprobing with primary antibodies.

2.11. ELISA for HGF

The concentration of HGF in conditioned media of OSE and ovarian cancer cells was measured using the Quantikine HGF immunoassay kit following the manufacturer's

instruction. HGF standard (0-8000 pg/ml) and samples were pipetted into the wells of a microplate that was pre-coated with a mouse monoclonal anti-HGF antibody, and incubated at room temperature for 2 h. After washing away any unbound substances, a horseradish peroxidase-linked polyclonal antibody specific for HGF was added. Following thorough washing, a substrate solution was added to the wells. The product of the enzymatic reaction was determined after 30 min from the absorbance at 450 nm using a microplate photoreader (Bio-Tek Instruments Inc.). HGF standards and all samples were assayed in duplicate, and in some cases, samples were also concentrated two- to three-fold using Centricon 30 tubes (Amicon) before the assay. The mean intra- and inter-assay variabilities are reported to be 5.6% and 7.0% respectively, and a wide variety of peptide hormones, cytokines and growth factors do not cross-react in this assay. The assay measures both HGF and its precursor, pro-HGF. The minimum detectable dose of HGF is 40 pg/ml.

2.12. Cell growth assay

2,000 cells/well were plated in 96-well plates in medium containing 10% FBS. After 24 h, the cells were serum starved with 0.1% FBS overnight, and then treated without (control) or with 5-20 ng/ml HGF in the presence of 2% FBS. To elucidate the underlying signal transduction mechanisms, groups of cultures were pretreated with or without PD98059 (50 μM) or LY294002 (25 μM) or rapamycin (20 nM) or SB203580 (10 μM) for 30 min prior to the addition of recombinant HGF (20 ng/ml) in 2% FBS-containing medium, with a daily change of medium containing both HGF and inhibitors. Cells were grown for 6-8 days and fixed in 4% paraformaldehyde for at least 30 min. Cell numbers were determined colorimetrically by the Methylene Blue assay (Elliott and Auersperg, 1993). In brief, cells were incubated for 30 min with 1% w/v Methylene Blue in 0.01 M borate buffer (pH 8.5).

After extensive washing in borate buffer, the dye was eluted with 100 μl 1:1 v/v ethanol and 0.1 M hydrochloric acid, and the absorbance at 630 nm, which is a linear function of cell number, was measured by a microplate photoreader (Bio-Tek Instruments Inc.).

2.13. Cell scatter assay

Confluent monolayers of OSE were carefully wounded with a rubber scraper. Cultures were serum starved with 0.1% FBS, and the cells were treated in the presence or absence of 20 ng/ml HGF. Scatter assays for other cell lines were performed as described by Stoker *et al.* (Stoker *et al.*, 1985). Approximately 5,000 cells/well were plated in a 24-well plate in medium containing 5% NCS overnight. Cultures were incubated with medium containing 2% NCS for 30 min with or without PD98059 (50 μM) or LY294002 (25 μM) or rapamycin (20 nM) or SB203580 (10 μM) before adding 20 ng/ml HGF. After a 24-h incubation, the degree of scattering was assessed visually. The experiments were repeated three times and representative fields were photographed.

2.14. Photography

Cultures were routinely examined using a Leitz Diavert inverted photomicroscope and photographed on Kodak Technical Pan 2415 black and white films. Immunofluorescence staining was visualized by a Zeiss Axiophot epifluorescence microscope and photographed using Kodak Tmax 400 black and white film or Fujichrome Provia color slide film.

2.15. Densitometry and statistical analysis

The difference in mean ages of women with and without family histories was compared using the unpaired t-test. The differences in expression levels of E-cadherin, HGF or Met between NFH-OSE and FH-OSE were compared for significance using the Fisher's exact test. The statistical analysis of E-cadherin expression levels for each passage of cultures of these two groups were compared by one-way ANOVA and Tukey's least significant difference post test.

Images from DIG chemiluminescent detection and ECL autoradiograms were captured using NIH Image and Adobe software. Cell growth assays were administered in six replicates, and each experiment was performed in a minimum of three different cases. Data were presented with standard deviations ($\pm$S.D.), and compared by one-way ANOVA and Tukey's least significant difference post-test.

To determine whether differences in specific protein kinase expression at different stages of ovarian neoplastic progression were present, the Kruskal-Wallis test was applied.

Differences were considered significant at $P<0.05$. All statistical evaluations were performed using the Prism statistical software package (GraphPad Software, San Diego, CA).

3. RESULTS

3.1. Morphology and growth pattern of OSE

As shown in Fig. 3, the morphology and growth pattern of OSE cells can be categorized into 5 classes: (a) compact epithelial cells forming a cobblestone pattern. These comprised the majority of cells in all primary cultures (passage (p.0); (b) large flat epithelial cells seen in >90% of all cultures in p.2-3; (c) atypical, fibroblast-like cells found in p.3 or beyond in 80% of the NFH-OSE cultures, while >90% of the FH-OSE cultures remained epithelial. These fibroblast-like cells were keratin positive indicating their origin in OSE (Auersperg *et al.*, 1994; Dyck *et al.*, 1996); (d) polygonal epithelial cells with distinct borders, (e) elongated, irregularly shaped overlapping cells that sometimes formed colonies appearing as whorls, which were present in primary culture and, rarely, in p.1. Such colonies represented up to 5% of the cells in culture, while the bulk of cultured OSE cells were cobblestone or flat epithelial. Categories (d) and (e) were found in approximately 5% of NFH-OSE cases but in >50% of FH-OSE cases ($P<0.001$). Category (e) colonies were strikingly similar to the colonies that are formed in primary culture by oviductal or endometrial cells under conditions that do not permit the formation of polarized monolayers (Kervancioglu *et al.*, 1994; Loke *et al.*, 1995; Sugawara *et al.*, 1997). These colonies stained uniformly and intensely for CA125 and E-cadherin, whereas the bulk of cultured OSE cells with cobblestone, flat epithelial, or atypical fibroblast-like morphologies were CA125- and E-cadherin-negative. On the basis of their morphological and functional resemblance to cultured Mullerian duct-derived epithelia, category (d) and (e) colonies appear to be derived from metaplastic OSE. Category (e) colonies usually formed in primary cultures, rarely in p.1 and never in later passage. It is unknown at present whether the distinctive colonies in
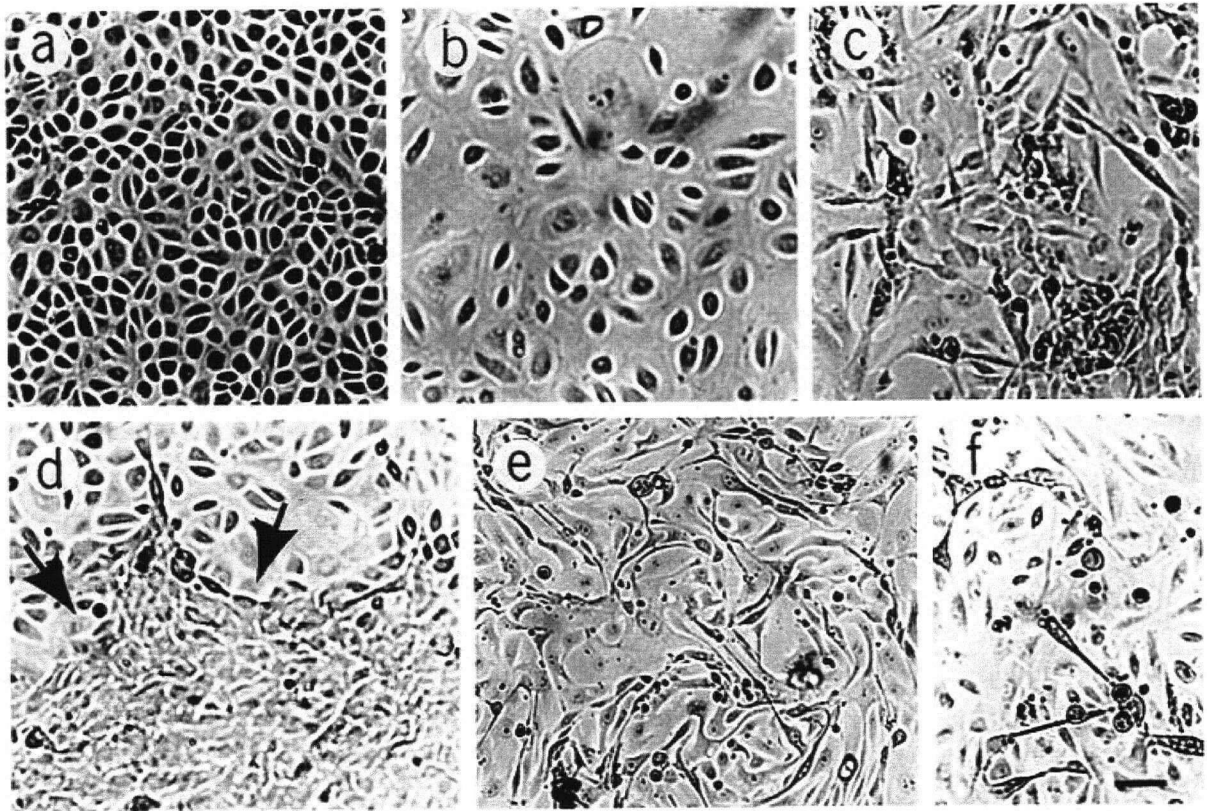
FIGURE 3 - Morphology and growth pattern of OSE cultures. Monolayers of compact epithelial cells forming a cobblestone pattern (a); large flat epithelial cells (b); monolayered or stratified atypical, fibroblast-like cells (c); polygonal epithelial cells with distinct borders, as delineated by arrows with cobblestone cells above (d); elongated cells that sometimes formed colonies appearing as whirls, signifying metaplastic OSE and elongated cells with characteristic cytoplasmic projections on top of a monolayer of compact epithelial cells (f). Such cells were commonly E-cadherin positive by immunofluorescence. *Bar,* 30 μm.

category (e) seen in primary culture give rise to groups of monolayered polygonal cells in category (d) in p.1 and p.2. Elongated cells with characteristic cytoplasmic projections which were found in early passages on top of epithelial monolayers are probably also derived from metaplastic colonies (Fig. 3f).

3.2. Constitutive and conditional cadherin expression in OSE

NFH-OSE cultures from 7 women at p.0-4 and FH-OSE cultures from 3 women at p.1 were stained for P- and N-cadherin. Immunofluorescence microscopy revealed that P-cadherin was consistently negative in all NFH-OSE and FH-OSE cultures regardless of the passages and the morphologies of the cells (Figs. 4b, c). In contrast, N-cadherin was detected in all 4 NFH-OSE (p.1-4) and 5 FH-OSE (p.0-1) cultures (Figs. 4e, f). Within these cultures, N-cadherin was present in almost all cells, regardless of morphologies, growth patterns or passages. However, the pattern of N-cadherin staining varied with cell morphology. It was consistently localized at the cell to cell borders of cobblestone OSE monolayers (Figs. 3a, 4e), but was found between overlapping fibroblast-like OSE cells with a fibrillar staining pattern (Figs. 3c, 4f). Moreover, N-cadherin staining was also demonstrated in metaplastic OSE colonies (data not shown). In contrast to these uniform distribution patterns of P-cadherin and N-cadherin, the presence of E-cadherin correlated with the morphology and growth pattern of the cells, with the number of passages in culture, and with the patients' family histories. NFH-OSE cultures from 27 women and FH-OSE cultures from 9 women at p.0-5 were examined for the expression of E-cadherin. OSE cells with a cobblestone epithelial morphology or fibroblast-like morphology were consistently E-cadherin negative regardless of passage number (Figs. 3a, 5b; 3c, 5c). E-cadherin was
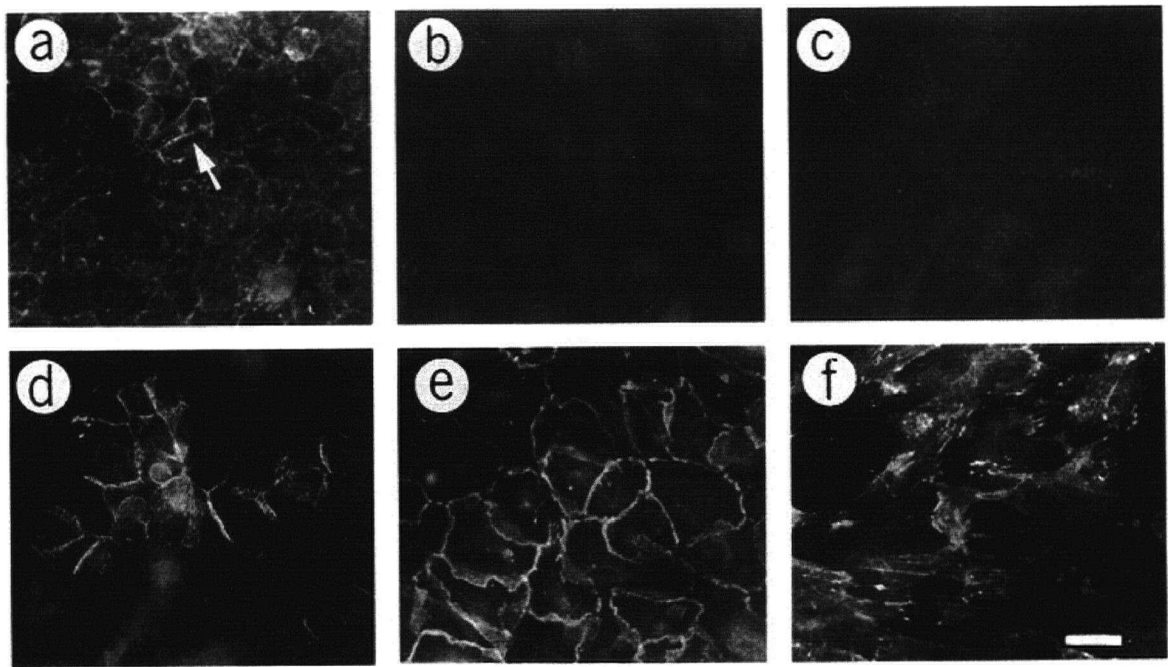
FIGURE 4 - P- and N-cadherin immunofluorescence in OSE, and ovarian cancer lines CaOV-3 and OVCAR-3. P-cadherin staining was consistently present in CaOV-3, particularly in colonies of compact epithelial cells (a, arrow). Both cobblestone and atypical fibroblast-like OSE cells lacked P-cadherin (b, c). In OVCAR-3 cultures, small groups of cells stained for N-cadherin (d). Both cobblestone and atypical OSE cells expressed N-cadherin homogenously (e, f). Note that the N-cadherin staining was localized between cell to cell borders in cobblestone OSE cells, but was found between overlapping atypical fibroblast-like OSE cells with a staining pattern reminiscent of stress fibers. *Bar,* 30 μm.

FIGURE 5 - E-cadherin immunofluorescence in NFH-OSE, FH-OSE and OVCAR-3 cells. OVCAR-3 with prominent E-cadherin staining at the cell-cell contact sites was used as a positive control (a). OSE cells with a cobblestone epithelial morphology (b) or atypical fibroblast-like morphology (c) were consistently E-cadherin negative. E-cadherin protein was detected in atypical cells overlying monolayers (d), and in metaplastic OSE cells (e and f), particularly in regions where OSE cells overlapped (e, arrows). In (e), stratification causes some cells to be out of focus. *Bar*, 10 μm.
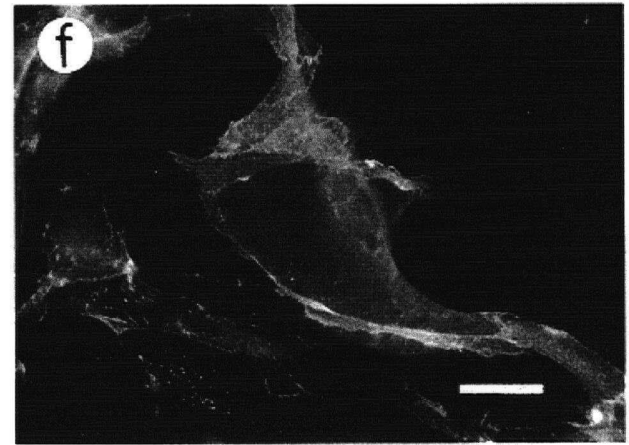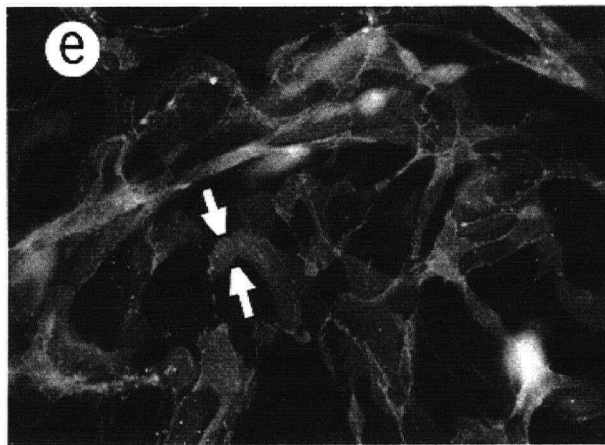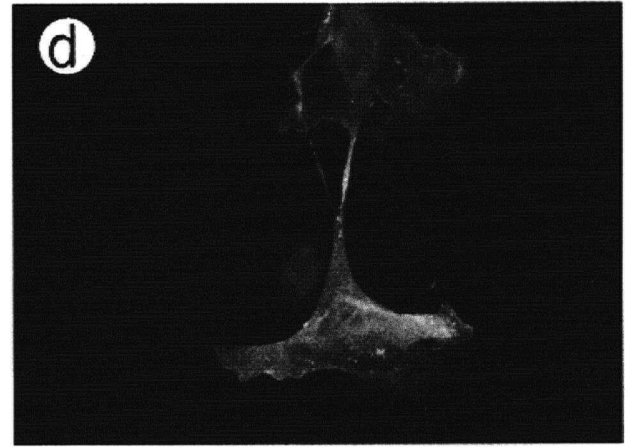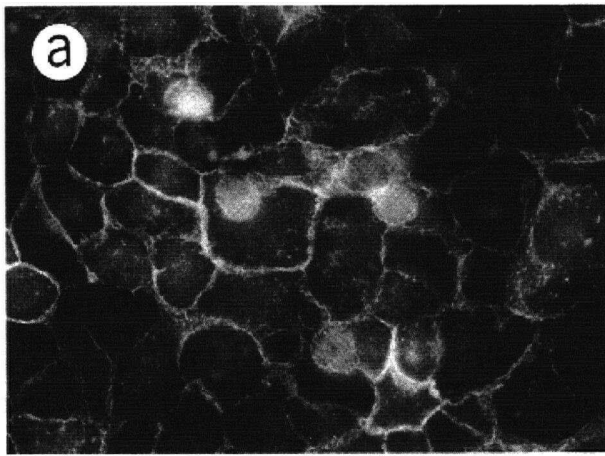
present on individual cells that were situated on top of monolayers (Figs. 3f, 5d). The strongest and most consistent E-cadherin staining occurred in metaplastic colonies where it was found between polygonal cells and, particularly, in regions of overlap between adjacent cell edges (Figs. 3e, 5e, f). Thus, E-cadherin was predominantly associated with areas of cell-cell adhesion that exceeded the limited lateral contact between flat, monolayered cells. As shown in Fig. 7, E-cadherin protein was expressed in cultures from a higher proportion of women (P<0.001), in a higher percentage of cells (P<0.001) and over more passages (P<0.001) in FH-OSE than in NFH-OSE cultures.

The ovarian carcinoma lines OVCAR-3 and CaOV-3 differed from the OSE and from each other in their cadherin expression. In OVCAR-3 cultures, less than 5% of cells stained for N-cadherin (Fig. 4d) and P-cadherin (data not shown) while CaOV-3 lacked N-cadherin completely (data not shown) but expressed P-cadherin homogeneously (Fig. 4a). Staining for E-cadherin was prominent in both OVCAR-3 (Fig. 5a) and CaOV-3 (data not shown).

To further examine E-cadherin expression, Western blot analysis was performed. The results of Western blot analysis were consistent with the immunofluorescence studies (Fig. 6). The E-cadherin specific band of 120 kDa could not be detected in any of the normal OSE cultures in p.1-5 including epithelial and fibroblast-like OSE cells (Fig. 6, lanes 4-6), whereas OVCAR-3 demonstrated a strong signal (lane 7). E-cadherin protein was also found in OVCAR-3 even at a total protein concentration three times lower than in OSE lanes (data not shown). In contrast to NFH-OSE, E-cadherin protein was detected in some FH-OSE cases (Fig. 6, lanes 1-3).

To determine whether enhanced intercellular contact would induce E-cadherin expression, NFH-OSE cultures from 6 women at p.1-4 were cultured on polyHEMA. The

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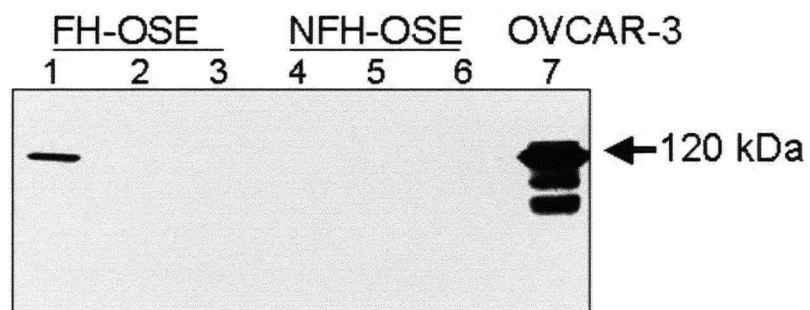
FIGURE 6 - Western blot analysis of E-cadherin protein expression
in FH-OSE, NFH-OSE and OVCAR-3 using HECD-1 antibody. Lane
1, OSE-201F p.2; lane 2, OSE-215F, p.4; lane 3, OSE-153F, p.5;
lane 4, OSE-143 p.4; lane 5, OSE-242 p.1; lane 6, OSE-232 p.2;
lane 7, OSE-202 p.5 and lane 8, OVCAR-3 as positive control.
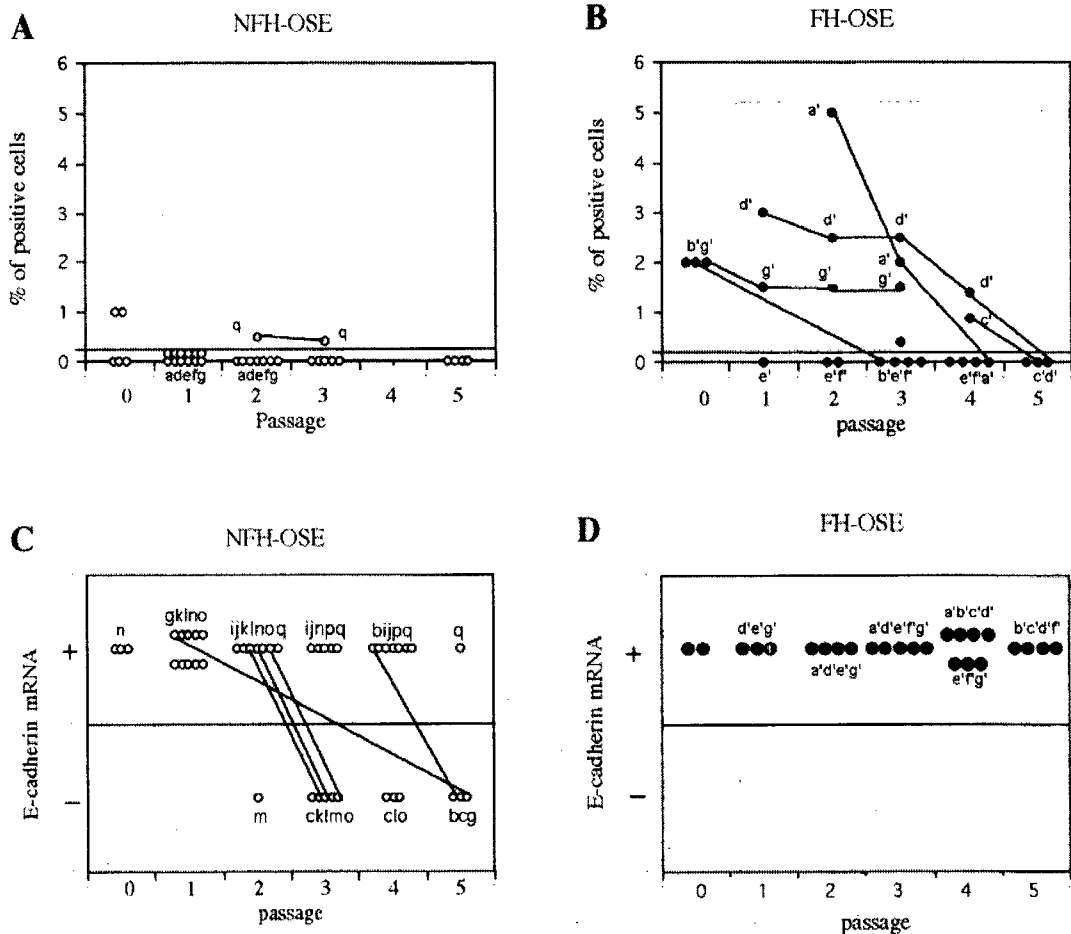
FIGURE 7 - A comparison of E-cadherin expression in NFH-OSE (○) (A and C) FH-OSE (●) (B and D) cultures. E-cadherin expression is compared at both protein (A and B) and mRNA (C and D) levels. In cases where cells from the same patient were tested in more than one passage, the symbols are indicated by the same *letters*, and those that show a decrease in E-cadherin expression with passaging are connected by *lines. Solid line*, cutoff level for 0% of positive cells (a and b) and absence of mRNA (c and d). Note that the E-cadherin positive cells are present in a higher proportion of cases (P<0.001), in a higher percentage of cells (P<0.001) and over more passages (P<0.001) in FH-OSE than in NFH-OSE. E-cadherin mRNA also persists to senescence in FH-OSE cultures, but not in NFH-OSE cultures.

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cells formed aggregates instead of monolayers on these substrata but junctional E-cadherin expression was only demonstrated in OVCAR-3 (Fig. 8a) and not in OSE cultures (Fig. 8c). Fluorescence visible on OSE culture (Fig. 8c) is likely to be non-specific instead of cytoplasmic E-cadherin staining, since it was also observed when normal goat serum was applied (Fig. 8e). The absence of E-cadherin in OSE aggregates was confirmed by Western blot analysis (data not shown).

In an effort to characterize E-cadherin mRNA expression in OSE cultures, total RNA was subjected to RT-PCR using primers specific for the E-cadherin transcript (Fig. 9A). The samples were scored as either E-cadherin present (+) or absent (-) (Figs. 7C and D). Amplicons of E-cadherin of 250 bp were detected in all 3 (100%) NFH-OSE cultures in p.0, and in 18/19 (94%) cultures from 14 women in p.1-2, but only in 14/25 (56%) cultures from 14 women in p.3-5 (Fig. 7C). In contrast, all 25 (100%) FH-OSE cultures from 9 women in p.0-5 expressed E-cadherin transcripts (Fig. 7D). Hence, E-cadherin mRNA was lost in about half the NFH-OSE cultures in late passage but persisted to senescence in all the FH-OSE cultures (P=0.001). If the results were compared by the number of cases (7/14 NFH-OSE and 7/7 FH-OSE), the difference remained significant (P=0.04). The presence of E-cadherin transcripts was independent of variation between epithelial and atypical cellular morphologies. These results were confirmed by Southern blot analysis of the PCR products using a non-radiolabeled E-cadherin specific probe (Fig. 9B). Transcripts were present in the ovarian carcinoma cell line OVCAR-3.

Among the above cases, 13 NFH-OSE cultures from 10 women and 20 FH-OSE cultures from 7 women were examined in parallel by immunofluorescence microscropy and by RT-PCR. In these cultures, E-cadherin mRNA was detected concurrently with immunoreactivity in 15% NFH-OSE cultures and in 50% FH-OSE cultures (P=0.06). In
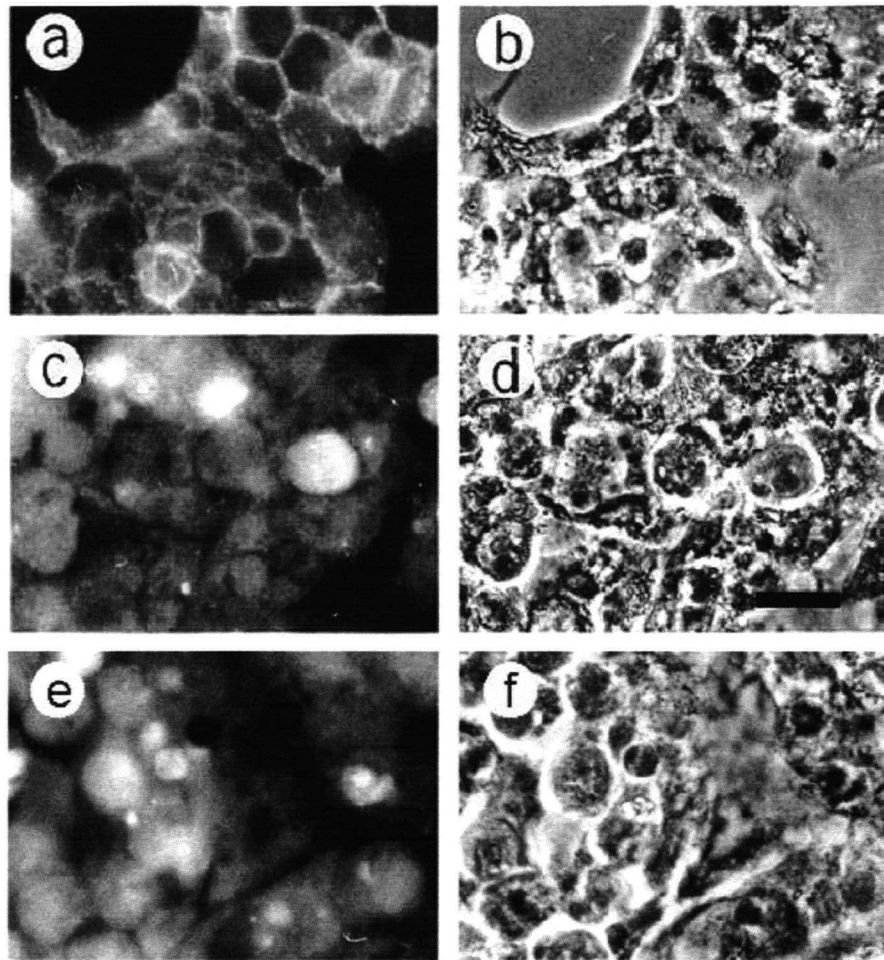
FIGURE 8 - Effect of polyHEMA on E-cadherin protein expression. Single cell suspensions of OVCAR-3 (a and b) and OSE cells (c and d) were grown on 0.8 mg/ml polyHEMA and stained for E-cadherin protein by immunofluorescence after they had formed aggregates. OSE cells that were incubated with normal goat serum instead of E-cadherin antibody were used as negative controls (e and f). Both (c) and (e) show background fluorescence due to the round cell shapes, but only in (a) is there specific fluorescence along the intercellular borders. *Bar*, 30 μm.
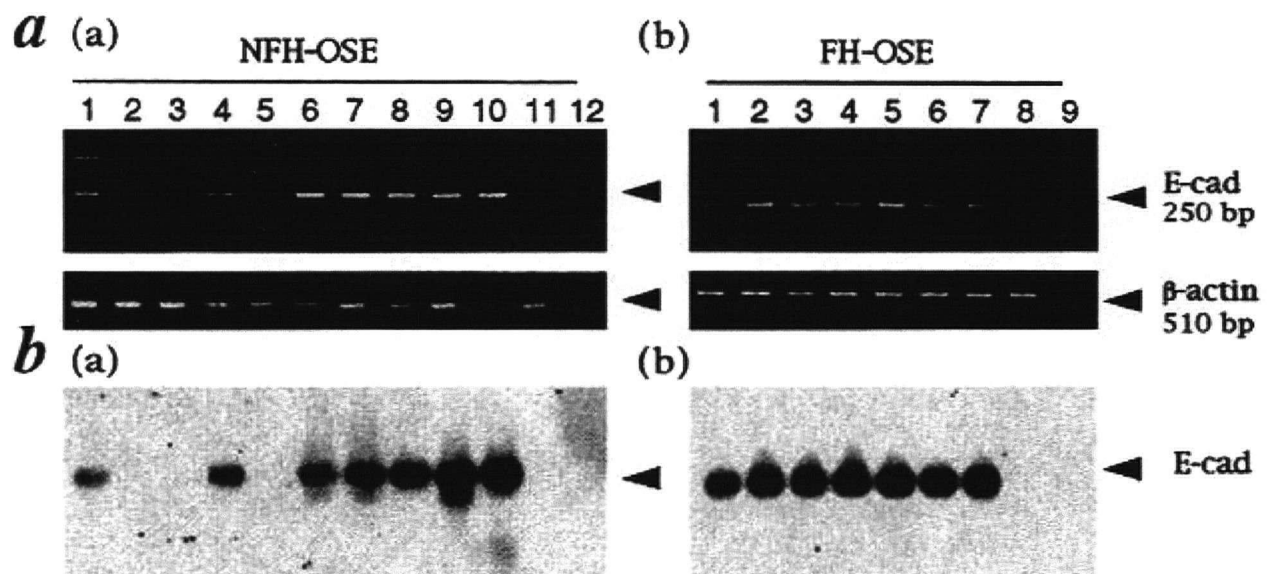
FIGURE 9 - A, Representative examples of E-cadherin mRNA expression by RT-PCR and B, Southern blot in NFH-OSE (a), and FH-OSE (b). (a), Lanes 1-2, OSE-84 p.4 and 5; lane 3, OSE-120 p.5; lanes 4-5, OSE-143, p.2 and 3; lanes 6-7, OSE-185 p.1 and 2; lane 8, OSE-202, p.4; lane 9, OSE-213 p.0; lane 10, OVCAR-3; lane 11, WI38 and lane 12, control. (b), Lane 1, OSE-178F p.4; lane 2, OSE-187F p.2; lane 3, OSE-195F p. 5; lanes 4-5, OSE-204F p.3 and 4; lane 6, OSE-215F p.0; lane 7, OVCAR-3; lane 8, WI38 and lane 9, control. β-actin was amplified from the same cases as an assessment for the cDNA quantity used as template in the PCR.

addition, E-cadherin mRNA in the absence of immunoreactivity was demonstrated in 46% NFH-OSE cultures and 50% FH-OSE cultures ($P>0.05$). Finally, neither mRNA nor immunoreactivity was detected in 39% of NFH-OSE cultures but in 0% of FH-OSE cultures ($P<0.05$).

To more fully characterize the expression pattern of the E-cadherin transcripts in OSE cells, ISH was performed. Both positive and negative results by ISH were consistent with the results of RT-PCR (Fig. 10). E-cadherin hybridization was positive in OVCAR-3. In human lung fibroblasts and in NFH-OSE cultures which were negative for E-cadherin mRNA by RT-PCR, the ISH signal was not discernible above background. In contrast, E-cadherin mRNA displayed a fairly homogeneous staining pattern and was present in almost all OSE cells in cultures that were positive by RT-PCR and with immunoreactive E-cadherin. Interestingly, it was equally abundant in cultures that were positive for mRNA by RT-PCR but lacked detectable immunoreactive E-cadherin. Therefore, the E-cadherin mRNA detected by RT-PCR in cultures that were negative by immunofluorescence appeared to be present in most cells and was not the product of amplification of signals from a small number of undetected E-cadherin positive cells. In OVCAR-3 as well, staining by ISH was more widespread than staining by immunofluorescence. The use of E-cadherin sense (Fig. 10), pBlueScript antisense (data not shown) and RNase pre-treatment (data not shown) controls confirmed that E-cadherin hybridization was indeed specific.
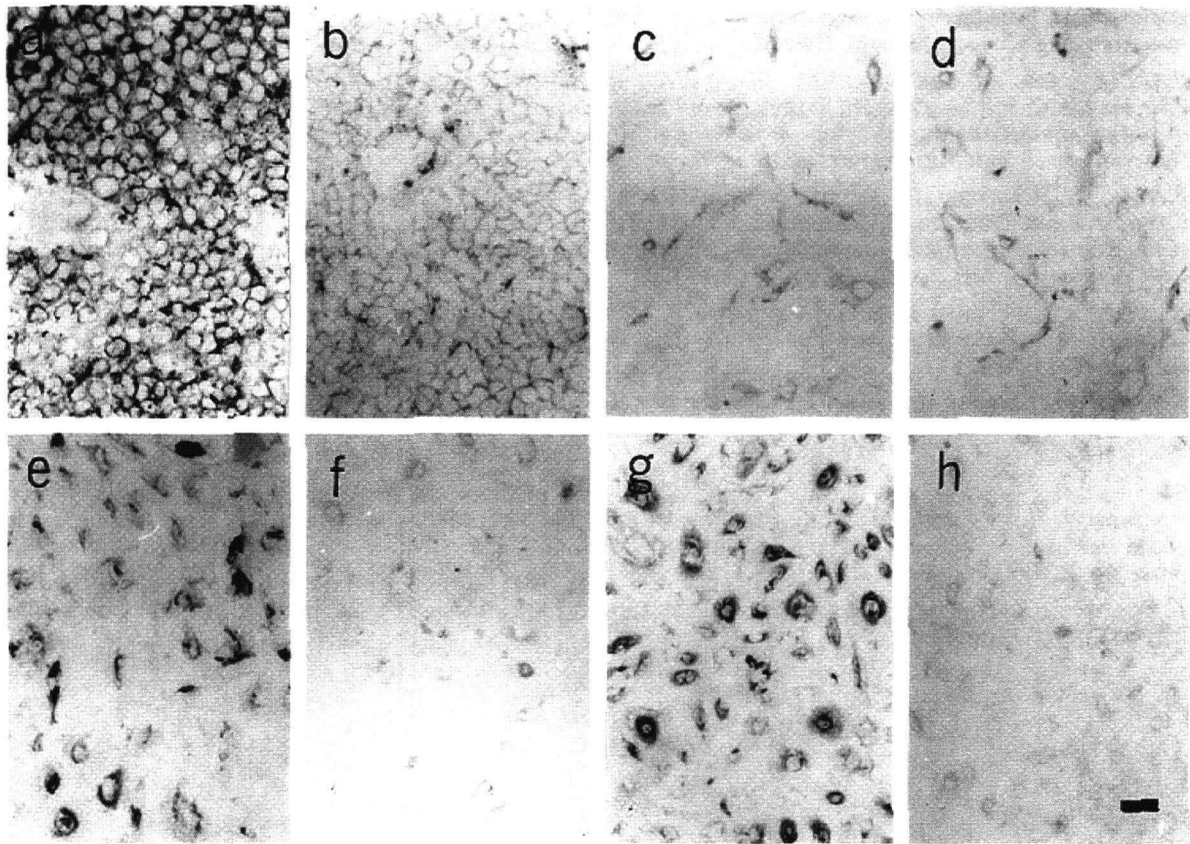
FIGURE 10 - *In situ* hybridization (ISH) of NFH-OSE, FH-OSE and OVCAR-3. The hybridization products, using E-cadherin riboprobes, were detected by the Digoxigenin colorimetric detection kit. a, antisense and b, sense signals of OVCAR-3; c, antisense and d, sense signals of NFH-OSE-143 p.4; e, antisense and f, sense signals of NFH-OSE-180 p.2; g, antisense and h, sense signals of FH-OSE-178F p.3. RT-PCR demonstrated E-cadherin mRNA in a-b, e-f and g-h but not in c-d. Immunofluorescence demonstrated E-cadherin protein in a-b, but not in c-h. *Bar*, 20 μm.

3.3. HGF-Met in normal OSE

3.3.1. Expression of Met and HGF in OSE

Immunoreactive Met protein was detected in 9/11 (81%) NFH-OSE cultures and in 4/4 (100%) FH-OSE cultures. Representative samples are shown in the upper panel of Fig. 11A. All Met positive NFH-OSE cultures had an epithelial phenotype and were in p.0 or at low passages (p.1-2), while two NFH-OSE cultures that were Met negative had a mesenchymal (fibroblast-like) phenotype and were in a late passage (p.4-5) (Fig. 11A, lane 4). In contrast, Met protein persisted to senescence in all FH-OSE cultures (Fig. 11A, lane 3). Met protein was also highly expressed in all three (100%) ovarian cancer cell lines. Met transcripts were always detected in parallel with E-cadherin transcripts. Unlike E-cadherin that seems to be regulated post-transcriptionally in some cases, Met protein was consistently detected along with its transcript in normal and neoplastic OSE (Fig. 11A, B). Using RT-PCR, transcripts of Met were detected in 20/21 (95%) of the NFH-OSE cultures in p.0-2, but only in 12/19 (63%) of the NFH-OSE cultures in p.3-5 (Fig. 11B and C). Consistent with our results of Western blot analysis, Met transcripts persisted to senescence in all 20 (100%) FH-OSE cultures, and in all 3 (100%) ovarian carcinoma lines (Fig. 11B and C). The difference in the expression of Met in prolonged cultures of FH-OSE and ovarian cancer cells as compared to NFH-OSE is statistically significant ($P<0.05$).

Transcripts of HGF were detected in 8/19 (42%) FH-OSE cultures from 9 women and in 2/3 (67%) ovarian carcinoma lines, but were undetectable in all 27 NFH-OSE cultures from 17 women (Fig. 12). To quantify the level of HGF expression in these cells, we performed ELISAs on conditioned media. HGF was measured in samples of 8 NFH-OSE, 5 FH-OSE and 2 ovarian cancer cell lines. Both positive and negative results by RT-PCR were consistent with the results of HGF ELISAs (Table 3). The median HGF level was 46.4

FIGURE 11 - Met expression in cultured human OSE. (A) Western blot analysis of Met (upper panel) & E-cadherin (lower panel) and (B) RT-PCR of Met (upper panel) & E-cadherin (lower panel) in NFH-OSE, FH-OSE and OVCAR-3. Lane 1, OSE-201F p.4; lane 2, OSE-215F p.2; lane 3, OSE-153F p.5; lane 4, OSE-143 p.4; lane 5, OSE-242 p.1; lane 6, OSE-232 p.2; lane 7, OSE-202 p.2 and lane 8, OVCAR-3. The 145 kDa and 190 kDa bands represent the β-subunit and the uncleaved precursor form of Met, respectively. (B, lower panel) β-actin was amplified from the same cases as an assessment for the cDNA quantity used as template in the PCR. Note that c-Met expression parallels E-cadherin expression at the mRNA level, but not at the protein level. (C) Comparison of Met expression in all NFH-OSE (□) and FH-OSE (■) cultures. Solid line, cut-off level for presence and absence of Met. Note that Met persists to senescence in all FH-OSE cultures but not in all NFH-OSE cultures.

A)

FH-OSE   NFH-OSE   OVCAR-3

1 2 3 4 5 6 7 8

190 kDa Met
145 kDa

120 kDa E-cadherin

B)

Met 320 bp

E-cadherin 250 bp

β-actin

C)

Passages 0-2   Passages 3-5

Presence

Absence

No. of cultures

□ NFH-OSE
■ FH-OSE

69
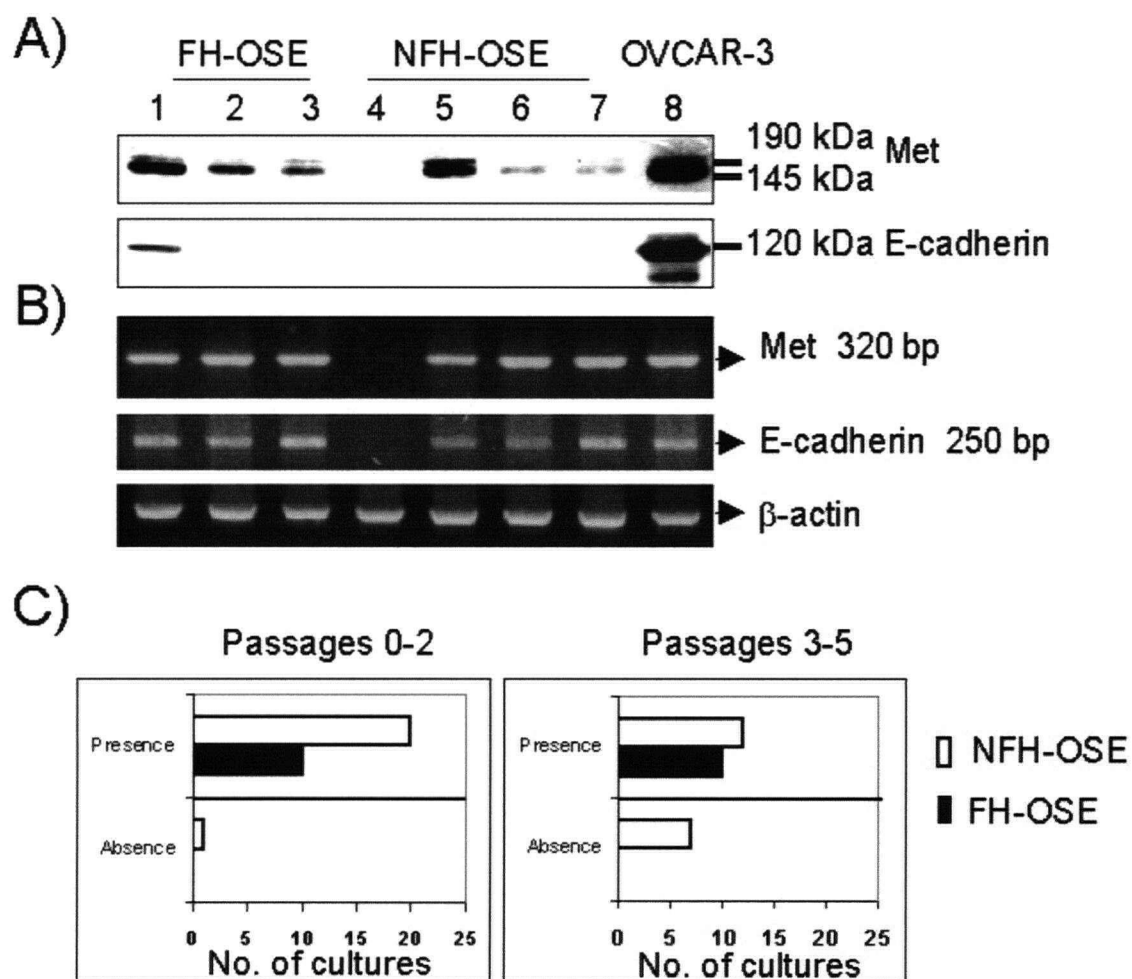
FIGURE 12 - Representative examples of HGF mRNA expression in cultured human OSE.

(A) RT-PCR (upper panel) and (B) Southern blot analysis of HGF in NFH-OSE, FH-OSE

and ovarian cancer cell lines. Lane 1, OSE-190; lane 2, OSE-120; lane 3, OSE-187F; lane

4, OSE-195F; lane 5, OSE-215F; lane 6, CaOV3; lane 7, OVCAR-3; lane 8, SKOV3; lane 9,

WI38; lane 10, HepG2; and lane 11, water control. (A, lower panel) b-actin was amplified

from the same cases as an assessment for the cDNA quantity used as template in the PCR.

(C) Comparison of HGF mRNA expression in NFH-OSE (□), FH-OSE (■) and ovarian

cancer cell lines (⊟). Solid line, cut-off level for presence and absence of HGF mRNA. Note

that HGF mRNA is more frequently detected in FH-OSE and ovarian cancer cell lines than

in NFH-OSE cultures.

A)

NFH-OSE   FH-OSE   OvCa

1   2   3   4   5   6   7   8   9   10   11

← HGF 380 bp

← β-actin

B)

← HGF

C)

Presence

Absence

No. of cultures

☐ NFH-OSE

■ FH-OSE

▤ Ovarian cancer cell lines

71
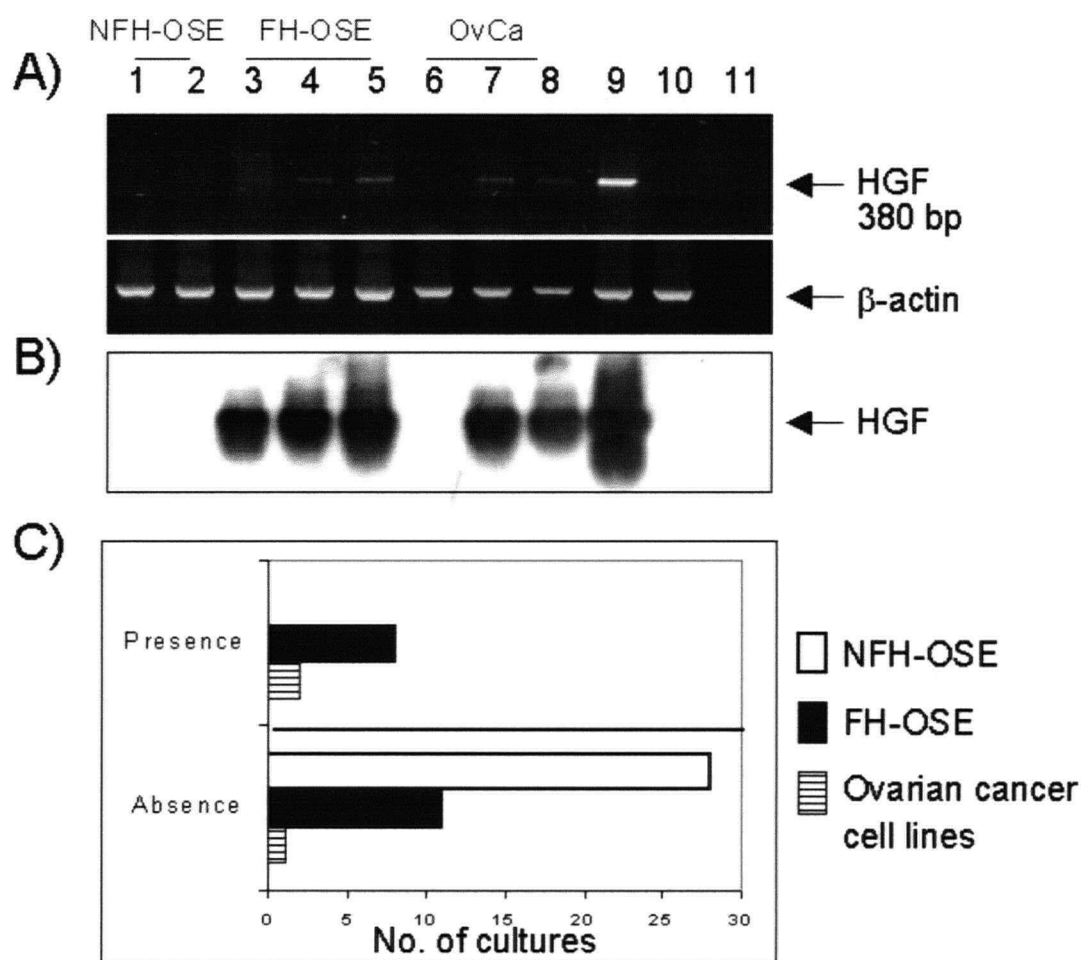
pg/ml (range 32-139.2 pg/ml) in FH-OSE cultures. HGF was expressed at a higher level in the ovarian cancer cell line SKOV-3 (309.8 pg/ml) than in OVCAR-3 (42.82 pg/ml) which was in the range of the OSE cultures (Table 3). In contrast, HGF protein was not detected in NFH-OSE cultures despite our effort to concentrate their conditioned media. We also compared the capacity to induce scatter activity (Stoker and Perryman, 1985) by the conditioned media. Media from FH-OSE and ovarian cancer cells induced moderate cell dissociation resulting in irregular contours of C-4II colonies (Wong *et al.*, 2000 [in press]), while intense cell dispersion and scattering were caused by the conditioned medium of the positive control cell line WI-38 (Fig. 13D-F). These effects corresponded to those of low and high concentrations of exogenously added HGF (Fig. 13A-C) and also to the difference in HGF concentrations in the conditioned media derived from different cell lines (Table 3). The identification of HGF in the conditioned media of FH-OSE and ovarian cancer cells as the scatter-inducing factor was confirmed by the inhibition of scatter activity of the conditioned media by neutralizing anti-HGF antibody (Fig. 13G, H). Similar results were observed with HGF-containing medium produced by WI-38 (Fig. 13I).

To overcome the limited number of low passage FH-OSE cells and to obtain sufficient protein, the SV40-immortalized IOSE-261F and IOSE-267F from women carrying known BRCA1 mutations were used for immunoprecipitation analysis (Maines-Bandiera *et al.*, 1992). Immunoblot analysis of IOSE-261F is showed in Fig. 14. Met receptor was highly reactive with anti-phosphotyrosine antibody after treatment with 20 ng/ml recombinant HGF (Fig. 14, lane 2). We also found that Met was reactive with anti-phosphotyrosine antibody without such treatment (Fig. 14, lane 1), suggesting that the receptor was activated in an autocrine fashion by endogenous HGF.

TABLE 3 - HGF levels in NFH-OSE, FH-OSE and ovarian cancer cell lines

Sample	Number of cases	HGF level (pg/ml)*	
		Median	Range
Normal OSE			
NFH-OSE	8	0	0
FH-OSE	5	46.4	32-139.2
Ovarian cancer cell lines			
SKOV3		309.8	
OVCAR-3		42.8	
Fibroblast cell line			
WI-38		6212.2	

The assay measured both HGF and its precursor, pro-HGF.

*All samples were assayed in duplicate, and the mean of each replicate was reported.
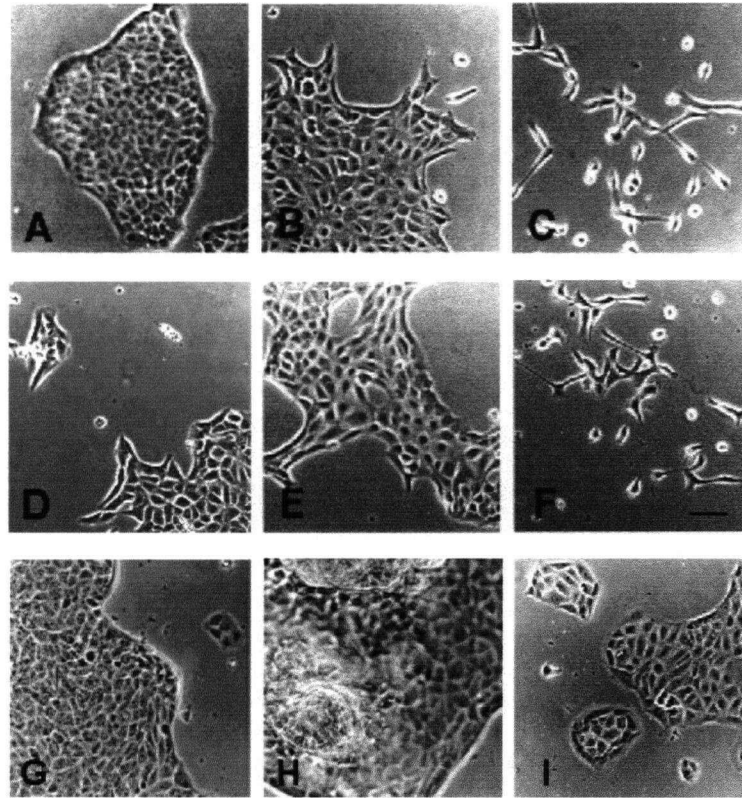
FIGURE 13 - Effect of conditioned media on scatter activity of C-4II cells. Colonies of C-4II cells were plated in 24-well plates and incubated at for 24 h in culture media containing 0 (A, control), 0.5 (B) or 10 (C) ng/ml recombinant HGF or in concentrated conditioned media of OSE-178F (D), SKOV-3 (E) or the positive control cell line WI-38 (F). Conditioned media of OSE-178F (G), SKOV-3 (H) or WI-38 (I) were incubated with neutralizing anti-HGF antibody (5 μg/ml). Bar, 50 μm.
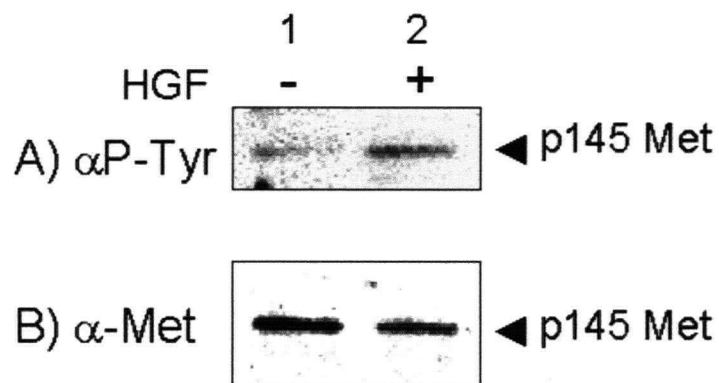
FIGURE 14 - Tyrosine phosphorylation of Met receptor. IOSE-261F were treated

without (lane 1) or with (lane 2) 20 ng/ml human recombinant HGF for 10 min. 1 mg

of protein was immunoprecipitated with DO-24 monoclonal antibody, resolved by

SDS-PAGE, and immunoblotted with anti-phosphotyrosine (A) or anti-Met (B).

3.3.2. Protein kinase regulation in OSE

8 NFH-OSE cultures and 6 FH-OSE cultures were examined for effects of HGF stimulation on kinase phosphorylation. In agreement with other reports (Khawaja *et al.*, 1998; Potempa and Ridley, 1998), HGF stimulation resulted in phosphorylation of ERK 2 and ERK 1, Akt1, Akt2 and the p70 S6K in normal and malignant OSE, as revealed by their reduced mobilities on SDS-PAGE gels (Fig. 15). Interestingly, in all (100%) FH-OSE cultures and in the ovarian cancer cell line OVCAR-3, the downstream effectors of PI3K Akt2 and p70 S6K, but not Akt1, were phosphorylated even in the absence of HGF stimulation. This is in contrast to NFH-OSE, where less than 50% of the cultures expressed constitutively phosphorylated Akt2 and p70 S6K (Fig. 15c, d). Other PI3K downstream regulators such as GSK3β and ILK were also studied. The antibodies used did not selectively recognize phosphorylated forms of these kinases. There was no difference in the expression of GSK3β and ILK between NFH-OSE and FH-OSE cultures before and after HGF stimulation (Fig. 15e, f).

3.3.3. HGF is mitogenic in normal OSE

To determine whether HGF had a mitogenic effect of on OSE, cells were cultured with or without HGF stimulation and cell growth was determined colorimetrically by the Methylene Blue assay (Elliott and Auersperg, 1993). The absorbance is a linear function of cell number. The OSE cell doubling time in the presence of 2% FBS is 7-9 days. Fig. 16 shows the dose-response effect of HGF for OSE. The optimal concentration of recombinant HGF for OSE cell proliferation was 10-20 ng/ml, and 5 ng/ml or more resulted in statistically significant increases in cell growth ($P<0.05$) (Fig. 16). All 7 early-passage NFH-OSE

FIGURE 15 - Effects of HGF stimulation on kinase activation assessed by phosphorylation-induced reductions of kinase mobilities on Western blots. Treatment with 20 ng/ml HGF resulted in the phosphorylation and apparent activation of the (a) p44 ERK1 and p42 ERK2 MAPKs, (b) Akt1, (c) Akt2 and (d) p70 S6K in NFH-OSE, FH-OSE and OVCAR-3. The bottom band represents unphosphorylated form of the kinases, whereas the upper bands represent different phosphorylated forms. (*)ERK2, (**) ERK1 and phosphorylated ERK2, (***) phosphorylated ERK1. HGF-induced activation of ERK2 is assessed by a reduction in the density of (*) unphosphorylated ERK2. The antibodies used for (e) GSK3β and (f) ILK did not selectively recognize phosphorylated forms of these kinases. Note that phosphorylated forms of (C) Akt2 and (D) p70 S6K are present in FH-OSE and OVCAR-3 even in the absence of HGF stimulation.

NFH-OSE   FH-OSE   OVCAR-3

HGF   -   +   -   +   -   +

a) ERK1/2   ***
                                             **
                                             *

b) Akt1

c) Akt2

d) p70 S6K

e) GSK3β

f) ILK

78
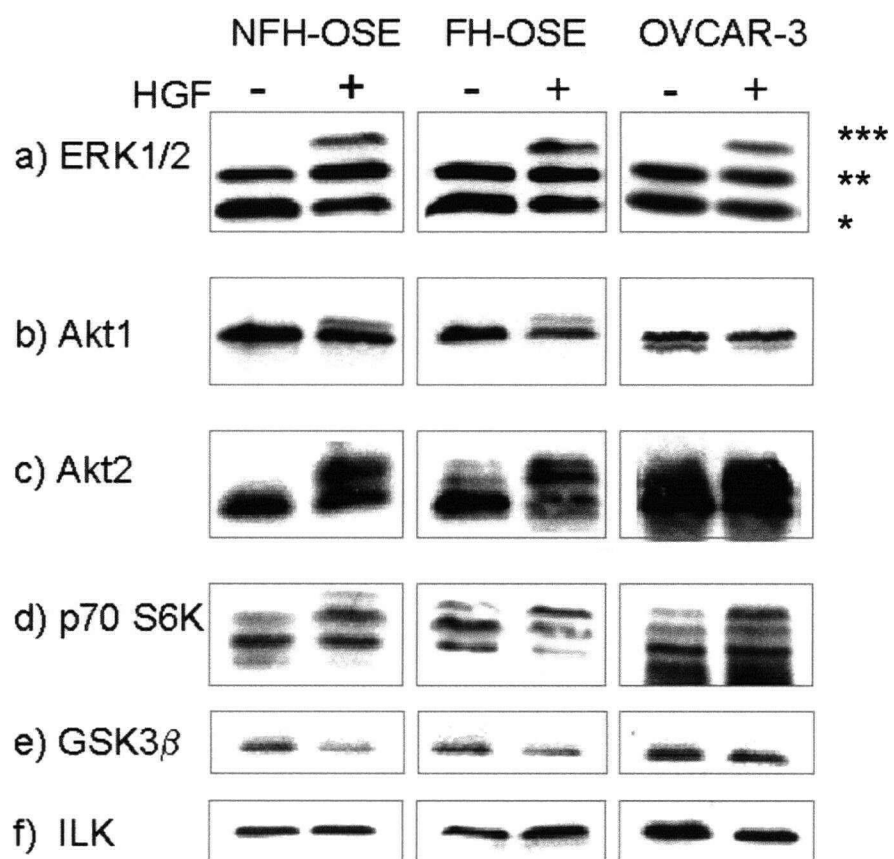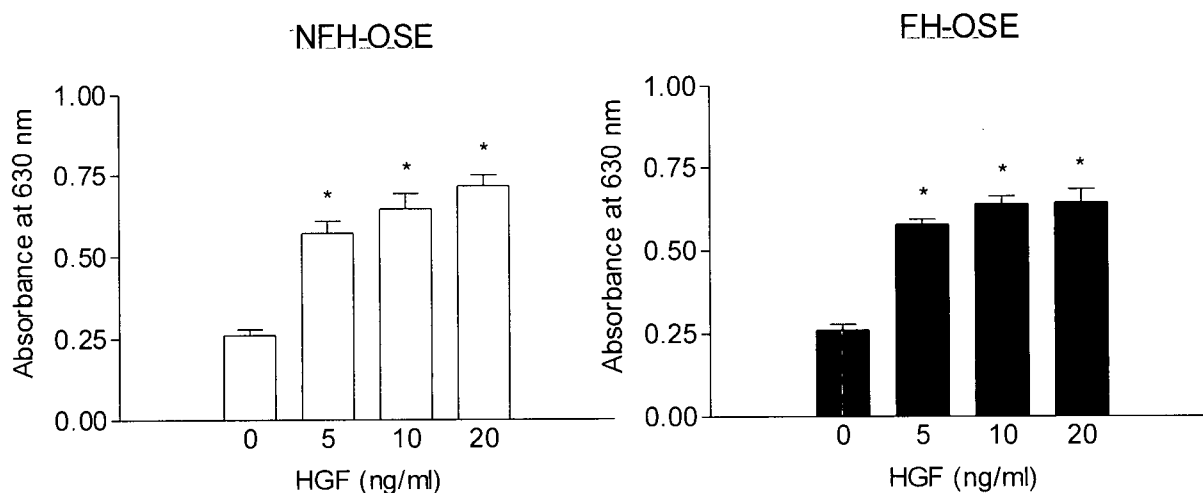
FIGURE 16 - Dose-response effect of HGF on cell proliferation of one representative case of NFH-OSE and FH-OSE. Cells were cultured in medium containing 2% FBS in the presence of HGF (5, 10 and 20 ng/ml) for 6-8 days. Cell numbers were determined colorimetrically by the Methylene Blue assay as described in Materials and Methods. Values in this graph represents means ± S.D. of six replicates of a representative OSE culture. *, indicates that a value is significantly different from the control value (0 ng/ml HGF) ($P<0.05$). The optimum concentration of HGF in stimulating OSE cell proliferation was 10-20 ng/ml.
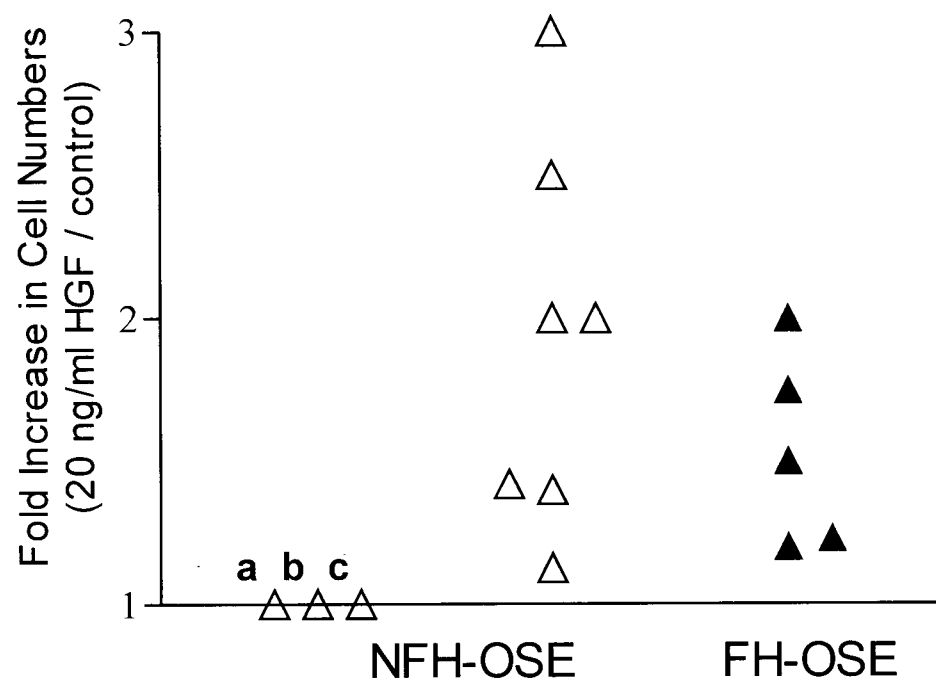
FIGURE 17 - Effect of HGF on cell proliferation of NFH-OSE (Δ) and FH-OSE (▲). Each symbol represents an individual case. Cell numbers were determined colorimetrically by the Methylene Blue assay as described in Materials and Methods. Data are presented as fold increase in cell number of HGF-stimulated (20 ng/ml) cultures of the control (0 ng/ml HGF). a, b and c represent NFH-OSE cultures that did not express Met and did not respond to HGF.

cultures (p.1-3) and all 5 FH-OSE cultures (p.1-2) demonstrated increases in cell growth in the presence of recombinant HGF. Those late-passage NFH-OSE cultures (p.4-7) that did not express Met had no growth response to HGF (Fig. 17, symbols a-c). Although there was no statistically significant difference, HGF-stimulated FH-OSE cultures appeared to have a more limited proliferative response than NFH-OSE cultures (Fig. 17).

By blocking the upstream MEK 1 and 2 with PD98059 (50 μM) or inhibiting mTOR/FRAP with rapamycin, HGF-activated OSE cell proliferation was abolished (Fig. 18) (P<0.05). All OSE cultures were most sensitive to the growth inhibitory effects of LY294002 (25 μM) (P<0.05), indicating that the PI3K signaling pathway is important in OSE cell growth and survival. LY294002 also significantly inhibited serum-induced OSE cell proliferation. Inactivation of p38 MAPK by SB203580 (10 μm) had no effect on HGF-stimulated OSE cell growth. The HGF-induced OSE proliferative response was neutralized by the use of anti-HGF antibody (Fig. 18) (P<0.05).

3.3.4. Motogenic and morphogenetic effects of HGF in normal OSE

To investigate the possible role of HGF as a motogen, confluent cultures were wounded with a scraper and the effect of HGF on cell motility was examined. HGF had no observable effect on either cell motility or morphology in wounded OSE cultures (data not shown).

3 NFH-OSE cases and 4 FH-OSE cases were analyzed for their morphogenetic responses in collagen gels. Both groups of OSE cells dispersed, assumed a spindle-shaped morphology within collagen gels independently of HGF (Fig. 19a, b) and eventually died.

On growth factor-reduced Matrigel, normal OSE cells initially formed aggregates that were joined to each other via branching structures (Fig. 20a, b), and later as
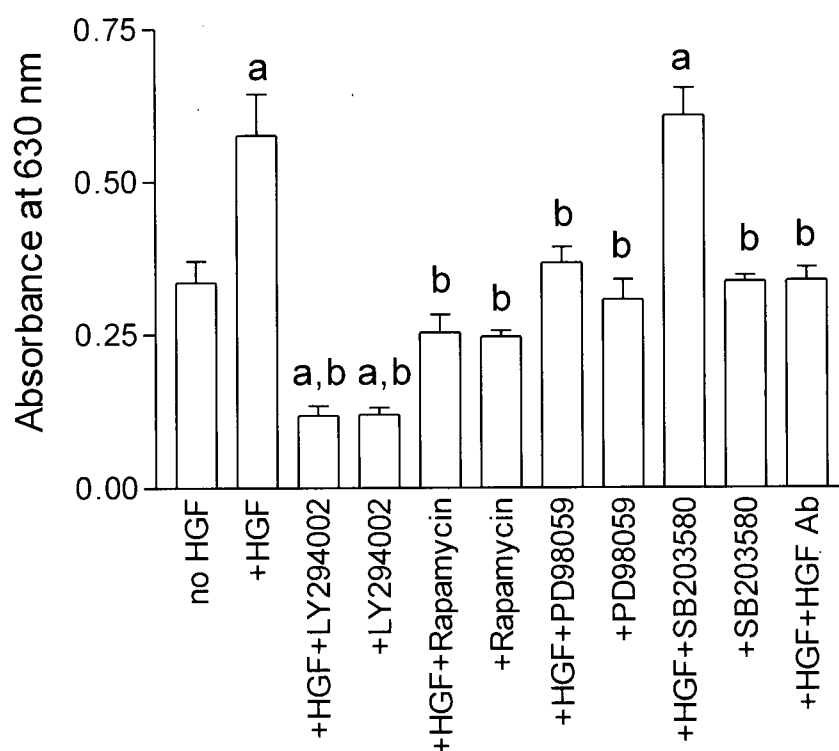
FIGURE 18 - Effect of the inhibitors to specific protein kinases on HGF-induced OSE cell proliferation. NFH-OSE cells were treated with 25 μM LY294002, 20 nM rapamycin, 50 μM PD98059, 10 μM SB203580 or 5 μg/ml neutralizing HGF antibody in the absence or presence of HGF (20 ng/ml) in medium containing 2% FBS. Cultures were serum starved with 0.1% FBS overnight before any treatment with HGF and inhibitors. Values in this graph represent means ± S.D. of six replicates of a representative OSE culture. a, b, indicate that a value is significantly different from the value in 0 ng/ml and 20 ng/ml HGF, respectively (P<0.05).
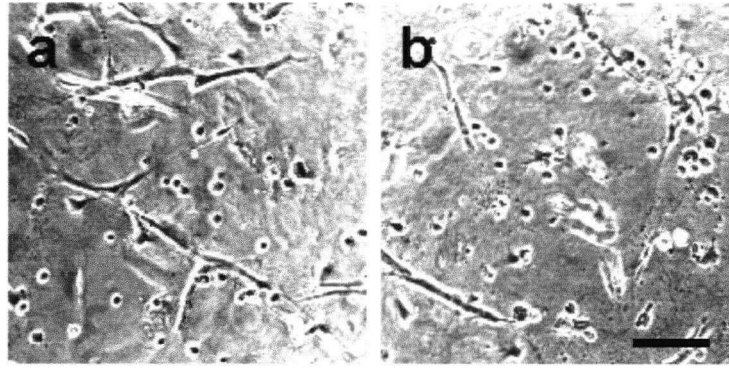
Figure 19 - Effect of HGF on OSE in rat-tail derived collagen gel. Single cell

suspensions of OSE were grown in collagen gel without (a) or with (b) 20 ng/ml

HGF. The OSE cells became dispersed and assumed a spindle-shaped

morphology independently of HGF. *Bar*, 100 μm.

Figure 20 - Effect of HGF on OSE on Matrigel. Single cell suspensions of OSE cells were grown on growth factor-reduced Matrigel without (a, c) and with (b, d) 20 ng/ml HGF for 2 (a, b) and 6 (c, d) days. OSE cells initially formed aggregates that were joined to each other via branching structures (a, b), and later formed individual round aggregates independently of HGF (c, d). These aggregates sometimes revealed cyst-like structures (e, f), that were particularly obvious when those structures were formed around cotton fibers (f). Circular rims and stress lines of Matrigel near OSE cell aggregates (arrowheads), suggested contraction of the matrix by OSE cells (c-f). *Bar,* 100 μm.
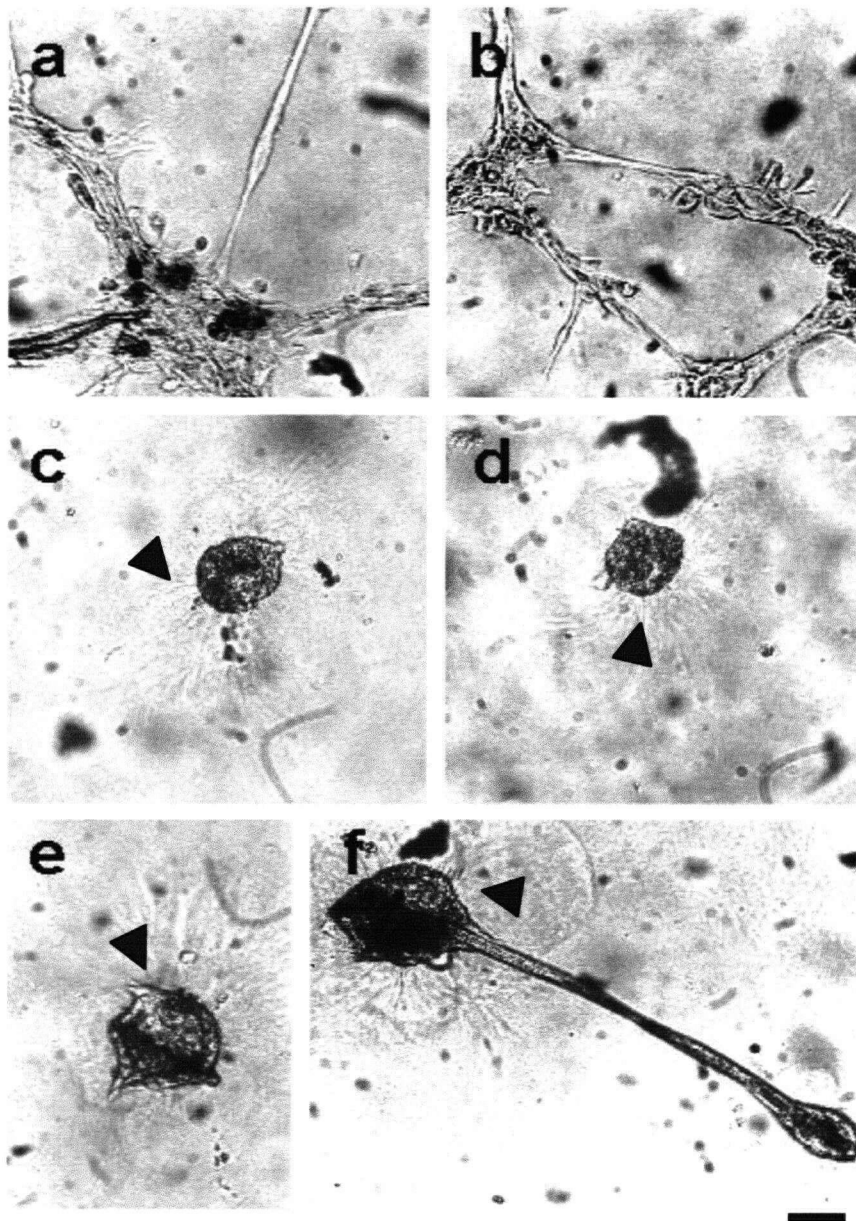
individual aggregates independently of HGF (Fig. 20c, d). OSE cell aggregates sometimes revealed cyst-like structures, that were particularly obvious when those structures were formed around cotton fiber (Fig. 20e, f). Circular rims and stress lines of Matrigel near OSE cell aggregates suggested degradation and contraction of the matrix by OSE cells (Fig. 20c-f). In contrast to OSE aggregates that were grown on regular Matrigel matrices which invaded the Matrigel and finally attached and spread on the underlying plastic (Kruk et al., 1994), those that were cultured on growth factor reduced Matrigel remained on the apical surface.

3.4. Changes in the responses to HGF and in protein kinase regulation with neoplastic progression of OSE

The contribution of HGF and protein kinases in the neoplastic progression of OSE was also studied. In addition to normal OSE and ovarian cancer cell lines, cell lines immortalized with SV40 large T antigen (IOSE-29) (Maines-Bandiera et al., 1992) and their E-cadherin transfected derivatives (IOSE-29EC and its derived tumor cell line IOSE-29EC/T4), which are tumorigenic (Auersperg et al., 1999; Ong et al., 2000), were compared as representatives of progressive stages in ovarian carcinogenesis (Fig. 2).

3.4.1. Changes in the responses to HGF with neoplastic progression of OSE

3.4.1.1. Enhanced HGF-Met expression in SV40-E-cadherin transfected OSE

Met was weakly expressed in normal OSE, IOSE-29 and neo-controls (Fig. 21A, lanes 1-3). The expression level of Met was enhanced in both IOSE-29EC and IOSE-29EC/T4 cell lines (Fig. 21A, lanes 4-5) and even more so in the ovarian cancer cell lines (Fig. 21A, lanes 6-8).
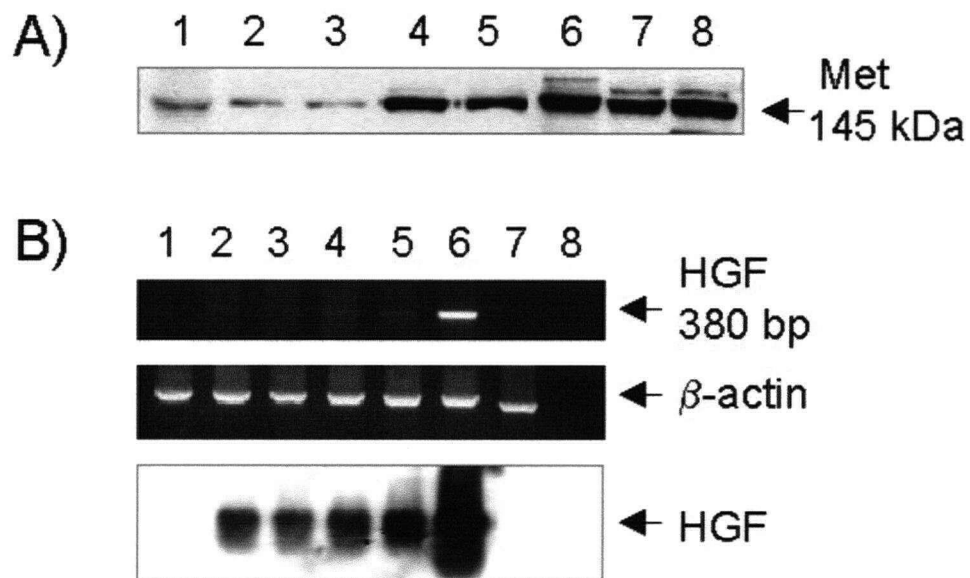
FIGURE 21 - Met and HGF expression in cultured human OSE at different progressive stages in ovarian carcinogenesis. (A) Western blot analysis of Met. Lane 1, normal OSE; lane 2, IOSE-29; lane 3, IOSE-29neo; lane 4, IOSE-29EC; lane 5, IOSE-29EC/T4 and lane 6, CaOV-3; lane 7, OVCAR-3 and lane 8, SKOV-3. The 145 kDa band represents the β-subunit of the Met receptor. (B) RT-PCR (upper panel) and Southern blot analysis (lower panel) of HGF. Lane 1, normal OSE; lane 2, IOSE-29; lane 3, IOSE-29neo; lane 4, IOSE-29EC; lane 5, IOSE-29EC/T4; lane 6, WI-38; lane 7, HepG2 and lane 8, water control. β-actin (middle panel) was amplified from the same cases as an assessment for the cDNA quantity used as template in the PCR. Note very faint HGF bands in lanes 2-5 were detected by RT-PCR and the presence of the signals was confirmed by Southern blot analysis.

HGF mRNA was not found in normal OSE (Fig. 21B, lane 1), but was expressed in IOSE-29EC and IOSE-29EC/T4 (Fig. 21B, lanes 4-5) and the parental IOSE-29 and neo-transfectants (Fig. 21B, lanes 2-3). Compared to the positive cell line WI-38 (Fig. 21B, lane 6) that expressed high levels of HGF, only very faint HGF mRNA signals were detected in IOSE-29, IOSE-29neo, IOSE-29EC and IOSE-29EC/T4 (Fig. 21B, lanes 2-5) by RT-PCR. The results were confirmed by Southern blot analysis using a non-radiolabeled, HGF-specific cDNA probe (Fig. 21B, lower panel). HGF protein was expressed at similar levels in IOSE-29EC (90.6 pg/ml) and IOSE-29EC/T4 (73.9 pg/ml), but was undetectable in IOSE-29 by ELISAs (Table 4). To determine whether the HGF protein synthesized by IOSE-29EC and IOSE-29EC/T4 was bioactive, scatter assay was performed. Media conditioned from IOSE-29EC/T4 induced moderate cell dissociation resulting in irregular contours of C-4II colonies (Fig. 22C), whereas intense cell dispersion and scattering were observed in the conditioned medium of WI-38 fibroblasts (Fig. 22D). These differences in responses correlated with the HGF concentrations in the conditioned media derived from different cell lines, as reported in Table 4.

To further investigate the possibility of an autocrine HGF-Met signaling in E-cadherin expressing IOSE cells, the phosphorylation status of Met was examined. Basal Met auto-phosphorylation was observed in IOSE-29EC/T4 (Fig. 23A, lane 1) and also in IOSE-29 that expressed HGF mRNA. In both lines, Met was highly tyrosine-phosphorylated in the presence of HGF stimulation (Fig. 23, lanes 2 and 4).

TABLE 4 - HGF levels in IOSE, IOSE-EC and ovarian cancer cell lines

Sample	HGF level (pg/ml) Mean
Normal OSE (as reported in Table 3)	undetectable
SV40-immortolized OSE	
IOSE-29	undetectable
SV40-E-cadherin transfected OSE	
IOSE-29EC	90.6
Tumor cell line derived from IOSE-29EC	
IOSE-29EC/T4	73.9
Ovarian cancer cell lines	
SKOV3	309.8
OVCAR-3	42.8
Fibroblast cell line	
WI-38	6212.2

The assay measured both HGF and its precursor, pro-HGF.

*All samples were assayed in duplicate, and the mean of each replicate was reported.
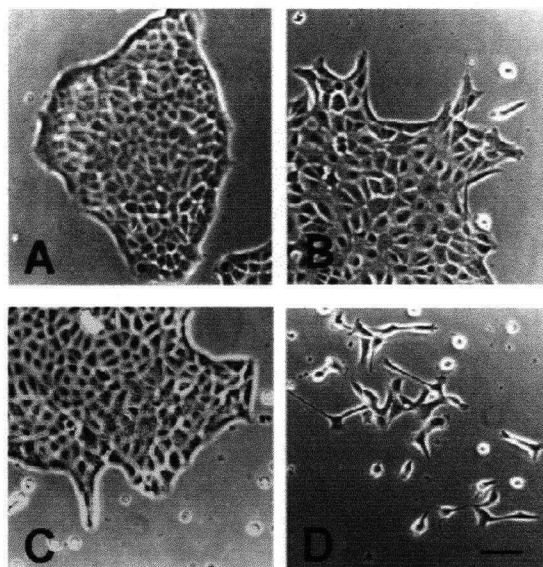
FIGURE 22 - Effect of conditioned media on scatter activity of C-4II cells. Colonies of C-4II cells were plated in 24-well plates and incubated at 37°C for 24 h in culture media containing 0 (A, control) and 0.5 ng/ml recombinant HGF (B) or in concentrated conditioned media of IOSE-29EC/T4 (C) or the positive control cell line WI-38 (D). *Bar*, 50 μm.
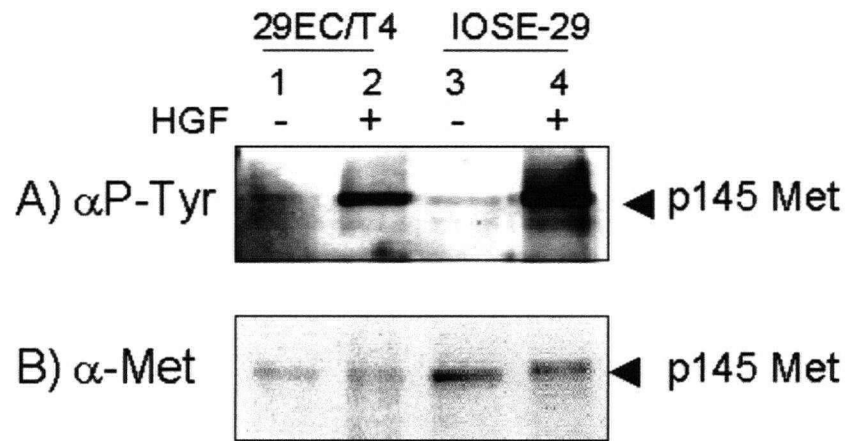
FIGURE 23 - Tyrosine phosphorylation of Met receptor. IOSE-29EC/T4 (lanes 1 and 2) (1 mg) and IOSE-29 (lanes 3 and 4) (2 mg) were treated without or with 20 ng/ml human recombinant HGF for 10 min. Clarified protein was immunoprecipitated with DO-24 monoclonal antibody, resolved by SDS-PAGE, and immunoblotted with anti-phosphotyrosine (A) or anti-Met (B). In both lines, HGF stimulated tyrosine phosphorylation of Met (lanes 2 and 4).

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3.4.1.2. Effect of HGF on cell proliferation of (pre)neoplastic OSE

HGF stimulated proliferation of the preneoplastic IOSE-29 cells and the ovarian cancer cell line OVCAR-3, but inhibited growth of the neoplastic IOSE-29EC and IOSE-29EC/T4 cells (Fig. 24).

3.4.1.3. HGF stimulates scattering in SV40-E-cadherin transfected OSE

Scattering was not assessed in IOSE-29neo cells, which had a spontaneous dispersed phenotype and growth pattern (Figs. 25a, b). None of the protein kinase inhibitors had an observable effect on these cells (Figs. 25c-f). HGF induced scattering in IOSE with constitutively active E-cadherin, i.e. IOSE-29EC (Fig. 26b) and IOSE-29EC/T4 (data not shown). Pretreatment of cells with PD98059 (Fig. 26c) or LY294002 (Fig. 26d) completely blocked scattering, whereas SB203580 (Fig. 26e) had no effect. HGF-induced scattering was also reduced by rapamycin (Fig. 26f). This is the first time that HGF has been shown to cause scattering via rapamycin-sensitive pathways in any cell type. In contrast to IOSE-29EC, HGF had no observable effect on the ovarian cancer cell line (OVCAR-3), which also expresses E-cadherin (Fig. 27b).

3.4.1.4. HGF induces branching morphogenesis/invasion in collagen gels

In collagen gels, IOSE-29 cells dispersed, and assumed a spindle, fibroblast-like morphology independently of HGF (Figs. 28a, b). The kinase inhibitors also had little effect except for LY294002 which caused cell death. Such a scattered phenotype was confirmed in paraffin sections (Figs. 28c, d). When E-cadherin expressing IOSE cells were suspended in collagen gels under control conditions, these cells formed epithelial colonies and round
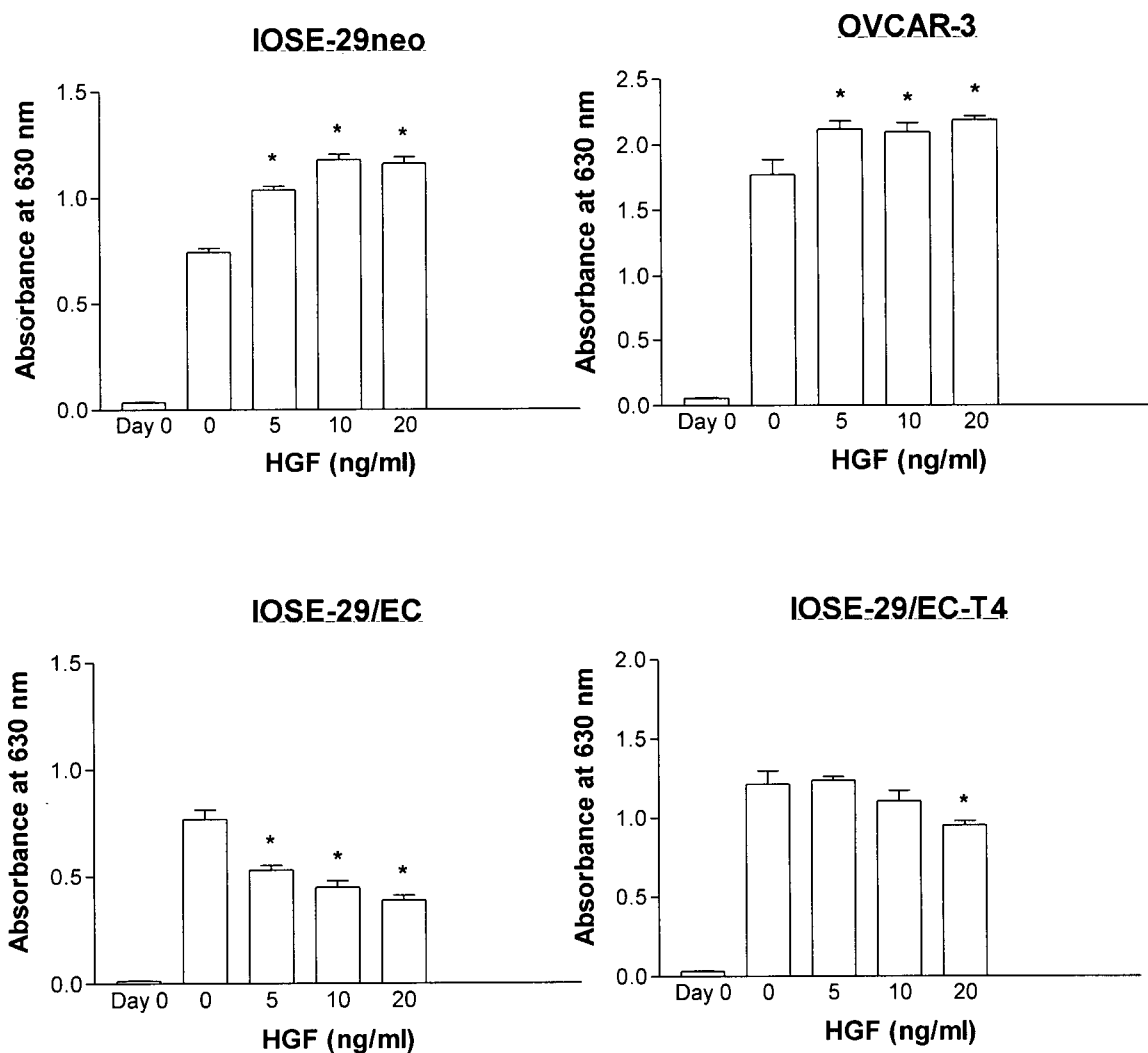
FIGURE 24 - Effect of HGF on cell proliferation of IOSE-29neo, IOSE-29EC, IOSE-29EC/T4 and OVCAR-3. Cells were cultured in medium containing 2% NBS in the presence of HGF (5, 10 and 20 ng/ml) for 6-8 days. Cell numbers were determined colorimetrically by the Methylene Blue assay as described in Materials and Methods. Values in this graph represents means ± S.D. of six replicates of a representative culture. Day 0 represents cultures that were harvested after serum starvation with 0.1% FBS overnight but before any treatment with HGF while '0' represents cultures maintained for 6-8 days in 2% NCS without HGF. *, indicates that a value is significantly different from the control value (0 ng/ml HGF) ($P<0.05$).

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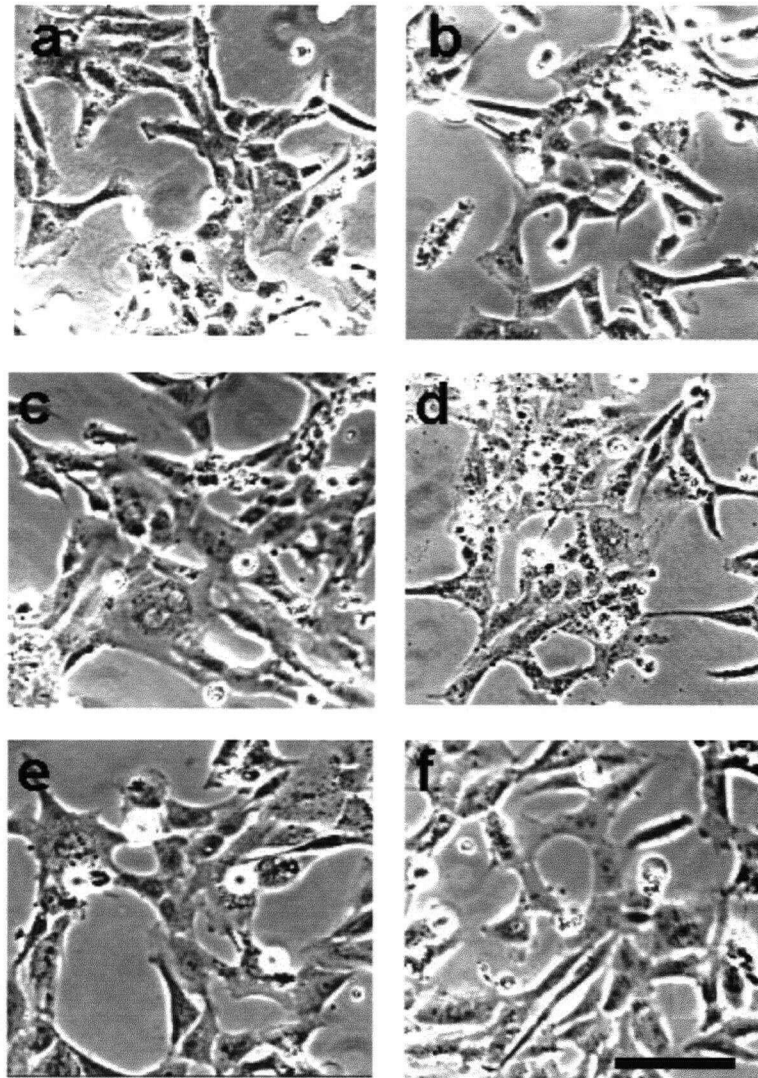
FIGURE 25 - Morphological effects of MEK1 or PI3K inhibition in HGF-induced scattering in IOSE-29neo. IOSE-29neo cells were treated without (a) or with 20 ng/ml HGF (b). Cells were pretreated (30 min) with the kinase inhibitors PD98059 (50 μM) (c) or LY294002 (25 μM) (d) or rapamycin (20 nM) (e) or SB203580 (10 μM) (f), and 20 ng/ml HGF was then added for 24 h. *Bar*, 30 μm.
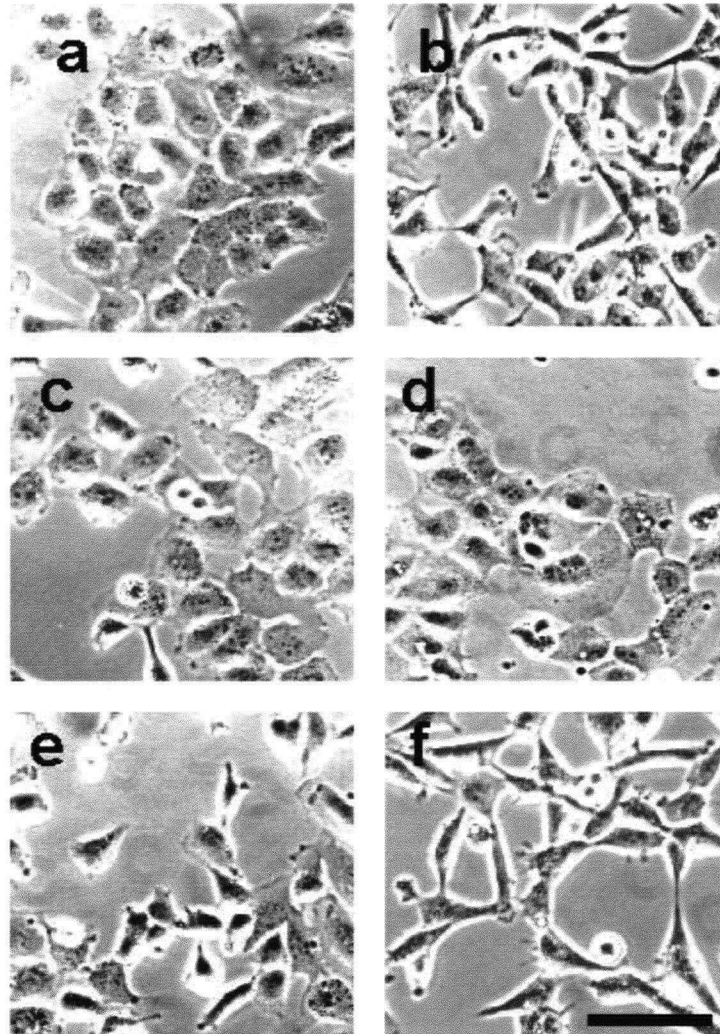
FIGURE 26 - Morphological effects of MEK1 or PI3K inhibition in HGF-induced scattering in IOSE-29EC. IOSE-29EC cells were treated without (a) or with 20 ng/ml HGF (b). Cells were pretreated (30 min) with the kinase inhibitors PD98059 (50 μM) (c) or LY294002 (25 μM) (d) or rapamycin (20 nM) (e) or SB203580 (10 μM) (f), and 20 ng/ml HGF was then added for 24 h. *Bar*, 30 μm.
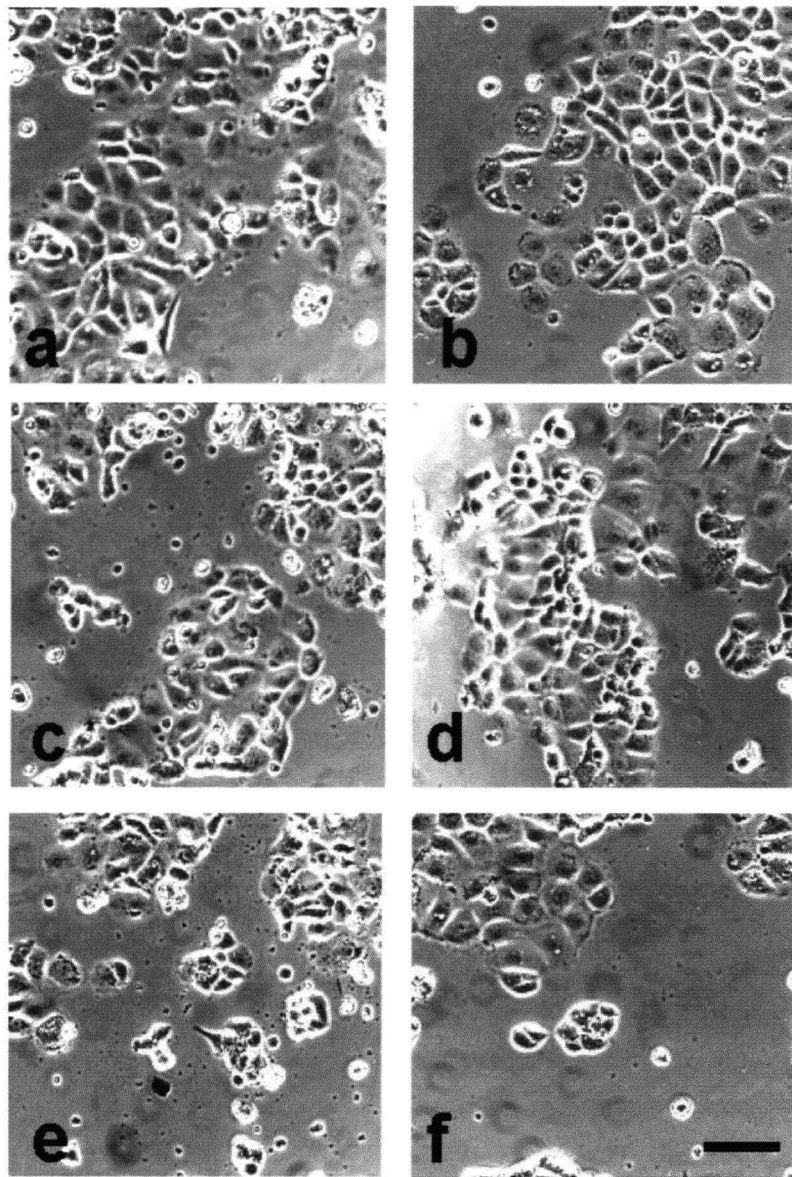
FIGURE 27 - Morphological effects of MEK1 or PI3K inhibition in HGF-induced scattering in OVCAR-3. OVCAR-3 cells were treated without (a) or with 20 ng/ml HGF (b). Cells were pretreated (30 min) with the kinase inhibitors PD98059 (50 μM) (c) or LY294002 (25 μM) (d) or rapamycin (20 nM) (e) or SB203580 (10 μM) (f), and 20 ng/ml HGF was then added for 24 h. *Bar*, 15 μm.

FIGURE 28 - Branching morphogenesis in collagen gels induced by HGF in IOSE-29neo. IOSE-29neo cells dispersed, and assumed a spindle, fibroblast-like morphology independently of HGF (a, control; b, with 20 ng/ml HGF). Such a dispersed phenotype was confirmed in paraffin-embedded sections (c, control; d, with 20 ng/ml HGF). Cells were coincubated with HGF (20 ng/ml) and the kinase inhibitors PD98059 (50 μM) (e), LY294002 (25 μM) (f), rapamycin (20 nM) (g) or SB203580 (10 μM) (h) for 21 days. *Bar*, 50 μm.
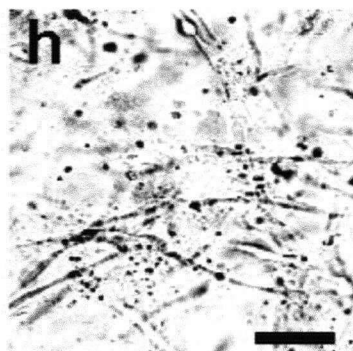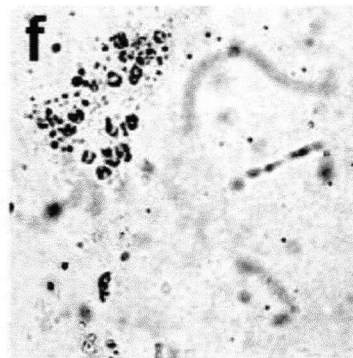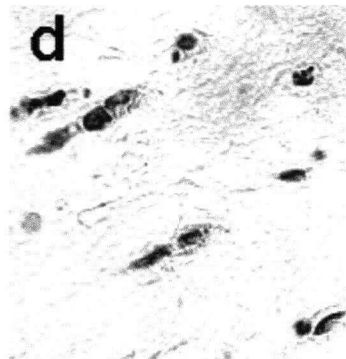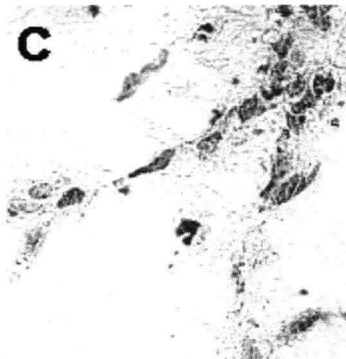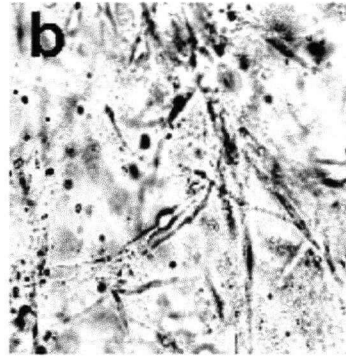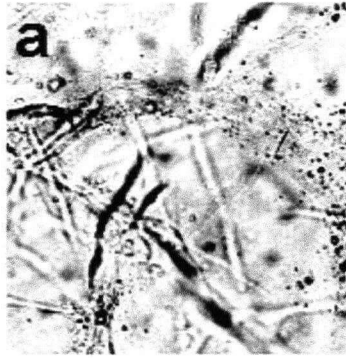
aggregates that sometimes revealed cystic structures (Figs. 29a, c). HGF induced the formation of branching tubules, which were characterized by a single layer of flat-to-cuboidal cells lining either an elongated or round lumen (Figs. 29b, d). These tubular structures were observed more frequently in HGF-treated IOSE-29EC/T4 than in IOSE-29EC cultures. Although both PD98059 (Fig. 29e) and rapamycin (Fig. 29g) inhibited the morphogenetic events of HGF on IOSE-29EC/T4 cells, rapamycin appeared to be more potent than PD98059. HGF did not induce branching morphogenesis in the ovarian cancer cell line OVCAR-3, which also expresses E-cadherin. However, bigger colonies were observed in HGF-treated OVCAR-3 cultures compared to control cultures, suggesting that HGF may promote growth of OVCAR-3 cells (Figs. 30a, b). Coincubation of either PD98059 or rapamycin with HGF blocked the effect of HGF on OVCAR-3 cells (Figs. 30c, e). LY294002 treatment resulted in cell death of all OSE derivatives, including OVCAR-3 (Figs. 28f, 29f, 30d), indicating that the PI3K signaling pathway is important in OSE cell survival. SB203580 had no inhibitory effect on HGF activities on IOSE-29EC/T4 (Fig. 29h) or OVCAR-3 cells (Fig. 30f).

3.4.1.5. Effect of HGF on E-cadherin expressing OSE cells on Matrigel

On growth factor-reduced Matrigel, IOSE-29EC/T4 cells formed branching aggregates, which contained multifocal cavitations, independently of HGF (Figs. 31a, b). IOSE-29EC/T4 also formed cystic structures on Matrigel (Fig. 31c), particularly around cotton fibers (Fig. 31d). Unlike in collagen gels, HGF not only stimulated growth but also induced dome or cyst formation of OVCAR-3 cells that were grown on Matrigel (Fig. 32b), suggesting that cell-substrate interaction may modify HGF-induced mophogenetic responses.

FIGURE 29 - Branching morphogenesis in collagen gels induced by HGF in IOSE-29EC/T4 cells. IOSE-29EC/T4 cells formed round aggregates which sometimes revealed cystic structures in control cultures (a, c). HGF induced tubule formation in these cells (b). Histological sections of HGF-treated cultures revealed a single layer of flat-to-cuboidal cells lining either an elongated or round lumen (d). Cells were coincubated with HGF (20 ng/ml) and the kinase inhibitors PD98059 (50 μM) (e), LY294002 (25 μM) (f), rapamycin (20 nM) (g) or SB203580 (10 μM) (h) for 21 days. *Bar*, 50 μm.
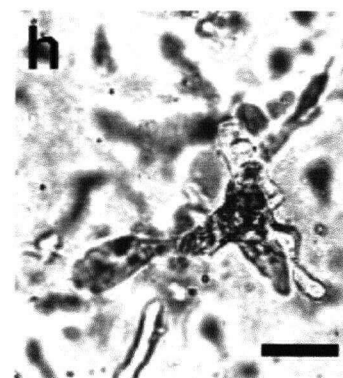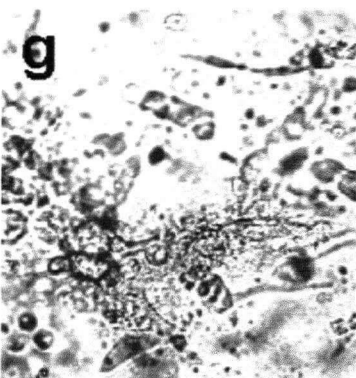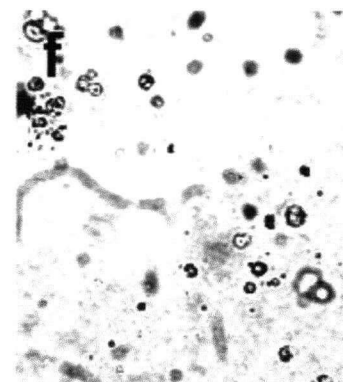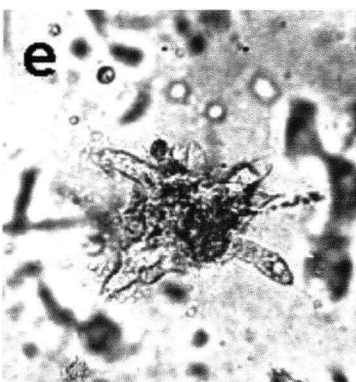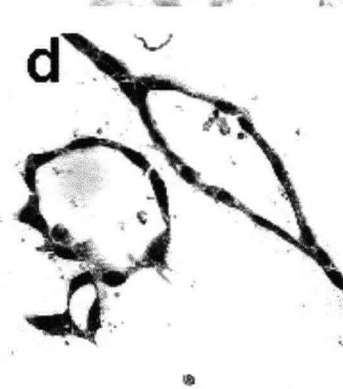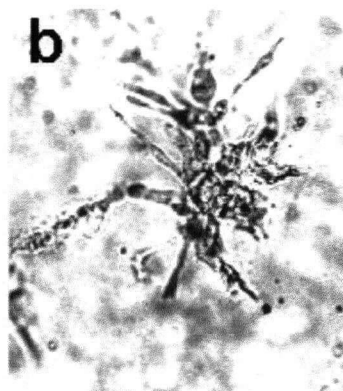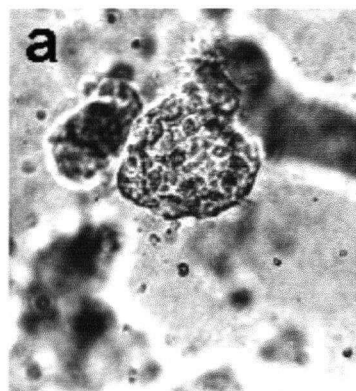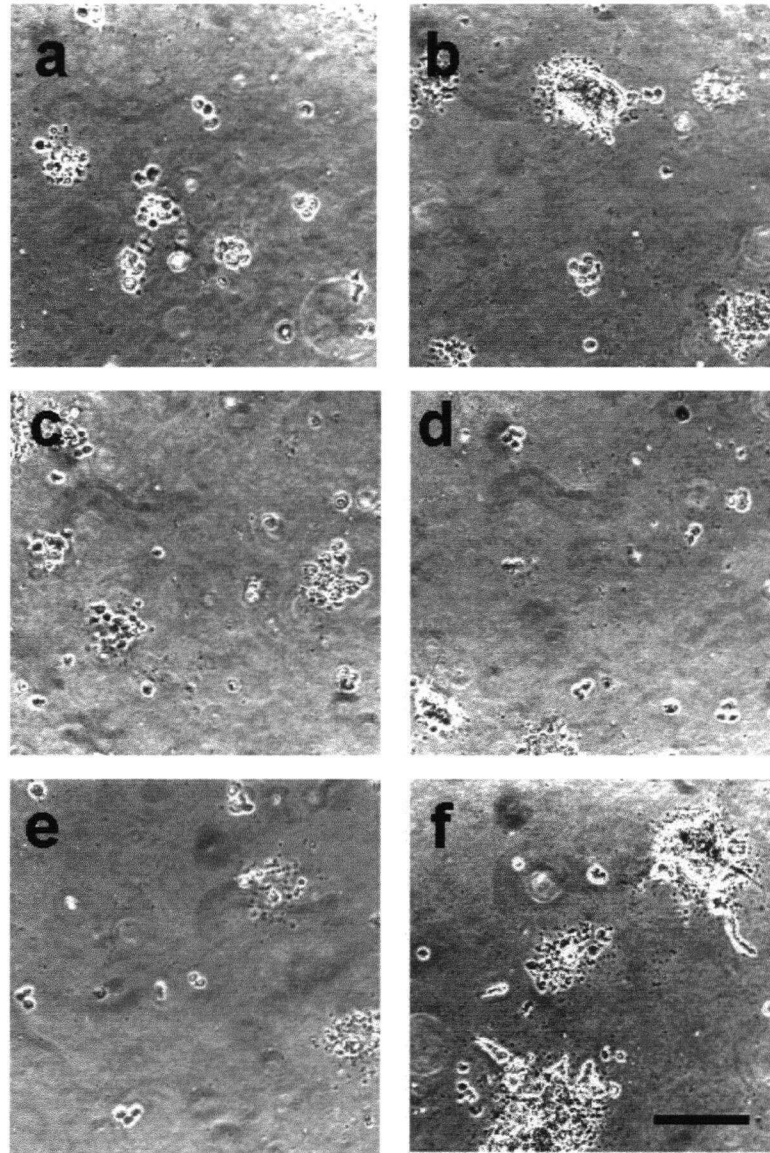
FIGURE 30 - Morphogenesis in collagen gels induced by HGF in OVCAR-3. OVCAR-3 cells

were treated without (a) or with (b) 20 ng/ml HGF and coincubated with the kinase inhibitors

PD98059 (50 μM) (c), LY294002 (25 μM) (d), rapamycin (20 nM) (e) or SB203580 (10 μM)

(f). Note bigger colonies in (b) and (f) for 21 days. *Bar*, 50 μm.

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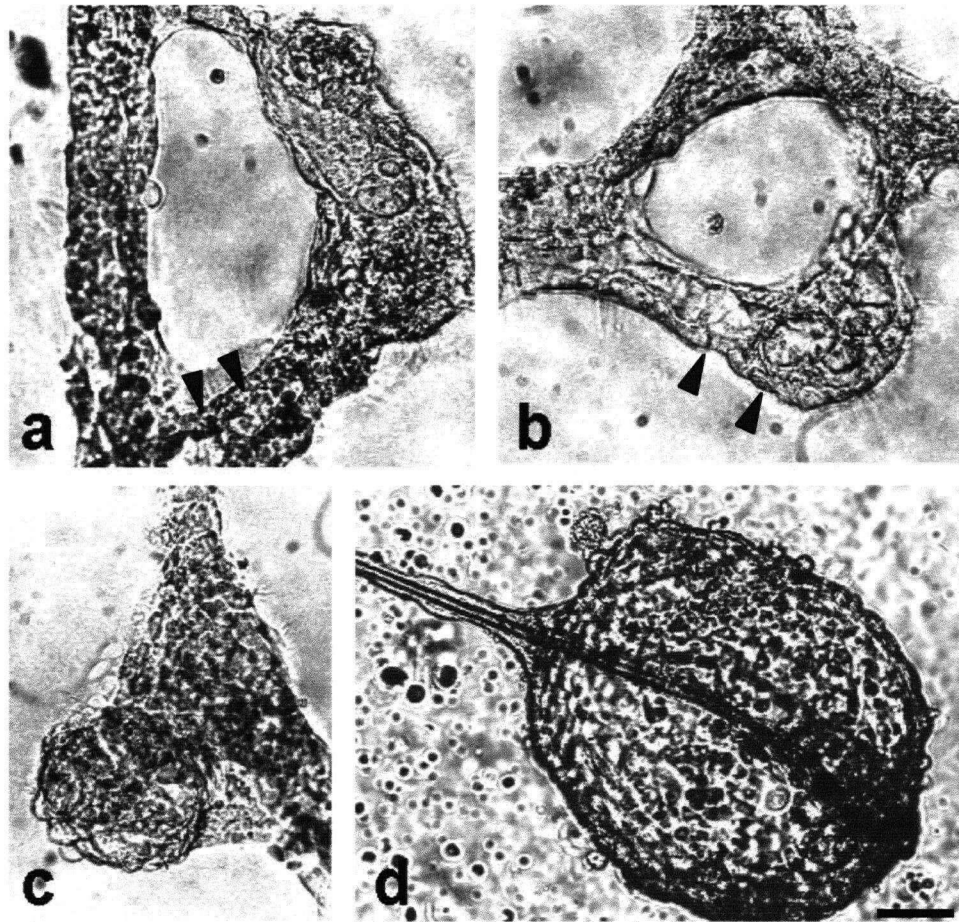
FIGURE 31 - Morphological effect induced by HGF in IOSE-29EC/T4 on Matrigel. IOSE-29EC/T4 cells formed branching aggregates, which contained multifocal cavitations (arrowheads), independently of HGF (a, control; b, with 20 ng/ml HGF). Cells also formed cystic structures on Matrigel (c), particularly around cotton fibers (d). *Bar*, 100 μm.
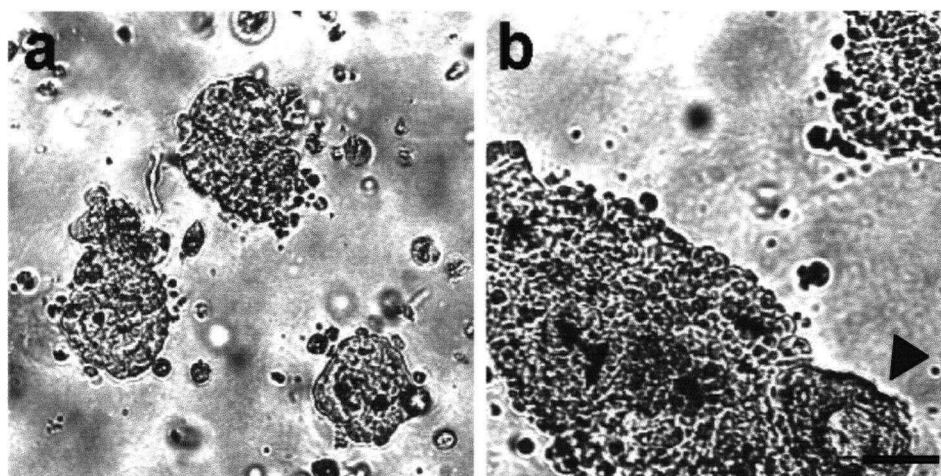
FIGURE 32 - Morphological effect induced by HGF in OVCAR-3 on Matrigel. HGF

induced both cell growth and dome or cyst formation (arrowhead) in OVCAR-3 cells

that were grown on Matrigel (b) as compared to control cultures (a). Note bigger

colonies in b compared with a. *Bar*, 100 μm.

3.4.1.6. Kinetics of HGF induced phosphorylation of ERK

Western blot analysis revealed that ERK MAPKs were phosphorylated within 10 minutes following HGF stimulation (Fig. 33). While an 80% or more reduction of HGF-induced ERK1 phosphorylation was observed in 30 min in IOSE-29neo and OVCAR-3, a similar reduction was found in IOSE-29EC and IOSE-29EC/T4 only after 60 min ($P<0.05$) (Fig. 33). These results suggest that ERK phosphorylation was sustained longer in E-cadherin transfected IOSE cells than in IOSE-29neo and OVCAR-3 cells. HGF also stimulated prolonged phosphorylation of Akt1, Akt2 and p70 S6K in IOSE-29neo, IOSE-29EC and IOSE-29EC/T4 (Fig. 34). The phosphorylation of these kinases peaked at 5-10 min and was still elevated 1 h after HGF stimulation (Fig. 34).

3.4.1.7. HGF stimulates multiple signaling pathways

The involvement of various regulators in HGF stimulation was analyzed. The phosphorylation of protein kinases was determined by their reduced mobilities on SDS-PAGE gels. HGF appeared to phosphorylate protein kinases that include ERK1 and ERK2 MAPKs, Akt1, Akt2 and p70 S6K, but not PHAS-1, in all OSE derivates (Fig. 35A-E). HGF-induced ERK phosphorylation was suppressed by PD98059 (Fig. 35A, lanes 4, 9, 14). LY294002 blocked the phosphorylation of PI3K, as illustrated by its profound inhibitory effects on Akt, p70 S6K and PHAS-1 (Figs. 35B-E, lanes 4, 9, 14). The phosphorylation of p70 S6K could be inhibited by PD90859 (Fig. 35D, lanes 3, 8, 13), suggesting that MEK accounts in part for the FRAP-mediated p70 S6K activity in response to HGF stimulation.

FIGURE 33 - Prolonged phosphorylation of ERK1 in OSE with constitutive E-cadherin expression. IOSE-29neo, IOSE-29EC, IOSE-29EC/T4 and OVCAR-3 were stimulated with HGF (20 ng/ml) for the indicated times. A: Expression of phosphorylated ERK2 (a), ERK1 and phosphorylated ERK2 (b) and ERK1 (c). B: Graphic representation of ERK1 activity is shown as the percentage density of the upper band of the total ERK (ERK1+ERK2) densitometric values. Data are mean ± S.D. of three separate experiments.

A

IOSE-29neo   IOSE-29EC   IOSE-29EC/T4   OVCAR-3

Time (min) 0 5 10 15 30 60 0 5 10 15 30 60 0 5 10 15 30 60 0 5 10 30 60

c —
b —
a —

B

Legend:
- ☐ IOSE-29neo
- ▨ IOSE-29EC
- ▦ IOSE-29EC/T4
- ■ OVCAR-3

Y-axis: ERK1 Activity (as % of ERK1+ERK2)

X-axis: Time after HGF treatment (min)

0.0   5.0   10.0   15.0   30.0   60.0
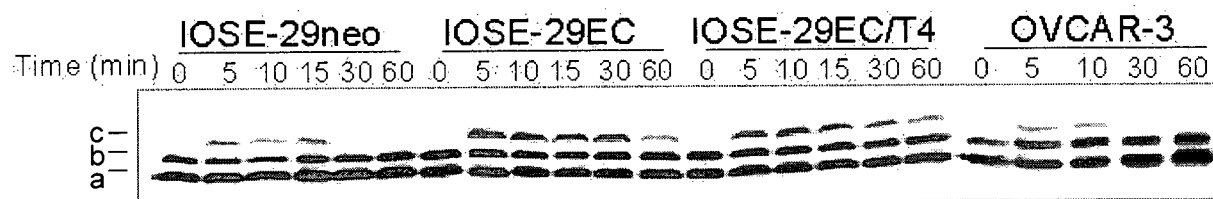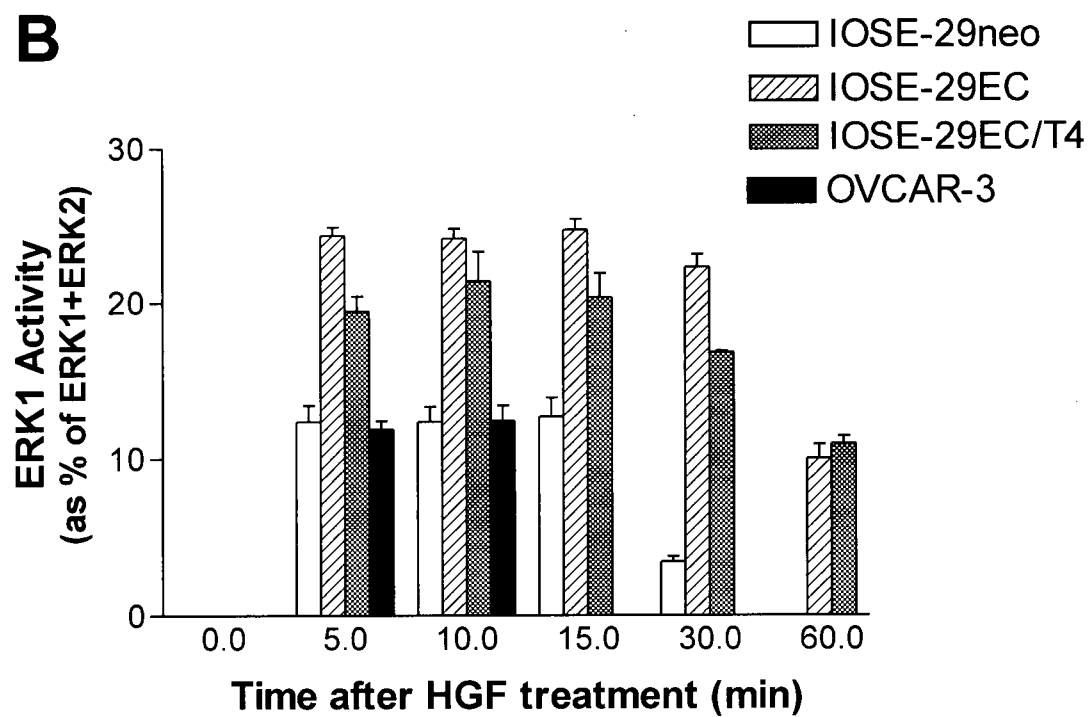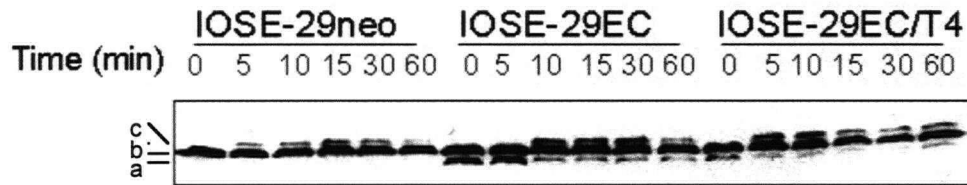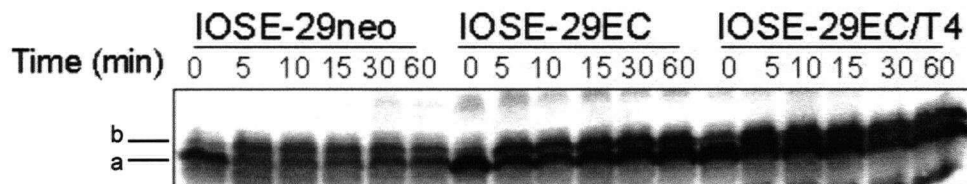
A) Akt1
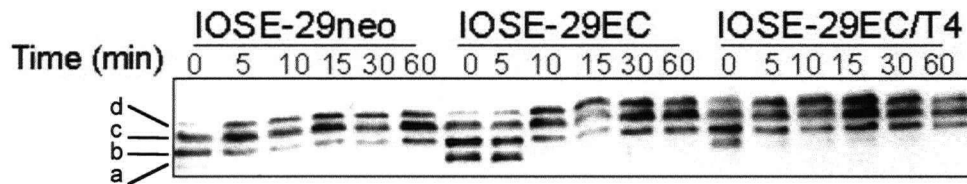
B) Akt2

C) p70 S6K

FIGURE 34 - Prolonged phosphorylation of Akt1, Akt2 and p70 S6K in IOSE-29neo, IOSE-29EC and IOSE-29EC/T4. Cells were stimulated with HGF (20 ng/ml) for the indicated times. Phosphorylation of (A) Akt1, (B) Akt2 and (C) p70 S6K was examined by their reduced mobilities on SDS-PAGE gels. In each case, the bottom band (a) represents unphosphorylated forms of the kinases, whereas the upper bands (b-d) represent different phosphorylated forms.
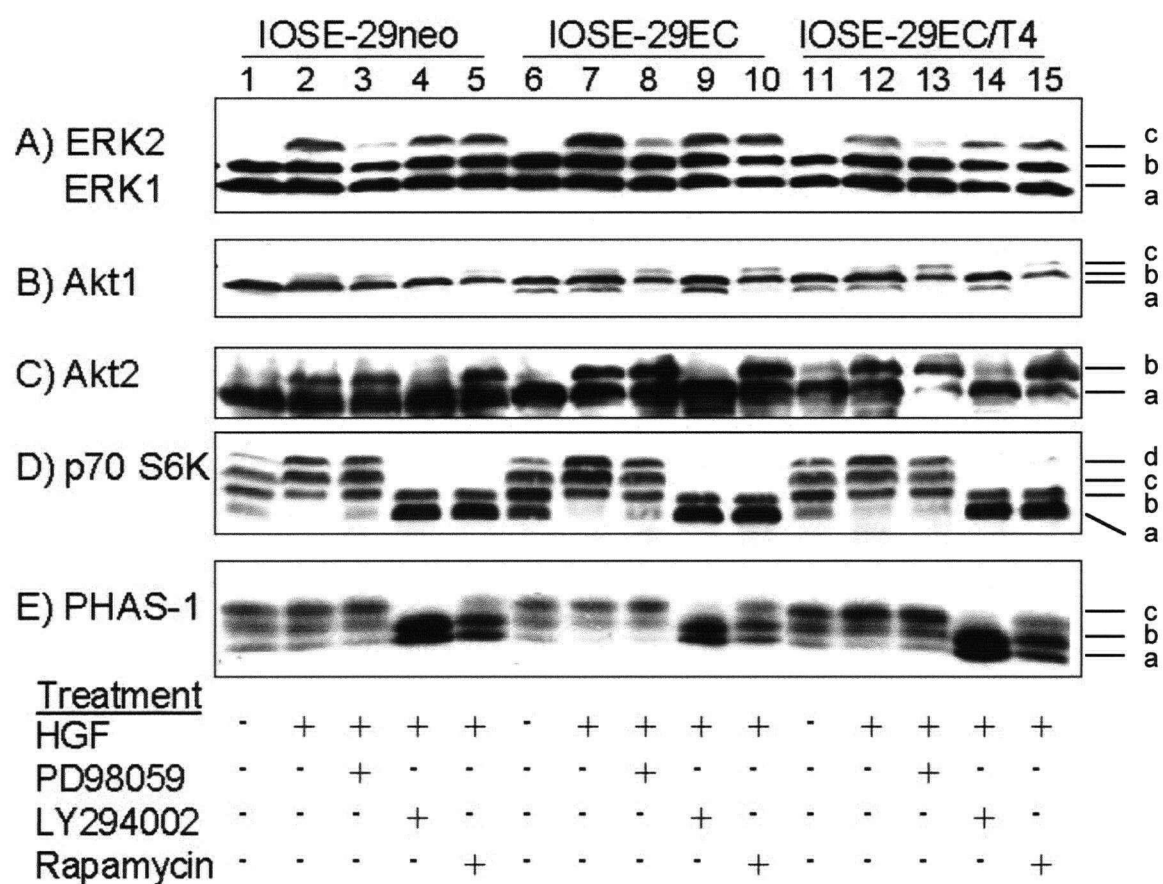
FIGURE 35 - Effects of HGF stimulation on kinase phosphorylation as revealed by reductions of kinase mobilities on Western blots. Treatment with 20 ng/ml HGF resulted in the phosphorylation of the (A) p42 ERK2 and p44 ERK1 MAPKs, (B) Akt1, (C) Akt2 and (D) p70 S6K, but had no effect on (E) PHAS-1, in IOSE-29neo, IOSE-29EC, and IOSE-29EC/T4. To determine whether MAPK and PI3K phosphorylation were specific responses to HGF, cells were pretreated (30 min) with the kinase inhibitors PD98059 (50 μM), LY294002 (25 μM) or rapamycin (20 nM). The bottom band (a) represents the unphosphorylated form of the kinases, whereas the upper bands (b-d) represent the phosphorylated forms.

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Interestingly, although PHAS-1 is a downstream target of FRAP *in vitro*, rapamycin did not reverse the phosphorylation of PHAS-1 (Fig. 35E, lanes 5, 10, 15). Rapamycin completed blocked the phosphorylation of p70 S6K (Fig. 35D, lanes 5, 10, 15). Pretreatment with SB203580 had no affect on both the MAPK and PI3K cascades (data not shown).

3.4.2. Changes in protein kinase regulation with the neoplastic progression of OSE

3.4.2.1. Changes in protein kinase expression

The expression of a variety of protein kinases were studied immunologically in normal, preneoplastic (SV40 large T antigen-immortalized, non-tumorigenic) and neoplastic OSE (SV40-E-cadherin transfected, tumorigenic OSE and ovarian cancer cell lines), which represent OSE cells with different genetic backgrounds and at different stages of neoplastic progression (Fig. 2). SV40 large T-immortalized OSE cells are characterized as preneoplastic OSE, since these cells have an increased growth potential and reduced serum dependence, but are non-tumorigenic and express several characteristics of normal OSE (Maines-Bandiera *et al.*, 1992). The immunoreactivity was quantified by densitometry on autoradiographs of immunoblots. Their relative intensities are summarized in Table 5. As shown in Fig. 36, high levels of PKG were found in normal and preneoplastic OSE, but were absent in neoplastic OSE (P<0.001). In contrast to PKG expression, MEK6 was absent in normal and preneoplastic OSE, and detected exclusively in neoplastic OSE (P<0.001). p38 MAPK, a downstream effector of MEK6, was present in OSE, became upregulated in preneoplastic OSE and was expressed at twofold-increased values in neoplastic OSE (P<0.05). As observed in a variety of tumors, levels of casein kinase II (CK2) were substantially higher (two- to threefold) in preneoplastic and neoplastic OSE than in normal OSE (P<0.05). CDK1 was expressed at relatively low levels in normal OSE, and

its expression levels were increased by more than tenfold in preneoplastic and neoplastic OSE ($P<0.001$). Expression of downstream regulators of PI3K, including Akt1, Akt 2 and p70 S6K, were twofold to fivefold higher in neoplastic SV40-E-cadherin transfected OSE and in some, but not all, ovarian cancer cell lines than in normal and preneoplastic OSE ($P<0.05$) (Fig. 37). GSk3β was present in normal OSE, became upregulated in preneoplastic OSE and was expressed at substantially higher levels in neoplastic SV40-E-cadherin transfected OSE and the ovarian cancer cell line OVCAR-3 ($P<0.05$) (Fig. 36). In contrast to OVCAR-3, the ovarian cancer cell line SKOV-3 expressed GSk3β at a level comparable to that of normal OSE (Table 5). Classical PKC-α, β, which were abundant in SV40-E-cadherin transfected OSE cells, were expressed at a fivefold reduced levels in normal OSE and absent in ovarian cancer cell lines ($P<0.05$) (Fig. 36). The regulation of other members of the MAPK cascades, including ERK1 and ERK2, were also studied. However, there was no difference in the expression of these protein kinases among normal, preneoplastic and neoplastic OSE ($P>0.05$) (Fig. 37). Although expression of Rsk1 was slightly higher (1.2-fold) in preneoplastic and neoplastic OSE than in normal OSE, the difference was not significant ($P>0.05$) (Fig. 36). There was no difference in the expression of these protein kinases with or without HGF stimulation.

3.4.2.2. Regulation of protein kinase phosphorylation

The phosphorylation of protein kinases was revealed by their reduced mobilities on SDS-PAGE gels. Downstream regulators of PI3K, such as Akt 2 and p70 S6K, were constitutively phosphorylated in neoplastic OSE, and to a lesser degree in preneoplastic cells, but not in normal OSE (Figs. 37). Conversely, a significant amount Akt1 was

TABLE 5 - EXPRESSION PROFILE OF PROTEIN KINASES IN NORMAL, PRENEOPLASTIC AND NEOPLASTIC OSE

Cell type	ERK1/ ERK2	Rsk1	Rsk2	PKC-α,β	ILK	Akt1	Akt2	GSK3β	p70 S6K	PKG	MEK6	p38 MAPK	CDK1	CK2
Normal OSE														
NFH-OSE-250 p.0														
NFH-OSE-299 p.1														
NFH-OSE-300 p.1														
NFH-OSE-190 p.2														
NFH-OSE-277 p.2														
NFH-OSE-281 p.2														
NFH-OSE-288 p.2														
NFH-OSE-294 p.2														
NFH-OSE-135 p.3														
NFH-OSE-242 p.3														
NFH-OSE-233 p.1														
FH-OSE-236 p.1														
FH-OSE-293 p.1														
FH-OSE-204 p.2														
FH-OSE-215 p.2														
FH-OSE-286 p.3										N/S	N/S			
Preneoplastic, non-tumorigenic SV40 large T antigen immortalized OSE														
IOSE-29										N/S	N/S			
IOSE-29neo										N/S	N/S			
IOSE-120										N/S	N/S			
IOSE-120neo										N/S	N/S			
Neoplastic, tumorigenic SV40-E-cadherin transfected OSE and ovarian cancer cell lines														
IOSE-29EC										N/S	N/S			
IOSE-29EC/T4										N/S	N/S			
CaOV-3				N/S						N/S	N/S			
OVCAR-3				N/S						N/S	N/S			
SKOV-3				N/S										

p., passage; N/S, no signal

The length of the bars is proportional to densitometric readings of individual cases of normal OSE and means with standard deviation preneoplastic and neoplastic OSE, which are representative of 3 separate experiments.

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FIGURE 36 - Protein kinase expression of normal, preneoplastic (IOSE-29) and neoplastic

OSE (IOSE-29EC, IOSE-29EC/T4 and OVCAR-3) with or without HGF stimulation (20

ng/ml) assessed by Western blots. Expression of the PKG, MEK6, p38 MAPK, CK2, CDK1,

GSK3β, PKC-α,β and Rsk1 in OSE, IOSE-29, IOSE-29EC, IOSE-29EC/T4 and OVCAR-3.

Note that PKG is expressed only in OSE and IOSE-29, whereas MEK6 is found only in

IOSE-29EC, IOSE-29EC/T4 and OVCAR-3. Expression of p38 MAPK, CK2, CDK1 and

GSK3β increases with the neoplastic progression of OSE. High levels of PKC-α,β are found

in IOSE-29EC and IOSE-29EC/T4. The expression of these protein kinases does not

change in response to HGF stimulation. n.d., not determined.

	Normal	Preneoplastic	Neoplastic		
	OSE	IOSE-29	29EC	29EC/T4	OVCAR-3
HGF	− +	− +	− +	− +	− +

PKG

MEK6

p38MAPK

CK2

CDK1   n.d.   n.d. n.d.

GSK3β

PKC-α, β

Rsk1
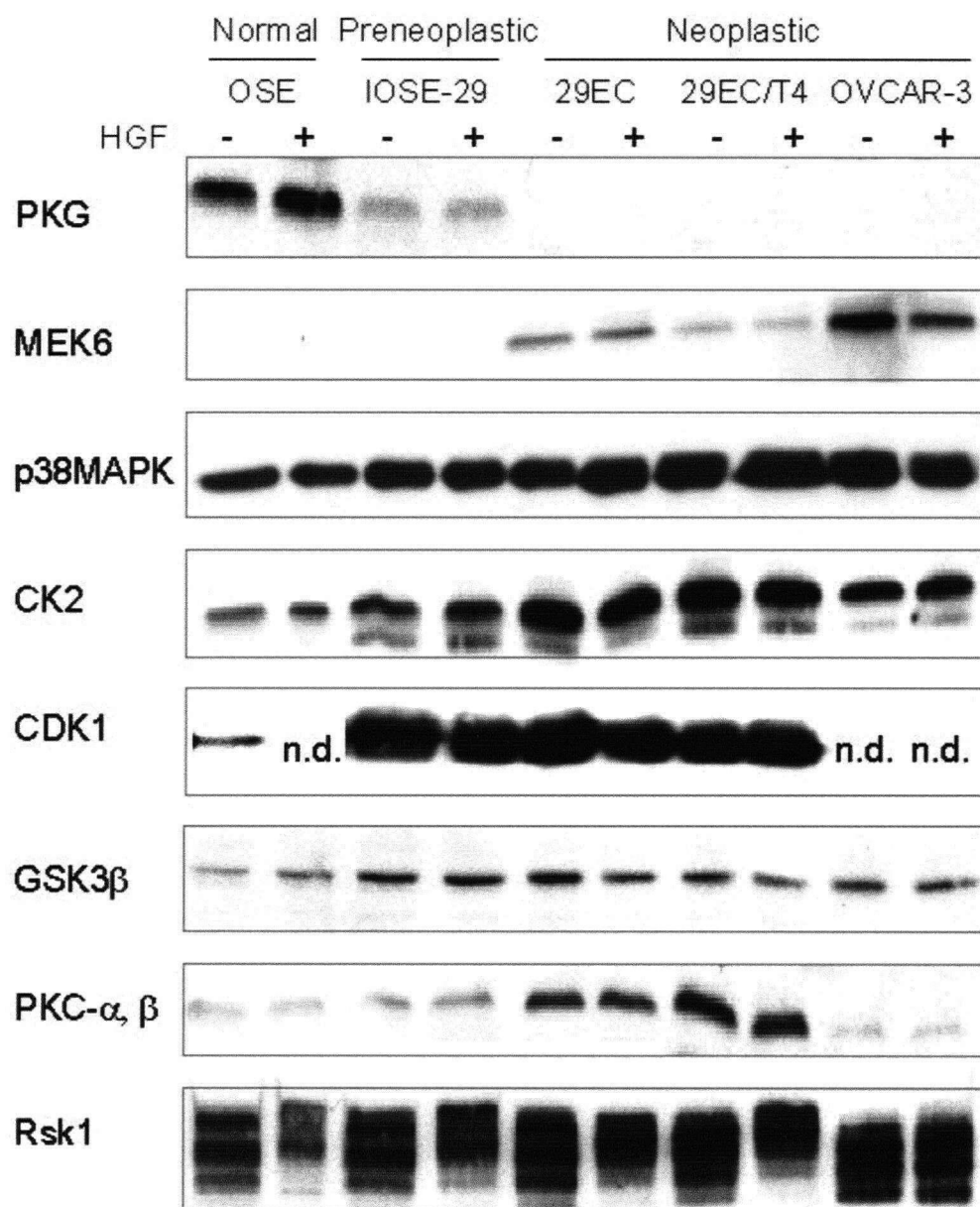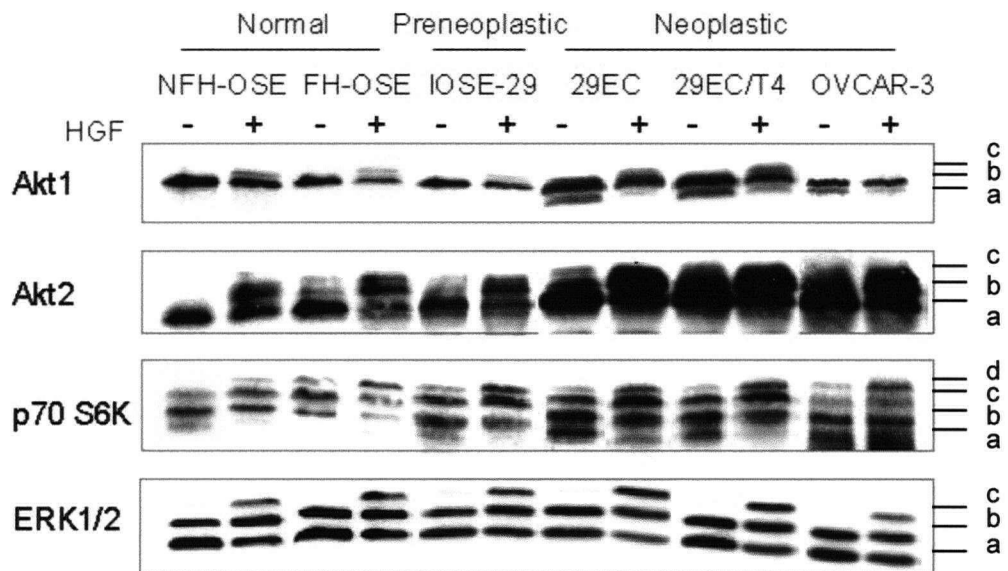
FIGURE 37 - Effects of HGF stimulation on kinase phosphorylation of normal (NFH-OSE, FH-OSE), preneoplastic (IOSE-29) and neoplastic OSE (IOSE-29EC, IOSE-29EC/T4 and OVCAR-3) assessed by phosphorylation-induced reductions of kinase mobilities on Western blots. Treatment with 20 ng/ml HGF resulted in the phosphorylation of the (a) Akt1, (b) Akt2, and (c) p70 S6K, (d) p44 ERK1 and p42 ERK2 MAPKs in NFH-OSE, FH-OSE, IOSE-29, IOSE-29EC, IOSE-29EC/T4 and OVCAR-3. The bottom band (a) represents the unphosphorylated form of the kinases, whereas the upper bands (b-d) represent the phosphorylated forms. Note that phosphorylated forms of (c) Akt2 and (d) p70 S6K are present in FH-OSE, preneoplastic and neoplastic OSE even in the absence of HGF stimulation. High expression levels of Akt2 and p70 S6K are found in neoplastic OSE.

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dephosphorylated in neoplastic OSE, as compared to normal and preneoplastic OSE (Fig. 37). Phosphorylation of ERK1 and ERK2 was found in OSE and all its derivatives only upon growth factor stimulation (Fig. 37).

The regulation of protein kinases among three human ovarian cancer cell lines CaOV-3, SKOV-3 and OVCAR-3 was also compared. Despite similarities in the expression of most protein kinases, differences in the expression of Akt2 and GSK3β were found among these lines. While these kinases were present at high levels in OVCAR-3, their levels were relatively low in SKOV-3 (Fig. 38). HGF had no effect on the expression of these kinases in all ovarian cancer cell lines.

3.5. PI3K in OSE: a balance between mitotic arrest and apoptosis

Treatment with the PI3K inhibitor LY294002 for 24 h had no effect on IOSE-29neo (Fig. 39b), but induced apoptosis in the ovarian cancer cell line OVCAR-3 (Fig. 39f). Unexpectedly, the inhibitor induced mitotic arrest at metaphase in IOSE-29EC and IOSE-29EC/T4 cells, which could be recognized by their distinct metaphase plates (Fig. 39d). As shown in Table 6, mitotic spread counts using mitosis-specific antibodies and the DNA dye Hoechst 33258 (Sauve *et al.*, 1999) showed IOSE-29EC populations to consist of only 2% of cells in metaphase in control cultures, but contained 20-25% of metaphase cells following LY294002 treatment, a difference that was statistically significant ($P<0.05$). LY294002 had little or no effect on apoptosis in IOSE-29EC. In contrast, LY294002 induced both mitotic arrest at metaphase and apoptosis in a second E-cadherin transfected line IOSE-118EC and the ovarian cancer cell line
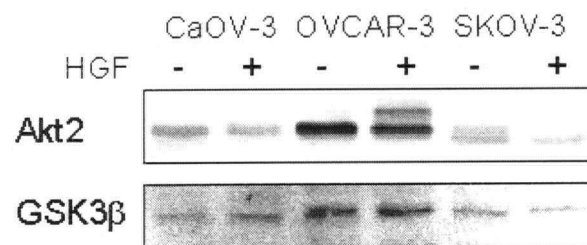
FIGURE 38 - Differential expression of (a) Akt2 and (b) GSK3β among ovarian cancer cell lines CaOV-3, OVCAR-3 and SKOV-3. Note that high levels of (a) Akt2 and (b) GSK3β are present in OVCAR-3, but these kinases are expressed at low levels in SKOV-3. HGF activated Akt2 in OVCAR-3, but not in CaOV-3 and SKOV-3.
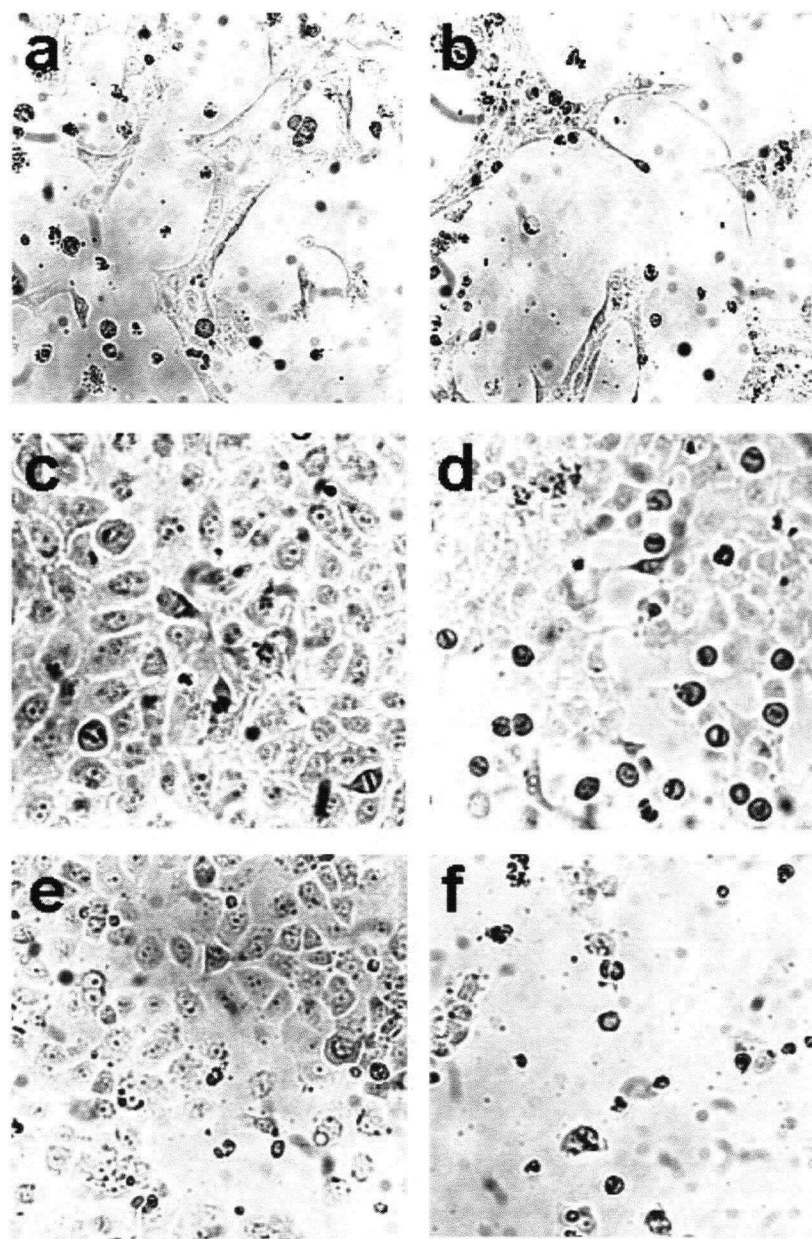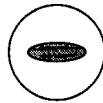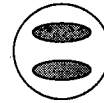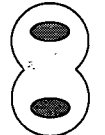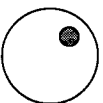
FIGURE 39 - Effect of the PI3K inhibitor LY294002 on IOSE-29neo, IOSE-29EC and OVCAR-3. IOSE-29neo (a and b), IOSE-29EC (c and d) and OVCAR-3 (e and f) cells were treated without (a, c, e) or with (b, d, f) 25 μM LY294002 for 24 h. The inhibitor has no effect on IOSE-29neo (b), but causes metaphase arrest in IOSE-29EC (d) and apoptosis in OVCAR-3 (f).

TABLE 6 - Mitotic spread count of SV40-E-cadherin-transfected OSE cells and ovarian cancer cell line with or without LY294002 treatment

Cell type	Treatment	Mitosis				Apoptosis
		Prophase	Metaphase	Anaphase	Telophase	
IOSE-29EC	Control	<1%	2%	<1%	<1%	0%
	LY294002	<1%	20-25%	<1%	<1%	<1%
IOSE-118EC	Control	<1%	2-3%	<1%	1%	2%
	LY294002	<1%	9-10%	<1%	<1%	12-13%
OVCAR-3	Control	<1%	2-3%	<1%	<1%	2%
	LY294002	<1%	6%	<1%	<1%	10%

Cells were treated with or without 25 μM LY294002 for 24 h, and stained with the DNA dye Hoechst 33258. DNA fluorescence of cells at different stages in mitosis was assessed according to Sauve *et al.* (1999).

1,000 cells were counted per sample.

OVCAR-3. LY294002-treated IOSE-118EC preparations contained a greater number of cells in metaphase (9-10%) as well as apoptosis (12-13%) compared to control cultures, which contained only 2% of cells in metaphase (Table 6). LY294002 induced mitotic arrest in 6% of OVCAR-3 cells and apoptosis in 10% of the cells ($P<0.05$). However, in the absence of any apoptosis-specific markers, the number of apoptotic cells may have been underestimated (Table 6). The proportion of IOSE-29EC cells arrested in metaphase depended on the concentration of the inhibitor, and increased in a dose-dependent manner (Fig. 40). The maximal percentage of metaphase cells was found in IOSE-29EC cells subjected to 50 μM LY294002 (Figs. 40e). Further increase in the inhibitor concentration resulted in increasing cell debris, indicative of cell damage (Fig. 40f). This mitotic arrest phenomenon was further confirmed by fluorescence staining using antibodies to mitosis-specific antigens that include nucleolin, histone H3 and β-tubulin (Figs. 41, 42). Staining with anti-β-tubulin antibody also showed intact mitotic spindles in metaphase cells from LY294002-treated preparations, similar to the control cultures, suggesting that the inhibitor presumably acts through a novel mechanism independently of microtubule disruption (Fig. 42). Throughout these experiments, LY294002 appeared to diminish cell-substratum adhesion of the IOSE-29EC (Fig. 40). Interestingly, fluorescence staining of β-tubulin revealed a more spherical morphology of metaphase cells in LY294002-treated IOSE-29EC cultures compared to control cultures (Fig. 42). Moreover, LY294002 also modulated SKOV-3 cells to a more spindle-shaped and irregular phenotype (Fig. 43). Together, these observations suggest that LY294002 decreases adhesion between cells and cell-substratum adhesion. These changes may or may not account for its effect on cell cycle progression.

FIGURE 40 - Dose-dependent effect of the PI3K inhibitor LY294002 on IOSE-29EC. IOSE-29EC cells were treated with (a) 0, (b) 1, (c) 10, (d) 25, (e) 50 and (f) 100 µM LY294002 for 24 h. There is a correlation between the number of mitotic arrest cells and the concentration of LY294002. Note debris, indicative of cell damage, in (f).
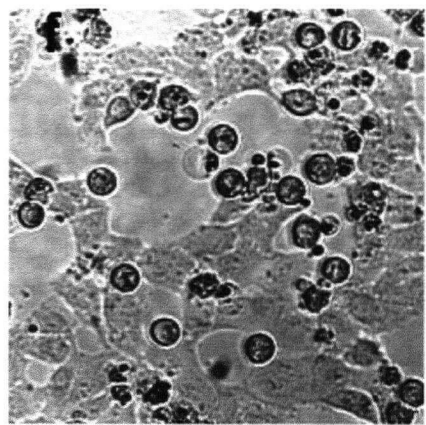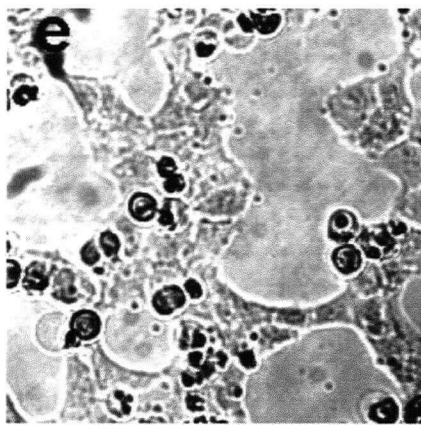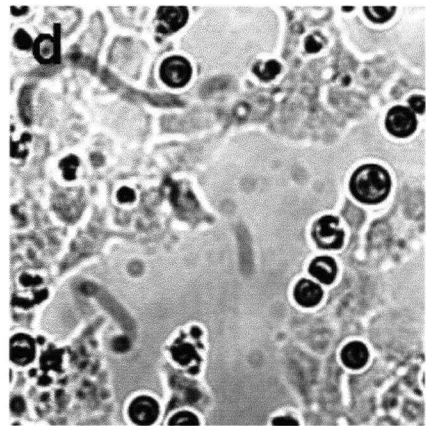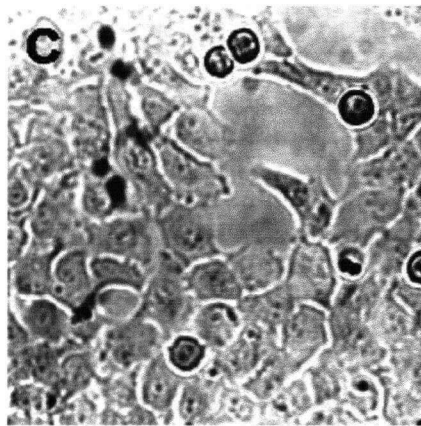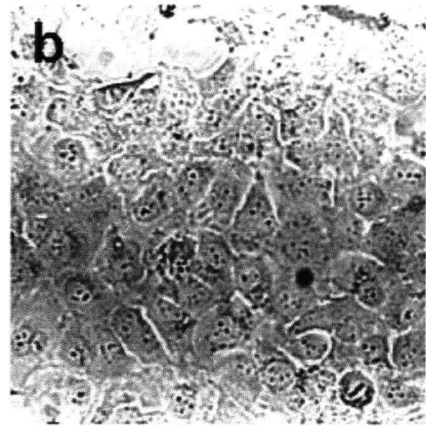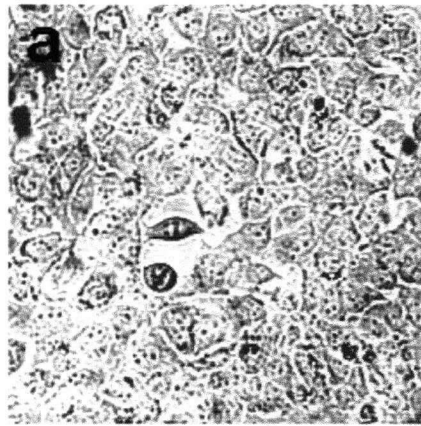
FIGURE 41 - DNA, nucleolin and histone H3 fluorescence in IOSE-29EC. IOSE-29EC cells were arrested at mitosis in response to LY294002 treatment. As revealed by Hoechst DNA staining, there were fewer cells in LY294002-treated cultures (b) than in the controls (a). There were also more nucleolin and histone H3 stained cells in LY294002-treated cultures (d, f) than in the controls (c, e).
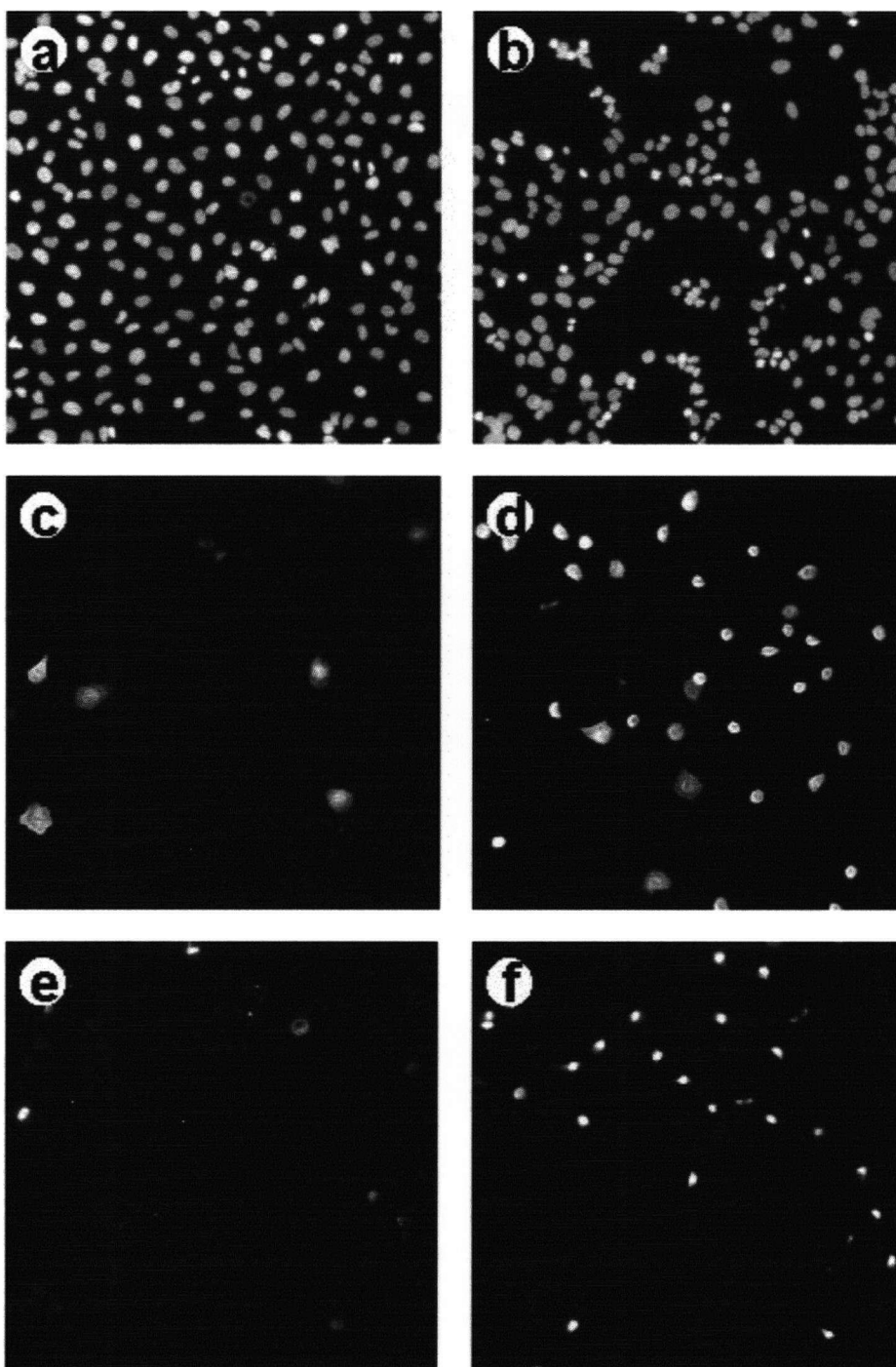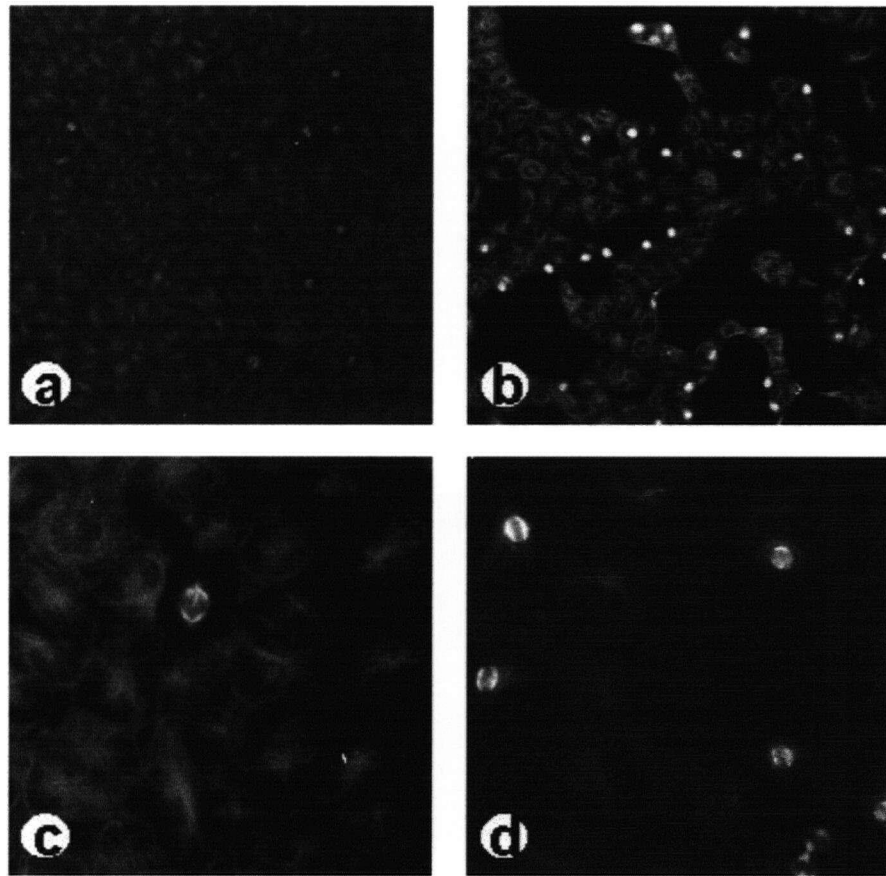
FIGURE 42 - β-tubulin immunofluorescence in IOSE-29EC. IOSE-29EC cells were treated without (a, c) and with (b, d) the PI3K inhibitor LY294002. Note that β-tubulin immunofluorescence revealed intact mitotic spindle staining even in the presence of LY294002 (b, d). Low magnification (x125), a and b; high magnification (x500), c and d.
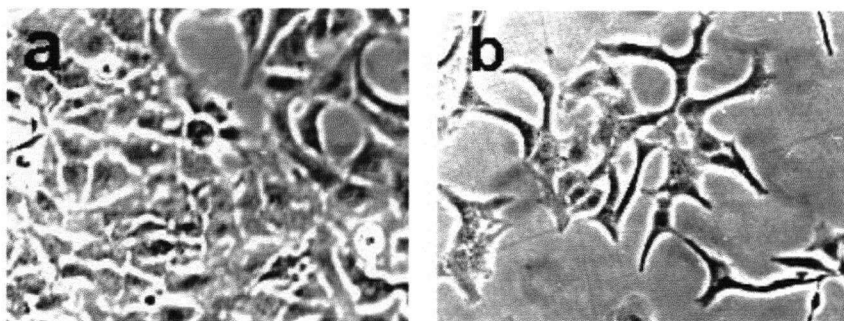
FIGURE 43 - Effect of the PI3K inhibitor LY294002 on the ovarian cancer cell line SKOV-3. Treatment of 25 μM LY294002 for 24 h on SKOV-3 (b) decreased adhesion between cells and cell-substratum adhesion, as compared with the controls (a).

4. DISCUSSION

The adult OSE is a simple mesothelium which, paradoxically, acquires the complex histological and functional characteristics of oviductal and endometrial epithelia in the course of neoplastic progression (Scully, 1995). The frequency and consistency of this aberrant differentiation suggest that such characteristics confer a selective advantage on the transforming cells. However, in spite of its potential importance, the factors controlling this process are unknown. At the cytological and molecular levels, epithelial differentiation depends critically on specific intercellular and histological structural interrelationships which, in turn, are based on complex cell recognition mechanisms and adhesive interactions (Redfield *et al.*, 1997). In the present study, some of these regulatory mechanisms, and their effects on normal and neoplastic OSE, were investigated.

4.1. Constitutive and conditional cadherin expression in OSE

Some of the cadherins that mediate these interactions have the capacity to induce differentiation by mechanisms which include competition for mediators of signal transduction as well as direct effects of intercellular adhesive interactions (Yap *et al.*, 1997; Redfield *et al.*, 1997). In the human ovary, E-cadherin is sparse in normal OSE but changes in amount and distribution very early in ovarian neoplastic progression (Maines-Bandiera and Auersperg, 1997; Sundfeldt *et al.*, 1997). This change is temporally in keeping with a possible inductive role of this adhesion molecule in the aberrant epithelial differentiation of OSE. In the present study, we used a culture system to define more precisely the relationship of E-cadherin expression to various OSE phenotypes and to other cadherins that mediate adhesion between these mesothelial cells. The results suggest that the epithelial integrity of OSE is maintained by N-cadherin, while E-cadherin regulates cell

shapes and interrelationships. E-cadherin expression increases with neoplastic progression and, importantly, is already enhanced in overtly normal OSE from women with strong family histories of breast/ovarian cancer. Therefore, changes in E-cadherin expression might indeed coincide with, or even preceed, the stage of neoplastic progression when aberrant differentiation is induced.

In vivo, E-cadherin protein is undetectable in flat normal OSE that makes up the bulk of the epithelium, but is enhanced between cuboidal and, especially, columnar cells. It is also prominent in benign metaplasia, in premalignant dysplastic lesions, in benign neoplasms and in ovarian carcinomas (Peralta Soler et al., 1997; Sundfeldt et al., 1997). In addition to these quantitative changes, there is a progression of changes in the polarity of E-cadherin distribution: it is not fully polarized between cuboidal OSE cells, as shown by immunostaining on both apical and lateral surfaces (Maines-Bandiera and Auersperg, 1997). In metaplastic, columnar OSE, E-cadherin is polarized, i.e. limited to lateral intercellular spaces. Stratification of OSE in vivo is one of the earliest histologic indicators of premalignant changes (Scully, 1995). In such lesions, as in overtly neoplastic OSE, polarity tends to be lost again and E-cadherin is distributed over all cellular surfaces. In the present study, the abundance and distribution of E-cadherin in culture similarly correlated with cell shapes, cellular interrelationships and neoplastic progression. Immunoreactive E-cadherin was lacking in flat OSE monolayers, and the negative results of our Western blot analysis demonstrated that this lack was due to the absence of protein rather than to an abnormal distribution secondary to other defects. E-cadherin appeared sporadically between closely packed, cuboidal cells, and was widely distributed over the contacting surfaces of overlapping cells. Cells in colonies derived from metaplastic OSE were intensely E-cadherin positive.

The increase in E-cadherin with flat-to-columnar shape changes and cell overlap raised the question whether increased intercellular contact induced E-cadherin, or whether an increased presence of E-cadherin enhanced intercellular adhesion. In the present study, polyHEMA caused the OSE cells to form solid aggregates but this increase in intercellular contact did not induce detectable E-cadherin. Similarly, E-cadherin expression of choriocarcinoma cells is not altered by aggregation on either Matrigel or polyHEMA, as compared to control cultures on plastic (Hohn et al., 1996). Probably, other extra- and intracellular modulators, notably integrins, modulate E-cadherin expression, intercellular adhesion, and the reorganization of OSE. It is likely that E-cadherin was functional in the OSE cultures because (1) E-cadherin staining was localized to the cell-cell borders, suggesting that E-cadherin was associated with the catenins and assembled at the adherens junctions; (2) when OSE monolayers were treated with EDTA, the cells dissociated suggesting that a calcium-dependent form of cell adhesion was inactivated.

RT-PCR demonstrated E-cadherin mRNA in some FH-OSE and NFH-OSE cultures which lacked detectable E-cadherin protein. In such cases, ISH demonstrated abundant E-cadherin mRNA in almost all cells, indicating that the positive PCR results were not based on amplification of signals from a small cell subpopulation that had escaped detection by immunofluorescence microscopy. Rather, the ISH results suggest that E-cadherin may be regulated posttranscriptionally. A mechanism for rapid posttranscriptional regulation of E-cadherin expression would be advantageous in an epithelium which overlies the ovary, i.e. an organ that is unusually pliable and prone to frequent rapid changes in size and contours. A similar lack of correlation between cadherin proteins and transcript expression, suggestive of posttranscriptional regulatory mechanisms, has been reported in the regenerating epithelium of the human gastrointestinal tract, in developing retina, in renal

carcinomas and in thyroid malignancies (Brabant *et al.*, 1993; Hanby *et al.*, 1996; Shimazui *et al.*, 1996).

Factors regulating E-cadherin expression in female reproductive tissues appear to involve hormonal controls, since estrogen and progesterone were reported to increase E-cadherin mRNA level in the immature mouse ovary and uterus *in vivo* (MacCalman *et al.*, 1994a, b). Recently, the transcription factor *snail* has been demonstrated as a repressor of E-cadherin gene expression in fibroblasts and some E-cadherin negative epithelial tumor cell lines through its binding to the E-boxes present in human E-cadherin promoter (Battle *et al.*, 2000; Cano *et al.*, 2000). It remains to be investigated whether a similar regulatory mechanism influences E-cadherin expression in human OSE in the course of neoplastic transformation.

We and others (Dyck *et al.*, 1996; Salazar *et al.*, 1996) previously demonstrated increased Mullerian epithelial differentiation in overtly normal OSE from women with hereditary breast/ovarian cancer syndromes (FH-OSE). In the present study, E-cadherin protein and mRNA were present in a significantly higher proportion of cases, in a higher percentage of cells and for more passages in culture in FH-OSE than in OSE from the general population (NFH-OSE). These differences again illustrate the increased presence and stability of epithelial differentiation in OSE cells from women with hereditary ovarian cancer syndromes. Of the 11 FH-OSE cases where mRNA and protein expression were compared, 7 had known BRCA1 mutations while genetic analysis of the remaining 4 women was incomplete. It is important to note that similar results were obtained in these two groups of cultures. This suggests that either all 11 women carried BRCA1 mutations, or that increased familial risk for ovarian cancer is associated with similar changes in differentiation

in more than one hereditary ovarian cancer syndrome. It is also possible that there are coseggregating high fequency low penetrance genes involved.

Taken together, these data confirm that OSE can be added to the list of mesodermally derived epithelia, such as renal epithelium and granulosa cells, that express N-cadherin. Furthermore, we show that increased E-cadherin expression, in common with other altered epithelial differentiation markers, defines the OSE phenotype of women who are at high risk of developing ovarian cancer. Importantly, the early onset of its increased expression is in keeping with the hypothesis that E-cadherin may be an inducer of Mullerian epithelial differentiation in transforming OSE.

4.2. Autocrine regulation of HGF-Met in human ovarian cancer and normal OSE from women with strong family histories of ovarian cancer

Normal OSE has a limited commitment to the peritoneal epithelial phenotype, as indicated by the retention of mesenchymal features and, in culture, a propensity to undergo epithelio-mesenchymal conversion (Auersperg *et al.*, 1994). In contrast to carcinomas arising from most other epithelia that lose differentiation during neoplastic transformation, OSE-derived carcinomas are distinguished by an aberrant epithelial differentiation indicating more stable and complex epithelial characteristics. HGF is an essential regulator of cell growth and motility and, importantly, it has the capacity to induce cyst formation that resembles (pre)neoplastic ovarian lesion with enhanced epithelial morphogenesis (Tsarfaty *et al.*, 1992, 1994). In the human ovary, Met is expressed in normal OSE but becomes upregulated in well-differentiated ovarian carcinomas (Di Renzo *et al.*, 1994; Moghul *et al.*, 1994; Huntsman *et al.*, 1999), suggesting that changes in Met expression might coincide with the stage of neoplastic progression when aberrant differentiation is induced. In the

present study, a culture system was used to investigate whether the predisposition to develop ovarian cancer in women with hereditary ovarian cancer syndromes involves changes in the expression of HGF-Met by the tissue of origin of epithelial ovarian cancer, the OSE. The results demonstrated differential regulation of HGF and its receptor Met in overtly normal human OSE from women with (FH-OSE) and with no (NFH-OSE) family histories of ovarian cancer. While the Met receptor is present in short-term cultures and downregulated in prolonged cultures of NFH-OSE, it is stabilized in FH-OSE cultures at all passages, similar to ovarian carcinoma lines. Therefore, some of the factors that alter Met levels in the malignant progression of ovarian surface epithelial tumors (Di Renzo *et al.*, 1994; Moghul *et al.*, 1994; Huntsman *et al.*, 1999) may already be found in FH-OSE. This phenotypic manifestation of FH-OSE again illustrates the increased stability of epithelial differentiation in women with hereditary ovarian cancer syndromes (Auersperg *et al.*, 1995; Dyck *et al.*, 1996).

RT-PCR demonstrated that Met mRNA was always detected in parallel with that of E-cadherin, which also becomes upregulated and stabilized in ovarian carcinomas and, to a lesser degree, in FH-OSE. However, while normal OSE expressed E-cadherin mRNA but lacked detectable protein (see section 4.1), Met mRNA was consistently detected along with its protein, suggestive of a differential posttranscriptional regulation of these two epithelial markers in OSE. Normal mesothelial cells, which share a common embryological origin and anatomical environment with OSE, lack Met (Klominek *et al.*, 1998). This difference suggests that expression of Met receptor might be a feature characteristic of coelomic epithelium derivatives at the urogenital ridge through local differentiation.

This study demonstrated, for the first time, the coexpression of HGF and Met in most cases of FH-OSE and ovarian cancer cells, and constitutive tyrosine-phosphorylation of the

Met receptor in the absence of exogenous HGF, suggesting an autocrine regulatory action in these cultured cells, and perhaps *in vivo*. HGF is also found in malignant ovarian cystic and ascitic fluid from women with ovarian carcinomas (Sowter *et al.*, 1999). In contrast, HGF mRNA and protein were rare in NFH-OSE cultures. Therefore, while the expression of Met by OSE is in keeping with the presence of this receptor in most epithelia, the presence of HGF may represent a very early or predisposing step in ovarian surface epithelial neoplastic transformation. Factors regulating HGF expression in female reproductive tissues appear to involve hormonal controls, since estrogen was reported to increase HGF mRNA levels in mouse ovary (Liu *et al.*, 1994). In a similar manner, Met gene expression can be regulated by cytokines and hormones (Moghul *et al.*, 1994). Estrogen and progesterone have been shown to dramatically induce Met mRNA expression in human ovarian carcinoma cells (Moghul et al., 1994).

In vivo, HGF is found in human ovarian cyst and ascites fluid, and the concentration of that HGF is amongst the highest in human biological fluids (Sowter et al., 1999). Since FH-OSE and ovarian cancer cells synthesized only small amounts of HGF, the high levels found in malignancies may be primarily derived from fibroblasts and cells of the immune systems, which are the normal sources of this factor. It has been shown that extracellular processing by proteases in response to tissue damage and tumor progression is required to activate the HGF precursor, pro-HGF (Miyazawa *et al.*, 1996; Jiang *et al.*, 1999). This raised the question whether HGF produced by cultures of FH-OSE and ovarian cancer cell lines was biologically active or whether the growth factor was secreted as an inactive precursor which would require *in vivo* factors for its subsequent activation. Our results show that it is likely that HGF produced by these cultures was functional because (1) Met was constitutively phosphorylated without HGF treatment; (2) their conditioned media induced

scattering of C-4II colonies, and that scattering was partly inhibited by neutralizing anti-HGF antibody. Exogenous HGF has been demonstrated to stimulate proliferation and motility in ovarian cancer cells, and these properties may contribute to the peritoneal dissemination during ovarian tumor progression (Corps et al., 1997; Parrott and Skinner, 2000).

HGF is a pleiotropic factor that evokes complex biological responses. Accordingly, its receptor tyrosine kinase Met activates various signal transduction pathways. In the present study, HGF activated multiple signaling molecules of the MAPK and PI3K pathways in both normal and malignant OSE. The phosphorylation of protein kinases was revealed by phosphorylation-induced mobility reduction on SDS-PAGE gels in the study. Alternatively, phosphospecific antibodies can be used. Akt2 and p70 S6K, which are downstream effectors of PI3K, were constitutively phosphorylated in ovarian cancer cell lines. Importantly, such phosphorylation was also found in all FH-OSE in the absence of HGF, but only in 50% of NFH-OSE cultures. These phosphorylation changes have been previously shown to correlate with the activation of protein kinases (Kim et al., 1998). Recent findings indicate that the PIK3CA gene, which encodes the p110α catalytic subunit of PI3K, is amplified in most human ovarian carcinoma lines. It was suggested that this amplification led to increased PI3K activity (Shayesteh et al., 1999). In the absence of evidence for PIK3CA amplification in FH-OSE cells, and since HGF could activate PI3K, the activation of PI3K in our FH-OSE cultures suggests that this change might be the result of alternative molecular mechanisms that may or may not involve HGF. The consitutitve phosphorylation of Akt2 and p70 S6K in 50% of NFH-OSE cases, which lacked detectable HGF, suggesting that activation of PI3K can occur independently of an HGF-Met autocrine activity. Probably, bioactive cytokines, which can activate PI3K, modulate PI3K activity in OSE and ovarian cancer cells in an autocrine (and paracrine) manner. Alternatively, there is a correlation

between E-cadherin engagement at the adherens junctions and the activation of PI3K (Pece *et al.*, 1999). E-cadherin is expressed in metaplastic and neoplastic OSE and in FH-OSE (see section 4.1), suggesting a possible significant role of enhanced E-cadherin expression in autonomous PI3K signaling during the (pre)neoplastic transformation of OSE.

HGF stimulated cell proliferation of human OSE cells. These cells were cultured in serum-supplemented medium that contains fibronectin. This result is in accordance with the observation that HGF is mitogenic in rat OSE when these cells are plated on a fibronectin-like extracellular matrix (RGD peptide) (Hess *et al.*, 1999), and that fibronectin-integrin binding, which maintains basal levels of PI3K-dependent Akt activity, promotes cell survival (Khwaja *et al.*, 1997). Though the OSE of the normal ovary mostly remains as a stationary tissue, it is capable of rapid mitosis to accommodate changes in size of the ovary during the menstrual cycle and to repair the rupture sites generated after ovulation. The mitogenic property of HGF in culture may have a similar effect on OSE cell proliferation *in vivo* during the post-ovulatory repair process. HGF-stimulated FH-OSE cultures appeared to have a more limited proliferative response than NFH-OSE cultures, suggestive of an autocrine HGF-Met regulation in FH-OSE. The present findings showed that in human OSE, the MAPK signaling pathway and a rapamycin-sensitive p70 S6K pathway are equally important regulators of the mitogenic action of HGF on these cells.

Together, the present findings support other data which indicate that HGF plays a role in normal OSE physiology (Gulati and Peluso, 1997; Parrott and Skinner, 2000). The variation in HGF levels that we observed among NFH-OSE, FH-OSE and neoplastic OSE suggests a possible route for autocrine regulation by HGF-Met in neoplastic OSE and, importantly, in overtly normal OSE from women who are genetically predisposed to ovarian cancer. In analogy with the behavior of other cell types where an autocrine HGF-Met loop

has been implicated in tumorigenic transformation (Vande Woude *et al.*, 1997; To and Tsao, 1998; Jiang *et al.*, 1999), the appearance of HGF-Met expression in FH-OSE may reflect increased autonomy of differentiation and growth controls that represents an early step in the (pre)neoplastic progression of OSE and enhances the susceptibility of these cells to malignant transformation.

4.3. Changes in the responses to HGF and protein kinase regulation with the neoplastic progression of OSE

E-cadherin changes in amount and distribution very early in ovarian neoplastic progression (Maines-Bandiera and Auersperg, 1997; Sundfeldt *et al.*, 1997), suggesting a possible inductive role of this adhesion molecule in the aberrant differentiation of OSE which accompanies ovarian carcinogenesis. To test this hypothesis, we transfected the mouse E-cadherin gene into simian virus (SV) 40 large T antigen-immortalized, non-tumorigenic OSE (IOSE-29) and generated line IOSE-29EC (Maines-Bandiera *et al.*, 1992; Auersperg *et al.*, 1999). Constitutive E-cadherin expression in this cell line not only restored normal epithelial characteristics that had been lost in culture but the cells also acquired epithelial markers that signify progression to a neoplastic form (Auersperg *et al.*, 1999). Moreover, these cells exhibit tumorigenicity in SCID mice, and from such tumors, we generated the ovarian cancer cell line IOSE-29EC/T4 (Ong *et al.*, 2000). This cell culture system (Fig. 2), in addition to normal OSE and ovarian cancer cell lines, was used to define more precisely the expression and contribution of (1) HGF and (2) protein kinases to various OSE phenotypes at different progressive stages and its inter-relationships with E-cadherin.

4.3.1. HGF in the neoplastic progression of OSE

In vivo, Met is expressed in normal OSE but becomes upregulated in well-differentiated ovarian carcinomas (Di Renzo *et al.*, 1994; Moghul *et al.*, 1994; Huntsman *et al.*, 1999), suggesting that changes in Met expression might coincide with the stage of neoplastic progression when aberrant differentiation is induced. In contrast, the expression of HGF was rare in normal OSE but became persistent in ovarian cancer cells (see section 4.2). Thus, the coexpression of HGF and Met, suggestive of an autocrine stimulatory loop, may play an important role in ovarian carcinogenesis. Our data demonstrated that E-cadherin-expressing OSE cells and ovarian cancer cells expressed significantly higher levels of endogenous HGF concomitantly with Met, as compared to normal OSE and non-tumorigenic IOSE cells. Therefore, the coexpression of HGF and Met, which may be induced by the constitutive expression of E-cadherin, is present in parallel with other potentially E-cadherin-related neoplastic characteristics, such as resistance to anoikis (Hermiston and Gordon, 1995; Kantak and Kramer, 1998), which may confer a selective advantage in the pathogenesis of ovarian cancer.

HGF possesses a number of characteristics that are essential for the development of ovarian cancer and metastasis. These include its morphogenetic capacity to induce tubule or cyst formation, which occur in (pre)neoplastic ovarian lesions (Tsarfaty *et al.*, 1992, 1994). HGF is also a potent mitogen and migratory factor. In this study, HGF caused scattering exclusively in IOSE-29EC and IOSE-29EC/T4, but had virtually no effect on either IOSE-29 cells that had a mesenchymal, scattered phenotype to begin with, or the ovarian cancer cell line OVCAR-3. In contrast, the capability of HGF signaling to stimulate epithelial morphogenesis was found in OSE cells that express E-cadherin. While HGF induced branching in E-cadherin transfected OSE in collagen gels, a similar morphogenetic

effect was only observed in OVCAR-3 cells that were grown on Matrigel. Therefore, a functional E-cadherin-catenin junctional complex may be required, but insufficient, for HGF-induced epithelial differentiation in OSE (Brinkmann *et al.*, 1995; Meiners *et al.*, 1998). Probably, other extra- and intracellular modulators, notably integrins, modulate OSE responses to HGF.

HGF stimulates cell-cell dissociation primarily by inhibiting cadherin-mediated cell adhesions in epithelial tissues, and the physical interaction between the Met receptor and E-cadherin at intercellular junctions may facilitate the regulation of cell adhesion by HGF (Crepaldi *et al.*, 1994; Hiscox and Jiang, 1999). Tyrosine phosphorylation of β-catenin appears to be an important regulatory signal for HGF-induced scattering of epithelial cells (Weidner *et al.*, 1990; Shabamoto *et al.*, 1994; Hiscox and Jiang, 1999). However, the possible involvement of tyrosine phosphorylation of other substrates associated with the complex, such as p120ctn or still unidentified proteins should not be excluded (Takeda *et al.*, 1995; Gumbiner, 2000). The data showed that the ability of E-cadherin transfected OSE to scatter and branch, indicative of cell dissociation and invasiveness, was significantly enhanced by HGF treatment. The enhancement in invasiveness is likely coordinated with the induction of plasminogen activator and metalloproteinases-1 and -3, which contribute to matrix remodeling (Pepper *et al.*, 1992; Dunsmore *et al.*, 1996). Therefore, a similar effect of HGF on these cells *in vivo* may promote ovarian cancer cells to spread and disseminate into the peritoneal cavity (Corps *et al.*, 1997; Ueoka *et al.*, 2000).

The present study showed that both MAPK and PI3K were required for the scattering in IOSE-29EC and IOSE-29EC/T4 cells. While both the MAPK and PI3K cascades activated by Ras are important in modulating cell-cell interactions, PI3K also seems to be critical in reorganizing the actin cytoskeleton (Khwaja *et al.*, 1998; Potempa and Ridley, 1998).

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Ectopic expression of Tiam1, an exchange factor for the Rho GTPase Rac, inhibited HGF-induced scattering, suggesting that Tiam-Rac signaling plays a role in cell dissociation (Hordijk et al., 1997). In this study, we showed that rapamycin can inhibit HGF-mediated scattering, suggesting that this process can be mediated through the p70 S6K, which is also a PI3K effector. The present data also indicate that MEK is involved in the activation of p70 S6K, but the link between MEK1/MAPK and p70 S6K remains unclear (Ballou et al., 1991; Ming et al., 1994; Sakaue et al., 1995; Lenormand et al., 1996). Recent evidence suggests that MAPK may play a more direct role in ECM degradation (Webb et al., 2000). Thus, in addition to its ubiquitous effect on cell proliferation, the MAPK pathway may be involved in the remodeling of cell-cell and cell-substrate adhesion, which are important factors for tissue morphogenesis. In contrast to rat cardiac myocytes that require p38 MAPK activity for differentiation (Davidson and Morange, 2000), the inherent insensitivity of E-cadherin expressing OSE to p38 MAPK inhibition indicates that the p38 MAPK pathway is not required to drive HGF-mediated morphological effects in these cells.

The temporal aspects of signaling are important in defining cellular responses (Marshall, 1995; Pumiglia and Decker, 1997). For instance, a sustained activation of ERK MAPKs by nerve growth factor has been implicated in differentiation and growth arrest, whereas a transient activation by EGF is associated with proliferation in PC12 cells (Marshall, 1995). In contrast, several publications appear to be in conflict with these observations (Cowley et al., 1994; Cressman et al., 1996). In fibroblast and other cell types, the prolonged activation of ERK MAPK is associated with proliferation and not differentiation (Cowley et al., 1994; Cressman et al., 1996; Sellers et al., 1999). Transient versus sustained ERK activation has also been implicated in the induction of apoptosis and maintenance of cell survival (Ishikawa and Kitamura, 1999; Kimura et al., 1999). In this

study, ERK activation was prolonged and sustained in IOSE-29EC and IOSE-29EC/T4 cells, whereas it was more transient in OVCAR-3 cells. HGF was mitogenic in OVCAR-3, but inhibited the growth of E-cadherin transfected OSE cells. The inhibition of cell proliferation in the E-cadherin transfected OSE was associated with the capability of HGF-Met signaling to induce branching ducts, indicative of enhanced epithelial differentiation, in collagen gels. In contrast, HGF did not induce branching morphogenesis in OVCAR-3. These data are in keeping with the idea that the specificity of tyrosine kinase receptor signaling that contributes to differentiation or proliferation depends on the duration of ERK activation, and that different kinetics of ERK phosphorylation cause secondary changes in transcription factor activation. Although a sustained ERK activation is not a mandatory condition for the promotion of differentiation, our data are in favor of the argument that a more persistent ERK activity is associated with the differentiated phenotype observed in SV40-E-cadherin transfected OSE. There are two unusual findings in the present study: firstly, the sustained phosphorylation (>1 h) of protein kinases, such as ERK MAPKs, Akt/PKB and p70 S6K, induced by HGF in OSE. Similar prolonged phosphorylation of ERK MAPKs and Akt/PKB induced by HGF is also shown in MDCK cells. These observations are in contrast to the rapid time course of phosphorylation and dephosphorylation (30 min - 1 h) induced by many other growth factors that include EGF, illustrating the unique property of HGF (Khawaja *et al.*, 1997). Secondly, unlike other cell types that utilize different receptor tyrosine kinase signaling pathways for differentiation and proliferation, HGF itself can generate different kinetics of ERK activation, which alter gene expression and selectively lead to different outcomes in OSE at progressive stages of ovarian carcinogenesis.

Together, these data demonstrate that coexpression of HGF and Met is oncogenic in OSE and that is likely a causative factor in the inappropriate expression of mitogenic,

motogenic and morphogenic signals. Differences in signaling events of proliferation versus differentiation of HGF on OSE cells at different progressive stages of transformation could be explained by two different scenarios, which are not mutually exclusive, (1) different levels and kinetics of ERK phosphorylation and (2) different cell context based on different genetic backgrounds of these cells. The initiation of scattering and branching morphogenesis in SV40-E-cadherin transfected OSE in response to HGF suggests that E-cadherin and HGF interact in the epithelial differentiation of ovarian neoplastic transformation.

4.3.2. Protein kinase regulation in the neoplastic progression of OSE

Increasing numbers of human cancers are known to involve mutations, over-expression, or malfunctioning of protein kinases and phosphatases, and their regulators and effectors. This study focused on the tracking of a wide range of protein kinases in epithelial ovarian carcinogenesis using normal OSE and ovarian cancer cell lines, and an experimental model that represents the progressive stages in ovarian neoplastic transformation. An important concept in signaling specificity is signal thresholds. In many systems, a two-fold decrease in the level of a signaling protein can be sufficient to abrogate signaling, and conversely a two-fold increase can initiate signaling (Hunter, 2000). In the present study, some of the most profound changes in the expression profile of protein kinases in ovarian carcinogenesis were observed for cGMP-dependent protein kinase (PKG) and MEK6. It is interesting to note that there was a downregulation of PKG but an upregulation of MEK6 in the development of ovarian cancer. PKG regulates calcium concentration and myosin light chain phosphorylation, which alters smooth muscle cell tone (Surks *et al.*, 1999). These observations have given rise to speculations that PKG may function in a similar manner in OSE which exhibits contractile activity during the post-

ovulatory repair of the ovarian surface. Contraction of OSE cells may also play a role in the shrinkage of the ovaries that occurs with age and results in their typical convoluted shape and the formation of the OSE-lined clefts and inclusion cysts. PKG regulates cell proliferation in different cell types, and this activity is mediated through the Ras-MAPK pathway (Suhasimi *et al.*, 1998). Moreover, PKG may activate Ras via CNrasGEF, a guanine nucleotide exchange factor, in response to elevations of intracellular cGMP (Pham *et al.*, 2000). These observations suggest possible mechanisms for growth regulation of OSE through PKG signaling. Altered expression of MEK6 in neoplastic OSE was associated with an increased expression of p38 MAPK, a downstream regulator of MEK6, suggesting that the MEK6/p38 MAPK pathway, which is an important component of the stress response required for the homeostasis of a cell, may play an important role in the neoplastic progression of OSE. This study also showed enhanced expression of casein kinase II (CK2) in the course of ovarian carcinogenesis. Increased expression of CK2 has been linked with neoplastic transformation because CK2 activity and protein levels are commonly elevated in solid human tumors and transformed cell lines (Prowald *et al.*, 1984; Munstermann et al., 1990; Pinna and Meggio, 1997; Guerra and Issinger, 1999). Though little is known concerning the acute regulation of this protein kinase, Sayed *et al.* demonstrated that p38 MAPK can directly activate CK2 in an allosteric mechanism, and that interaction regulates cell cycle progression, DNA repair and apoptosis through p53 (Sayed *et al.*, in press). Though there is currently no definite explanation for the expression profile of these kinases in OSE, the present data suggest that while PKG is associated with normal OSE functions, MEK6, p38 MAPK and CK2 are central to oncogenic OSE transformation.

The phosphorylation of protein kinases was revealed by phosphorylation-induced mobility reduction on SDS-PAGE gels in the study. Alternatively, phosphospecific antibodies

can be used. Although phosphorylation changes have been previously shown to correlate with the activation of protein kinases (Kim *et al.*, 1998), *in vitro* kinase assays will definitely provide a better understanding of the kinase activities. Akt2 and p70 S6K, which are downstream regulators of PI3K, were not only over-expressed but also phosphorylated in neoplastic OSE, including SV40-E-cadherin transfected OSE and ovarian cancer cell lines, in the absence of HGF stimulation. Importantly, such phosphorylation was also found in preneoplastic IOSE and overtly normal FH-OSE, but only in 50% of NFH-OSE, suggesting that the PI3K cascade may be responsible for the propensity of OSE to undergo neoplastic transformation. Introduction of E-cadherin to SV40-transfected OSE caused changes in a variety of protein kinases that were found among ovarian cancer cells, but not in normal and SV40-immortalized OSE. These variations include the downregulation of PKG, the acqusition of MEK6 expression, and the expression of high levels of Akt2 in SV40-E-cadherin transfected OSE and ovarian cancer cell lines compared to normal and SV40-immortalized OSE. This supports our hypothesis that E-cadherin contributes in an important way to ovarian carcinogenesis. Since E-cadherin mediated cell contacts prevent anoikis (Hermiston and Gordon, 1995; Kantak and Kramer, 1998), it is intriguing to speculate that E-cadherin contributes to tumorigenesis of SV40-E-cadherin transfected OSE by stabilizing SV40-induced genetic abnormalities, which may confer a selective advantage to the transforming OSE. The present study demonstrated constitutive phosphorylation of Akt2 and p70 S6K in SV40-E-cadherin transfected OSE and ovarian cancer cell lines, and that PI3K activity may regulate the survival and transforming signals of E-cadherin in these cells. Though Akt2 appears to play a more important role than Akt1 and Akt3 in malignant transformation, a high proportion of dephosphorylated Akt1 was observed consistently in E-

cadherin transfected OSE and ovarian cancer cell lines. However, the mechanism responsible for this phenomenon and its biological significance remain to be defined.

Though in many cases non-tumorigenic SV40-immortalized OSE retained phenotypic properties of normal OSE, an important issue that is frequently overlooked are the differences among these OSE. Examples of such differences in terms of protein kinase networks, listed in this report, include elevated levels of CDK1 and p38 MAPK, reduced levels of PKG, and activated Akt2 and p70 S6K which were present in ovarian cancer cells, but were already found, to a lesser degree, in SV40-transfected OSE. Multiple lines of evidence indicate that SV40 immortalization is associated with the aberrant regulation of cell cycle regulatory proteins. Cells transduced with large T antigen exhibits elevated expression of various cyclin-dependent kinase subunits including CDK1 (Oshima *et al.*, 1993). Large T antigen has also been shown to transactivate the human cdc2 promoter and stimulates DNA synthesis (Chen *et al.*, 1996). The differences in protein kinase regulation found between normal and SV40-transfected OSE provide an additional molecular distinction between these cells, and show that SV40-immortalized OSE do not completely resemble normal OSE. Rather, these cells represent a progressive stage in ovarian malignant transformation.

The study also compared the regulation of protein kinases among three human ovarian cancer cell lines, CaOV-3, SKOV-3 and OVCAR-3. Although they are all derived from serous ovarian adenocarcinomas, each line possesses some distinguished characteristics, which illustrate the heterogeneity found among ovarian carcinomas. For instance, OVCAR-3 expresses estrogen and androgen receptors which are absent in the other two lines, and over-expression of HER-2/neu is observed only in SKOV-3. Our data showed high expression levels of Akt2 and GSK3β in OVCAR-3 compared with SKOV-3,

suggesting that PI3K is over-expressed in OVCAR-3. This is in agreement with previous findings which indicate that OVCAR-3 has a greater copy number of the *PIK3CA* gene, which correlates with PI3K activity, than line SKOV-3 (Shayesteh *et al.*, 1999).

Together, the data demonstrate that different repertoires of downstream signaling proteins are correlated with phenotypic manifestations of OSE at progressive stages in the development of ovarian cancer. The accumulating knowledge about signaling networks and the kinase patterns involved in ovarian cancers will permit the identification of predictive markers for early detection and the development of specific therapeutic pharmacological modulators of protein kinases as a potential management strategy for ovarian cancer patients.

4.4. PI3K: a balance between mitotic arrest and apoptosis

PI3K has been implicated in ovarian carcinogenesis. The over-expression of the *PIK3CA* gene, which encodes the p110α subunit of PI3K, results in an increase in PI3K activity in 40% ovarian cancers. Treatment with the PI3K inhibitor LY294002 decreases proliferation and increases apoptosis in many ovarian cancer cell lines but not in normal OSE or IOSE lines (Shayesteh *et al.*, 1999). Unexpectedly, this study showed that LY294002 blocked metaphase-anaphase transition of mitosis in neoplastic SV40-E-cadherin transfected OSE, but induced apoptosis and some metaphase arrest in the ovarian cancer cell line OVCAR-3. The relationships between the mechanisms that induce cell cycle delay and those that induce apoptosis have not yet been worked out. Although mitotic arrest physiologically occurs in meiosis in oocytes, such arrest is unusual for somatic cells and probably detrimental. Most LY294002-arrested OSE cells exited mitosis and entered apoptosis after exposure of at least 24 hr or longer, or at doses above 50 µM.

However, the biochemical events leading to apoptosis following mitotic arrest are complex, poorly understood and appear to vary among cell types (Jordan *et al.*, 1996). Among these as yet undefined factors, the p53 status has been implicated to influence cell-cycle progression following mitotic arrest (Sorger *et al.*, 1997).

Microtubule-active drugs, which include paclitaxel (taxol) and vinca alkaloids (vinblastine and vincristine), constitute one of the most important classes of chemotherapeutics (Rowinsky and Donehower, 1991; Wilson and Jordan, 1995). Taxol stabilizes microtubules, while vinblastine dramatically depolymerizes them (Woods *et al.*, 1995; Jordan *et al.*, 1996). In addition, taxol appears to induce a temporary G1 block in non-transformed cells (Trielli *et al.*, 1996). In contrast to microtubule-active drugs, such as taxol and vinblastine, β-tubulin immunofluorescence staining revealed intact microtubules in IOSE-29EC cells following LY294002 treatment. These data suggest that LY294002 inhibits mitotic progression independently of microtubule inactivation, presumably through a novel mechanism as yet to be determined.

Anaphase is typically initiated shortly after the last chromosome has aligned at the metaphase plate and is connected to microtubules of both spindle poles. The kinetochore complex that includes BUBR1 has been implicated in the regulation of this process. Mitosis-promoting factor (MPF) is a protein kinase that comprises a catalytic subunit p34 Cdc2 and a regulatory subunit cyclin B. Degradation of cyclin B mediated by protein phosphatase 2A (PP2A) and activation of cAMP-dependent protein kinase (PKA) by MPF has been implicated in the metaphase to anaphase transition. In view of the intact mitotic spindles, alterations in the regulation of cell cycle specific protein kinases and kinetochores as a result of unaligned chromosomes may be relevant to the unexplained metaphase block in LY294002-treated SV40-E-cadherin transfected OSE cells.

The implication of a PI3K inhibitor in the mediation of metaphase arrest in neoplastic SV40-E-cadherin transfected OSE is a surprising outcome of this study. Though it is not clear why PI3K inhibition causes mitotic arrest in some ovarian cancer cells and apoptosis in others, these findings underline the importance of PI3K in ovarian carcinogenesis and the potential influence of the drug as a chemotherapeutic agent.

5. SUMMARY

The ovarian surface epithelium (OSE) -derived epithelial ovarian carcinomas encompass a diverse, biologically complex group of malignant neoplasms with a dismal clinical prognosis. There is an urgent need for a better understanding of regulatory mechanisms that control growth, motility and differentiation of their source, the OSE, for better means to therapeutically inhibit cancer progression, and for the identification of new, clinically useful detection markers. In this study, changes in E-cadherin, HGF and in the regulation of various protein kinases were examined in (1) OSE in women with hereditary ovarian cancer syndromes and (2) OSE at different, progressive stages of transformation, in an attempt to study the contribution of these factors to preneoplastic changes in the OSE, and to functions of normal and neoplastic OSE.

Our data are in agreement with other studies, which suggest that FH-OSE is altered phenotypically before the loss of both copies of tumor suppressor genes, such as BRCA1. Our data suggest that these phenotypic alterations might associate with hereditary ovarian cancer syndromes in general rather than with BRCA1 gene mutations specifically. Therefore, there may be other coseggregating high frequency low penetrance genes involved. Our results, which suggest that enhanced E-cadherin and autocrine HGF-Met activity occur in FH-OSE, shed light on very early, or even preceding steps in ovarian carcinogenesis. In view of the expression of HGF in FH-OSE and ovarian cancer cells but not in normal OSE, observed in the present study, it will be of interest to determine whether circulating HGF is already found in the serum and biological fluids of women with hereditary ovarian cancer syndromes. This may emerge as a novel and potentially useful ovarian tumor marker.

Cadherins and HGF are central to normal and neoplastic OSE functions. Our immunofluorescence results suggest that the epithelial integrity of OSE is maintained by N-cadherin, while E-cadherin regulates cell shape. The mitogenic activity of HGF on normal OSE *in vitro* suggests that it may play a role in the wound repair of the ovarian surface following ovulatory ruptures *in vivo*. Met is overexpressed in malignant OSE, thus these cells may have an augmented response to HGF. HGF induced the formation of tubules and cysts, which indicate enhanced epithelial differentiation, in neoplastic E-cadherin expressing OSE cells. Modulation of intercellular adhesive interactions by HGF in E-cadherin expressing neoplastic OSE may play a role in the events leading to tumor cell dissemination, and the HGF-stimulated branching responses might facilitate tumor cell invasion *in vivo*. Since HGF may play a role as a key regulatory molecule in ovarian cancer development and progression, antagonism of this molecule thus presents a potential strategy in the management and treatment of ovarian cancer patients.

The effects of HGF are mediated by signaling through the Met receptor, and activation of different intracellular signaling pathways ultimately leading to various cellular responses. The identification of signal transduction pathways specific to the responses may prove useful for the inhibition of HGF activities in clinical practice.

This study has highlighted changes in the expression and regulation of signaling networks and the proteins involved in ovarian cancer development. Though there is still much to understand concerning both the molecular and cellular events which accompany changes in protein kinase regulation, the data present interesting and valuable information for future research. The accumulating knowledge in this area will permit the identification of predictive markers for early detection and the development of potent and specific pharmacological modulators of signaling that can be used therapeutically.

The observation that the PI3K inhibitor causes metaphase arrest in neoplastic SV40-E-cadherin transfected OSE is a surprising outcome of this study. Though the mechanism is not clear, the findings underline the importance of PI3K in ovarian carcinogenesis and the potential influence of the drug as a chemotherapeutic agent.

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