HUMAN OVARIAN SURFACE EPITHELIAL CELLS IN CULTURE: CHARACTERIZATION AND MATRIX INTERRELATIONSHIPS

by

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ABSTRACT

The human ovarian surface epithelium (HOSE) is thought to give rise to over 85% of human ovarian carcinomas. In spite of its clinical importance, experimental systems for investigations of HOSE are lacking and available culture methods have yielded limited success. In this study, HOSE from normal ovarian biopsy specimens was used: 1) to improve and simplify the methodology for HOSE culture; 2) to further characterize HOSE cells; 3) to characterize and isolate an ovarian-derived extracellular matrix (ECM); and 4) to study the interactions between cultured HOSE cells and ECM as a means to examine the dynamic, pleomorphic, and morphogenetic nature of HOSE.

Improved tissue culture techniques, developed during the course of this study, allowed for a better method for the culture of HOSE cells and the decontamination of mold-infected cultures. An improved explantation method was developed which takes advantage of the tenuous attachment of HOSE to underlying tissues: the surface epithelium is scraped off the ovarian surface, generating epithelial fragments which produce monolayers in culture, with little contamination by other cell types. The scrape method is superior to the explant method previously described in terms of speed, simplicity, higher purity of cultures, and increased cell yield. This improved culture system provided conditions for the in depth studies of HOSE described herein. A technique for the elimination of mold contamination by a simple one-step Percoll gradient centrifugation was developed, using the fungus *Cladosporium* as a prototype and intentionally infected cell lines. Centrifugation in Percoll did not affect cell morphology, growth, or the cells' ability to produce ECM. Percoll decontamination may be a generally applicable technique for decontamination of mold infected cultures.

Characterization of HOSE cells in culture showed that they are variably positive for mucin and contain lipid, vimentin, and keratin subtypes #7, 8, 18 and 19 like their *in vivo* counterparts. Additionally, HOSE cells produce basement membrane and stromal ECM components as demonstrated by the presence of laminin and collagen types I, III, and IV in HOSE cultures.

ECM derived from rat ovarian surface epithelial cell cultures (ROSE 199-ECM) also consisted of basement membrane and stromal matrix components as shown by the presence of laminin, fibronectin, and collagen types I and III. This predominantly fibrillar ECM supports the attachment, spreading, and even growth of several cell types. Although HOSE cells remain epithelial, but dispersed throughout the ROSE

199-ECM, HOSE cells plated on a combination of ROSE 199-ECM and collagen gel contract the matrices into organoids providing evidence that HOSE cells are capable of physically remodelling ECM.

Examination of HOSE-ECM interactions revealed that substrata influence HOSE morphology, growth, and proteolytic activities. On plastic and fibrin clots, HOSE cells formed epithelial monolayers while they are spindle-shaped on rat tail tendon-derived collagen gels. On Matrigel, the cells form aggregates that penetrate and lyse the gel. HOSE cells grow rapidly on plastic, remain stationary on collagen gels and fibrin clots and eventually decrease in number when maintained on Matrigel. The different morphologic phenotypes did not translate into the expression of different integrins at the cell surfaces except for collagen gels. With the exception of cells on collagen gels, HOSE surveyed for various integrins express receptors for vitronectin (VNR), collagen, laminin, and fibronectin (VLA-2,-3,-5, and β1), but lack β4 and VLA-6, a laminin receptor. There appears to be a downregulation of integrins when cells are maintained on collagen gels. Conditioned medium of HOSE cultures on these substrata contains neutral proteases: 1.) chymotrypsin-like and elastase-like activities that are inversely related to growth; and 2.) a 30 KD and a 42 KD gelatinase.

These results suggest that normal HOSE cells play an active role in ECM remodelling, that is, its deposition, degradation, and physical reorganization. These capabilities may be important for normal ovarian development and normal adult functioning such as ovulation. Furthermore, while proteolytic activities and invasiveness are characteristics of the malignant phenotype, they also appear to be part of the normal HOSE phenotype. With regards to why HOSE is a preferred site of cancer development, it is perhaps aberrations of these functions that contribute to pathological states.

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LIST OF ABBREVIATIONS

AB ..... Alcian blue
AH ..... Ammonium hydroxide
APMA ..... p-Aminophenylmercuric acetate
BAPNA..... N-Benzoyl-DL-arginine p-nitroanilide
BCIP..... 5-Bromo-4-chloro-3-indolyl phosphate p-toluidine salt
BSA ..... Bovine serum albumin
CE..... Coelomic epithelium
CEA..... Carcinoembryonic antigen
COLL..... Rat tail tendon-derived collagen gel
DAB..... Diaminobenzidine
DC ..... Deoxycholate
DM ..... Defined medium
DMF ..... N,N-Dimethylformamide
DMSO ..... Dimethyl sulfoxide
ECM..... Extracellular matrix
EDTA ..... Ethylene-diaminetetra-acetic acid
EGF..... Epidermal growth factor
EHS ..... Matrigel
FB ..... Fibrin clot
FBS ..... Fetal bovine serum
FITC ..... Fluorescein isocyante conjugated
FN ..... Fibronectin
FT..... Freeze thaw
HBSS..... Hank's balanced salt solution
HC ..... Hydrocortisone
H&E..... Hemotoxylin and eosin
H_2O_2..... Hydrogen peroxide
HOSE ..... Human ovarian surface epithelium
HRP..... Horseradish peroxidase
HUDF..... Human dermal fibroblasts
LN ..... Laminin
MDPF..... 2-Methoxy-2,4-diphenyl-3(2H) furanone
MeOH..... Methanol
NBD..... Phallacidin

NBT..... Nitro blue tetrazolium
OSE..... Ovarian surface epithelium
p..... Passage number
PA..... Plasminogen activator
PAI-1..... Plasminogen activator inhibitor type 1
PAS..... Periodic acid Schiff
PBS..... Phosphate buffered saline
PC-1..... Pederson's fetuin
PD..... Placental-derived matrix
PH..... Phalloidin
PL..... Plastic
PMSF..... Phenylmethyl-sulfonyl fluoride
RLF..... Rat lung fibroblasts
ROSE..... Rat ovarian surface epithelium
ROTC..... Rhodamine isocyante conjugated
RT..... Room temperature
SAANA..... N-Succinyl-ala-ala-ala p-nitroanilide
SAPNA..... N-Succinyl-ala-ala-pro-phe p-nitroanilide
SDS-PAGE..... Sodium dodedecyl sulfate polyacrylamide gel electrophoresis
SE..... Surface epithelium
SEM..... Scanning electron microscopy
SIP..... Stock iso-osmotic Percoll
TBS..... Tris-HCl
TEM..... Transmission electron microscopy
TTBS..... TBS containing 0.05% Tween-20
VNR..... Vitronectin receptor
WM..... Waymouth's medium
1^{o}..... Primary
2^{o}..... Secondary

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INTRODUCTION

A. Overview

The human ovary is invested with a simple, yet dynamic, epithelium which is intimately involved with ovarian development, function, and pathology. During development, the human ovarian surface epithelium (HOSE) undergoes periods of intense proliferation and invades the ovarian stroma as the sex cords to become associated with oogonia and contribute to the formation of the primordial follicles. In the adult, HOSE undergoes cyclic changes in morphology related to ovulation. As the preovulatory follicle matures, HOSE is flattened due to mechanical stresses and at ovulation, the continuity of HOSE is disrupted. Although there is little evidence to suggest that HOSE plays a role in the initiation of ovulation, following ovulation, HOSE cells proliferate at the edges of the ovulatory wound, migrate over the wound site, and contribute to the repair of the ovulatory defect. When repair is completed, the HOSE returns to its original, resting morphology of a simple cuboidal epithelium. The attrition of follicles during menopause results in ovarian remodelling and shrinkage so that the senile ovary appears wrinkled and the ovarian surface is folded into a gryus-like appearance. Further, with increasing age the number of HOSE-lined crypts and cysts increases.

Yet, the most notable change in HOSE is its transformation to malignancy which is responsible for over 85% of ovarian cancers, despite the fact that HOSE comprises only a minute fraction of the total ovarian mass. HOSE-derived carcinomas are common, and in general, have a poor prognosis. In spite of its clinical importance, studies on the biology of normal HOSE and the role of HOSE in ovarian carcinogensis have been severely limited by the lack of experimental systems. Animals have contributed little to the understanding of HOSE-derived malignancies because, with few exceptions, ovarian cancers in animals do not arise in the ovarian surface epithelium. Most of the information about ovarian carcinogenesis is based upon histopathological examination. These studies suggest that early malignant changes occur in HOSE-lined cysts and invaginations rather than on the ovarian surface.

Undoubtedly many factors contribute to the dynamic nature of HOSE such that it is capable of proliferation, migration, invasion, cyst formation, and wound repair, but one common factor appears to be an interaction between HOSE and extracellular matrix. This report examines the dynamic, pleomorphic, and morphogenetic nature of HOSE by: 1) improving HOSE culture methodology; 2) further characterizing

HOSE cells; 3) isolating and characterizing an ovarian-derived matrix; and 4) studying the interactions between cultured HOSE cells and various extracellular matrices.

B. HOSE *In Vivo*

(1.) Embryology

The dynamic nature of HOSE is evidenced throughout life. Even early in development HOSE cells demonstrate pleomorphism, a proliferative capacity, and invasive behaviour. Gonadal development is first seen around the 5th week of development with the formation of the indifferent gonad that is covered with coelomic epithelium (CE) and which develops on the medial side of the mesonephros. The CE proliferates, possibly in response to induction by mesonephric cells (Wartenberg '82) to form a bulging structure referred to as the gonadal ridge. Cords of surface epithelial cells, the primary sex cords, penetrate the mesenchyme of the indifferent gonad and form a rudimentary rete ovarii in female embryos. While the primary sex cords in female embryos normally regress and degenerate, vestiges of the rete ovarii occur as epithelial cords in the region of the hilum of the ovary while the cortex of the indifferent gonad will differentiate into the cortex of the adult ovary (Byskov '86, Moore '82).

At 4-5 months of development the epithelium covering the fetal ovary (i.e. HOSE) contributes to the formation of the somatic components of the ovarian cortex. Following intense mitotic activity HOSE cells become stratified (Gondos '75, Motta & Makabe '82, Nicosia '83, Pinkerton et al. '61). It is suggested that the stimulus for such proliferation in the fetal ovary may be the onset of steroid production and secretion by the interstitial cells (Gondos '75). At the light microscopic level the thickening of the ovarian surface epithelium into a multilayered structure is usually uniform in nature, but occasionally irregular papillary projections are present. In this situation, HOSE cells can be oriented in many different directions and exhibit pleomorphism. Further, groups of HOSE cells, referred to as the secondary sex cords, invade and penetrate the fetal ovary, associate with germ cells which have migrated from the yolk sac, and contribute to the formation of primordial follicles. Whether follicular cells are derived solely from HOSE or mesonephric cells or from both (Byskov '86, Gondos '75, Hirshfield '91, Nicosia '83, Wartenberg '82) is unclear so that the contribution of HOSE to the various components of the adult ovary is still controversial. With the formation of the primodial follicles completed, HOSE

is reduced to a simple epithelium and remains relatively quiescent throughout the remainder of development and childhood.

While the mesonephric kidneys are the fetal functional kidneys in both sexes and the mesonephric ducts give rise to the male genital ducts and their associated derivatives, it is the paramesoneophric or Mullerian ducts which give rise to the genital ducts and their associated derivatives in female embryos (Moore '82). The paramesonephric ducts arise as invaginations of the same CE that gives rise to the ovarian surface epithelium just laterally to the mesonephric ducts and in close proximity to the site of development of the ovary. The edges of the invaginations approach each other and fuse to form the paramesonephric ducts proper. Cranially, the ducts open into the coelomic cavity which will be the future peritoneal cavity. The cranial unfused portions of these ducts will develop into the oviducts. The paramesonephric ducts run caudally parallel to the mesonephric ducts. In the caudal region they come together in the midline and fuse forming the uterovaginal primordium, i.e. the primordium for the epithelium of the uterus and part of the vagina.

Hence arises an important relationship. The CE gives rise to both the HOSE and Mullerian duct, so that the lining of the Mullerian duct derivatives and HOSE are embryologically closely related. This proximity is reflected in the various directions of Mullerian differentiation pursued by the HOSE when it undergoes neoplasia (Blaustein '77a). The most frequent forms of metaplasia, in order of decreasing frequency, are to cells resembling oviduct, endometrium, and endocervix (Blaustein '77a,b, Scully '70,'77). This is of clinical importance in the interpretation of the phenotypes of HOSE-derived ovarian carcinomas.

(2.) Adult Morphology and Function

HOSE remains relatively unchanged throughout childhood. At the light microscopic level, the normal resting HOSE (such as prior to ovulation) investing the adult ovary is a single layer of squamous to columnar epithelium depending upon its position relative to follicles, corpora lutea, and crypts (Anderson et al. '76, Balboni '80, Centola '80,'83, Motta et al. '80, Papadaki & Beilby '71, Van Blerkom & Motta '79). The ovarian surface epithelium is also separated from underlying ovarian structures by a basement membrane below which is a layer of dense collagenous material referred to as the tunica albuginea and in humans the ovarian surface epithelium is tenuously attached to its underlying tunica albuginea (Anderson et al. '76, Clement '87, Van Blerkom & Motta '79). Although rare in human ovaries, surface papillae

interrupt the otherwise smooth ovarian surface in other animals such as rabbits (Motta et al. '80, Nicosia et al. '89, Nicosia & Johnson '84b, Van Blerkom & Motta '79). Areas of the surface of the human ovary are occasionally invaginated into the subjacent cortical layers in the form of crypts, cords, and inclusion cysts lined by HOSE cells (Motta et al. '80, Nicosia & Nicosia '88, Van Blerkom & Motta '79, Young et al. '89). Crypts are hollow, tubular invaginations in which the lumens open directly onto the surface of the ovary. The cells lining the crypts are continuous with HOSE on the ovarian surface and are generally cuboidal or columnar in shape (Papadaki & Beilby '71). Some invaginations of HOSE terminate as small, irregular solid masses or cords of cells that lack a lumen and are directly connected to the ovarian surface. In addition to crypts and cords, HOSE-lined inclusion cysts occur in the cortical stroma which are not in contact with the surface HOSE (Blaustein '81b, Van Blerkom & Motta '79). Characteristics of HOSE *in vivo* include the presence of: glycogen; glycosaminoglycans; epithelial mucin; lipid (Blaustein '84, Blaustein & Lee '79); glycoproteins (Guraya '80); epidermal growth factor receptors (Berchuk et al. '91); 17-β-hydroxysteroid dehydrogenase activity (Blaustein & Lee '79); keratin (Czernobilsky '85, Czernobilsky et al. '84), and vimentin filaments (Czernobilsky '85).

While appearing uniform at the light microscopic level, Gillet et al. ('91) demonstrated the pleomorphic nature of HOSE *in vivo* . SEM examination of ovarian samples revealed two morphologically distinct types of HOSE cells. Both cell types are common to all surfaces of the ovary including the distending preovulatory follicle. Type A cells are cuboidal and covered with numerous microvilli while type B cells are flat squamous cells with fewer microvilli. It is suggested that type B cells are the result of squamous metaplasia in response to surface injury associated with ovulation. Once repair is completed and the ovulatory defect is re-epithelialized, zones of type B cells become permanantly established. Other ultrastructural studies reveal that the apical surface of HOSE is generally covered by numerous microvilli of uniform size and shape (Anderson et al. '76, Blaustein & Lee '79, Gillet et al. '91, Motta & Van Blerkom '80, Van Blerkom & Mota '79). The microvilli may be branched and are coated with glycocalyx (Motta & Van Blerkom '80). Although HOSE is continuous with the peritoneal mesothelium at the mesovarium, it is functionally and morphologically somewhat different from mesothelial linings (Andrews & Porter '73, Behbehani et al. '82, Blaustein '84, Whitaker et al. '80a, '80b, '82). For example, a single cilium is seen on some HOSE cells (Anderson et al. '76, Nicosia & Nicosia '88, Papadaki & Beilby '71, Van Blerkom & Motta '79), a feature lacking on

mesothelial cell surfaces. Mesothelial cells also lack 17-β-hydroxysteroid dehydrogenase activity. Adjoining lateral cell borders of HOSE cells are connected by desmosomes along the length of the cell and by incomplete tight junctions near the apical free surface of the cell (Anderson et al. '72,'76, Blaustein & Lee '79, Motta et al. '80, Papadaki & Bielby '71). Anderson et al. ('72) demonstrated the occurence of incomplete tight junctions in the mammalian ovarian surface epithelium when horseradish peroxidase injected intraperitoneally made its way into the ovary. Gap junctions have been observed in the ovarian surface epithelium of the rabbit ovary (Anderson et al. '76), but no specific reference has been made to their presence in HOSE.

Functionally, HOSE appears to provide a protective and lubricated covering over the ovary as denuding the ovary of its HOSE has been implicated in the pathogenesis of adhesion formation (Gillet '91). Histochemical and ultrastructural studies indicate that HOSE cells are involved in both secretory (presence of RER, Golgi complexes, secretory vesicles) and endocytotic (presence of endocytotic pits, lysosomes, multivesicular bodies) activities so that HOSE may be involved in the transport of material to and from the peritoneal cavity (Papadaki & Beilby '71). Further, an increased number of lysosomes has been noted in HOSE just prior to ovulation with apparent basal release of the lysosomal contents at ovulation suggesting that HOSE may produce proteolytic enzymes which play a role in follicular rupture by the dissolution of the follicular apex at ovulation (Bjersing & Cajander '75, Cajander & Bjersing '77). However, direct evidence for the production of proteases by HOSE cells has not yet been reported. The presence of 17 β-hydroxysteroid dehydrogenase activity has been reported in HOSE (Auersperg et al. '84, Blaustein & Lee '79) and 3-β-hydroxysteroid dehydrogenase and 17-β-hydroxysteroid dehydrogenase activities have been reported in the ovarian surface epithelium of mice, rats, and rabbits (Rembiszewska & Brynczak '85) indicating that the ovarian surface epithelium is capable of progestin and androgen metabolism. However, HOSE does not appear to be capable of steroid synthesis. HOSE cells show an absence of cholesterol or its esters (Guraya '80) as well as steroidogenic enzymes, such as P450 side chain cleavage activity, necessay for *de novo* steroid synthesis (Blaustein & Lee '79, Hoyer '80, Rembiszewska & Brynczak '85).

At puberty the onset of menses marks the beginning of the reproductive phase of life. From then on the adult ovary undergoes repetitive periods of folliculogenesis and steroidogenesis for the development of a mature follicle and oocyte which is released at ovulation. For ovulation to occur the follicle must penetrate formidable

barriers. These include, from inward to outward, granulosa cells, the basement membrane underlying the granulosa cells, layers of theca interna and externa, the ovarian stroma, the tunica albuginea, the basement membrane underlying HOSE, and finally HOSE itself. Structural alterations at ovulation restricted to the stigma area in the follicular wall have been reported in mammals and avians. There is a loosening and decomposition of the collagen fibers and glycosaminoglycans in the tunica albuginea and thecal layers (Jackson et al. '91, Yoshimura et al. '87) of the preovulatory follicle. The degradation of glycosaminoglycans and collagen fibers in the stigma region of the preovulatory follicle prior to ovulation is thought to reduce the tensile strength of the ovulatory barrier, and hence, loosen the barrier and ease ovulation.

In ovulation the oocyte transverses the surrounding connective tissue and basement membrane barriers which have been disrupted by the proteolytic activities of the follicular cells. This process could be considered similar to tumour invasion, in which malignant cells disrupt basement membranes. Therefore, it is not surprising that collagenolytic (including type IV) activities are found in the follicular fluid (Puistola et al. '86). In many species including humans, rats, rabbits, and chickens, there is an increase of many proteases including stromal collagenases, type IV collagenases, and plasminogen activator as ovulation approaches, followed by a decrease in protease production after ovulation (Cajander '89, Cajander et al. '89, Curry et al. '86, Politis et al. '90a,b, Puistola et al. '86, Tilly & Johnson '87). While ovulation requires protein synthesis (Brannstrom et al. '89), protease production in the ovary appears to be hormonally regulated and can be induced in human and rat follicular fluid following the administration of LH or hCG (Curry et al. 88, '89, Dhanasekaran & Moudgal '88, Reich et al. '85b, Thomas & Sernia '90, Yoshimura et al. '87). The importance of proteases such as collagenase in ovulation is evidenced where the application of collagenase inhibitors completely inhibits ovulation (Brannstrom et al. '88, Reich et al. '85a, Yoshimura et al. '87). Interestingly, a preovulatory increase in both serum-derived and tissue-derived protease inhibitor activities parallels the proteolytic and collagenolytic changes (Zhu & Woessner '91). Metalloproteinase inhibitors acting on collagenase, gelatinase, and proteoglycanase have also been found in the follicular fluid and their levels change with follicular development (Curry et al. '88, '89, '90). Likewise, plasminogen activator inhibitor activity has been found in increasing amounts associated with follicular maturation (Politis et al. '90a,b). Like protease activities, inhibitor activities in the ovary correlate with the hormonal environment. That is,

concentrations of inhibitors increase with increasing estradiol and progesterone concentrations and thus, with the stage of folliclular development (Curry et al. '88, '89, Ny et al. '85). The major source of protease and inhibitor production appears to be the granulosa cells (Cajander et al. '89, Fukumoto et al. '81, Ny et al. '85). Concomitant increases in both proteases and their inhibitors have been proposed to maintain proteolytic homeostasis and provide localized control of extracellular matrix degradation (Unemori et al. '90). Follicular degradation may occur due to an excess of enzyme in relation to its inhibitor at the apex of the follicle. For example, Fukumoto et al. ('81) reported that collagenase activities in the apex of the follicle were higher than at the base of the follicle throughout the ovarian cycle.

In addition to changes occuring in the maturing follicle related to ovulation, HOSE undergoes dramatic morphological and functional changes, such as proliferation and migration, related to the processes occuring in the ovarian cortex (Papadaki & Beilby '71). These changes may occur in response to hormonal changes (Van Blerkom & Motta '79). For example, the rat ovarian surface epithelium has been shown to be susceptibile to hormones (Hamilton '80) and to exhibit estrogen receptors (Adams & Auersperg '83). As the preovulatory follicle matures, it causes distension of the overlying tunica albuginea and HOSE. HOSE is composed of cuboidal cells with numerous microvilli at the base of the preovulatory apex, but is flattened with fewer microvilli on the lateral and apical surfaces of the protruding follicle due to mechanical stresses (Dietl et al. '87, Motta & Van Blerkom '80). Intercellular accumulations of fluid appear to be correlated to disruption of the apical wall of the preovulatory follicle (Dietl et al. '87). Cajander and Bjersing ('77) and Rawson and Espey ('77) noted the accumulation of numerous lysosomes in the cytoplasm of the HOSE cells just prior to ovulation and the subsequent disappearance of lysosomes shortly after ovulation. In addition, irregular infoldings of the basal plasma membrane indicated that the active substance, presumably proteolytic enzymes, had been released by exocytosis to contribute to the dissolution of the follicular apex before rupture. Others have also implicated HOSE in the production of proteolytic enzymes responsible for the degradation of the surrounding stroma (Guraya '80, Okamaura et al. '80). While the contributions of HOSE to the initiation of ovulation remain unknown, the net result is that the continuity of HOSE is disrupted at ovulation.

Following ovulation, repair is initiated to the ovulatory wound composed of plasma clot, follicular fluid, and cell debris (Nicosia '83, Van Blerkom & Motta '79). The ovulatory defect is invaded by stromal cells which produce ECM components that act

as a scaffold for the adjacent ovarian HOSE cells (Nicosia '83, Papadaki & Beilby '71, Harrison & Weir '77). HOSE proliferates at the wound edges and migrates over the wound site as very flattened cells essentially devoid of microvilli. When repair is completed and the ovulatory defect is re-epithelialized, HOSE returns to its resting morphology (Gillet et al. '91, Motta & Van Blerkom '80). During the reproductive period the ovarian surface becomes increasingly scarred with each subsequent ovulation.

If fertilization and implantation of the ovum occurs, the corpus luteum of that ovulatory cycle does not regress, but is transformed into a temporary endocrine gland, the corpus luteum of pregnancy, which produces progesterone for six weeks and then degenerates (Balboni '83, Nicosia '83). Ultrastructurally, in the pregnant condition, HOSE differs from HOSE in the non-pregnant condition in that its basal surface is deeply infolded and penetrates well into the underlying tunica albuginea (Papadaki & Bielby '71). There appear to be few if any cytoplasmic differences except for the presence of more lipid droplets (Papadaki & Bielby '71).

Attrition of follicles during menopause results in ovarian remodelling and shrinkage so that, grossly, the senile ovary appears wrinkled and cerebriform in appearance (Blaustein '77a, Nicosia '83, '87, Papadaki & Beilby '71, Sauramo '52). HOSE follows the contours of the gryus-like senile ovary and often lines surface invaginations or crypts that can penetrate deep into the ovarian cortex. Further, an increased number of HOSE-lined inclusion cysts and other anomalies such as papillary or cord-like projections are common in the post-menopausal ovary (Clement '87, Nicosia '83, '87). Histopathological studies suggest that HOSE lining such crypts and cysts is frequently atypical and appears to be the site of early malignant changes in HOSE (Scully '70,'77). Lastly, many reports suggesting that HOSE is lost from the ovarian surface in the post-menoapusal ovary and limited to lining crypts and cysts (McKay et al. '61, Sauramo '52) appear to be erroneous. Rather, the absence of HOSE from the ovarian surface can be attributed to ovarian handling with the resultant removal of HOSE from the ovarian surface (Clement '87, Gillet '91).

C. The Role of HOSE in Ovarian Cancer

Over 80% of ovarian cancers are thought to arise from HOSE (Czernobilisky '85, Fox '80, Nicosia & Nicosia '88, Parmley & Woodruff '74, Scully '70, '77, Young et al. '89) despite the fact that HOSE comprises only a small fraction of the total

ovarian mass. HOSE-derived carcinomas are commom, and in general, have a poor prognosis. For example, HOSE-derived carcinomas are the 5th most frequently occuring malignancy among American women (Richardson et al. '85). Although less frequent than cervical and endometrial cancers, the mortality attributed to ovarian cancers far exceeds that of both the others combined. The 5-year survival of patients with ovarian cancer is no better than 37% (Nicosia & Nicosia '88). The poor prognosis is largely due to inadequate means to detect ovarian cancers in early stages, so that generally by the time the initial diagnosis is made, the cancer is inoperable. Ovarian carcinoma cells express epidermal growth factor receptors at the cell surfaces and receptor expression is associated with poor prognosis (Berchuk et al. '91, Rodriguez et al. '91). Additionally, HOSE-derived tumours respond poorly to the chemotherapy methods currently available. Epidemiologic studies indicate that racial, geographic, and genetic (Christian '71, Dazois et al. '71, Kinbrough '29, Liber '50, Lingeman '74, Weiss '80) factors may be involved. Also, it appears that ovarian cancers increase with ovulatory age (Casagrande et al. '79, Joly et al. '74, Papadaki & Beilby '71, Weiss '80), that is, the total time in a woman's reproductive life during which her ovarian cycle is not surpressed by pregnancy, lactation, use of oral contraceptives, and certain pathologies. Environmental agents including polycyclic aromatic hydrocarbons, industrial pollutants, smoking, asbestos, talc, and infectious agents may be involved in HOSE carcinogenesis (Cramer et al. '82, '83, Gerard et al. '78, Henderson et al. '71, Longo et al. '79, Mattison & Thorgiersson '78, Newhouse et al. '72, Weiss '80).

In spite of its clinical importance, studies of the biology of normal HOSE and the role of HOSE in ovarian carcinogenesis have been severely limited by the lack of experimental systems. Most information about ovarian carcinogenesis is based upon histopathological examinations of ovaries that usually come from women with disease. These histopathological studies suggest that early malignant changes occur in HOSE-lined crypts or inclusion cysts in the ovarian stroma rather than on the ovarian surface (Scully '70, '77). The close embryonic relationship between HOSE and Mullerian duct derivatives is exemplified in the epithelium lining inclusion cysts. These common inclusion cysts histologically exhibit a variety of Mullerian-type epithelia including cuboidal, columnar, secretory, ciliated, and squamous cells. However, their common characteristic is that they form more or less rounded, dilated structures consisting of a single layer of epithelium surrounding a central lumen. Little has been reported about the contents of inclusion cysts. Further, inclusion cysts are normally well separated from each other by abundant stroma. An

example of such a common ovarian inclusion cyst is illustrated in figure 7. The literature on the formation and pathogenesis of inclusion cysts is also sparse although following examination of sections of 1000 normal ovaries Radisavljevic ('77) suggested that inclusion cysts are almost always initiated by ovulation. The prevailing theory is that as a woman approaches menopause, and sometimes earlier in her reproductive life, but rarely before puberty, the HOSE extends downward into the ovarian stroma to form surface epithelial inclusion cysts. So, basically, with age there is an invagination or indentation of the HOSE which may be subsequently nipped off by surrounding stroma. Cancers, then, are thought to arise from HOSE invaginations which may be continuous with the outer surface or which may be isolated and located within the stroma. Some researchers consider this process of inclusion cyst formation as a return of the surface epithelium to a state which recapitulates Mullerian duct development (Blaustein '77a, Scully '77).

While the predominant theory is that the development of common ovarian inclusion cysts is post-pubertal and associated with normal and abnormal ovulations or with rearrangements in the postmenopausal ovary, inclusion cyst formation is not only a late occuring event. The existence of ovarian inclusion cysts in fetal ovaries has been reported (Blaustein '81b, Meizner et al. '91). This suggests, then, that there may be two groups of ovarian inclusion cysts in the adult human ovary. One develops with advancing age as a result of normal and abnormal ovulations and the other occurs during fetal development.

Fetal inclusion cysts as one of a possible dual origin of ovarian inclusion cysts is an attractive idea and it may relate to the re-expression or increased expression of onco-developmental genes during carcinogenesis. For example, increased carcinoembryonic antigen (CEA) serum levels are used diagnostically as tumour markers. The veritability of this marker is seen in mucinous cystadenocarcinomas where 77% of the patients with this carcinoma show elevated CEA serum levels (Blaustein et al. '82). Moreover, Blaustein et al. ('82) found that CEA was present in fetal inclusion cysts, papillary cystadenomas, and papillary serous cystadenocarcinomas. While this does not prove the origin of tumours from these cysts, it demonstrates a shared characteristic, CEA production, among all three.

The exact mechanism by which the HOSE extends downward into the ovarian stroma to form inclusion cysts is not known. It remains spectulative as to whether there is an active ingrowth and lumen-formation by the normal HOSE or whether inclusion glands form passively through rearrangement of the ovarian surface through ovulation, scarring, or shrinkage of the ovary with age. It is also not known what

conditions must be present for inclusion cyst formation and further progression to neoplasms.

Interestingly, a variety of HOSE-derived inclusion cysts have been reported. For example, Kerner et al. ('81) distinguished between common ovarian epithelial inclusion cysts described above and a particular type of HOSE-derived inclusion cysts found in patients with endometriosis. Although of the same origin as the common ovarian epithelial inclusion cysts (i.e. from HOSE), these ovarian mesothelial inclusions were smaller that common ovarian inclusion cysts and consisted of closely packed cell nests lacking a central lumen found in common HOSE-lined cysts. These ovarian mesothelial inclusions were found to occur deeper in the cortex than common inclusion cysts. The reason for the appearance of these mesothelial inclusion cysts only with endometriosis is not known, but is not fortuitous as such inclusions were not found in any of the ovaries examined without endometriosis. The occurence of these mesothelial inclusions and endometriosis suggests that a common stimulus could be responsible for the development of both conditions.

D. Development of Culture Conditions for HOSE

To date there is no direct evidence that human ovarian carcinomas arise in the HOSE. The inference of HOSE as the source of ovarian epithelial cancers is based primarily on the histopathological finding of premalignant and malignant changes in the HOSE of ovaries which may sometimes be seen to be in direct continuation with fully malignant tumours.

Animal models have contributed little to the understanding of HOSE cancers because ovarian tumours in species other than man most typically arise in granulosa, theca, stromal, or germ cells (Cotchin '77, Fox '80, Marchant '80, Murphy '80, Stevens '80). Ovarian surface epithelial-derived tubular adenomas in mice with a genetic deletion of oocytes (Murphy '80), adenocarcinomas in hens and adenomas and carcinomas in dogs (Marchant '80) are the exceptions. Ovarian epithelial tumours can be induced in rodents by irradiation (Miller '72) and carcinogenic chemicals (Marchant '80, Miller '72, Murphy '80, Normura '77). However, because of the small amount of ovarian surface epithelium present and the complex cellular composition of the ovary in these models, it is difficult to be certain of the precise origin of the malignant cells in these few studies. Thus, there is no satisfactory

animal model for the study of the role of surface epithelium in human ovarian carcinogenesis.

Rat ovarian surface epithelial cells were the first culture model for ovarian surface epithelium (Adams & Auersperg '81, Hamilton et al. '80). These cells have since been shown to have estrogen receptors (Adams & Auersperg '83, Hamilton et al. '80) and hydroxysteroid dehydrogenase activity (Adams & Auersperg '81). Androgens (testosterone and 5-dihydrotestosterone) stimulate their growth while antiestrogens inhibit their growth (Hamilton et al. '80, '83). Further, rat ovarian surface epithelial cells are susceptible to transformation by oncogenic retrovirus (Adams & Auersperg '81). More recently, methods for the isolation and culture of rabbit ovarian surface epithlium have been reported (Nicosia et al. '84, '85, Piquette & Timms '90, Setrakian et al. '90). In this species, several protein hormones (hCG, FSH, LH, and prolactin) stimulate surface epithelial cell growth (Olsterholzer et al. '85a,b). These cells are also susceptible to mineral fiber irritation (Nicosia & Johnson '84a) and demonstrate morphological plasticity (Nicosia et al. '85, '89).

Until recently, attempts to culture normal HOSE have been unsuccessful, although cells from benign HOSE-derived tumours have been grown in culture and established tumour-derived cell lines have been easily maintained *in vitro* (Courtenay '80). In 1984, Auersperg et al. first reported the growth of HOSE in primary culture adapted from the culture methods originally developed for rat cells. HOSE cells were grown in plastic dishes using Waymouth's medium 752/1 + 25% fetal bovine serum (FBS) from explants of ovarian surface from biopsy material obtained from premenopausal women undergoing surgery for non-malignant disorders. HOSE cells were distinguished from other morphologically similar cells (endothelial and mesothelial) and from other ovarian cells by the presence of keratin and 17-β-hydroxysteroid-dehydrogenase.

While the initial growth of HOSE under the conditions described above was limited and unpredictable, further work improved these methods (Siemens & Auersperg '88). Of several culture media examined, the medium best suited for the serial cultivation and clonal growth of HOSE contained medium 199:MCDB 202 mixed 1:1 supplemented with 15% FBS, 20 ng/ml epidermal growth factor (EGF), and 0.40 ug/ml hydrocortisone. In this medium, HOSE; 1) underwent 20-25 population doublings before senscence; 2) had a population doubling time of approximately 48 hours in the log phase of growth; 3) could be subcultured 8 to 10 times; 4) had a seeding efficiency of up to 53%; and 5) had a cloning efficiency of up to 13%.

Rodriguez et al. ('91) also demonstrated the mitogenic effect of EGF on cultured HOSE cells as well as the presence of EGF receptors on the cell surfaces. Yet, there is no relationship between the number of EGF receptors and responsiveness to the mitogen. More recently, rete ovarian epithelial cells from primary cultures of ovaries from twenty-week old fetuses have been grown in chemically-defined, serum-free conditions (Dubeau et al. '90) and HOSE cells from adult women have been maintained in serum-free medium (Elliott & Auersperg '90).

E. Rationale/Objective

Recent work developed the culture conditions to sustain growth of HOSE in monolayer and allowed propagation of sufficient cell numbers for subsequent studies on normal HOSE growth (Siemens & Auersperg '88). However, a persistant problem was contamination by ovarian cells other than HOSE cells which was difficult to eliminate in explant-derived cultures. The objectives of this project were: 1.) to improve and simplify the methodology for HOSE culture; 2.) to further characterize HOSE cells; 3.) to characterize and isolate an ovarian-derived matrix; and 4.) to study the interactions between cultured HOSE cells and extracellular matrix as a means to examine the dynamic, pleomorphic, and morphogenetic nature of HOSE that might account for normal physiological changes and for cyst formation. The response of HOSE to various three-dimensional substrata was examined in the hope that this might mimic HOSE-ECM interactions naturally occuring in the ovary and provide insight as to why HOSE is a preferred site of cancer development. The work reported here, then, may provide a better understanding of HOSE physiology and of the etiology of ovarian epithelial cancers and be of clinical relevance as it may provide useful information for the diagnosis of ovarian carcinomas.

MATERIALS AND METHODS

A. Tissue Culture

(1.) Tissues

Thirty five biopsy specimens of normal ovary were obtained at surgery from women undergoing surgery for nonmalignant gynecological disorders (ages 21 to 66 years). The specimens were collected under aseptic conditions and transported to the laboratory in Waymouth's 752/1 medium (WM; Sigma, St. Louis, MO) with 10% or 15% fetal bovine serum (FBS; Hyclone, UT) at ambient temperature. Each specimen was assigned a code name to maintain the patient confidentiality.

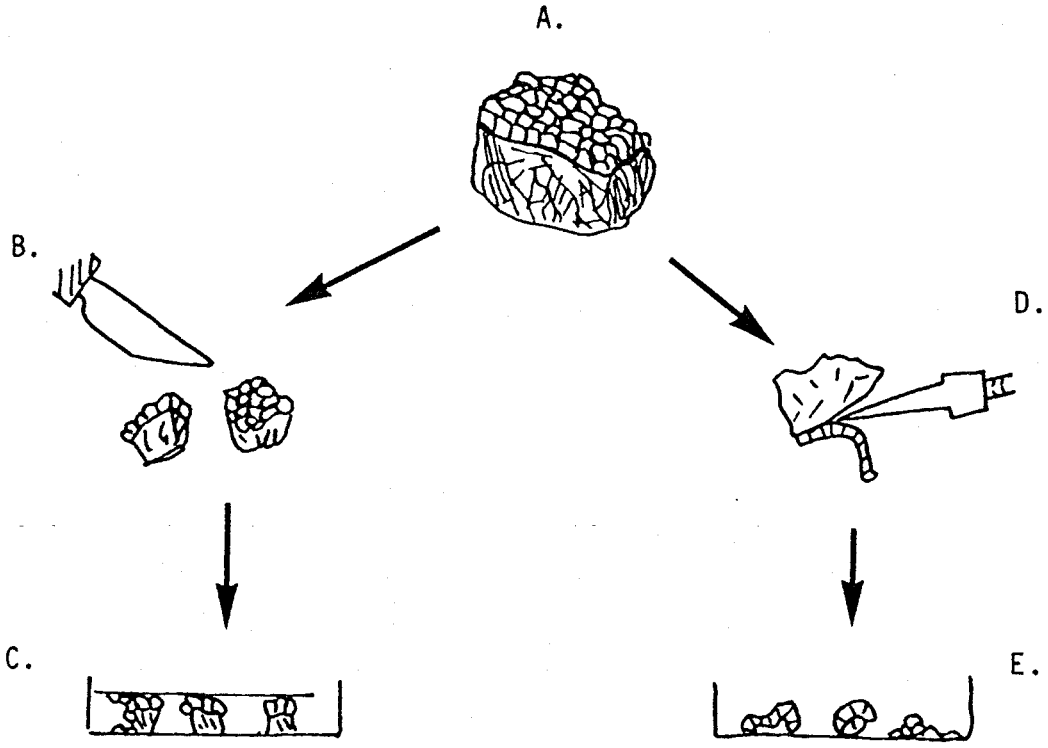
(2.) HOSE Cultures Established by Explantation from Biopsy Material

The first 9 ovarian specimens were processed for culture by the explantation method of Auersperg et al. ('84). Briefly, the tissue was transferred to a glass dissecting dish containing routine culture medium, Medium 199 (Sigma): MCDB 202 (Irvine Scientific, Irvine, CA) mixed 1:1 and supplemented with 15% FBS and 25 ug gentamicin/ml (GIBCO, Grand Island, NY) or 100 IU penicillin G (GIBCO) and 100 ug streptomycin (GIBCO). Under a dissecting microscope, extraneous stroma was trimmed from beneath the ovarian surface (figure 1). The tissue was then cut into small pieces or explants 1-2 mm^3. These explants were placed, ovarian surface facing up, into 35 mm culture dishes (Corning, Corning NY), weighed down with coverslips and glass cloning cylinders, and two mls medium was gently added. The explants were arranged near the periphery of the coverslips to allow adequate exchange of nutrients and gases. In addition, the culture medium used to collect and prepare the tissues was spun at 1000 RPM (180g) in a clinical centrifuge and the cells within these washings were plated into a separate culture dish.

In total 469 explants were cultured in this manner. Cell outgrowths extended out onto the culture dishes and coverslips. The outgrowths were identified as epithelial (either typical cobblestone epithelial or atypical epithelial) or fibroblastic (fusiform cells arranged in parallel arrays) by the morphological criteria of Siemens & Auersperg ('88). When HOSE outgrowths began to approach each other, the coverslips were removed and the explants transferred to new dishes. The explants were again covered with coverslips and cloning cylinders and immersed in two ml medium to obtain additional HOSE growth. In most cases, however, outgrowths

Figure 1. Diagramatic Culture of Human Ovarian Surface Epithelium

(A), Ovarian biopsy specimens are obtained at surgery, transported to the laboratory in sterile culture medium, and examined under a disecting microscope so that only areas devoid of anomalies are chosen for subsequent culture. In the explantation method (B,C), biopsies are trimmed of excess stroma and cut with a scapel into 1-2 mm^3 explants (B) which are placed epithelial side up in a culture dish and weighed down (C). HOSE outgrowths extend out onto the dish or overlying coverslip. In the scraping method (D,E), the ovarian biopsy is held, surface side down, over a culture dish containing medium. The surface epithelium is scraped firmly 2 to 3 times with a rubber policeman (D). Scraping generates sheets of HOSE and these are rinsed from the scraper and biopsy surface into one or two culture dishes (E). The epithelial sheets attach to the culture dish and HOSE cells grow out from the attached sheets.


A.
B.
C.
D.
E.

contained contaminating fibroblasts. Contaminating fibroblasts were removed from cultures by either scraping the fibroblasts off with a rubber policeman or cutting the fibroblastic outgrowths from the coverslips with sterile scissors. Both of these methods of decontamination were only temporarily successful. Eventually fibroblastic contamination returned. Often, fibroblasts overgrew HOSE cultures. Dishes badly contaminated with fibroblasts were discarded. Because of the problems associated with HOSE cultures derived from explants: 1) fibroblastic overgrowth; 2) low HOSE cell yield; and 3) laborious technique, attempts were made to improve the culture of surgical specimens.

(3.) HOSE Culture Purification by Differential Adhesion to Collagen Gel

To increase the purity of cultured HOSE cells from fibroblastic contaminants, the possibility that HOSE cells might demonstrate differential adhesion to rat tail collagen gel as compared to that of stromal fibroblasts was examined. Rat tail tendon-derived collagen gels were prepared according to the method of Emerman and Pitelka ('77) and described in Materials and Methods section D. (1.). HOSE from 2 flasks of Welch HOSE passage 4 (p4) and one flask of Solo HOSE p1 containing mixtures of epithelial and fibroblastic cells, were plated onto the unrimmed collagen gels. At T=0, 10, 15, 20, 25, 30, 40, 45, and 60 minutes following plating, the cultures were observed, photographed, and the non-adherant (floating) cells were collected and replated onto coverslips. At the end of the experiment, the cells adherent to the collagen gels were harvested as described in Materials and Methods section D. (1.) and plated onto coverslips. The following day all coverslips were fixed and stored in -20^{o}C methanol until they were stained for keratin by immunofluorescence with anti-keratin antibodies AE1 and AE3 as described in Materials and Methods B.(2.) (b.).

(4.) Culture Purification by Percoll Density Gradient Centrifugation

(a.) Purification of Mixed HOSE Cultures

A second approach to purifying mixed epithelial and fibroblastic HOSE cultures was to separate epithelial and fibroblastic cell populations by Percoll density gradient centrifugation. Mixed cultures were obtained from 6 different cases (Anon, Solo, Sub, Mac, Bent, Welch) ranging from p1 to p4. As controls, a line of mesothelial cells (LP-9) (Connell & Rheinwald '83) was used to represent an equivalent of the epithelial component of HOSE cultures while human dermal fibroblasts (HUDF) or

rat lung fibroblasts (RLF) were used to represent equivalents of the fibroblastic component of the HOSE cultures.

In addition to contaminating stromal fibroblasts, the addition of epidermal growth factor (EGF) to HOSE cultures causes a modulation of epithelial HOSE cells to a fibroblast-like morphology (Siemens & Auersperg '88). This phenotypic modulation makes it difficult to identify EGF/HC treated fibroblast-like HOSE cells from contaminating stromal fibroblasts. Experiments to test if the EGF/HC-treated fibroblast-like HOSE cells have different cell densities from epithelial HOSE cells or from stromal fibroblasts were performed in the following manner. LP-9 cells were centrifuged in Percoll under the following conditions: 1.) LP-9 cells maintained with 20 ng/ml EGF + 0.04 ug/ml HC for 5 days prior to Percoll centrifugation to establish fibroblastic LP-9 populations (Connell & Rheinwald '83) reminiscent of EGF/HC modulated HOSE cells; 2.) LP-9 cells were maintained without EGF/HC to represent epithelial HOSE cells; and 3.) LP-9 revertants, that is cells modulated to a fibroblastic-like morphology when grown with EGF/HC and then allowed to revert to an epithelial morphology following 3 days in culture without EGF/HC. HUDF were also treated with and without EGF/HC to see if EGF/HC altered HUDF cell densities.

(b.) Continuous Percoll Gradients

Stock iso-osmotic Percoll (SIP) was made by mixing 9 parts Percoll (Pharmacia, Uppsala, Sweden) with 1 part of 10x complete Hanks' Balanced Salt Solution (HBSS, GIBCO). Twenty five percent and 40% SIP were prepared by further dilution with 1x HBSS. Thirty six ml of 25% or 40% SIP was placed in a 40 ml round bottomed polycarbonate test tube (Nalgene, Richester, NY). RLF, mixed HOSE, HUDF or LP-9 cells, each in 100 ul HBSS supplemented with 1%FBS, were gently layered on top of the Percoll solution and centrifuged at 10,000 g for 30 minutes at RT in a Sorvall Superspeed RC2-B centrifuge equipped with an SS34 rotor. The density of the cell bands was determined with density marker beads (Pharmacia) in parallel gradients. Following centrifugation, each cell band was collected with a Pasteur pipet and plated directly, without washing, into tissue culture dishes lined with glass coverslips. The following day the cultures were examined morphologically and the coverslips fixed in -20^{o}C methanol and stained by immunofluorescence for keratin as described in Materials and Methods B.(2.) (b.).

(c.) Percoll Decontamination of Mold Infected Cultures

In the course of one of the Percoll experiments described above, it was noted that Percoll treatment appeared to separate cells from mold infections. To examine the possible use of Percoll centrifugation as a means to decontaminate cultures of mold and yeast infections more closely, decontamination experiments were carried out on two cell types: an immortalized, non-tumorigenic rat ovarian surface epithelial cell line ROSE 239 (Adams & Auersperg '85) and HUDF. ROSE 239 grow as epithelial monolayers and produce basement membrane and interstitial extracellular matrix components (Auersperg et al. '91b). All cultures were maintained in the absence of any fungicides.

An environmental mold and an environmental yeast were obtained by exposing opened petri dishes of culture medium to the circulating air within a tissue culture incubator. The infecting mold was identified as belonging to the fungal *Cladosporium* species and the yeast was identified as *Torulopsis candida*. Both occur commonly in the environment. On two separate occasions, confluent ROSE 239 and confluent HUDF cultures were intentionally infected with mold and yeast for 24 hours. Contaminated, as well as uncontaminated cultures, were prepared for Percoll treatment by rinsing the cultures three times with complete HBSS and resuspending 3-9 x 10^6 single cells in 100 ul of complete HBSS supplemented with 1% FBS. These suspensions of ROSE 239 and HUDF were gently layered on 40% SIP and centrifuged as described in Materials and Methods A.(4.)(b.). Following centrifugation the cells were counted, tested for viability by trypan blue exclusion, and plated directly, without washing, into tissue cuture dishes. To compare the effects of high speed Percoll centrifugation on cell morphology, growth, and extracellular matrix production with the effects of centrifugation as used in routine subculturing, uninfected ROSE 239 and HUDF cells were spun for 5 minutes at 100g in a counter-top clinical centrifuge. These cells, designated LoC, were compared with uninfected, mold-infected, and yeast-infected cultures centrifuged at 10,000g in 40% SIP, designated HiC, HiM, and HiY respectively. To ensure that decontamination had taken place following Percoll treatment, HiM cultures of ROSE 239 and HUDF were maintained for up to one month without antifungal agents.

To assess the effects of the various centrifugation treatments on cell growth, ROSE 239 and HUDF cells collected following centrifugation (HiC, LoC, and HiM) were plated at 5000 cells/12 mm well. Cultures were maintained for 12 days and the medium was changed as required. Cells from triplicate wells were harvested at intervals and counted.

ROSE 239 and HUDF, collected from all centrifugation treatments, were grown on glass coverslips and stained by immunofluorescence for extracellular matrix production to examine the effect of centrifugation treatments on the differentiation of ROSE 239 cells and HUDF cells. HUDF cultures were supplemented daily for one week with 50 ug/ml ascorbic acid (GIBCO) and HUDF cultures and ROSE 239 cultures were stained for collagen type I as described in Materials and Methods B. (2.) (a.). ROSE 239 cultures were also stained for laminin as described in Materials and Methods B. (2.) (c.).

(5.) HOSE Cultures Established by Scraping Biopsy Material

An improved method to culture HOSE cells was established by taking advantage of the tenuous attachment of HOSE to its underlying tissues (Kruk et al. '90). By this method, the biopsy specimen was examined under a dissecting microscope and areas devoid of anomalies, blood vessels, and papillae were selected for culture. The entire specimen was rinsed with medium, held surface down over a 35 mm culture dish containing two ml of culture medium and the ovarian surface was scraped firmly two to three times with a white rubber scraper (Canlab, Mississauga, ONT) attached to a glass rod. Scraping generated sheets of HOSE cells and these were rinsed from the scraper and the biopsy surface into one or two culture dishes (figure 1). Like the explantation method, the medium used for the transport and the rinsing of the specimen was collected, centrifuged, and cells plated into a separate 35 mm dish. Cultures were left undisturbed for 48 hr and incubated as described in Material and Methods A. (6.).

(6.) Media and Culture Conditions

All cultures were incubated at 37°C in a humidified incubator with 5% CO_2:95% air. Initially, HOSE cells were routinely grown and maintained in Medium 199:MCDB 202 (Irvine Scientific, Irvine, CA) (1:1) with 15 % fetal bovine serum (FBS, Hyclone, UT) (199:202/15%FBS) supplemented with either 25 ug gentamicin (GIBCO)/ml or 100 IU penicillin (GIBCO) and 100 ug streptomycin (GIBCO). In the course of this study, Medium 202 became unavailable commercially, so Medium 202 was replaced with Medium 105 (Sigma) (personal communication, Dr. J. Rheinwald) (199:105/15%FBS). In some cases, 20 ng/ml epidermal growth factor (EGF, Daymar Laboratories, Toronto, ONT) and 0.04 ng/ml hydrocortisone (HC, Sigma) were added to cultures to increase the growth rate and growth potential (Siemens & Auersperg '88).

For culture in low-serum containing medium HOSE cells were maintained in Medium 199:105 (1:1) supplemented with 1%FBS (199:105/1%FBS) or 0.5% FBS (199:105/0.5%FBS). For culture in medium containing 0.05% serum proteins (199:105/PC-1) Medium 199:105 (1:1) was supplemented with 2% Pederson's fetuin (PC-1, Ventrex, 500ug/ml), 15 ug/ml insulin (Sigma), 200 ug/ml transferrin (Sigma), 0.33 ug/ml ethanolamine (Sigma), 20 ng/ml phosphatidylcholine (Sigma), and 0.1 ug/ml lipoic acid (Sigma) (Elliott & Auersperg '90). For culture in defined medium (199:105/DM) (Elliott & Auersperg '90), HOSE cells were maintained in Medium 199:105 (1:1) supplemented with insulin (15ug/ml), transferrin (200ug/ml), ethanolamine (0.33ug/ml), phosphatidylcholine (200 ng/ml), lipoic acid (0.1ug/ml), HC (400 ng/ml), and purified fetuin (Hyclone, 500 ug/ml).

The following cell lines were also used in this study: 1.) the mesothelial cell line, LP-9 (from Dr. J. Rheinwald) and human dermal fibroblasts (HUDF) maintained in medium 199:105/15% FBS; 2.) immortalized HOSE cells (IOSEVan) maintained in medium 199:105 supplemented with 5% FBS; 3) rat ovarian surface epithelial cell lines 199 (ROSE 199) and ROSE 239 (Adams & Auersperg '85), ROSE 199 subclones E11/A4 and C8/D10, cervical carcinoma cells C4-I and C4-II (Auersperg et al. '89) and rat lung fibroblasts (RLF) were maintained in Waymouth's medium 752/1 (WM) (Flow laboratories, VA) supplemented with 10% FBS.

To harvest or subculture cells, cultures were dissociated in 0.06% trypsin (250:1, GIBCO) and 0.01% ethylene-diaminetetra-acetic acid (EDTA, Biological Research Laboratories, Burlington, ONT) in calcium/magnesium-free Hanks' balanced salt solution (HBSS) and spun at 100g in a clinical centrifuge for 5 minutes. HOSE cells, LP-9 cells, IOSEVan cells, and HUDF cells were frozen under liquid nitrogen in WM + 25% FBS + 10% dimethylsulfoxide (DMSO, BDH) while all other cell types were frozen under liquid nitrogen in WM + 10% FBS + 10% DMSO. All cell counts were made using a hemocytometer and cell viability was determined by trypan blue dye exclusion. Cultures were routinely examined using a Wild M40 inverted photomicroscope or a Leitz Laborlux K inverted photomicroscope and photographed on Kodak 2415 Technical Pan black and white film.

B. Culture Characterization

For histological, immunocytochemical, and immunofluorescent studies cells were grown to the desired densities on glass coverslips. HOSE cultures between passages

0-4 were considered early passage cultures, while those between passages 6-10 were considered late passage HOSE cultures. Stained specimens were examined either on a Zeiss Photomicroscope II or on a Zeiss Axiophot photomicroscope and photographed using Kodak T-Max black and white film or Kodak Ektachrome colour slide film.

(1.) Histology

The histochemical procedures used were those described by Culling ('74).

(a.) Oil Red O for Neutral Fats

Cultured HOSE cells, from 5 separate cases, 2 early passage and 3 late passage cultures, were stained with Lillie and Ashburn's Isopropanol Oil Red O method as described in Culling ('74). Immediately prior to use a saturated stock solution of 0.5% Oil Red O in isopropyl alcohol was diluted 3:2 with distilled water and filtered with Whatman #1 filter paper. Unfixed cultures were rinsed briefly with HBSS, stained for 20 minutes with the diluted solution of Oil Red O, rinsed quickly with 70% alcohol, washed for 3 minutes in running tap water, counterstained lightly with hematoxylin for 2 minutes, rinsed in running tap water, and mounted in glycerine jelly or Gelvatol pH 7.2 (Monsanto) (O'Guin et al. '85).

(b.) Periodic Acid-Schiff (PAS) and Amylase + PAS for Neutral Sugars and Glycogen

Coverslips from 2 early passage and 3 late passsage HOSE cultures were fixed in formalin, brought to water, and oxidized with freshly made 1% aqueous periodic acid for 10 minutes. The specimens were washed in running tap water for 10 minutes, treated with pararosaniline Schiff reagent for 30 minutes, washed again in running tap water for 10 minutes. The coverslips were counterstained briefly with hematoxylin, differentiated in base, rinsed in water, dehydrated in alcohol, cleared in xylol, and mounted.

Treatment with human saliva (amylase) for 60 minutes prior to periodic acid oxidation constituted glycogen digestion so that staining attributable to glycogen was selectively removed. Control slides consisted of sections of human ovarian tissue obtained from the Dept. of Pathology, UBC.

(2.) Immunofluorescence Staining

(a.) Collagen Types I, III, IV

To promote collagen secretion, HOSE cells of both early passage (4 cases) and late passage (3 cases) as well as HUDF were grown to confluence and maintained for one

week with daily additions of 50 ng/ml ascorbate (Gibco). The cultures were fixed for 15 minutes in 3.7% paraformaldehyde-PBS. After a 30 minute PBS wash, the cultures were treated with 0.1% Triton X-100 (Sigma) and then rinsed. The cultures were then incubated appropriately for 15 minutes at room temperature in 5% normal rabbit serum or 5% normal goat serum (both from Jackson Immunochemicals, PA) followed by 60 minutes at 37°C with appropriate primary antiserum. Primary antisera included sheep anti-type I collagen (1:200), rabbit anti-type IV collagen (1:50) (previously absorbed with 0.1 mg laminin (Sigma)/ml antiserum and 0.1 mg gelatin (Sigma)/ml antiserum) (both anti-type I and anti-type IV antiserum kindly provided by Dr. H.K. Kleinman), or goat anti-type III collagen (1:100) (Southern Biotechnologies, Burmingham, AL). After a 30 minute wash in PBS the cultures were stained with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-sheep IgG (1:400) (Miles Scientific, Naperville, IL), rhodamine isothiocyanate (ROTC)-conjugated goat anti-rabbit IgG (1:75) (Cooper Biomedical, Mississauga, ON), and FITC-conjugated rabbit anti-goat IgG (1:100) (Miles-Yeda Ltd., Elkhart, Ind.) respectively for 60 minutes. Following a 30 minute wash in PBS the specimens were mounted in Gelvatol pH 7.2 (O'Guin et al. '85). Antibody controls consisted of specimens stained with normal serum instead of the primary antiserum. ROSE 239 and HUDF served as control cells.

(b.) Keratin

As described previously (Auersperg et al. '84), the cultures were rinsed in PBS, fixed and stored in -20°C methanol (MeOH). The cultures were permeabilized with -20°C methanol:acetone (1:1) for 5 minutes and air dried. They were rehydrated in PBS for 30 minutes at room temperature and incubated at 37°C for 1 hour with the mouse monoclonal anti-keratin antibodies (hybridoma-conditioned medium) AE1 (1:5) and AE3 (1:2), generously provided by Dr. T.-T. Sun, or AE1 + AE3 (1:100) (Hybritech, SanDiego, CA). Regardless of the source, AE1 and AE3 antibodies were diluted in PBS containing 1% BSA (Sigma). Following incubation with the primary antibodies, the cells were washed for 30 minutes with PBS at room temperature, incubated with ROTC-conjugated goat anti-mouse IgG (1:20) (Hyclone) for 1 hour at 37°C, washed again for 30 minutes and mounted in Gelvatol pH 6.5. Controls consisted of cells stained with PBS instead of primary antibody. C-4I or C4-II cells served as positive controls and HUDF served as negative controls. Occasionally, cultures of human amniotic cells, which contain both keratin-positive epithelial cells and keratin-negative cells, were used as controls.

(c.) Laminin

Cells from 6 early passage HOSE and 5 late passage HOSE cases were stained for laminin (LN) using a modification of Auersperg et al. ('89). Coverslips were fixed in 3.7% paraformaldehyde/PBS, rinsed in PBS, permeabilized by treatment with 0.1% Triton X-100 for 3 minutes at room temperature, rinsed again in PBS, and then incubated with 5% normal goat serum for 15 minutes at room temperature. The coverslips were drained, incubated with rabbit anti-mouse laminin antiserum (1:150) (Biological Research Laboratories) for 1 hour at 37°C, washed in PBS for 30 minutes. The cells were then incubated with ROTC-conjugated goat anti-rabbit IgG (1:200) for 1 hour at 37°C, rinsed for 30 minutes with PBS, and mounted in Gelvatol pH 7.2. Controls consisted of cells stained with normal rabbit serum instead of primary antiserum and positive controls consisted of the rat ovarian surface epithelial cell line, ROSE 239, and HUDF were negative controls.

(d.) Plasminogen Activator Inhibitor (PAI-1)

Early (from 2 cases) and late (from 3 cases) passage HOSE cultures were stained for PAI-1 according to the method of Rheinwald et al. ('87). Cultures were fixed in -20°C methanol, air dried, and rehydrated in PBS as described for keratin immunofluorescence. The coverslips were incubated with rabbit anti-PAI-1 antiserum (1:25) (provided by Dr. Rheinwald) for 30 minutes at room temperature, washed in PBS for 15 minutes, incubated with ROTC-conjugated goat anti-rabbit IgG (1:100) for 30 minutes at room temperature, washed for 30 minutes, and mounted in Gelvatol pH 7.4. Controls consisted of cells stained with normal rabbit serum instead of primary antibody and cultures of human mesothelial cells LP-9 served as positive controls.

(3.)Staining for Mucin and Keratin

HOSE cultures were doubly stained for the epithelial markers, mucin and keratin according to the method of Wilson et al. ('83). HOSE cultures from two p3 and three p10 cases were rinsed with HBSS and fixed in either 4°C 70% alcohol or -20°C MeOH. The coverslips were brought to 95% alcohol and blocked for endogenous peroxidase activity with 0.6% hydrogen peroxide (H_2O_2, BDH) in MeOH for 30 minutes at room temperature and then washed in running water for 10 minutes and were subsequently incubated with human saliva (amylase) for 1 hour at 37°C and then washed again for 10 minutes in running water. The coverslips were then oxidized in 1% periodic acid for 10 minutes, washed in running water for 10

minutes, stained in Schiff reagent for 30 minutes and then washed again for 10 minutes in running water. The cultures were then rinsed in 0.05M Tris pH 7.2 and incubated with 5% normal goat serum in 0.05M Tris. The coverslips were drained and incubated at 37°C for 1 hour with mouse anti-human keratin antibodies AE1 (1:5) and AE3 (1:5). This was followed by a brief rinse with Tris, an incubation for 1 hour at 37°C with horse radish peroxidiase (HRP) conjugated goat anti-mouse IgG (1:100 or 1:200) (Biorad, Richmond, CA), a rinse with Tris, and the reaction developed with 3,3'diaminobenzidine (DAB, Sigma). The coverslips were develped for 1-20 minutes at room temperature in 0.24 g DAB dissolved in 400 ml Tris:0.24 ml of 50% H_2O_2. The reaction was stopped with water and the coverslips were counterstained in haematoxylin, rinsed in water, blued in 1.5% sodium bicarbonate (Sigma), washed in water, dehydrated, cleared, and mounted in Permount. Controls consisted of cells stained with PBS instead of primary antibody as well as cultures of C4-I cells and frozen sections of human cervix and oviduct which served as controls for keratin and mucin.

(4.) Scanning Electron Microscopy and Transmission Electron Microscopy

For transmission electron microscopy (TEM), specimens were fixed in 2.5% glutaraldehyde/0.1 M PO_4 buffer, post-fixed in 1% OsO_4/0.2M PO_4 buffer, dehydraded in an ethanol and propylene oxide series, embedded in Epon 812, sectioned, stained 15-20 min in uranyl acetate (2% in water) and lead citrate, and examined on a Zeiss EM 30 electron microscope. For SEM, specimens were fixed in the same solutions used for TEM. They were dehydrated in a graded series of aqueous ethanol solutions to 100% ethanol. The specimens were then critical point dried in a CPD 020 critical-point drying system using liquid carbon dioxide. Samples were mounted on stubs with silver paint, coated with gold using a SEMPREP 2 sputter coater, and examined in a Cambridge Steroscan 250-T electron microscope.

(5.) SDS-PAGE Western Immunoblots for Keratin and Vimentin

Preparations of enriched intermediate filaments (Achtstaetter et al. '86) were obtained from two cases of confluent HOSE cultures at early passage (p3) and from two cases of confluent HOSE cultures at late passages (p10). They were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 8.5% gels (Laemmli '70) using the high ionic strength buffer system of Thomas and Kornberg ('75). After electrophoresis, the gels were washed for 30 minutes at RT in transfer buffer and then, electrophorectically transferred to nitrocellulose (Towbin et al.

'79). The nitrocellulose was dried for 30 minutes, blocked for 1 hour at RT with 20 mM Tris-HCl buffer (TBS, pH 7.5) plus 3% BSA and 1% normal goat serum, and then incubated with either mouse monoclonal antibodies to all human acidic (AE1) keratins except #18 (1:10), mouse monoclonal antibodies to all human basic (AE3) keratins (1:10), or with mouse monoclonal anti-cytokeratin No. 18 (1:100) (Boehringer Mannheim Biochemica, Laval, QUE) in TBS containing 0.05% Tween-20 (TTBS) plus 1% BSA overnight at $4^{o}C$. After washing for 10 minutes in TTBS, the nitrocellulose was incubated for two hours at RT in goat anti-mouse horse radish peroxidase- (HRP) conjugated IgG (heavy and light chains, Biorad) diluted at 1:2000. After 2x10 minute TTBS and 1x10 minute TBS washes, the keratin bands were visualized with 4-chloro-1-naphthol (Sigma) prepared fresh by dissolving 60 mg in 20 ml cold methanol and mixing with 100 ml of TBS containing 60 ul of a 30% stock solution of hydrogen peroxide. The reaction was stopped with water.

For vimentin detection, the nitrocellulose sheets were blocked with 1% normal rabbit serum + 3% BSA in TTBS, incubated with goat anti-vimentin (1:10) (Polysciences Inc, Warrington, PA), followed by incubation with rabbit anti-goat alkaline phosphatase-conjugated IgG (1:2000) (Southern Biotechnologies), and the reaction developed in 100 ml of 0.1M $NaHCO_3$, 1 mM $MgCl_2.6H_2O$ carbonate buffer pH 9.5 containing 15 mg 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP, Sigma) in 1.0 ml N,N-dimethylformamide (DMF) plus 30 mg nitro blue tetrazolium (NBT, Sigma) in 1.0 ml 70% DMF. The reaction was stopped with EDTA/Tris-HCl pH 7.5.

The nitrocellulose sheets were later stained with amido black (0.1% in methanol:acetone:water, 45:10:45, vol%) to locate molecular weight markers. In addition to the molecular weight markers, the human cervical carcinoma cell line (C4-I) was used as a positive control for keratin and a negative control for vimentin. C4-I cells express keratins #5,6,8,16,18,19 (Auersperg et al. '89). HUDF were used as negative controls for keratin and positive controls for vimentin.

C. Ovarian-Derived Extracellular Matrix and HOSE Organoids

(1.) Preparation of ROSE 199-ECM

To pursue HOSE-ECM interactions, it was decided to characterize an ovarian-derived matrix, isolate such a matrix, and examine the response of HOSE cells to such a matrix. It was thought that HOSE might mimic the normal resting state of the ovarian surface if maintained on such an ovarian-derived matrix. To this end, ECM

was derived from the immortalized, non-tumorigenic rat ovarian surface epithelial cell line (ROSE 199). ROSE 199 secrete an extracellular matrix which, at the ultrastructural level, contains as one of its major components banded fibrils characteristic of interstitial collagen. ROSE 199 cells grow as a characteristic cobblestone monolayer, but, when crowded, these cells form ridges and multiple cell layers by virture of their ability to deposit ECM between cell layers. ROSE 199 subclone E11/A4 cells form ridges when crowded, but the ROSE 199 subclone C8/D10 forms cobblestone monolayers and remains monolayered when crowded.

For ECM production, cells were seeded sparsely in 35 mm petri dishes (Costar) or 12 well plates (NUNC) in WM + 10%FBS + 25 ug of gentamicin/ml (Gibco). It was found that ROSE 199 and ROSE 199 subclones E11/A4 and C8/D10 grew equally well in medium supplemented with either 10% FBS or 10% OMNI serum (Advanced Biotechnologies Inc, Columbia, MD), so when cultures became confluent, they were switched from WM supplemented with 10% FBS and gentamicin to WM supplemented with 10% OMNI and gentamicin. The cultures were maintained for 6 weeks postconfluence, and the medium changed as required.

Cell-free ECMs were prepared from ROSE 199 and ROSE 199 subclones E11/A4 and C8/D10 by modifications of one of three treatments, two of which were chemical and one which was a non-chemical procedure. Cell-free ECMs have been previously described by treatment with ammonium hydroxide (AH) (Gospodarowicz '84), sodium deoxycholate (DC) (Liotta et al. '80), and repeated freeze thaws (FT) (Carley et al. '88). ROSE cultures were rinsed briefly with sterile ddH_2O and then treated with either 20 mM AH for 5 minutes, 1% DC for 5 minutes, or three freeze thaws. The ECMs were thoroughly washed with ddH_2O and then with complete HBSS and finally, stored at 4^oC for up to one week.

To estimate the amount of ECM material produced by six week old post-confluent 35 mm cultures, triplicate ECMs prepared by all three treatments (AH, DC, FT) were dried in a 200^oC oven and weighed.

(2.) Characterization of ROSE 199-ECM

ROSE 199-ECMs were stained in situ or as frozen sections of detached matrix. ROSE 199-ECMs were examined by both SEM and TEM and ECMs were stained histologically according to the procedures of Culling ('74) or Kiernan ('81) for acidic and sulphated sugars, collagen, and reticulin. By immunofluorescence microscopy ECMs were stained for laminin and collagen type I to see if ROSE 199-derived ECM, like the ECM produced by HOSE cells, contained both basement

membrane and stromal matrix components. ROSE 199-ECMs were also either stained for DNA or doubly stained for actin and laminin. The presence of actin or DNA would be an indication of remaining cellular debris in the ROSE 199-ECM preparations. Additionally, ROSE 199-ECMs derived from all three preparative treatments were probed for laminin, fibronectin, and collagen types I, III, and IV by western immunoblotting.

(a.) Alcian Blue for Sulphated and Non-Sulphated Acidic Sugars

Formalin fixed sections of ROSE 199-ECM were rinsed in water, stained with either freshly filtered 1% Alcian Blue 8 GX in 3% acetic acid (pH 2.5) or 1% Alcian Blue 8 GX in 0.1 N hydrochloric acid (pH 1.0) for 30 minutes. The specimens were then dehydrated, cleared, and mounted.

(b.) Masson for Collagen

Frozen ROSE 199-ECMs were sectioned, fixed in formalin, rinsed in water, stained for 20 minutes in Weigert's iron haematoxylin, washed in water and differentiated in 1% acid alcohol. The sections were washed again in water, treated with 1% phosphomolybdic acid for 5 minutes and then stained for 5 minutes with 2.5% aniline blue in 2.5% acetic acid. The sections were differentiated in 1% acetic acid for 1-2 minutes, then dehydrated, cleared, and mounted.

(c.) Silver Staining for Reticulin

Frozen sections of ROSE 199-ECM were fixed in Bouin's fixative and stained for reticulin according to the method of Gordon & Sweets '36 (described in Kiernan '81). Sections were brought to water, oxidized for 1 minute in acid permanganate, washed in water, dipped in 1% oxalic acid, washed again, and treated for 10 minutes with an iron alum solution. Following further washing, the specimens were immersed briefly in an ammonical silver solution, rinsed in water, placed in a formaldehyde reducer solution for 30 seconds, and washed once again in water. The sections were then toned in 0.2% yellow gold chloride solution, washed in water, immersed in a sodium thiosulphate solution for 3 minutes, washed in water, and, finally, dehydrated and mounted.

(d.) Double Staining for Actin and Laminin

ROSE 199-ECMs derived by treatment with ammonium hydroxide were doubly stained for actin and laminin. Laminin staining would identify ROSE 199-ECM while

actin staining would serve as an indication of residual cellular debris present in ECM preparations. Frozen sections were fixed in paraformaldehyde (3.7%) for 15 minutes at RT and then plunged into -20^{o} C acetone for 5 minutes. The slides were then air dried and rehydrated for 10 minutes with PBS and incubated with 5% normal goat serum at RT for 15 minutes. Sections were then doubly stained with anti-laminin (as in Material and Methods 2. (c.)) and with 1.1uM NBD-phallacidin (NDB, Sigma) for 20 minutes at RT. After washing, the sections were incubated with secondary antibody, goat anti-rabbit ROTC-conjugated IgG (1:200), washed, and mounted. Control sections of ROSE 199-ECM and rat intestine, which served as a control, had the anti-laminin antibody replaced with normal rabbit serum and NBD-phallacidin neutralized with 82.5 ug/ml phalloidin (PH, Sigma).

(e.) Hoechst Staining for DNA

Frozen sections of AH-derived ROSE 199-ECMs, half of which were treated with 25 ug DNAse (Sigma)/ml WM at 37^{o}C for one hour and washed overnight in PBS, were frozen, sectioned, and fixed in formalin. The sections were washed in PBS for 30 minutes, stained for 3 minutes at room temperature with 0.02 ug/ml Hoechst 33258 bisbenzimide chromosomal dye (Sigma), washed in PBS for 15 minutes, and then mounted in Gelvatol pH 7.2.

(f.) Western Immunoblots for Laminin, Fibronectin, and Collagen

Preparations of ROSE 199 ECM from all treatments (AH, DC, FT) were subjected to separation by continuous SDS-PAGE in 12% acrylamide gels for fibronectin (FN) and laminin (LN). After electrophoretic transfer to nitrocellulose, the nitrocellulose was blocked with TBS + 0.5% milk powder (TBS milk) for 1 hour at RT and then incubated with anti-laminin (1:50) or anti-fibronectin (1:50) (GIBCO) antiserum overnight at 4^{o}C. After washing, the nitrocellulose was incubated with goat anti-rabbit HRP (1:500) (Biorad) in TBS milk. After further washing, LN and FN were visualized with 4-chloro-1-naphthol prepared as described for keratin westerns (Materials and Methods B. (5.)).

To detect collagen subtypes, ROSE 199 ECMs from all treatment types were subjected to separation by continuous SDS-PAGE in 8% acrylamide gels according to the method of Grinnell et al. ('89). After electrophoretic transfer to nitrocellulose, the nitrocellulose was blocked with TTBS + 3% BSA + 1% normal rabbit serum for 1 hour at RT and then incubated with anti-collagen antiserum against collagen types I (1:100), III (1:500), IV (1:500) in TTBS + 1% BSA overnight at 4^{o}C. After

washing, the nitrocellulose was incubated at room temperature in rabbit anti-goat (1:2000) (for collagen types III, IV) or rabbit anti-sheep (1:2000) (for collagen type I) alkaline phosphatase conjugated IgG (Southern Biotechnologies) diluted in TTBS + 1%BSA for 2 hr. After further washing, the collagens were visualized with BCIP/NBT as described for vimentin westerns (Materials and Methods B. (5.)).

(3.) Adhesion Assays

For a quantative determination of cellular adhesion to ROSE-ECMs, ROSE 199, HUDF, human HT-29 colon carcinoma, human $CAOV_3$ ovarian carcinoma, and human melanoma 6278 cells were grown to confluence (i.e. for up to one week) with 100 uCi methyl-^{3}H-thymidine (NEN, Boston, MA, 74 GBq/mmol 2.0 Ci/mmol)/ T-25 flask of cells. Five thousand to 10,000 radioactively labelled cells were seeded in 12 mm wells onto: (1) ECMs derived from six week old postconfluent ROSE 199; (2) ECMs derived from six week old postconfluent ROSE subclone cultures; and (3) uncoated plastic wells. The proportion of cells attached to ECM was determined by liquid scintillography counting of the radioactivity associated with the supernatant, the ECM, and the scraped underlying plastic fractions, collected at different time points. Early adhesion was at 10 minutes, intermediate adhesion between 20-30 minutes, and late adhesion, when most cells had adhered to plastic, was 45 minutes to 8 hours depending on the cell line. Non-adherent cells in the supernatant fraction as well as those scraped off the plastic underlying the ECM were collected as a pellet resulting from spinning at 10,000 rpm for 5 seconds in an Eppendorf centrifuge. Each fraction, along with the ECM with adherent cells, was removed from triplicate wells and placed into glass scintillation vials. The cells and ECMs were solubilized with Protosol (NEN) according to manufacturers instructions. Briefly, 0.5 ml of Protosol was added to each vial, sealed tightly, incubated at 55^{o}C for six hours with occasional shaking, followed by the addition of 100 ul of 30% hydrogen peroxide to decolourize the sample, and heated again for 30 minutes at 55^{o}C. Once the samples had cooled, 50 ul of glacial acetic acid (BDH) was added to neutralize the Protosol, followed by the addition of 14 ml Econofluor (NEN). The vials were shaken vigorously and allowed to equilibrate for one hour in a Philips PW 4700 scintillation counter before counting on a tritium channel. The percentage of cells attached to ECM at each time point was expressed as the percentage of radioactivity associated with the ECM fraction over the total radioactivity associated with the entire well and the results were expressed as the mean of triplicate wells $\pm$ the standard error of the mean.

For verification, concurrent with the liquid scintillography adhesion assays, either the cells remaining in the supernatant were counted with a hemocytometer or the wells were examined morphologically to estimate the proportion of nonadherant cells.

(4.) HOSE Organoids

The ROSE 199-derived ECMs were used in conjunction with collagen gels to make a complex three-dimensional tissue culture model that will be referred to as an organoid. The organoid set up was designed to mimic conditions that might crudely reflect a miniature ovary in culture. The components of this model are outlined in figure 26. To ensure that HOSE cells interacted with the components of the organoid model and not with the underlying plastic, wells were layered with 1% agarose (GIBCO), allowed to gel at RT, 0.4 ml collagen gel (prepared as described in Materials and Methods D. (1.)) was laid on top of the agarose layer and also allowed to gel. Finally, an AH-ROSE 199-ECM was deposited on top of the collagen gel. Single HOSE cell suspensions, from 11 different cases, varying from 50,000 to 300,000 cells were placed on top of the organoid set up. Organoids were incubated under standard conditions and medium changed as required. Organoids were periodically examined morphologically and were maintained until there was no further change in the amount of contraction. Organoid contraction was quantified by measuring the maximum and minimum axes of the organoids. The average diameter of each organoid was calculated and expressed as a percentage of the diameter of the culture well. The organoids were then fixed in alcohol and examined following either histological staining with haematoxylin and eosin or immunocytochemical staining for keratin.

Various culture conditions have been shown to affect collagen gel contraction in other systems (Anderson et al. '90, Bell et al. '79, Montesano & Orci '88, Schafer et al. '89). To test similar effects: 1.) organoids were seeded with varying numbers of HOSE cells; 2.) 24 hours following organoid set up the medium was replaced with medium containing 20 ng/ml EGF and 0.4 ug/ml hydrocortisone to stimulate HOSE proliferation; 3.) 24 hours after organoid set up, the medium was replaced with the low serum containing medium 199:105/PC-1 (see Methods A. (6.)); and 4.) some organoids were set up with NIH 3T3 fibroblasts in the collagen gel layer to mimic ovarian stroma.

D. Maintainance of HOSE on Various Biological Substrata

(1.) Preparation of Placental-Derived, Collagen, Fibrin Clot, and Matrigel Substrata.

The morphology and growth patterns of HOSE cells were compared on plastic (PL), placental-derived ECM (PD, Collaborative Research, Bedford, MA), rat tail tendon-derived collagen gel (COLL) (Emerman & Pitelka '77), PD-coated collagen gels (PD+Coll), fibrin clots (FB), and Matrigel (EHS, Collaborative Research) (Kleinman et al. '82). These substrata were chosen to represent some of the different types of ECM that HOSE may interact with *in vivo*. For example, placental-derived matrix (PD), a commercially available laminin rich ECM derived from placenta was employed to mimic basemement membranes. HOSE on rat tail tendon-derived collagen gels, which consist mostly of denatured collagen type I, might represent a situation where HOSE are in contact with the tunica albuginea or stromal ECM during invasion into the stroma. Fibrin clots were used to mimic the post-ovulatory defect within the follicle and Matrigel, which is made up of basement membrane components, was employed to mimic the basement membrane that HOSE normally rests upon. Also, by virtue of its jelly-like nature, Matrigel may mimic loose mesenchymal connective tissue comparable to that found in the developing ovary.

Collagen gels and Matrigel gels were prepared on ice using pre-chilled pipets and plates to prevent premature gelation. To prepare collagen gels, 1 part 0.34N NaOH was mixed with 2 parts 10x medium 199 and then added to 12 parts rat tail tendon-derived collagen gel solution (provided by Dr. J.T. Emerman). This solution was added to 24 well plates (NUNC) at 0.4 ml/well and allowed to gel overnight at 37^{o}C. One hour prior to use, the collagen gels were primed with complete medium, which was removed prior to the addition of cells. PD-coated dishes and PD-coated collagen gels were prepared by thawing stock solutions of PD-derived ECM at 4^{o}C and diluting the stock solution to 20 ug/ml with medium 199. On ice, 5 ug/cm^{2} PD solution was dispensed per plastic well and collagen gels, allowed to incubate at RT for 2 hours after which the excess PD solution was removed. Frozen Matrigel was thawed overnight at 4^{o}C, added undiluted at 0.4 ml well, allowed to gel at 37^{o}C for 30 minutes, and primed for one hour with complete medium before the addition of HOSE cells. Fibrin clots were prepared according to a modification of Kaibara et al. ('81). Stock solutions of fibrinogen (Sigma #F4883) at 20 mg/ml in ddH_2O were diluted in HBSS without calcium and without magnesium to 10 mg/ml and stored at -70^{o}C

until needed. To prepare fibrin clots, stock solutions of 10 mg/ml fibrinogen were thawed and added at 0.4 ml/well using 1 mg/ml poly-D-lysine (Sigma) coated pipets to prevent the fibrinogen from sticking to the pipets. Immediately afterwards, 0.25 ml of thrombin (Sigma #T6759) at 1.2 U/ml was quickly added to the fibrinogen in each well. The wells were swirled to mix the contents and immediately incubated at 37°C for 30 minutes for clot formation. As above, the fibrin clots were primed with complete medium for one hour prior to the addition of cells.

Single cell suspensions of HOSE from 12 different cases were plated onto these substrata and maintained for up to 12 days. The medium was changed as required. To harvest cells from the substrata, cells on PL & PD-coated plastic were collected by routine trypsinization in 0.06% trypsin (1:250)/0.01% EDTA. To harvest cells from Matrigel and fibrin clots, the culture medium was removed and the wells incubated at 37°C for 30 minutes with 0.5 ml/well of undiluted Dispase (Collaborative Research). This treatment dissolved both Matrigel and fibrin clots without damaging cells. After the dissolution of these substrata, the cells, often in clumps, were disociated into single cells suspensions by briefly resuspending the cell clumps in trypsin/EDTA. To recover cells from collagen gels, the gels were incubated at 37°C for 30 minutes in 4 mg/ml collagenase type II (Sigma) in DME:F12 (Sigma) supplemented with 1% BSA (Sigma). The dissolved collagen gels contained clumps of cells which were resuspended in trypsin/EDTA as described above to obtain single cell suspensions.

(2.) Morphological Examination

HOSE cells plated onto substrata were examined by phase microscopy and on paraffin sections stained with either haemotoxylin and eosin or immunocytochemically for keratin. Additionally, some HOSE cultures maintained on Matrigel were examined with scanning electron microscopy.

(3.) Immunoprecipitations for Integrins

The integrins expressed by HOSE cells on plastic, collagen gels, fibrin clots, and Matrigel, were examined from two low passage (p3) cobblestone epithelial cases (Van, Boo) and one late passage (p9) atypical epithelial case (Hed). Further, one of the low passage cases (Van) was tested again in passage 4. Due to differences in the numbers of cells obtained from each cases, depending upon the case, 0.5-2.6 x 10^6 cells were seeded per T-25 flask (Corning, NY) previously coated with 4 mls of collagen gel, fibrin clot, or Matrigel. However, each HOSE case was seeded at the

same density on all four substrata. The cultures were maintained for 48 hours. Then, the cells were photographed and surveyed for integrins as previously described (Dedhar et al. '87, '89, Dedhar & Saulnier '90). The cells were harvested, washed three times in PBS containing 1mM $CaCl_2$ and 1mM $MgCl_2$ and surface labelled with 5-7 ul (0.5mCi-0.7mCi) I^{125} (Amersham, Oakville, ONT) for 30 minutes at room temperature. The cells were washed three times with PBS and the cell pellet lysed in RIPA containing 1mM phenylmethyl-sulfonyl fluoride (PMSF, Sigma) for 30 minutes at 4^oC, then spun at 12,000 rpm for 15 minutes at 4^oC in an Eppendorf microfuge. The lysate was normalized to that volume that would give 6×10^6 cpm per immunoprecipitate reaction. An appropriate volume of lysate was incubated with anti-integrin antiserum for one hour at 4^oC. The primary antibodies used were: rabbit anti-vitronectin receptor (VNR), mouse anti-VLA2, anti-VLA3, anti-VLA5, anti-β4 (all from Telios, San Diego, CA), and rat anti-VLA6 (provided by Dr. C. Damsky, UCSF, CA). Following this incubation, 50 ul of Protein A Sepharose (Sigma) (0.1 g pre-swelled in 15 ml PBS, then pelleted and resuspended to 1 ml) were added to each immunoprecipitate and left to mix overnight at 4^oC. If the anti-integrin antiserum used had not been raised in rabbits, then 4 ul of rabbit anti-mouse antiserum (BIOCAN) or rabbit anti-rat antiserum (BIOCAN) were added to each immunoprecipitate at the same time as the addition of Protein A sepharose. The immunoprecipitates were each washed twice with 1 ml/wash of RIPA + 0.5M NaCl + PMSF (10 ul of 100mM PMSF per 1 ml RIPA) and followed with two washes of 1ml/wash of RIPA + PMSF. The antigen-antibody complexes in the final pellet were dissociated by boiling in 45 ul of sample buffer (200mM Tris-HCl pH 6.8 containing 3% SDS, 10% glycerol, and 0.001% bromophenol blue). Samples were analyzed by electrophoresis in 7.5% SDS polyacrylamide gels followed by autoradiography.

For time course studies, a large number of HOSE cells was required so a culture of Van HOSE (p2) was transfected by $CaCl_2$ precipitation using the SV40 large T antigen-containing plasmid PX-8 (Fromm & Berg '82) at 5 ug DNA per 35 mm culture dish. The transfected cells, IOSEVan, expressed large T-antigen, were cobblestone in morphology, keratin positive, and showed enhanced growth potential compared to untransfected cells. IOSEVan were cultured for 48 hours on plastic and collagen gel coated flasks. Cells were harvested, surface labelled, lysed, and immunoprecipated with rabbit anti-vitronectin receptor antiserum.

(4.) Growth Curves

To determine growth potential on the various substrata in short term culture, 15,000-20,000 cells from 3 HOSE cases (Any, Natty, Mull) were plated per well. Cells were harvested from triplicate wells at various intervals, counted, and counts expressed as the mean ± SEM.

(5.) Protease Production

For most protease assays, HOSE cells were plated onto PL, PD, FB, PD + COLL, Coll, EHS in complete culture medium 199:105/15%FBS. After 24 hours the culture medium was removed, the cultures washed with HBSS, and the medium replaced with: 1.) medium 199:105/1%FBS (3 cases-Any,Natty,Mull); 2.) medium 199:105/0.5%FBS (2 cases-Dose,Pere); or 3.) medium 199:105/PC-1 (2 cases-Dose,Pere). Conditioned media were collected following a further 48 hour incubation and tested for chymotrypsin, elastase, trypsin, plasminogen activator inhibitor, and collagenase type I activities. Further, chymotrypsin and elastase activities were compared in 2 cases (Dose,Pere) following 3 (D3) and 9 (D9) days of culture to see if proteases detected during the first few days of culture could also be detected later. Controls consisted of conditioned medium collected from substrata-coated wells alone, but treated identically to coated wells containing HOSE cells.

For initial gelatinase assays, cells from 2 cases (Casa & Gar) were plated for 24 hr on PL, FB, COLL, and EHS in medium 199:105/15% FBS. The cultures were washed with HBSS and the medium replaced with 199:105/DM. This conditioned medium was collected following a further 24 hr incubation, concentrated using Centricon 30 microconcentrators with a 30,000 MW cutoff (Amicon, Danvers, MA) and subjected to gelatin zymography. For subsequent gelatinase assays, cells from one case (Cello) were plated for 24 hr on PL, FB, COLL, and EHS in 199:105/DM. As above, the cultures were then washed with HBSS, incubated for a further 24 hr in 199:105/DM which was then collected, concentrated, and subjected to gelatin zymography.

(a.) Chymotrypsin, Elastase, and Trypsin Chromogenic Assays

Chymotrypsin, elastase, and trypsin-like activities in conditioned media were determined by spectrophotometric assay (Uitto et al. '89). Peptidase activities were assayed in 96 well flat bottomed plates (Linbro, Flow Lab) by incubating 50 ul conditioned media with 1mM chromogenic p-nitoanilide amino acid substrates (made as 40-50 mM stock solutions in DMSO) in a final volume of 100ul with 0.05M Tris

HCl/0.2M NaCl buffer (pH 7.8) for up to 24 hours at 37^{o}C with continuous shaking. The change in absorbance at 405 nm was recorded spectrophotometrically using a BIO-TEK microplate autoreader EL311 (Mandel Scientific Co. Ltd), was corrected for cell number and for the level of background activity associated with the controls, and recorded as the relative absorbance at 405 nm. The synthetic peptides N-succinyl-ala-ala-pro-phe p-nitroanilide (SAAPNA, Sigma), N-succinyl-ala-ala-ala p-nitroanilide (SAANA, Sigma), and N-benzoyl-DL-arginine p-nitroanilide (BAPNA, Sigma) were used to demonstrate chymotrypsin-like, elastase-like, and trypsin-like activities respectively. The results of triplicate wells are reported as the mean relative change in absorbance per hour per 10^4 cells.

(b.) Plasminogen Activator Assay

Conditioned media were also assayed for plasminogen activator (PA) activity using a casein-agarose diffusion plate assay (Uitto et al. '89). Agarose substrate tablets containing casein (Biorad) were soaked overnight in dH_2O to give a 1% agar gel containing casein. The agarose solution was boiled for 3 minutes until the solution became opaque and then allowed to cool to 50^{o}C. Twenty micrograms of plasminogen (Sigma) was quickly added per ml agarose solution. Fifteen ml of agarose/plaminogen solution was poured per 100 cm plastic petri dish (Corning), the solution swirled to cover the dish, and allowed to gel at room temperature. Once gelled, wells were bored into the agarose. Thirty ul of conditioned medium were added per well and incubated for up to 72 hours in a humidified chamber. When plasminogen is converted to plasmin by plasminogen activator, it degrades casein and forms a clear area of lysis in the agarose gel. Human urokinase (Sigma) at 0.2 U/ml and 0.02 U/ml was used as standard plasminogen activator activity. The reaction was stopped by fixing the agarose plates in 4% acetic acid for 1 hour at RT.

(c.) Collagenase Type I: ^{3}H-Collagen Type I Degradation

Fifty ul of conditioned medium was incubated with soluble ^{3}H-proline-labelled chicken type I collagen (provided by Dr. V.-J. Uitto, UBC, BC) in 0.05M Tris HCl buffer, pH 7.8, containing 0.2M NaCl and 5 mM $CaCl_2$ for 18 hours at 25^{o}C (Uitto et al. '89). The specific acitivity of the labelled collagen was 2 x 10^4 dpm/mg and to activate latent collagenase in that might be in the conditioned media, 1 mM p-aminophenylmercuric acetate (APMA, provided by Dr. V.-J. Uitto) was added to each assay. The degradation products of collagen were analyzed by SDS-PAGE using 10% acrylimide gels, followed by autoradiography.

(d.) Gelatin Zymography

Gelatinolytic activity was assayed by a modification of Heussen and Dowdle ('80). The conditioned media were mixed with sample buffer and applied directly, without prior heating or reduction, to 10% acrylamide gels containing 1 mg/ml 2-methoxy-2,4-diphenyl-3(2H)furanone (MDPF, Calbiochem #444899)-conjugated gelatin (Sigma, #G-6269) (O'Grady et al. '84 and provided by Dr. Uitto). Following electrophoresis, the SDS was removed from the gels by incubation in 50 mM Tris-HCl pH 7.5 containing 2.5% Triton X-100 and 0.02% NaN_3 for 30 minutes at RT followed by a second incubation at RT for 30 minutes in 50 mM Tris-HCl pH 7.5 containing 5 mM $CaCl_2$, 1 uM $ZnCl_2$, 2.5% Triton X-100 and 0.02% NaN_3. The gels were then incubated overnight at 37°C in 50 mM Tris-HCl pH 7.5 containing 0.2M NaCl, 5 mM $CaCl_2$, 1uM $ZnCl_2$, 0.02% NaN_3. The gels were fixed and stained in 50% MeOH/7% acetic acid containing 0.2% Coomassie Brillant Blue G250 (Sigma) and briefly destained in 10% acetic acid. The gelatinolytic activity was evident as clear bands against the blue background. MDPF conjugated to gelatin provided a means to monitor gel lysis under ultraviolet illlumination during incubation of the gels.

RESULTS

A. Improved HOSE Yield and Tissue Culture

(1.) HOSE Cultures Established by Explantation from Biopsy Material

HOSE from 9 ovarian biopsy specimens was prepared by the explantation method (Table 1). By three weeks of culture, 422 out of 469 explants had cellular outgrowths (90%). Only 150 (34%) of all the explants produced epithelial outgrowths. Outgrowths from 113 explants (24%) contained mixtures of epithelial and stromal fibroblasts and 151 out of the total explants (32%) produced completely fibroblastic outgrowths. Only 47 (10%) explants failed to grow HOSE cells.

Although cells grew out from explants, few cultures contained pure populations of HOSE cells. Described below are the attempts to overcome the major problems associated with this technique which are: 1.) labourious technique; 2.) low HOSE cells yield; and 3.) fibroblastic overgrowth.

(2.) HOSE Culture Purification by Differential Adhesion to Collagen Gel

HOSE cultures (from 2 cases, Solo p1 and Welch p4) that contained mixtures of epithelial and fibroblastic cells were plated onto unrimmed collagen gels. By one hour post plating most cells had attached to the collagen gels. Keratin immunofluorescence of the cells recovered from the gels as well as the non-adherent cells remaining in the supernatant revealed mixtures of keratin positive and keratin negative cells, often regardless of morphology (figure 2 and summarized in table 10). Therefore, the epithelial component of mixed HOSE cultures could not be separated from the fibroblastic component by differential adhesion to collagen gels. Controls of amniotic cells, which contain populations of keratin positive and negative cells, stained as mixtures of keratin positive and negative cells. All coverslips treated with PBS instead of primary antibody failed to stain.

(3.) Culture Purification by Percoll Density Gradient Centrifugation

(a.) Purification of Mixed HOSE Cultures

Following centrifugation, all cell types tested were visible as one or two bands in the range of 1.05-1.08g/ml (Table 2). HOSE cultures from 6 cases banded variably ranging from a broad band at 1.05-1.08g/ml to two bands at 1.05 & 1.06-

Table 1. HOSE Cultures Established by Explantation from Biopsy Material

Case	# Explants	# Outgrowths			
		# Epithelial	# Mixed	# Fibroblasts	# No Growth
Anon	35	14	0	21	0
Bent	115	53	10	45	7
Dew	82	1	39	40	2
Dose	44	39	0	0	5
Felice	46	5	30	11	0
Mac	30	11	9	7	3
Mal	40	9	17	10	4
Myc	20	0	7	8	5
Sub	57	26	1	9	21

Figure 2. Keratin Expression in HOSE Cells After Differential Adhesion

Photomicrographs representative of mixed HOSE cell populations derived from differential adhesion to collagen gels and stained by immunofluorescence for keratin. (A), Immunofluorescence of cells recovered from collagen gels demonstrate mixtures of keratin-positive and keratin-negative cells. (B), Phase microscopy of the same field as in A. Note that the presence of keratin is independent of either an epithelial or fibroblast-like cellular morphology. X475.

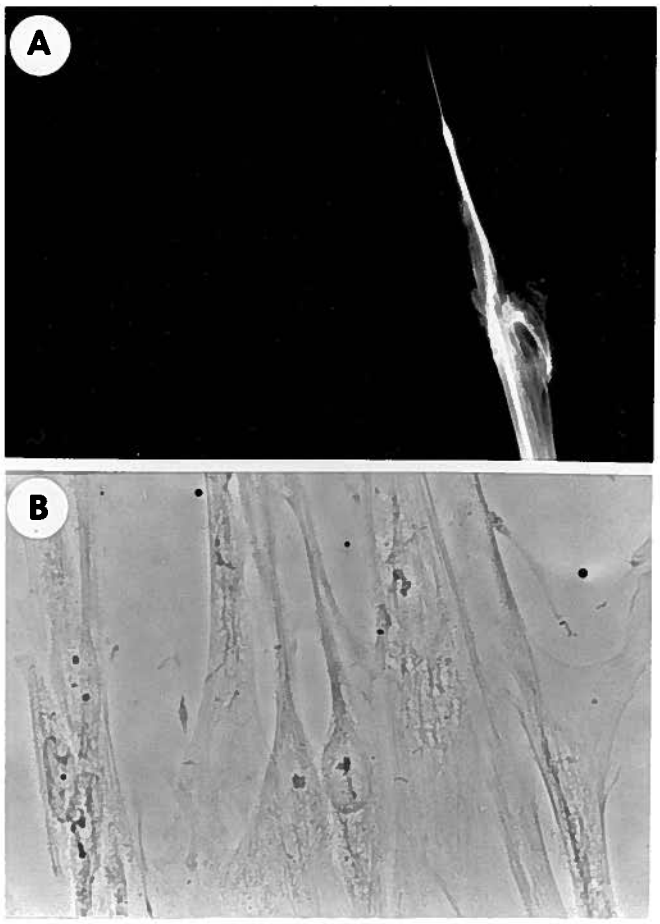

A
B

Table 2. Percoll Centrifugation to Separate Mixed HOSE Cultures

Mixed HOSE Cultures	% SIP	Centrifugation	Cell Band (g/ml)
Anon	25	10,000g 100'	1.05
Solo	25	10,000g 100'	1.06
Solo	40	10,000g 30'	1.05
Mac	40	10,000g 30'	1.05
Bent	40	10,000g 30'	1.07-1.08
Welch	40	10,000g 30'	1.05 & 1.06-1.07
Sub	40	10,000g 30'	1.05 & 1.06-1.07
Other Cell Types			
RLF	25	10,000g 100'	1.05-1.08
HUDF + EGF/HC	25	10,000g 30'	1.06
HUDF - EGF/HC	25	10,000g 30'	1.06
HUDF revertants	25	10,000g 30'	1.06
LP-9 + EGF/HC	25	10,000g 30'	1.06
LP-9 - EGF/HC	25	10,000g 30'	1.06
LP-9 revertants	25	10,000g 30'	1.06

'= minutes

1.07g/ml. RLF banded in a broad band at 1.05-1.08g/ml and HUDF and LP-9 cells banded as a discrete band at 1.06g/ml. The various EGF/HC treatments of LP-9 cells and HUDF did not alter the cells' density in gradient centrifugations.

The morphology of each HOSE cell band recovered following Percoll centrifugation resembled its parent culture. That is, bands recovered from mixed HOSE cultures demonstrated mixed epithlelial and fibroblastic phenotypes similar to cells recovered following differential adhesion to collagen gels. HOSE cultures stained as mixtures of keratin-positive and keratin-negative cells following centrifugation similar to the tests for differential adhesion on collagen gels where keratin staining was not restricted to any particular morphological phenotype. That is, some fibroblastic cells stained positively for keratin while others did not. Similarly, some epithelial cells stained positively for keratin and some did not. RLF and HUDF remained fibroblastic after centrifugation and LP-9 cells were fibroblastic or epithelial following centrifugation depending on whether they were maintained with or without EGF/HC respectively. RLF and HUDF failed to stain for keratin. LP-9 cells without EGF/HC or revertants uniformly stained positive for keratin following Percoll centrifugation, while those treated with EGF/HC did not stain. The results indicate that centrifugation in Percoll gradients did not separate the stromal fibroblasts in mixed HOSE cultures from the epithelial or fibroblastic HOSE component and that the addition of EGF/HC did not alter cellular density in Percoll gradient centrifugations. Coverslips stained with PBS instead of primary antibody failed to stain for keratin.

(b.) Percoll Decontamination of Mold Infected Cultures

While centrifugation in Percoll failed to separate stromal fibroblasts from HOSE cells in mixed cultures, it appeared to provide a method to remove mold infections from cultures. To develop this method, ROSE 239 and HUDF cells were intentionally infected with mold and yeast contaminants in an attempt to decontaminate the cultures by Percoll centrifugation. Following centrifugation, all cell types tested were visible as single bands with the lower limit of the heaviest band approximately 1-2 cm above the bottom of the centrifuge tubes. ROSE 239 banded at a density of 1.056-1.074 g/ml and HUDF banded at 1.048-1.056 g/ml, while the mold pelleted at the bottom of the test tubes. The percentage of ROSE 239 and HUDF cells recovered from Percoll centrifugation varied between 40-50%. However, the viability was 94% or better regardless of the centrifugation treatment employed.

Yeast infections, using *Torulopsis candida* as a prototype, could not be eliminated by Percoll gradient centrifugation. Also, Percoll centrifugation did not enhance decontamination of yeasts when used in conjunction with Fungizone (data not shown). This is similar to the results reported by Behrens and Paronetto ('84).

Figure 3 shows the presence of mold in ROSE 239 and HUDF cultures prior to Percoll centrifugation. Microscopic examination of cells recovered following Percoll centrifugation revealed no contaminants for up to one month in cultures maintained without antifungal agents (figure 3). The morphology of ROSE 239 and HUDF cells remained epithelial and spindle-shaped, respectively. There was no consistent difference in growth between the high speed-centrifuged Percoll-treated cultures and low speed-centrifuged controls (figure 4). After either treatment, ROSE 239 cells grew to confluence quickly and were subcultured weekly at a split ratio of 1:5 while HUDF, which grew more slowly, were subcultured every second week at a split ratio of 1:2.

Percoll treatment of mold-infected ROSE 239 and HUDF also did not alter the ability of these cells to produce and secrete extracellular matrix. ROSE 239 and HUDF from all treatments, HiC, LoC, and HiM, stained positively for collagen type I (figure 5). Similarly, ROSE 239 retained their capacity to secrete laminin and the pattern of extracellular matrix components was indistinguihable among centrifugation treatments (figure 5). All cultures failed to stain when incubated with normal serum and secondary antibody and HUDF failed to stain for laminin.

(4.) HOSE Cultures Established by Scraping Biopsy Material

While neither differential adhesion to collagen gel nor centrifugation in Percoll gradients yielded pure HOSE cultures, scraped-derived cultures were highly successful. In contrast to the explantation method, scraping generates epithelial fragments that settle and attach to plastic culture dishes and produce epithelial outgrowths (figures 1 and 6). Of the HOSE cultures established from 24 scraped biopsies (table 3), 3 (12.5%) were discarded due to contamination and one (4%) was discarded because it failed to provide any HOSE cells. HOSE cultures from scraped biopsies were scored for growth as described previously (Kruk et al. '90). 0 denotes no growth; +, some HOSE cells in primary culture, but little if any cell proliferation; ++, proliferation to 10^4-10^5 cells, adequate for short-term experiments and characterization, but the cells did not reach confluence, +++, cells reached confluence (i.e. approximately 10^5 cells/35 mm dish) in 2 to 4 weeks and underwent up to 12 population doublings in medium 199/MCDB105/15%FBS.

Figure 3. Cell Lines Before and After Percoll Decontamination

Phase micrographs of confluent ROSE 239 (A,B) and HUDF (C,D) before (A,C) and after (B,D) centrifugation in Percoll. X150.

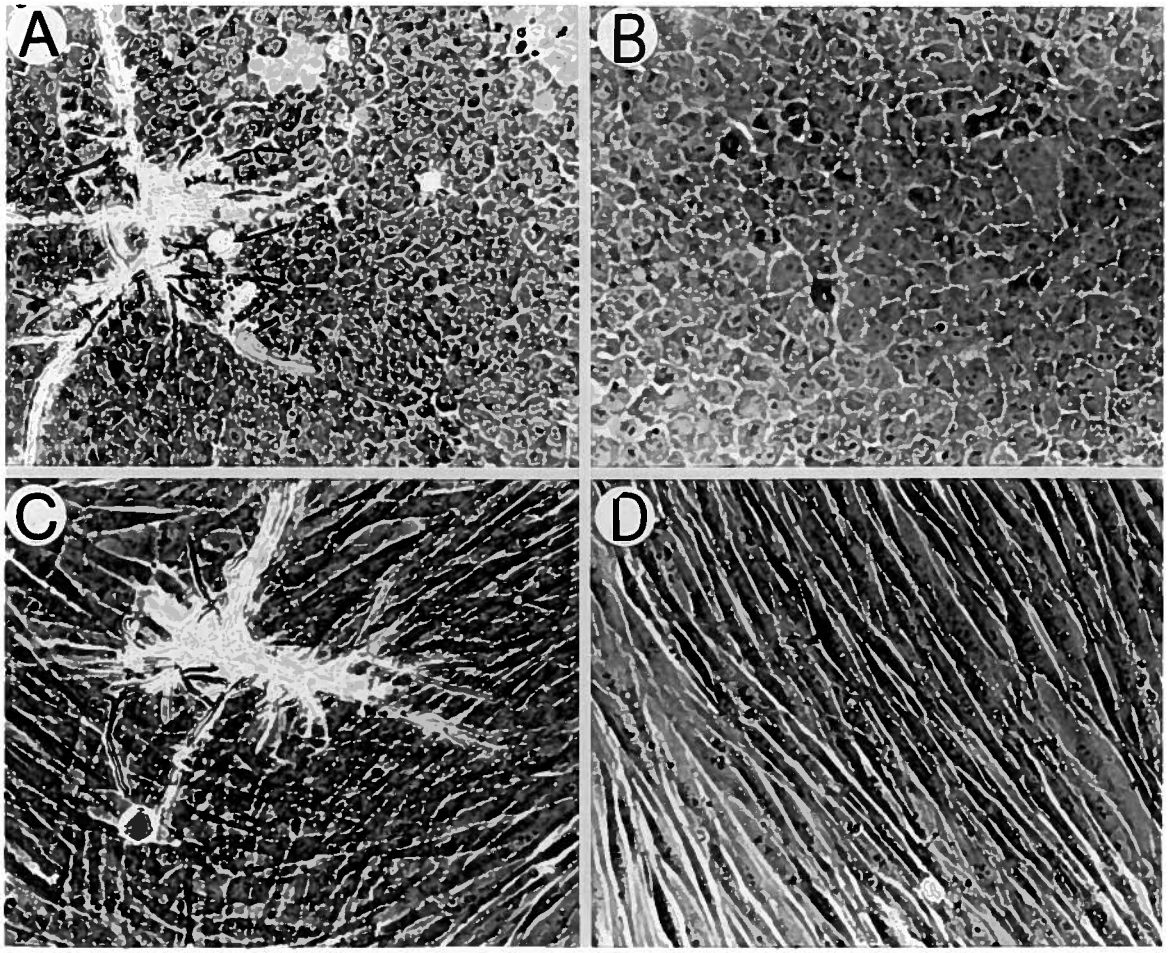

A
B
C
D

Figure 4. Effect of Centrifugation in Percoll on Cell Growth

Growth curves of ROSE 239 (A) and HUDF (B) following Percoll centrifugation of uninfected cultures (HiC), mold-infected cultures (HiM) and low speed centrifugation of uninfected cultures (LoC). ROSE 239 and HUDF, collected following centrifugation (HiC, LoC, and HiM) were plated at 5000 cells/12 mm well. Cultures were maintained for 12 days and the medium was changed as required. Cells from triplicate wells were harvested at intervals and counted. Triplicate counts are expressed as the mean $\pm$ SEM.

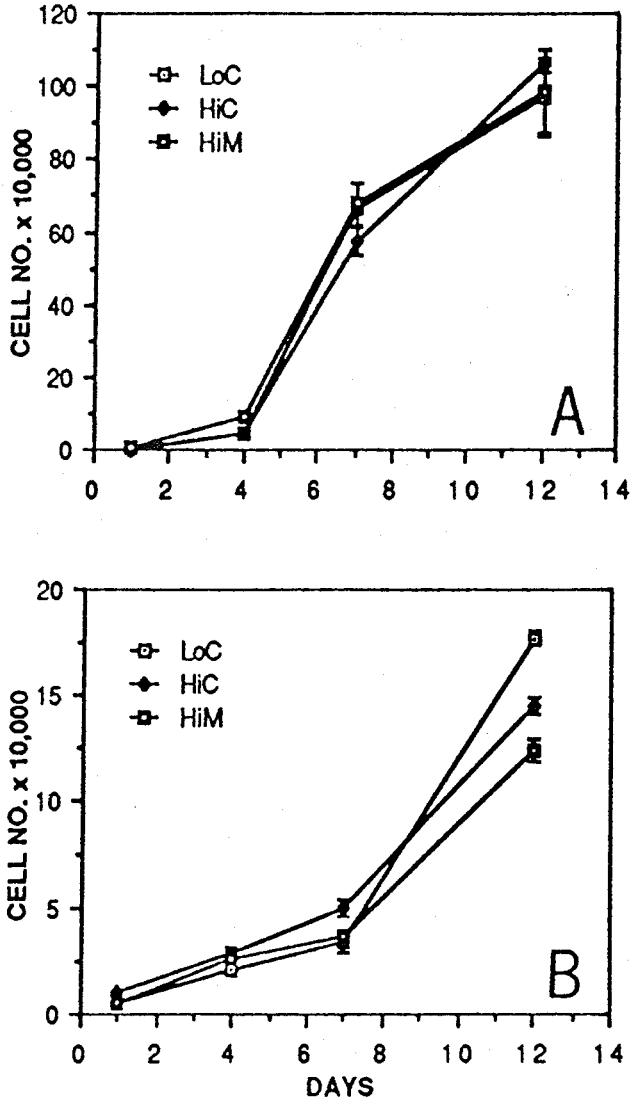

120
100
80
60
40
20
0
CELL NO. x 10,000
LoC
HiC
HiM
A
0
2
4
6
8
10
12
14
20
15
10
5
0
CELL NO. x 10,000
LoC
HiC
HiM
B
0
2
4
6
8
10
12
14
DAYS

Figure 5. Matrix Production Following Percoll Decontamination

Laminin (A) and collagen type I (B,C) in the extracellular matrix of ROSE 239 (A,B) and HUDF (C). Immunofluorescence microscopy. X450.

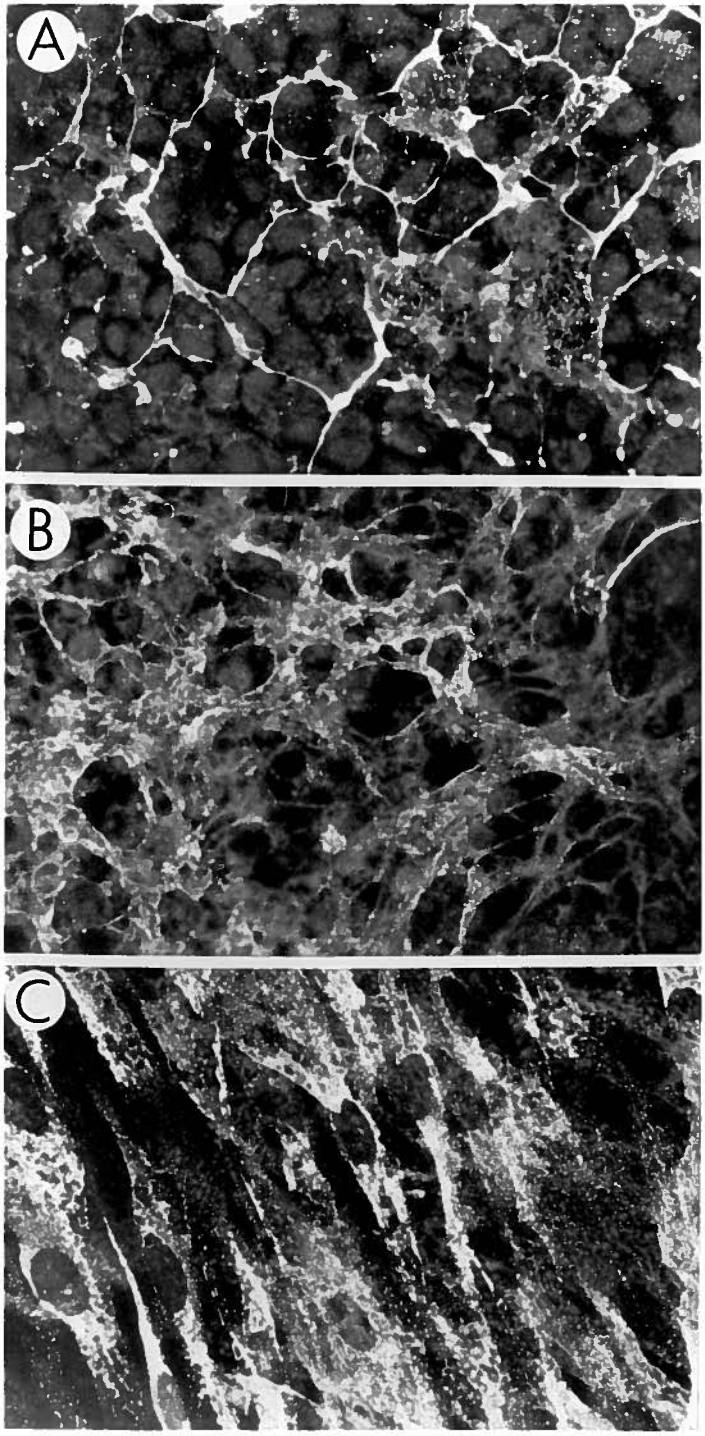

A
B
C

Figure 6. Establishment of HOSE Cultures by the Scrape Method

(A) An ovarian surface epithelial fragment newly detached from the ovary by scraping. Note red blood cells in the background; (B) 5-day-old outgrowth from an epithelial fragment; (C) 10-day-old primary HOSE culture, epithelial fragment in the center. Phase microscopy. X120.

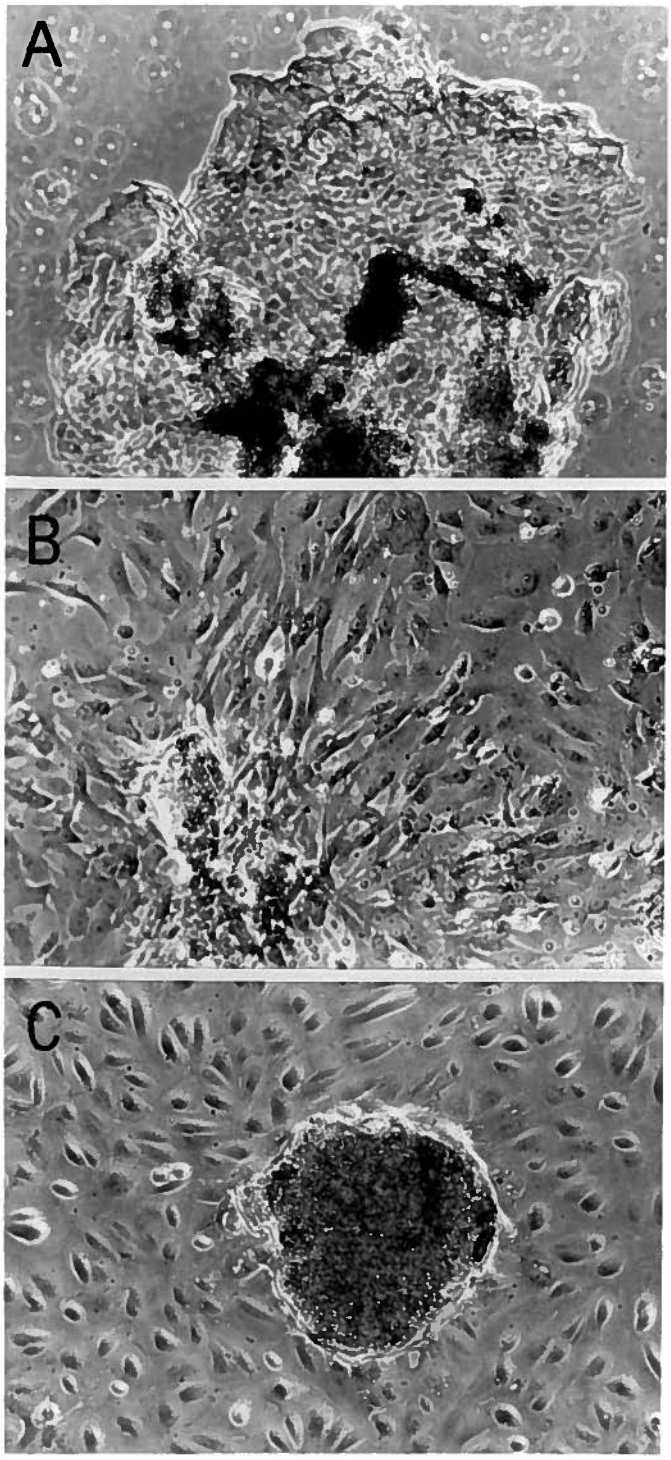

A
B
C

Table 3. HOSE Cultures Established by Scraping Biopsy Material

Case	Morphology in Primary Culture[1]	HOSE Cell Growth[2]
Ape	Flat, epithelial, cobblestone	+++
Any	Compact, epithelial, cobblestone	+++
Cal	Discarded due to contamination	-
Cap	Flat, epithelial, cobblestone	++
Casa	Compact, epithelial, cobblestone	+++
Cor	Flat, epithelial, cobblestone	++
Dose	Compact, epithelial, cobblestone	+++
Eng	Atypical epithelial	+
Fella		0
Ha	Flat, epithelial, cobblestone	+
Hig	Flat, epithelial, cobblestone	+
McHOSE	Discarded due to contamination	-
Mull	Flat, epithelial, cobblestone	+++
Nag	Flat, epithelial, cobblestone	++
Natty	Flat, epithelial, cobblestone	+++
Pere	Atypical epithelial	+++
Ric	Flat, epithelial, cobblestone	+
Ste	Atypical epithelial	++
Tea	Fibroblastic	+++
Tuc	Flat, epithelial, cobblestone	+++
Van	Compact, epithelial, cobblestone	+++
Very	Flat, epithelial, cobblestone	+++
Wee	Atypical epithelial	++
Yar	Discarded due to contamination	-

1= HOSE cultures were scored according to the morphological criteria of Siemens & Auersperg ('88).

2= 0, No HOSE growth; +, some HOSE cells in primary culture, but little if any cell proliferation; ++, proliferation to 10^4-10^5 cells, adequate for short-term experiments and characterization, but the cells did not reach confluence; +++, HOSE cells reached confluence (i.e. approximately 10^5 cells/35mm dish in 2 to 4 weeks and underwent up to 12 population doublings in Medium 199:MCDB 105/15% FBS.

16/24 cases or 67% showed good (++) to excellent (+++) growth with 11 cases or 46% showing excellent growth, and only 4 cases or 16.5% yielded few HOSE cells (+). Also, HOSE cultures from scraped biopsies were classified according to their morphology in primary culture (Auersperg et al. '84, Siemens & Auersperg '88). The vast majority of scrape-derived HOSE cultures, 19/20 or 95%, of cultures grew as epithelial cells. Of these epithelial cultures, 20% or 4/20 were compact cobblestone, 55% or 11/20 grew as flat epithlial cells, and 20% or 4/20 grew as atypical epithelial cells. Cells from only a single case were spindle-shaped and fibroblast-like. Therefore, in addition to the improved yield, the scrape method also greatly increased the purity of the cultures.

In contrast to procedures that use explants (Auersperg et al. '84) or enzymatic dissociation (Hamilton et al. '80) of the original tissue, the ovarian surface below the HOSE remains undisturbed by the scrape method. As a result, contamination by stromal and follicular cells was rare. Occasional groups of contaminating fibroblasts could be removed with a rubber scraper. Histologic examination of scraped specimens confirmed that scraping denuded 90 to 100% of the ovarian surface of HOSE with little disruption of underlying structures (figure 7). Thus, normal surface-lined HOSE is selected over cyst and invagination-derived HOSE which is frequently atypical. In addition to the improved yield and greater uniformity of the cultures, the scrape method has the obvious advantages of being considerably faster and simpler than either explant culture or culture of enzymatically dissociated tissues and HOSE cultures are now routinely cultured by the scrape method.

B. Characterization of HOSE Cells in Culture

(1.) To What Extent do Cultured HOSE Cells Reflect Their *In Vivo* Counterparts?

To characterize and compare HOSE cells in culture with what has been reported of their *in vivo* counterparts (McKay et al. '61, Blaustein '81a, Blaustein & Lee '79), HOSE cultures were stained for lipid, mucin, and keratin. Additionally, preparations of intermediate filaments from HOSE cultures were examined by western immunoblotting for keratin subtypes and vimentin.

Like their *in vivo* counterparts (Blaustein '81a, Blaustein & Lee '79), cultured HOSE cells from both early and late passage stained positively for lipid with Oil Red O indicating the presence of neutral fats as small red droplets at varying depths in the cytoplasm (figure 8). The amount of positive material varied among cells within

Figure 7. Section of a Scraped Ovarian Biopsy

All HOSE, except for a small area (arrowhead) has been removed. C, inclusion cyst lined with epithelium. Hematoxylin and eosin. X60.

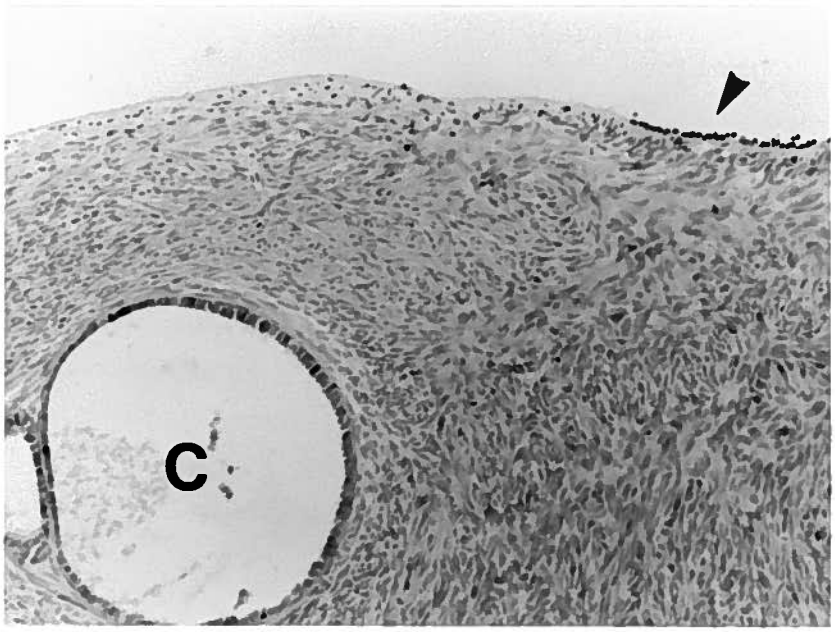
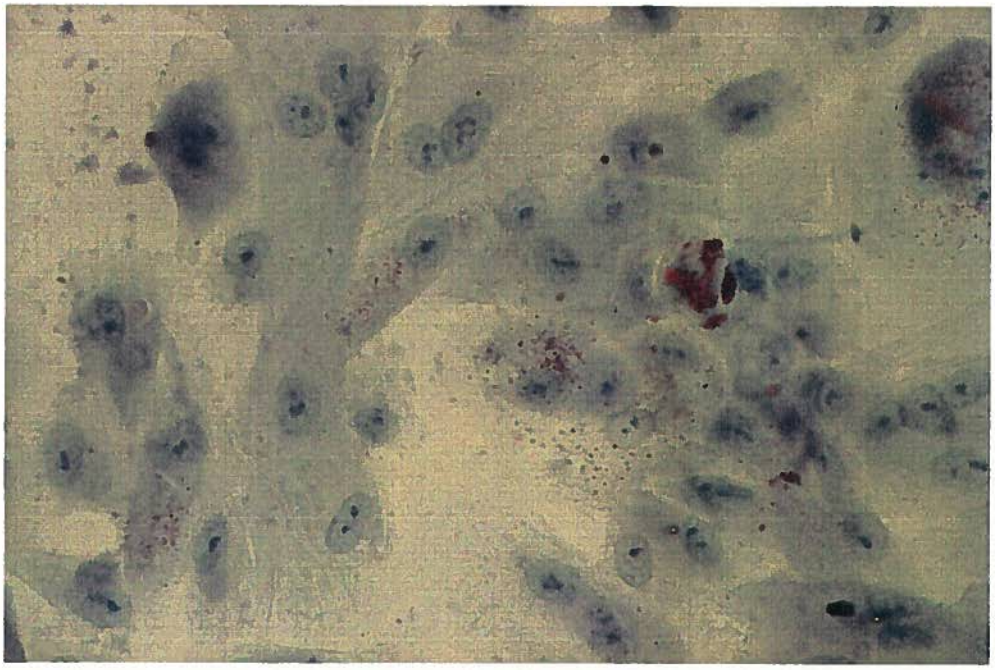

C

Figure 8. HOSE Cultures Stained with Oil Red O

HOSE cultures stain positively for Oil Red O as small droplets that appear singly or in clusters. X300.



cultures from a few droplets scattered in the cytoplasm to small clusters of lipid droplets to large accumulations found in groups of piled up cells.

Cultured HOSE, of both early and late passage, also stained intensely with routine PAS indicating the presence of neutral sugars, like their *in vivo* counterparts (McKay et al. '61). There are three distinct collections of PAS positive material: 1) fine, uniform, pink granular staining; 2) clusters of coarse, magenta granules at various locations in cells; and 3) large, dark pink vacuole-like areas adjacent to the nucleus (figure 9A). Following digestion with amylase (figure 9B), the clusters of coarse granules failed to stain demonstrating that they were glycogen. Occasional large vacuoles also failed to stain, but some mucin, defined as amylase-resistent PAS positive material, was detected in the large vacuoles. The cytoplasms of HOSE cells in ovarian sections also contained PAS positive amylase insensitive material indicating that both cultured HOSE cells and HOSE *in vivo* are variably positive for mucin.

The epithelial nature of both early and late passage HOSE cells was confirmed by the presence of keratin similar to previous reports (Auersperg et al. '84) and like their *in vivo* counterparts (Czernobilisky '85). However, the proportion of keratin-positive cells generally decreased with increasing passage (figure 10). Also, with increasing passage, HOSE cells modulated from an epithelial morphology to a more fibroblast-like phenotype (figure 10). HOSE cells incubated with PBS instead of primary antibodies failed to stain for keratin. As controls, C4-I cells stained positively for keratin while HUDF failed to stain for keratin.

Table 4 illustrates the degree of co-expression of mucin and keratin in HOSE cultures. HOSE cells, of both early and late passages, stained variably for mucin. HOSE cultures also contain variable amounts of keratin positive cells (11.25-66%). Mucin was present in less than 10% of HOSE cells, regardless of passage. The percentage of mucin vacuoles that were contained within keratin positive cells ranged from 43-100% and was not passage dependent. Furthermore, mucin and keratin expression were independent of morphology, as these markers of epithelial differentiation were found in epithelial and fibroblast-like HOSE cells. For controls, C4-I cells, oviductal epithelium, and cervical epithelium were keratin positive and the cervical epithelium was also positive for mucin.

To determine if the keratin subtypes expressed by HOSE cultures are the same as their *in vivo* counterparts as well as to determine if cultured HOSE cells co-express vimentin and keratin like their *in vivo* counterparts, preparations of intermediate filaments were probed by western immunoblotting. As shown in figure 11, low

Figure 9. HOSE Cultures Stained with PAS and Amylase + PAS

HOSE cultures stained positively with PAS as fine uniform pink granular material, clusters of coarse magenta granules, and large pink vacuole-like areas adjacent to the nucleus (A). Following digestion with amylase, PAS positive material (mucin) is restricted to large pink vacuole-like material in the peri-nuclear region (B). X300.

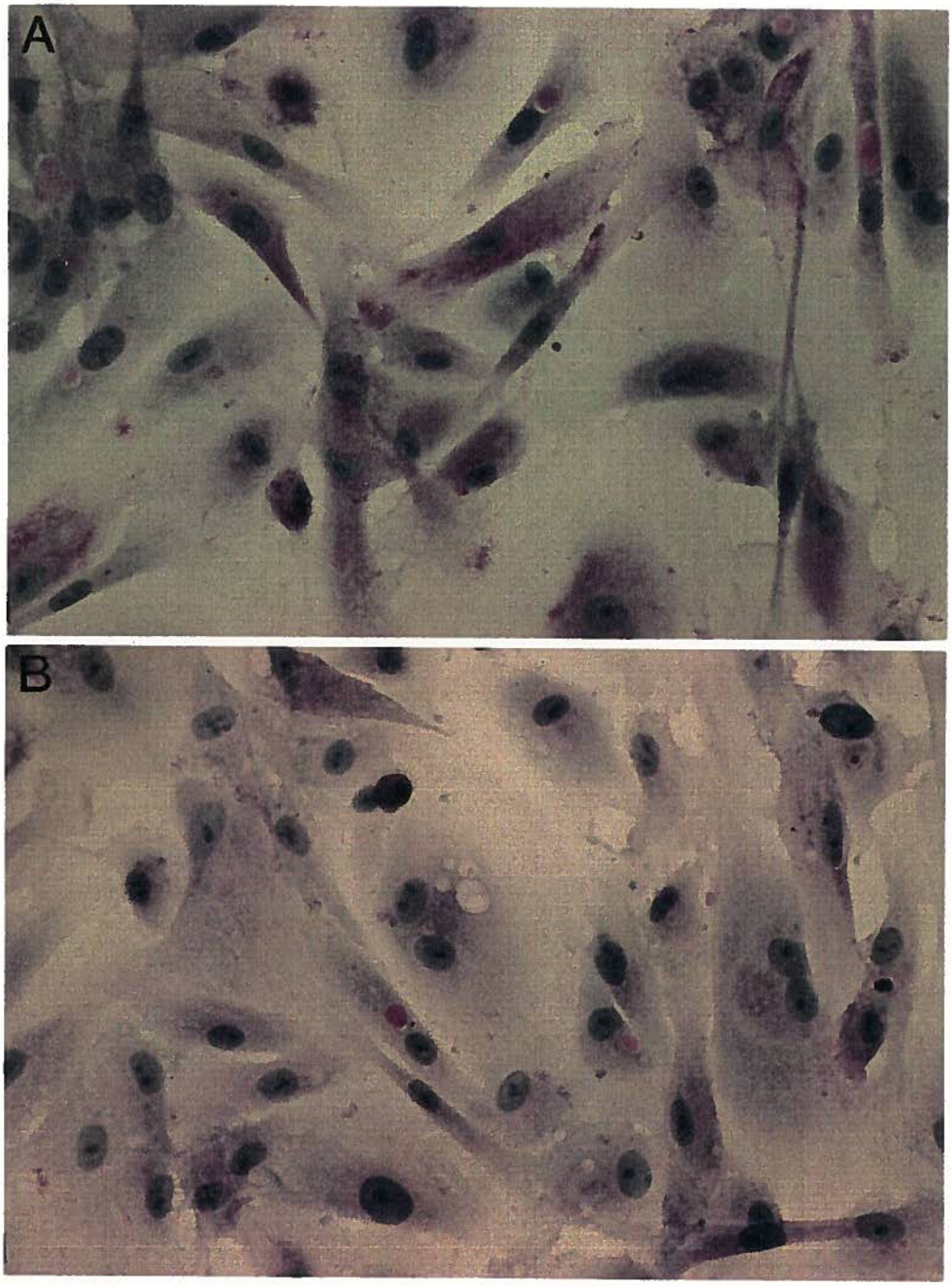

A
B

Figure 10. Keratin Expression in Early and Late Passage HOSE Cultures

Early passage HOSE cells are keratin positive (A) and epithelial (B) while late passage HOSE cells are largely keratin negative (C) and often fibroblast-like in morphology (D). A, C, Immunofluorescence microscopy. C, D, Phase microscopy. X300.

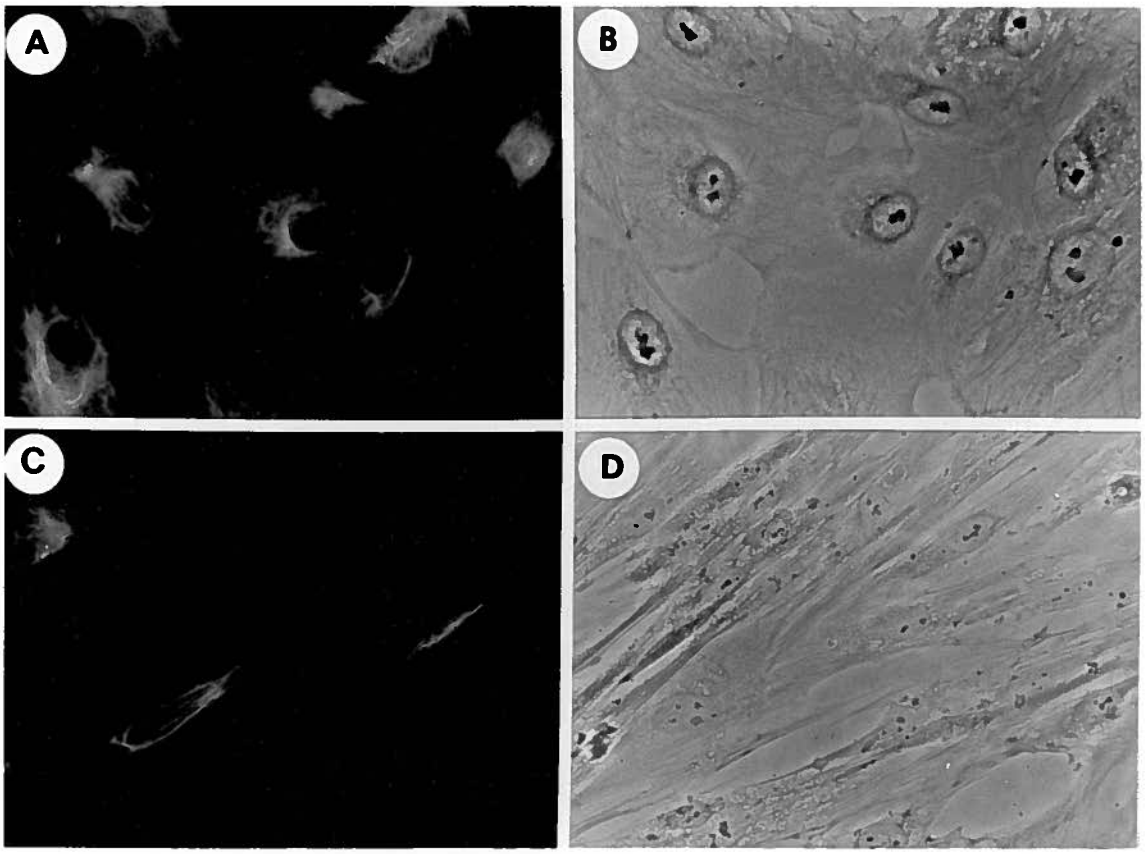
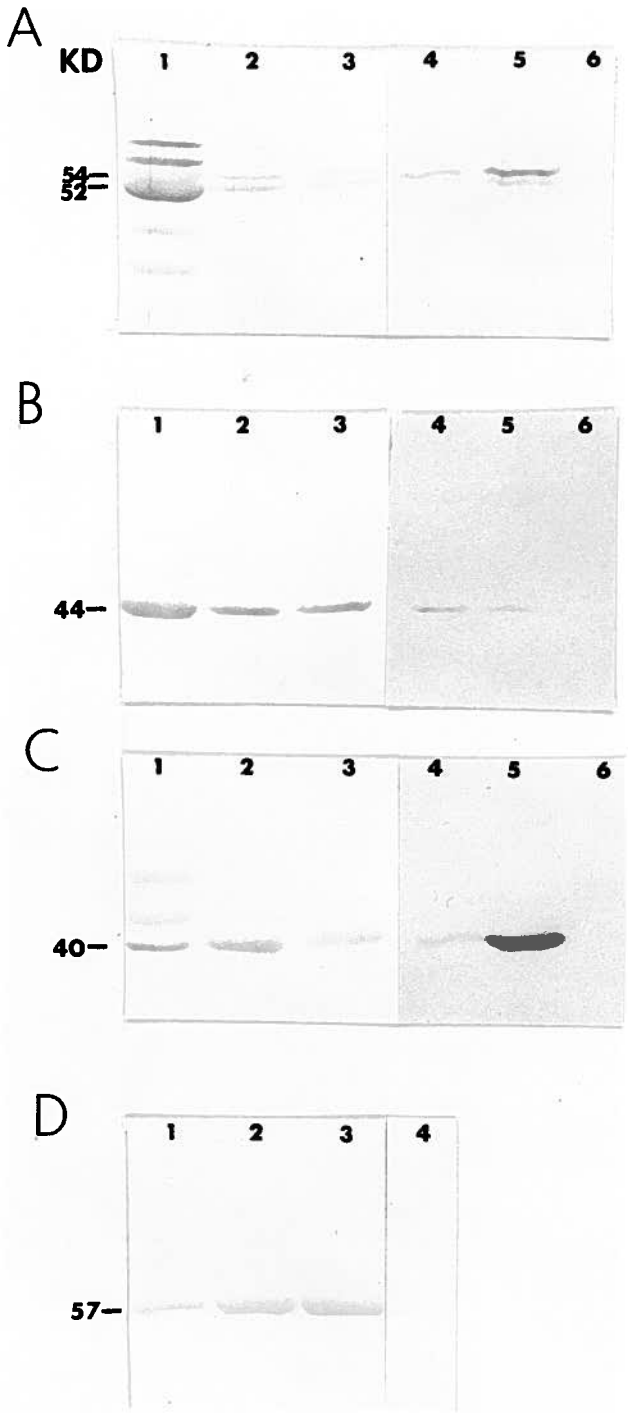

A
B
C
D

Table 4. Co-Expression of Keratin and Mucin in HOSE Cultures

Case	# Keratin Positive Cells (%)		# Mucin Positive Cells (%)		# Mucin Positive Cells that Express Keratin	
Any p3	124/243	(51)	16/243	(7)	10/16	(63)
Pere p3	157/237	(66)	11/237	(5)	9/11	(81)
Gar p10	12/42	(29)	2/42	(5)	2/2	(100)
Hed p10	20/85	(24)	7/85	(8)	3/7	(43)
Tync p10	18/160	(11)	16/160	(8)	5/16	(38)

Figure 11. Keratin and Vimentin Western Immunoblots

Preparations of intermediate filaments were subjected to SDS-PAGE followed by transfer to nitrocellulose. (A-C), lane 1, C4-I; lanes 2&3, early passage HOSE; lanes 4&5, late passage HOSE; lane 6, HUDF. (D), lane 1, HUDF; lanes 2&3 HOSE; lane 4, C4-1. Nitrocellulose sheets were immunoblotted for acidic keratins with AE-1 (A), keratin #18 (B), basic keratins with AE-3 (C), and vimentin (D). All HOSE cells express keratins of 40, 44, 52, and 54 KD, except for some late passage HOSE cells that lack the 44 KD keratin. HUDF serve as negative controls for keratin while C4-I cells serve as positive controls for keratin. All HOSE cells and HUDF express vimentin while C4-I cells do not.


A
KD
1
2
3
4
5
6
54
52
B
1
2
3
4
5
6
44
C
1
2
3
4
5
6
40
D
1
2
3
4
57

passage HOSE cells expressed keratins of 40, 44, 52, and 54 KDa corresponding to keratins #7, 8, 18, and 19 characteristic of simple epithelia (Moll et al. '82) and identical to the keratins expressed by the human peritoneal cells, LP-9 (Connell & Rheinwald '83) and HOSE *in vivo* (Czernobilisky '85). While keratins derived from one case of late passage HOSE cells expressed keratins #7, 8, 18, and 19 like low passage HOSE cells, the keratins derived from a second late passage HOSE case clearly expressed keratins #7 and #18 while keratin # 19 was barely detecteable and keratin # 8 was absent. This suggests that with increasing passage specific keratins are gradually lost. These results support the immunofluorescence studies which demonstrated decreased staining for keratin with increasing passage (figure 10). The cultured HOSE cells also co-express vimentin along with keratin (figure11). Vimentin was demonstrated as a single band at 57 KD. Human dermal fibroblasts were devoid of keratin, but demonstrated the presence of vimentin (figure 11). C4-I cells expressed keratins #5, 6, 8, 16, 18, 19 (figure 11), as demonstrated previously (Auersperg et al. '89), but C4-I cells did not express vimentin (figure 11).

(2.) Do HOSE Cells Produce Extracellular Matrix?

Further characterization of HOSE cells in culture involved staining by immunofluorescence for laminin and various collagens to determine if HOSE cells produce ECM.

Both early and late passage HOSE cells stained positively for laminin (figure 12) as compared to serum controls. Laminin was only present in the cytoplasm of HOSE cultures even where cultures were crowded. As positive controls, ROSE 239 cells stained intensely for laminin, in both the cytoplasm and in the extracellular matrix as has already been reported (Auersperg et al. '91b) while HUDF failed to stain for laminin.

Both early and late passage HOSE cells in culture stained more intensely with anti-collagen type IV antibodies than normal serum controls. Collagen type IV staining appeared as some staining over the cell bodies, but also as punctate material at the edges of cells, often forming an outline of the cells (figure 13). HOSE cells of both early and late passage keratin-positve cultures also stained more intensely for the stromal collagens types I and III, than their respective serum controls. Collagen type III was present as fine fibrillar material at the cell edges and as punctate and globular staining over the cell bodies (figure 14). Collagen type I stained as punctate material seen within the cell boundary and also occuring beyond the cell boundary

Figure 12. HOSE Cells Stained for Laminin

HOSE cells stain positively for laminin (A,B) compared to non-immune serum controls (C,D). A,C Immunofluorescence microscopy. B,D Phase microscopy of fields shown in A,C respectively. X400.

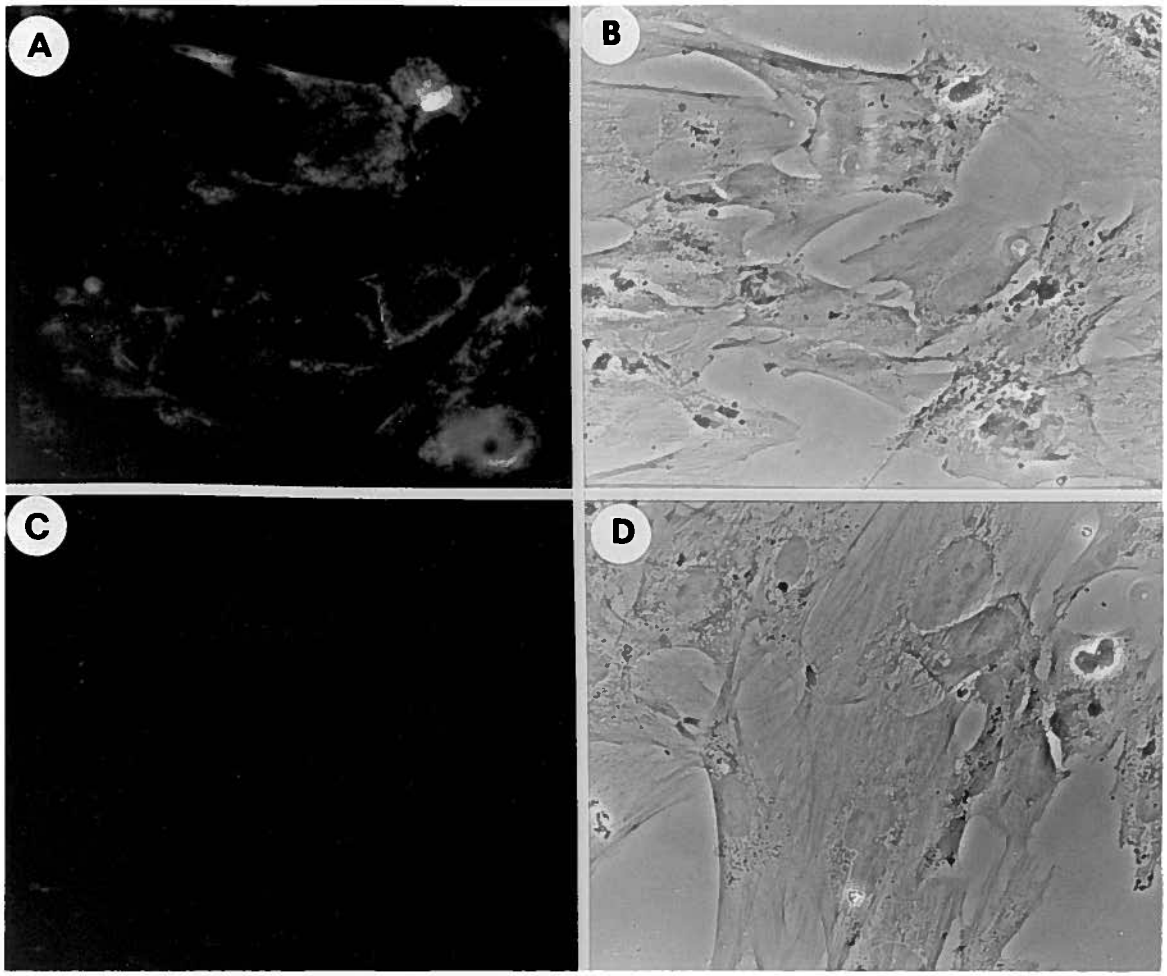

A
B
C
D

Figure 13. HOSE Cells Stained for Collagen Type IV

HOSE cells stain positively for collagen type IV as punctate material outlining the cells (A,B) compared to serum controls (C,D). A,C Immunofluorescence microscopy. B,D Phase microscopy of fields shown in A,C respectively. X575.

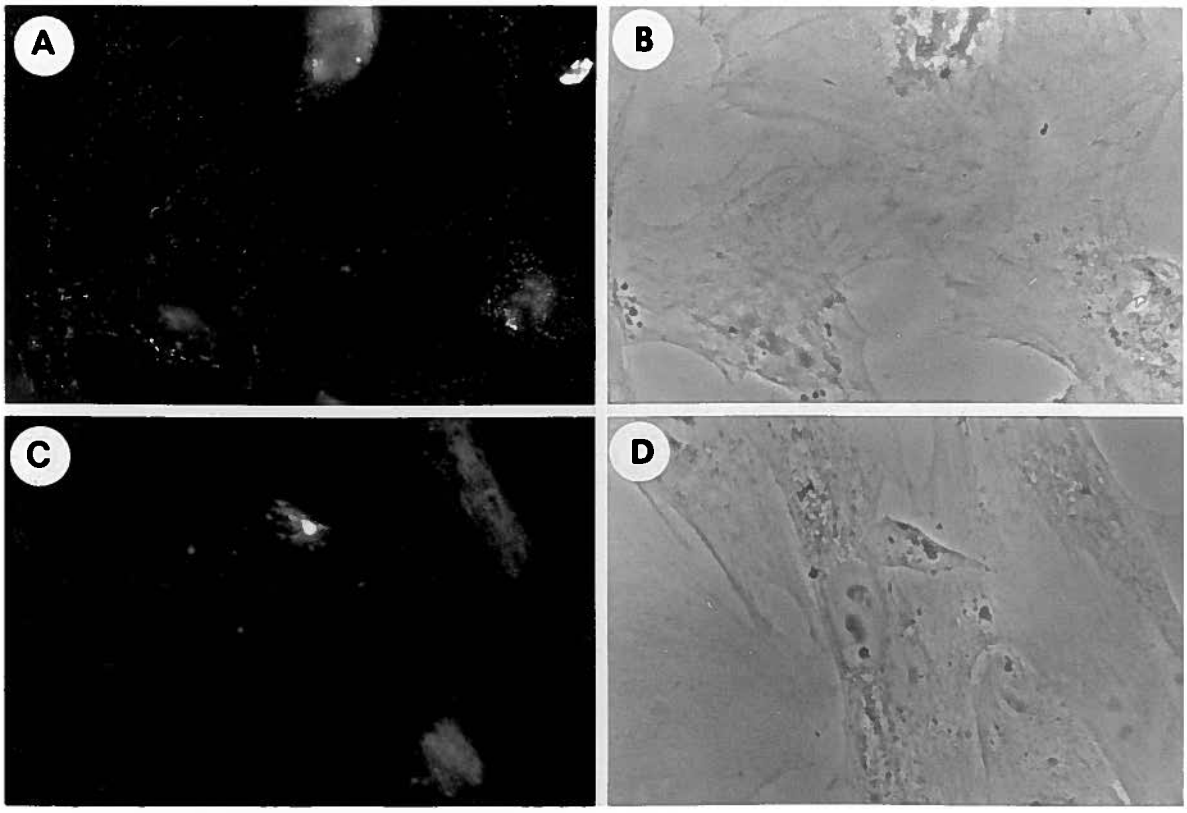
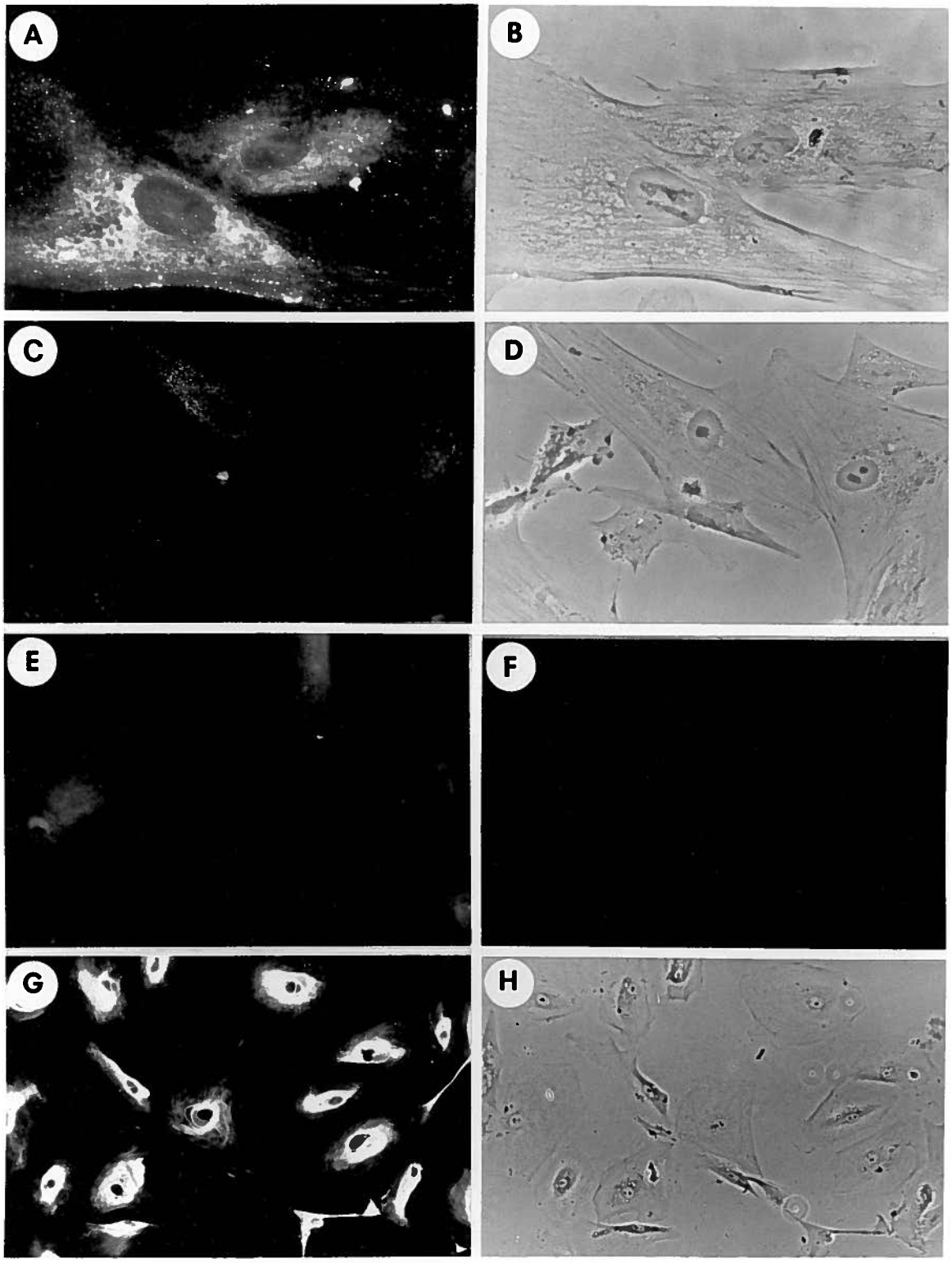

A
B
C
D

Figure 14. HOSE Cells Stained for Collagen Types I and III

HOSE cells stain more strongly for collagen types I (A,B) and III (C,D) than their respective serum controls (E,F). The epithelial nature of these HOSE cultures producing stromal collagen types I and III was confirmed by the presence of keratin in parallel cultures (G,H). A,C,E-G, Immunofluorescence microscopy. B,D,H, Phase microscopy. A,B,E, X575. C,D,F, X300. G,H, X100.


A
B
C
D
E
F
G
H

suggesting extracellular deposition. These results were consistent not only among HOSE cases, but also within HOSE cases. Gar HOSE in both p4 and p9 stained positively with anti-collagen antibodies against collagens types I and III. ROSE 239 and HUDF controls also stained more strongly for collagen types I and III than their serum controls. These results indicate that HOSE cells in culture clearly demonstrate the ability to deposit extracellular matrix components and that these components are of both basement membrane matrix and stromal matrix.

C. Ovarian-Derived ECM

(1.) What Are the Characteristics of the Ovarian-Derived ROSE 199-ECM?

A spontaneously-derived immortalized, but non-tumorigenic rat ovarian surface epithelial cell line, ROSE 199, was used as the material from which to isolate and characterize an ovarian-derived ECM. Throughout this study, the growth patterns of the cell lines ROSE 199 and their subclones, E11/A4 & C8/D10 remained constant and resembled those reported from the time of inital explantation (Adams & Auersperg '85 and unpublished results). When ROSE 199 cultures became crowded, they multilayered and formed ridged structures (figure 15A). SEM and TEM clearly demonstrated the multilayered nature of ROSE 199 cultures and the ECM interspersed between the cell layers (figure 15B, C). Due to the abundant ECM produced by ROSE 199 cultures, ROSE 199 cells were used to prepare and characterize an ovarian-derived ECM.

Treatment of ROSE 199 cultures with ammonium hydroxide (AH), deoxycholate (DC), or freeze thaws (FT) resulted in the gradual and progressive rounding and lysis of cells. As the cells lysed, the extracellular material became evident. ROSE 199-ECM was detected as fine wispy material one week post-confluence and became abundant with prolonged culture (figure 16). All ROSE 199-ECMs appeared similar when prepared by treatment with either AH or DC or FT, except for the presence of residual nuclei in FT preparations (figure 16). SEM depicted the ROSE 199-ECM as fibrous material interspersed with globular material (figure 17A). ECMs prepared by all the of three treatments examined at the ultrastructural level demonstrated striated fibrillar collagenous material of comparable size and shape, averaging 30 nm in diameter (figure 17B,C).

Sections of ROSE 199 cultures and ECMs of all types of preparations were stained histochemically for hematoxylin and eosin, Masson for collagen, and silver stain for reticulin. ROSE 199-ECMs were also stained for non-sulphated and sulphated sugars

Figure 15. ROSE 199 Cultures

Crowded ROSE 199 cultures become multilayered and form ridges (A). SEM further illustrates the multilayered nature of ROSE 199 cultures and that fibrillar ECM is deposited between the cell layers (B). TEM also illustrates the fibrillar matrix deposited in ROSE 199 cultures (C, From Adams & Auersperg '84). A, Phase microscopy, X40. B, SEM, X4000. C, TEM, X22,000.

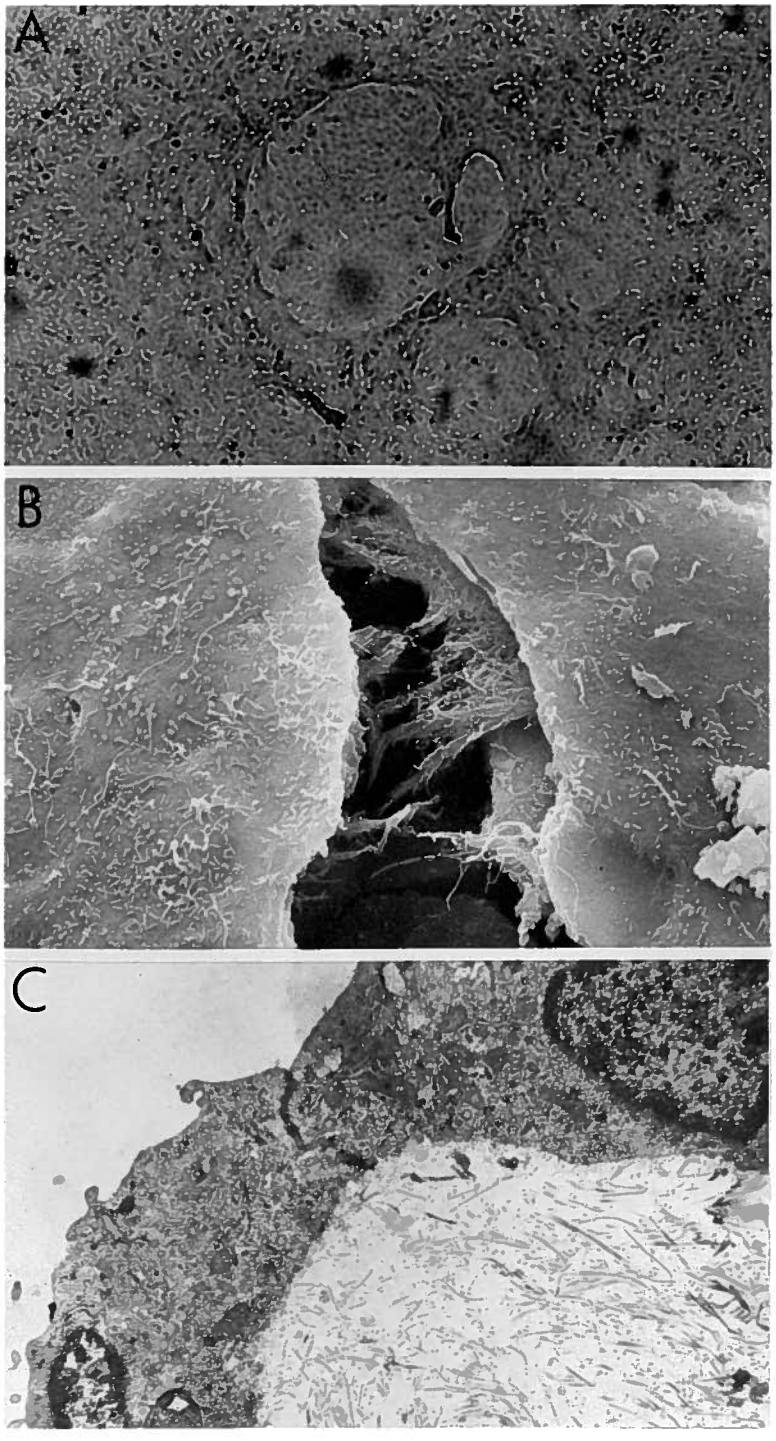

A
B
C

Figure 16. Light Microscopic Appearance of ROSE 199-ECM

In situ appearance of ROSE 199-ECMs prepared by treatment of ROSE 199 cultures with AH (A), DC (B), and FT (C). Toluidine blue. X250.

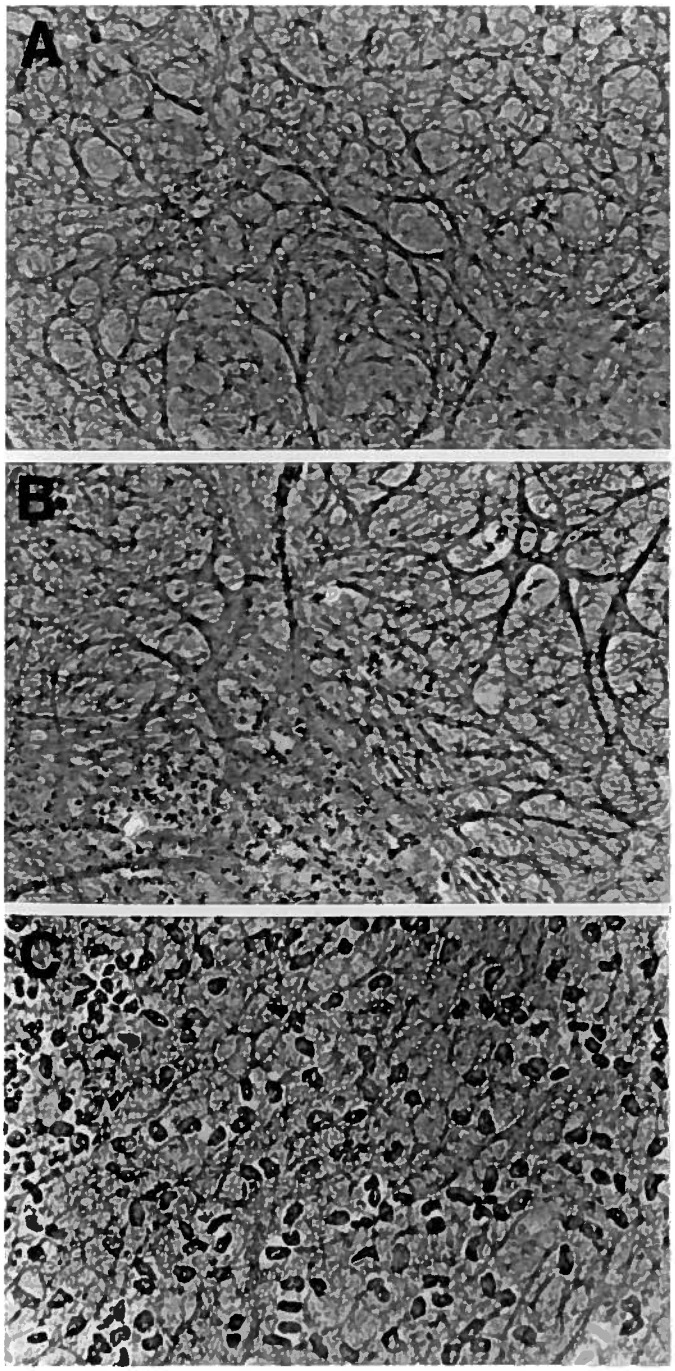
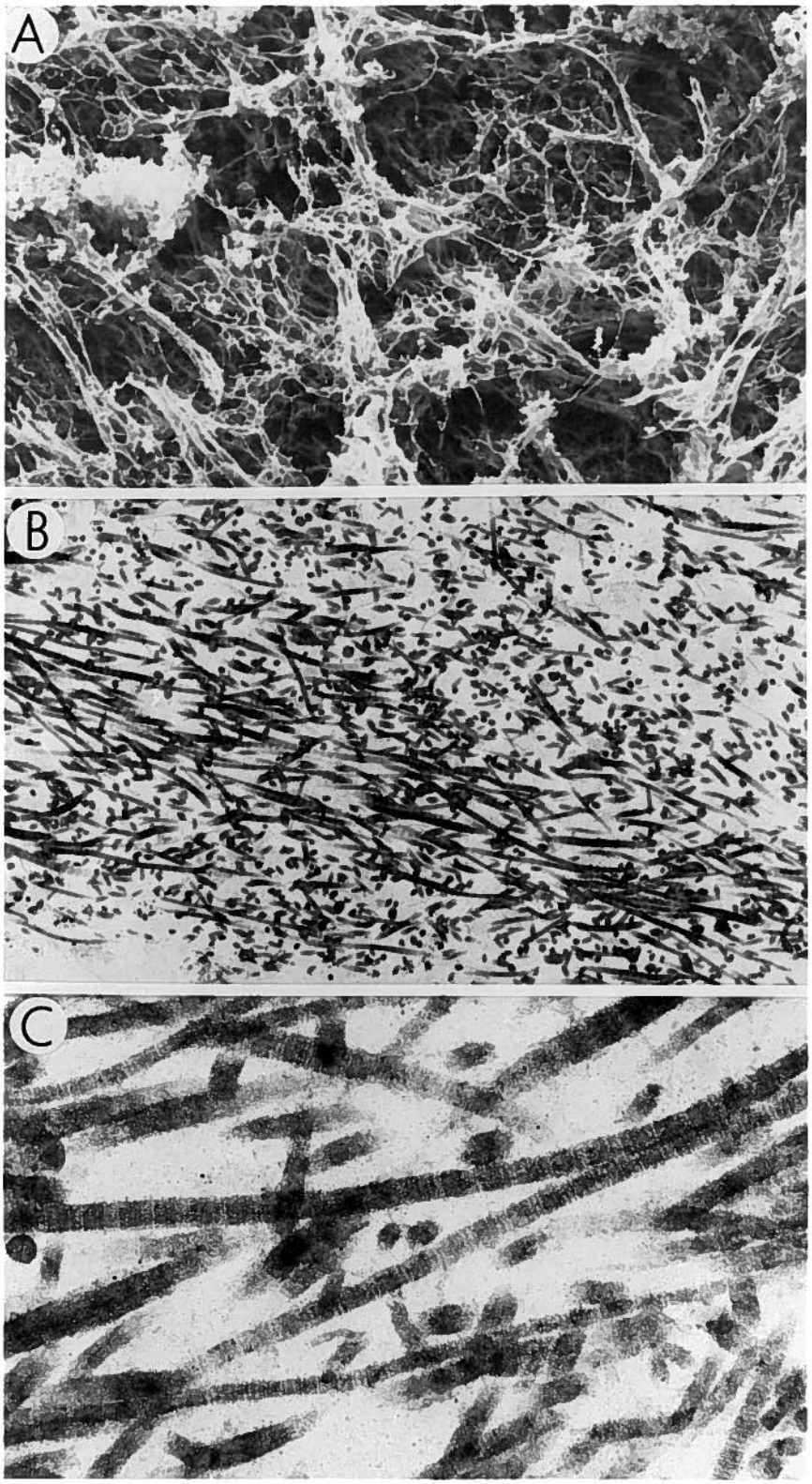

A
B
C

Figure 17. Ultrastructural Appearance of ROSE 199-ECM

SEM depicts ROSE 199-ECM as predominantly fibrillar with some globular material (A). TEM illustrates the striated fibers, characteristic of interstitial collagen, in ROSE 199-ECM (B,C). A, X5000. B, X12,000. C, X64,000.


A
B
C

with Alcian Blue at pH 2.5 and 1.0. The abundance of collagen in both ROSE 199 cultures and ECMs is seen as broad, uniform bands of staining with eosin and Masson stain (figure 18A-D). ROSE 199-ECMs also stain intensely for reticulin. The reticulin is evident as fine dark layers between cell layers in intact cultures (figure 18E, arrows), but somewhat more dispersed in ECM preparations (figure 18F). ROSE 199-ECMs were found to contain acidic sugars by positive staining with Alcian Blue at pH 2.5 (figure 18G) and that, at least, some of the acidic sugars are sulphated sugars as illustrated by an increased intensity of staining with Alcian Blue at pH 1.0 (figure 18H). ROSE 199-ECMs stained in situ for laminin and collagen type I by immunofluorescence stained more intensely than their respective serum controls (figures 19 & 21) demonstrating that ROSE 199-ECM contains basement membrane as well as stromal matrix components. As controls, ROSE 239 cells stained positively for laminin and failed to stain when the primary antibody was replaced with normal serum. Similarly, HUDF stained positively for collagen type I, but failed to stain when the primary antibody was replaced with normal sheep serum. HUDF also failed to stain for laminin. ROSE 199-ECMs doubly stained for laminin and actin demonstrated the presence of laminin, but not actin suggesting the absence of cellular debris in the ECM preparation (figure 20). However, ECMs stained histologically with hematoxylin demonstrated the presence of nuclear material contaminating ECM preparations (figure 18B&D, arrowheads). The presence of DNA in ECM preparations was confirmed following staining with Hoechst dye and there was a noticable decrease in DNA following treatment of ECMs with DNAse suggesting that at least some of the residual cellular debris can be removed from ECM preparations (data not shown).

The presence of laminin, fibronectin and collagen types I and III in all three types of ROSE 199-ECMs was confirmed by western immunoblots. Laminin and fibronectin were present as single bands on nitrocellulose with molecular weights 400 KD and 250 KD respectively (figure 22A, B). Collagen type III was visible in western blots as a single band at 95KD in all ROSE 199 ECM preparations, while the major bands associated with collagen type I occured as a doublet at 95 and 92KD compared to the 97 and 95 KD of the α1 and α2 chains of human collagen type I standard (figure 22C). Collagen type IV was not detected by western immunoblotting in any of the ROSE 199-ECMs (figure 22D).

ECMs derived from six-week post-confluent 35 mm cultures produced roughly 0.9 mg of material from AH and FT preparations, but only 0.5 mg of ECM material from DC preparations as determined by dry weights.

Figure 18. Histochemical Comparison of ROSE 199 Cultures and ECM

Sections of ROSE 199 cultures (A,C,E) and ECM (B,D,F,G,H) were stained histochemically with hematoxylin and eosin (A,B), with Masson stain for collagen (C,D), and with silver stain for reticulin (E,F). ROSE 199 cultures stained for reticulin were prepared from plastic sections as this preparation clearly demonstrated the fine layers of reticulin in ROSE 199 cultures (arrows in E). ECMs stained uniformly for acidic sugars (G) and as distinct linear material for sulphated sugars (H) with Alcian Blue pH 2.5 and 1.0 respectively. Note the hematoxylin stained material in ECMs indicative of nuclear remnants (arrowheads in B,D). X250.

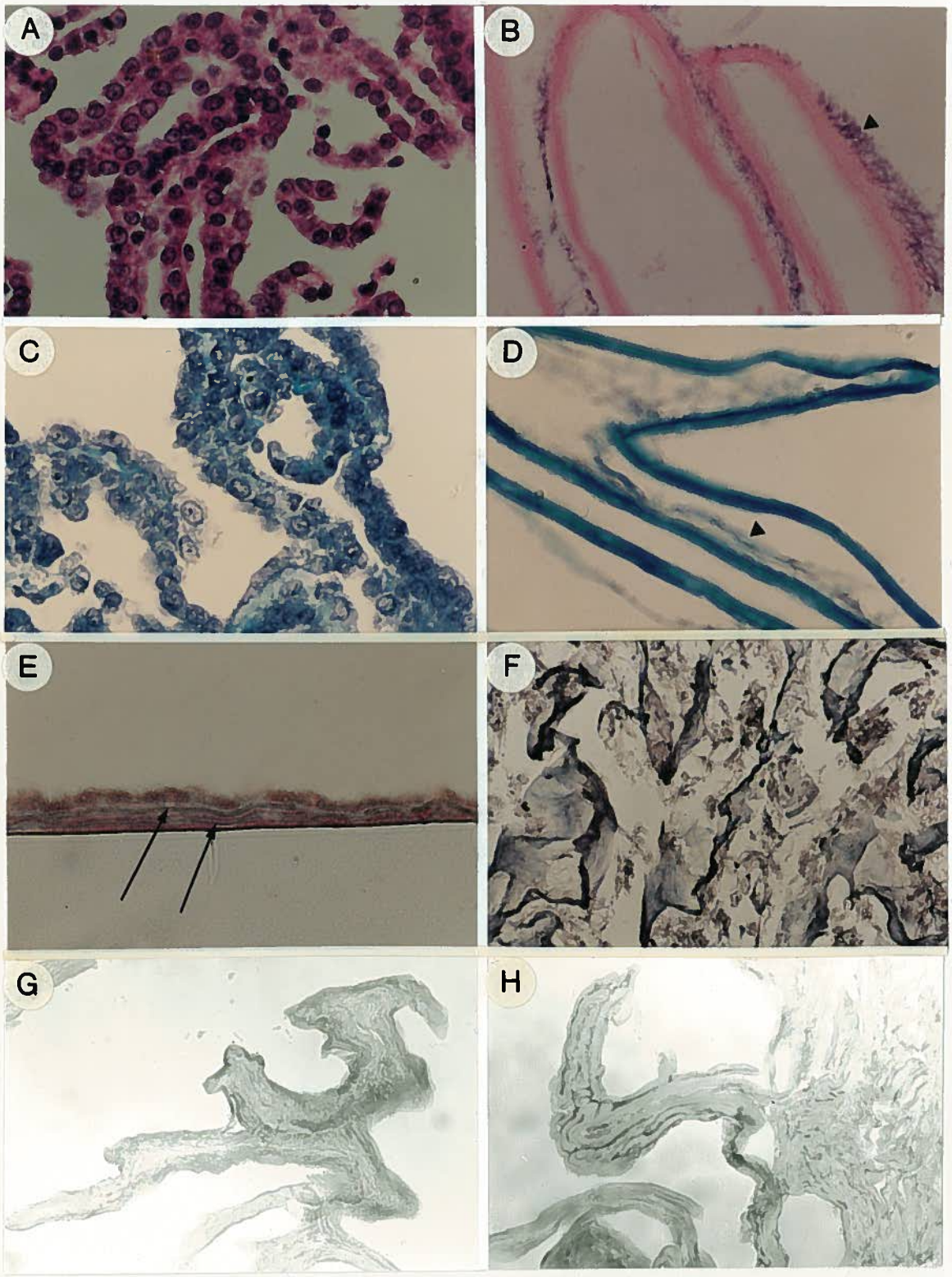

A
B
C
D
E
F
G
H

Figure 19. ROSE 199-ECM Stained for Laminin

ROSE 199-ECMs stained more intensely for laminin (A,B) than serum controls (C). A, Phase microscopy. B,C, Immunofluorescence microscopy. X300.

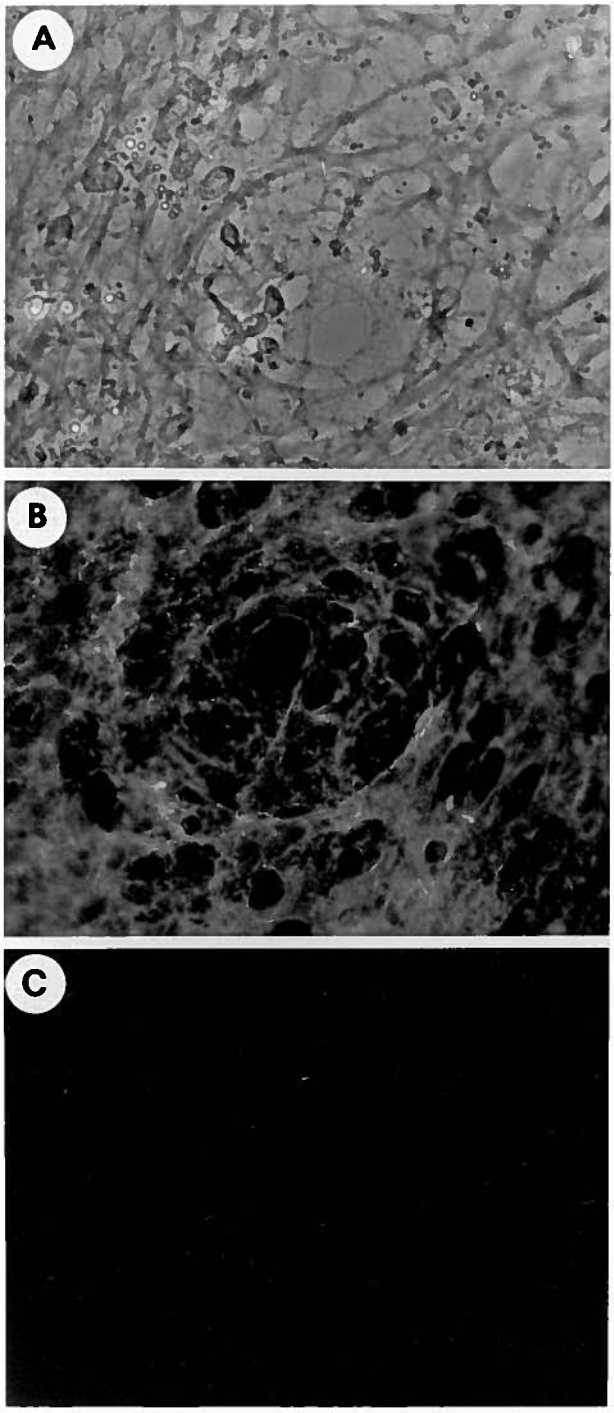

A
B
C

Figure 20. ROSE 199-ECM Doubly Stained for Laminin and Actin

Section of ROSE 199-ECM (A-C) stained for actin with NBD-phalloidin (B) and laminin (C). Controls sections of rat intestine were stained for actin with NBD-phalloidin (D), NBD-phalloidin neutralized with phallocidin (E), or treated with phallocidin (F). Control sections of rat intestine stained more intensely for laminin (G) than serum controls (H). A, Phase microscopy. B-H, Immunofluorescence microscopy. X200.

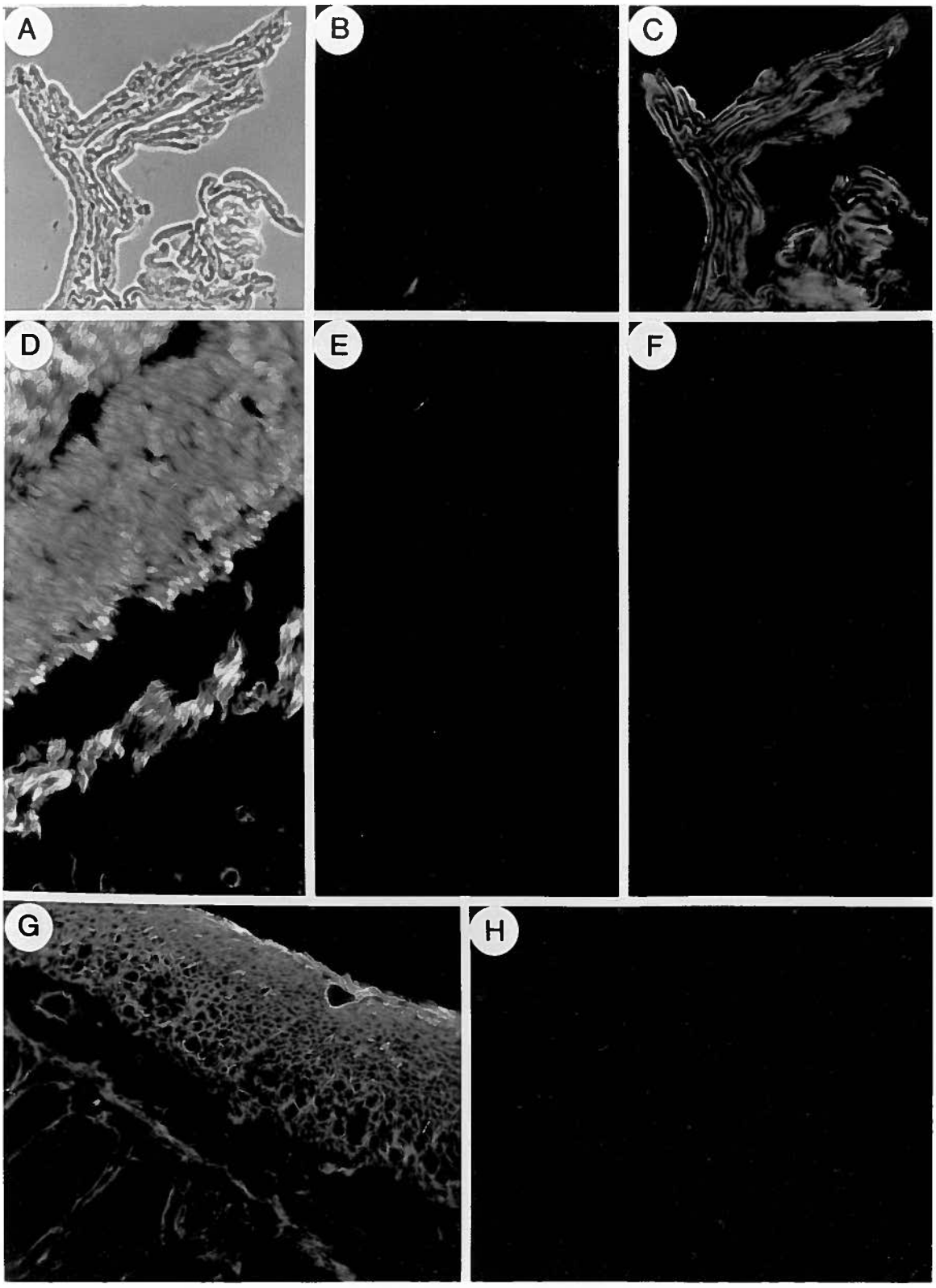

A
B
C
D
E
F
G
H

Figure 21. ROSE 199-ECM Stained for Collagen type I

ROSE 199-ECM stained more intensely for collagen type I (B) than serum controls (C). A, Same field as B, phase microscopy. B,C, Immunofluorescence microscopy. X325.

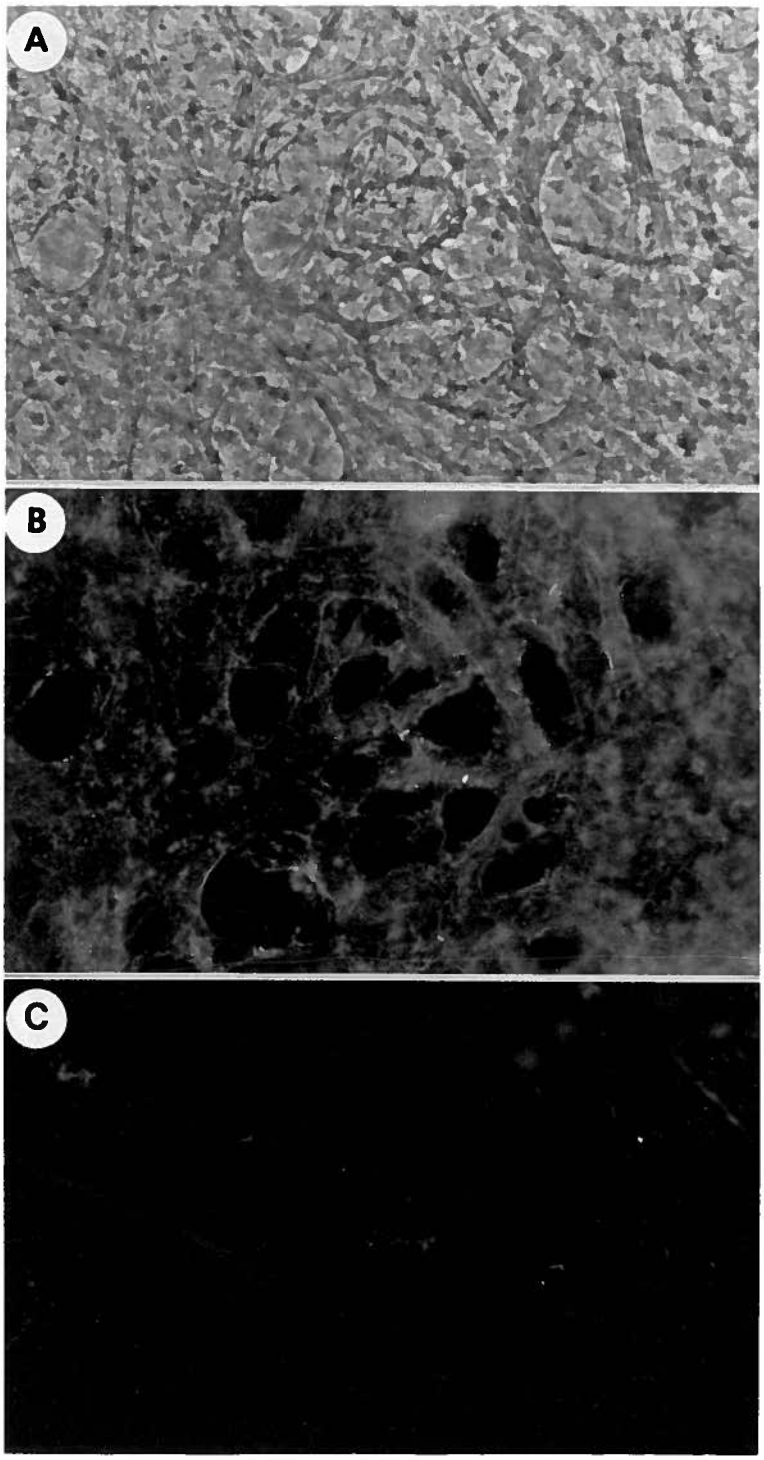
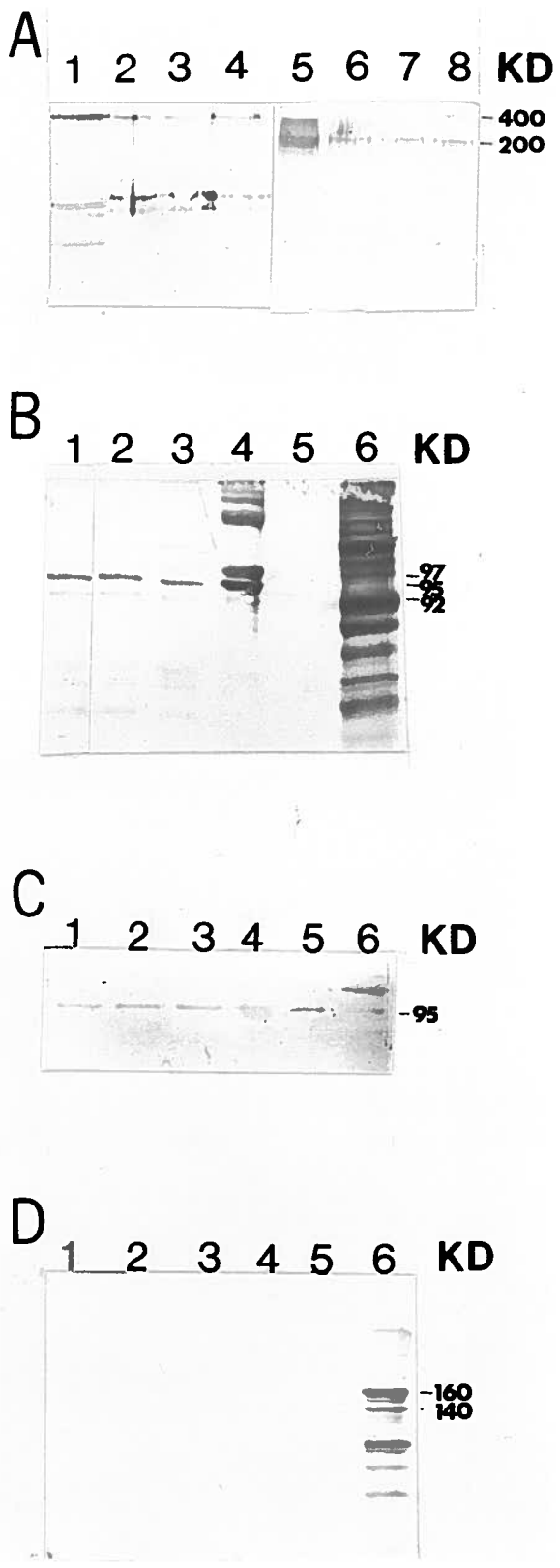

A
B
C

Figure 22. Western Immunoblots for Laminin, Fibronectin, and Collagen

ROSE 199-ECMs from all three preparative treatments were subjected to SDS-PAGE followed by transfer to nitrocellulose. The nitrocellulose was blotted for laminin, fibronectin, and collagen types I, III, and IV. (A), lane 1, laminin standard; lanes 2&6, AH-ROSE 199-ECM; lanes 3&7, DC-ROSE 199-ECM; lanes 4&8, FT-ROSE 199-ECM; lane 5, fibronectin standard. Lanes 1-4 were immunoblotted for laminin and lanes 5-8 were immunoblotted for fibronectin. (B-D), lane 1, AH-ROSE 199-ECM; lane 2, DC-ROSE 199-ECM; lane 3, FT-ROSE 199-ECM; lane 4, collagen type I standard; lane 5, collagen type III standard; lane 6, collagen type IV standard. Nitrocellulose sheets were immunoblotted for collagen types I (B), III (C), and IV (D). All ROSE 199-ECMs contain laminin, fibronectin, and collagen types I and III, but they lack collagen type IV.


A
1 2 3 4 5 6 7 8 KD
400
200
B
1 2 3 4 5 6 KD
97
92
C
1 2 3 4 5 6 KD
95
D
1 2 3 4 5 6 KD
160
140

(2.) Does ROSE 199-ECM Have Biological Activity?

Biological activity of ROSE 199-ECM was demonstrated by the adhesion, spreading, and replication of several cell types on ROSE 199-ECM as compared to plastic. As shown in figure 23, three catagories of activity were found, as determined by enhanced cellular adhesion to ROSE 199-ECM, by liquid scintillography assay: 1) all ECM preparations enhanced adhesion (ROSE 199, HT-29); 2) AH-ECM and FT-ECM were superior to DC-ECM and plastic ($CAOV_3$, melanoma 6278); 3) all ECM preparations resembled plastic (HUDF). Cell counts and morphological examination of cellular adhesion to ROSE 199-ECM were in agreement with these results. However, there were no differences in morphological appearance between plastic and ECM among the cell lines tested. Further, ROSE 199-ECM supported the growth of some cell lines as indicated by the presence of mitotic figures in cultures maintained on ROSE 199-ECM (figure 24). ROSE 199 cells plated onto ROSE 199-ECM adhered, spread, and grew over the entire ECM so that these cultures soon resembled normal ROSE 199 cultures which had never been depleted of cells.

To determine if the co-production of basement membrane and stromal matrix components is a feature of ROSE 199 cells and not due do the presence of a mixed, heteogeneous population of stromal and epithelial cells, two ROSE 199 subclones, E11/A4 and C8/D10 were examined. Both clones produce ECM that contains both basement membrane and stromal components, like their parental line. The ECM derived from the clones is also biologically active as it also supports the adhesion, spreading and growth of HUDF and ROSE 199 cells (data not shown).

(3.) What is the Response of HOSE Cells to ROSE 199-ECM?

HOSE atttach and spread on the ROSE 199-ECM, but they do not form a confluent, intact cuboidal epithelium like HOSE *in vivo*. They remain as single cells dispersed throughout the ROSE 199 matrix (figure 25).

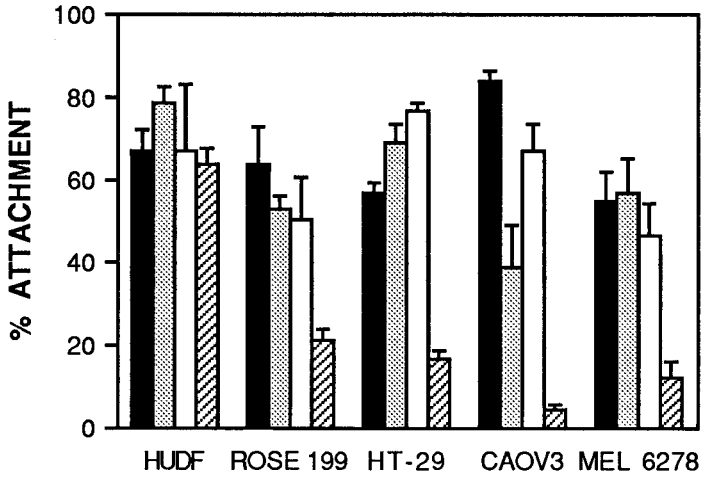
(4). HOSE Organoids: An Ovarian Tissue Culture Model

To prepare organoids (figure 26), plastic dishes were coated with a nonadhesive substance, 1% agarose, to encourage cells to interact with the overlying substratum rather than underlying plastic. HOSE cells and ROSE 199 cells plated onto agarose did not adhere to the surface and remained as floating aggregates. When transferred to plastic after 24 hr, cells remained viable as indicated by their ability to adhere to and spread onto the plastic (data not shown).

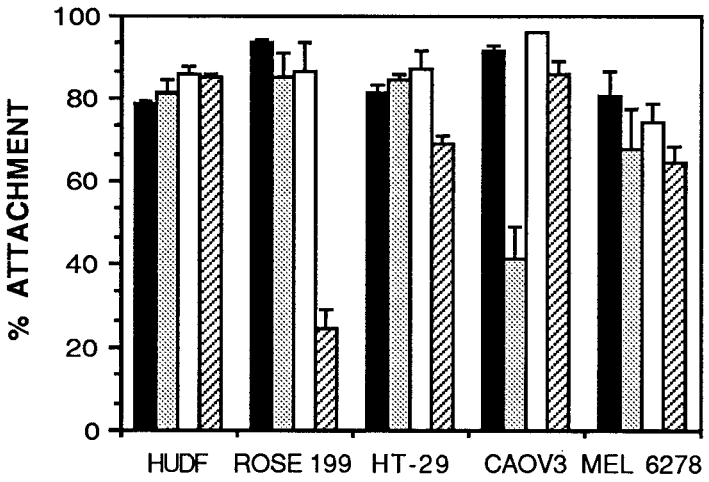
Figure 23. Cellular Adhesion to ROSE 199-ECM

Biological activity of ROSE 199-ECMs was shown by determining the percentage of HUDF, ROSE 199, human HT-29 colon carcimona, human CAOV3 ovarian carcimona, and human melanoma 6278 cells which attached to ROSE 199-ECMs compared to plastic. Early adhesion was measured 10 minutes, intermediate adhesion was measured between 20-30 minutes, and late adhesion was measured, depending upon the cell line, between 45 minutes to 8 hours after plating cells onto ROSE 199-ECMs and plastic. Cellular adhesion on plastic (striped bars) was compared with cells plated on AH-derived (closed bars), DC-derived (dotted bars), and FT-derived (open bars) ROSE 199-ECMs. All ECM preparations enhanced adhesion of ROSE 199 and HT-29 cells compared to plastic, AH-ECM and FT-ECM enhanced adhesion of CAOV3 and melanoma cells compared to DC-ECM and plastic, and HUDF adhesion to all ECMs was not different from adhesion to plastic.

EARLY ADHESION (10 Min)



INTERMEDIATE ADHESION (20-30 Min)



LATE ADHESION (45 Min - 8 Hr)

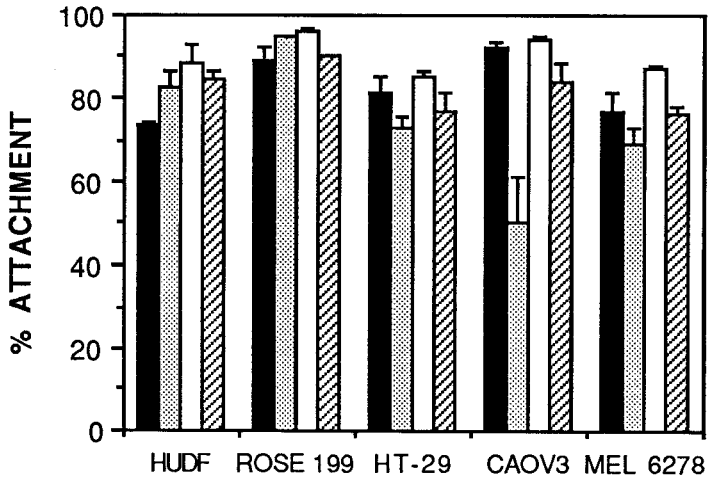


Figure 24. Cellular Spreading and Growth on ROSE 199-ECM

The attachment, spreading, and growth of several cell lines was examined on ROSE 199-ECM. (A), HUDF attached to ROSE 199-ECM and became dispersed throughout the matrix. (B), HT-29 cells attached and formed epithelial monolayers on ROSE 199-ECM. (C), CAOV3 cells also attached, spread, and grew as indicated by the presence of mitotic figures in the cultures (arrows). A&C, Cresyl violet, X300. B, phase microscopy, X200.

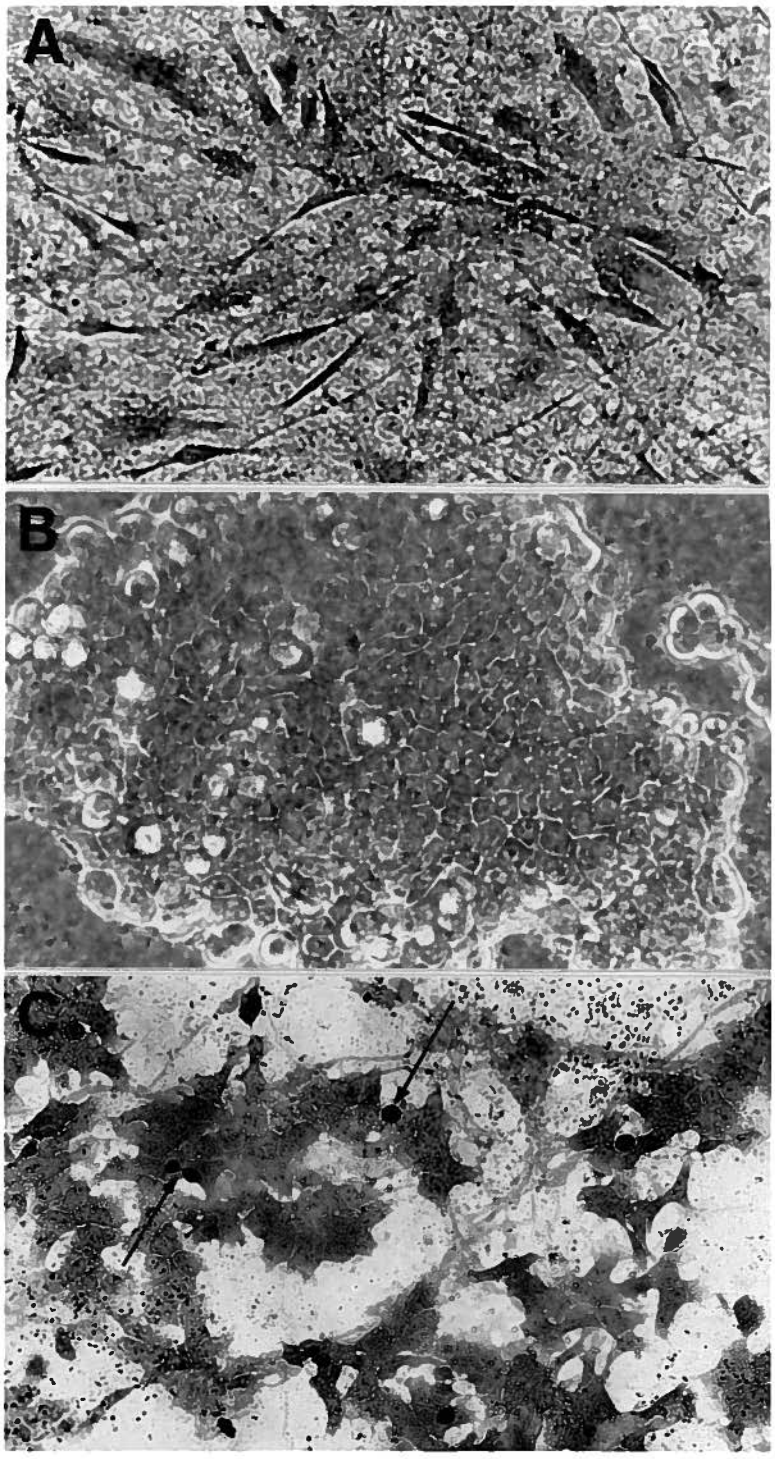

A
B
C

Figure 25. HOSE Cells on ROSE 199-ECM

While HOSE cells formed epithelial monolayers on plastic (A) they dispersed as single epithelial cells when plated on ROSE 199-ECM (B). Cresyl violet. X185.

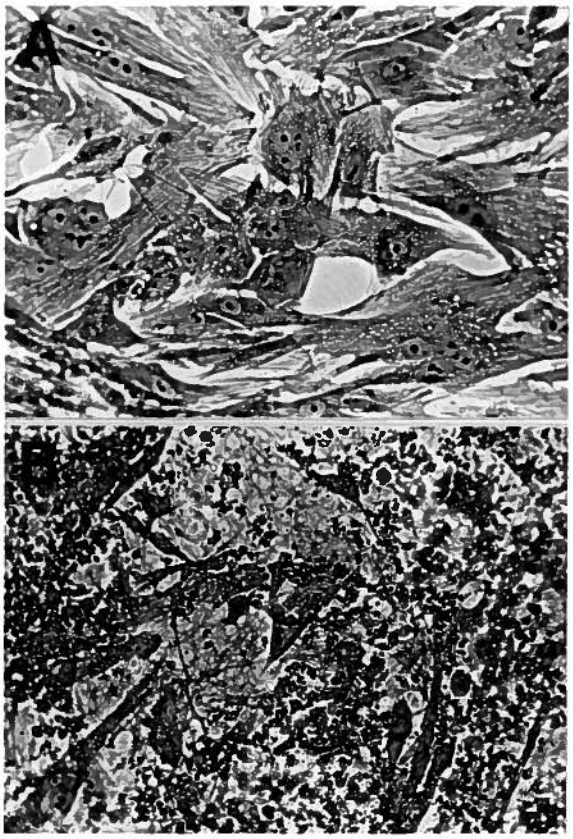
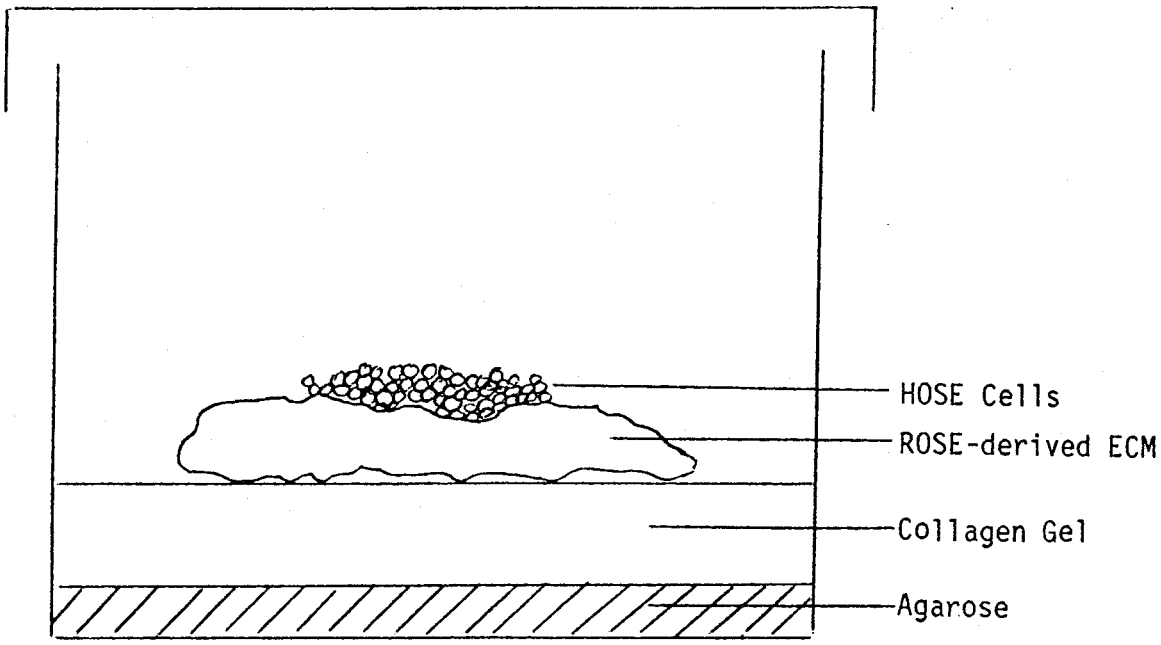


Figure 26. Organoid - Schematic Set up

A diagramatic cross-section through a culture dish illustrates the components of an organoid. Cultures dishes are first layered with agarose to prevent HOSE cells from attaching to the underlying plastic. A layer of rat tail tendon-derived collagen gel is then layered over the agarose and allowed to dry overnight. The collagen gel is primed for one hour with medium, then a ROSE 199-ECM is placed on top of the collagen gel, and finally, a suspension of HOSE cells is placed on top of the ROSE 199-ECM.


HOSE Cells
ROSE-derived ECM
Collagen Gel
Agarose

When HOSE cells were placed on ROSE 199-ECM coated collagen gels, the HOSE cells spread over the entire mixture of ROSE 199-ECM and unrimmed collagen gel and contracted the mixture into small organoids (figure 27), demonstrating their ability to physically remodel ECM. Figure 27A illustrates three organoids in varying degrees of contraction and a control well without HOSE cells. Cross-sections of organoids revealed that HOSE cells envelop the organoid, that the majority of HOSE cells remain on the surface of the organoids with only a few scattered cells found within the stroma, and that HOSE cells in organoids remain epithelial as they retain keratin (figure 27B). Table 5 summarizes the number of organoids prepared. Cells from three cases, Syk p2, Hack p1, Any p6, failed to contract organoids. Contraction appeared to occur only when ROSE 199-ECMs were in direct contact with and anchored to the collagen gels. In a few instances (Any p6, Pere p3, Sam p3, Solo p5) ROSE 199 ECMs remained floating when the organoids were prepared. However, they generally contracted if these floating ROSE 199-ECMs were anchored into the collagen gels, usually by placing a cloning cylinder on top of the ROSE 199-ECM for 24 hr. The ROSE 199-ECMs used in the organoids set up with Any HOSE cells p6 failed to attach to the collagen gels even following attempts to remedy this situation.

The degree to which HOSE cells contracted organoids varied among cases. However, the degree of contraction was directly related to the number of HOSE cells seeded per organoid (figure 28A & table 5). For example, HOSE cells seeded at densities less than 50,000 cell per organoid generally did not contract organoids maximally. The addition of EGF/HC or reduced serum-containing medium did not affect the degree to which HOSE cells contracted organoids (figure 28B). However, the inclusion of 3T3 fibroblasts within the collagen gel portion of the organoid markedly increased the overall degree of organoid contraction (figure 28B).

D.) HOSE-ECM Interactions

(1.) What is the Morphologic Response of HOSE Cells to Substrata?

Having demonstrated that HOSE cells can remodel ECM by production of ECM components and by physically modifying (contracting) ECM it was decided to expand upon HOSE-matrix interactions as a means to study HOSE pleomorphism and morphogenesis. The interactions between HOSE cells and simple substrata were examined because the complexity of the organoid model made such analyses difficult. To this end, HOSE cells were cultured on various substrata commonly used in tissue

Figure 27. Contraction into Organoids

When HOSE cells are plated onto ROSE 199-ECM coated collagen gels, the cells extend over both matrices and contract them into organoids. A, photograph of three HOSE organoids demonstrating different degrees of organoid contraction along with one control well devoid of cells (lower left well). The upper right well illustrates a maximally contracted organoid. Note that in the lower right well the cells are clearly contracting the outer, uniform, opaque collagen gel (arrowhead) about the centrally located dense ROSE 199-ECM (arrow). X2.5. B, a cross-section of an organoid stained immunocytochemically for keratin illustrates that the many of HOSE cells are on the surface of the organoid and retain keratin. X210.

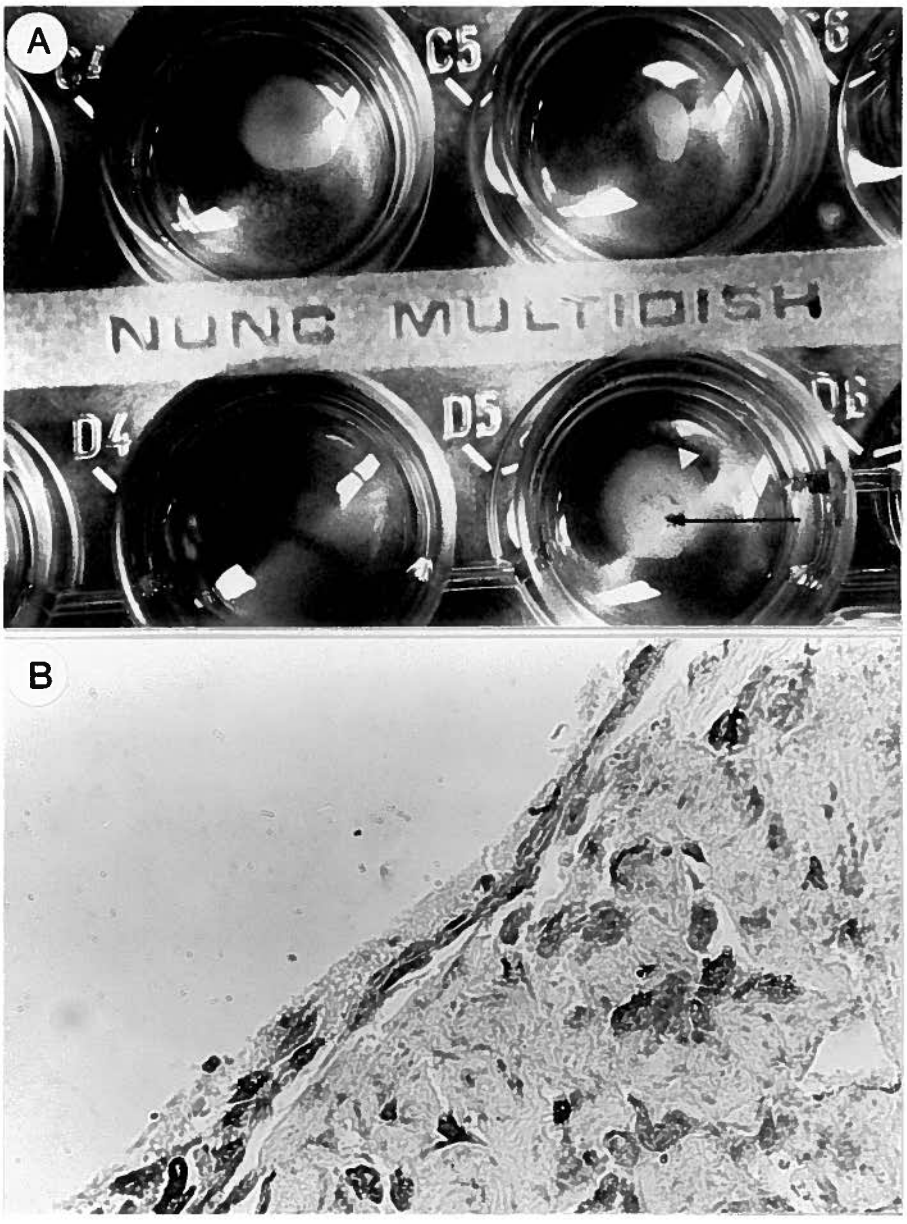
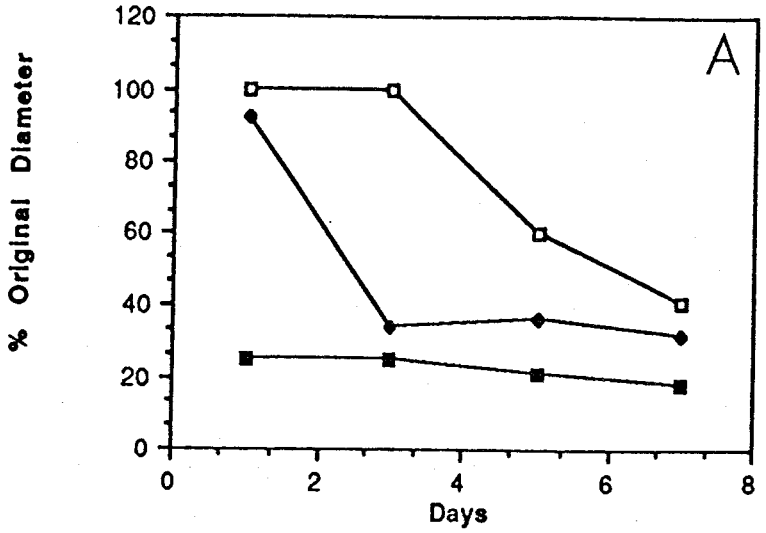

A
C5
NUNC MULTIDISH
D4
D5
B

Figure 28. Factors Affecting Organoid Contraction

The effect of cell number on the degree of organoid contraction was compared among organoids seeded with 5.7 x 10^4 cells (open squares), 1.4 x 10^5 cells (closed diamonds), and 3.0 x 10^5 cells (closed squares) (A). The degree of organoid contraction was compared among organoids maintained: in 199:105:15%FBS (open squares); in 199:105/15%FBS supplemented with 20 ng/ml EGF and 0.4 ng/ml hydrocortisone (closed diamonds); in 199:105/PC-1 (closed squares); with 3T3 fibroblasts (open diamonds) (B). The degree of organoid contraction was related to cell number (A) and, of the culture variables examined, only the addition of fibroblasts enhanced organoid contraction (B).


A
% Original Diameter
120
100
80
60
40
20
0
0
2
4
6
8
Days

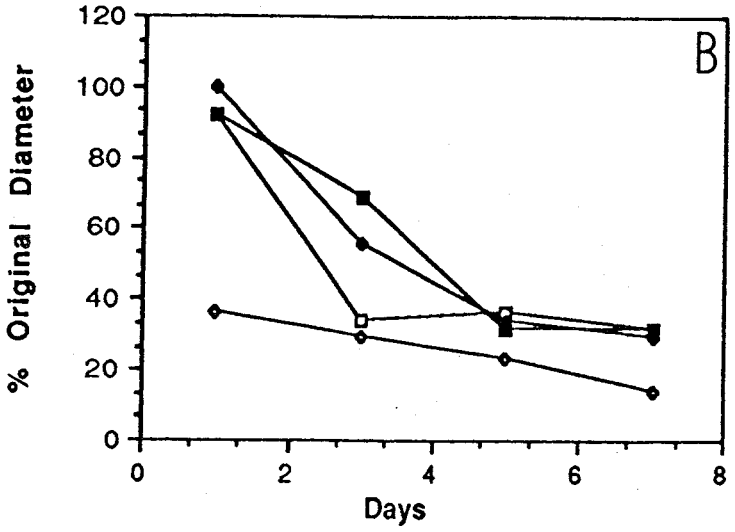

B
% Original Diameter
120
100
80
60
40
20
0
0
2
4
6
8
Days

Table 5. Contraction of HOSE Organoids

Case	# HOSE Cells Seeded per Orgaoid	# Contracted Orgaoids / # Organoids Set up	Degree of Contraction: % Original Diameter
Dose p4	200,000	4/4	10
Hack p1	100,000	0/3	100
Sub p3	50,000	3/3	75
Syk p2	50,000	0/2	100
Welch p4	80,000	2/2	13
Any p6	20,000	0/4[1]	100
Pere p3	100,000	2/3[2]	22
Sam p3	100,000	2/3[2]	15
Solo p5	100,000	5/7[3]	60
Natty p3	50,000	5/5	60
Natty p1	150,000	5/5	15
Gar p3	80,000	4/4	15
Gar p4	50,000	4/4[4]	30-42
Mehal p5	50,000	4/4[4]	23-35
Solo p5	170,000	4/4[4]	12-20
Van p5	250,000	4/4[4]	3-11
Pere p5	57,000	8/8[5]	25-37
Pere p8	140,000	8/8[5]	14-32
Pere p6	300,000	8/8[5]	3.5-5

1= All ROSE 199-ECMs failed to attach to the collagen gel, even following treatment with cloning cylinders.
2= One out of three ECMs was originally attached to the collagen gel, but following treatment with cloning cylinders, two out of three ROSE 199-ECMs were attached to the collagen gel.
3= Two out of seven ECMs were originally attached to the collagen gel, but following treatment with cloning cylinders, five out of seven ROSE 199-ECMs were attached to the collagen gel.
4= Each organoid was maintained under different conditions: 1) one in 199:105/15% FBS; 2) one in 199:105/15% FBS supplemented with EGF/HC; 3) one in 199:105/PC-1; and 4) one in 199:105/155 FBS with 3T3 fibroblasts added to the collagen gel component of the organoid. See also figure 28.
5= Same as in 4, except that each condition was set up in duplicate organoids.

culture which have been used elsewhere to stimulate and simulate cellular differentiation or morphogenesis (Emerman et al. '77, Hadley et al. '85, Taub et al. '90, Vigier et al. '89).

Within 2 days of culture on the various substrata HOSE cells from 12 different cases showed consistent changes in morphology (figure 29). On PL, PD, and FB, HOSE attached, spread, and formed flat cobblestone, epithelial monolayers. The response of HOSE to PD-ECM was no different from HOSE on plastic. While cells on fibrin were epithelial, they were more dispersed than on plastic. HOSE attached, spread, but assumed a spindle-shaped morphology on unrimmed collagen gels. HOSE plated upon PD-coated collagen gel demonstrated a phenotype identical to that on unrimmed collagen gel alone.

On Matrigel, HOSE cells formed aggregates that were joined to each other via branching structures (figure 29). Granular areas of Matrigel near HOSE cells suggested degradation of the Matrigel matrix by HOSE cells. This impression was supported by SEM and paraffin sections. SEM showed that HOSE aggregates became embedded and completely covered by Matrigel (figure 30A). HOSE cells cultured for one week or more on Matrigel invaded the Matrigel, and finally attached and spread on the underlying plastic (figure 30B). Cross-sections of HOSE on all substrata revealed that HOSE remained on the apical surface of all matrices (figure 31A) except Matrigel where nests of cells were found within and completely enveloped by the Matrigel (figure 31B,C). HOSE aggregates on Matrigel may represent a differentiated phenotype or an attempt at morphogenesis. The epithelial nature of HOSE was confirmed by the retention of keratin (figure 31C).

These results demonstrate specific morphological responses by HOSE cells to different culture conditions and may crudely mimic morphological changes in HOSE as they occur *in vivo*. The PD-ECM did not provide any advantage over plastic and so, after preliminary experiments, PD-ECM was eliminated from further experiments. Further, the results showed that HOSE cells in culture have the ability to remodel ECM not only by the contraction of organoids, but also by invasion of Matrigel.

(2.) Do Substrata Influence HOSE Cell Integrin Expression?

To determine if the different HOSE morphologies observed on the various substrata were related to the expression of different integrins, the pattern of specific integrins expressed at cell surfaces was examined. I^{125}-labeled HOSE cells from three separate cases (two low passage cobblestone cultures and one late passage atypical epithelial culture) were immunoprecipitated with several anti-integrin

Figure 29. Morphological Response of HOSE Cells to Substrata

Two days after plating, HOSE cells are epithelial on plastic (A) and fibrin clots (B) while they are spindle-shaped on collagen gels (C). On Matrigel, the cells form aggregates that are joined to each other via branching structures (D). Phase microscopy. X150.

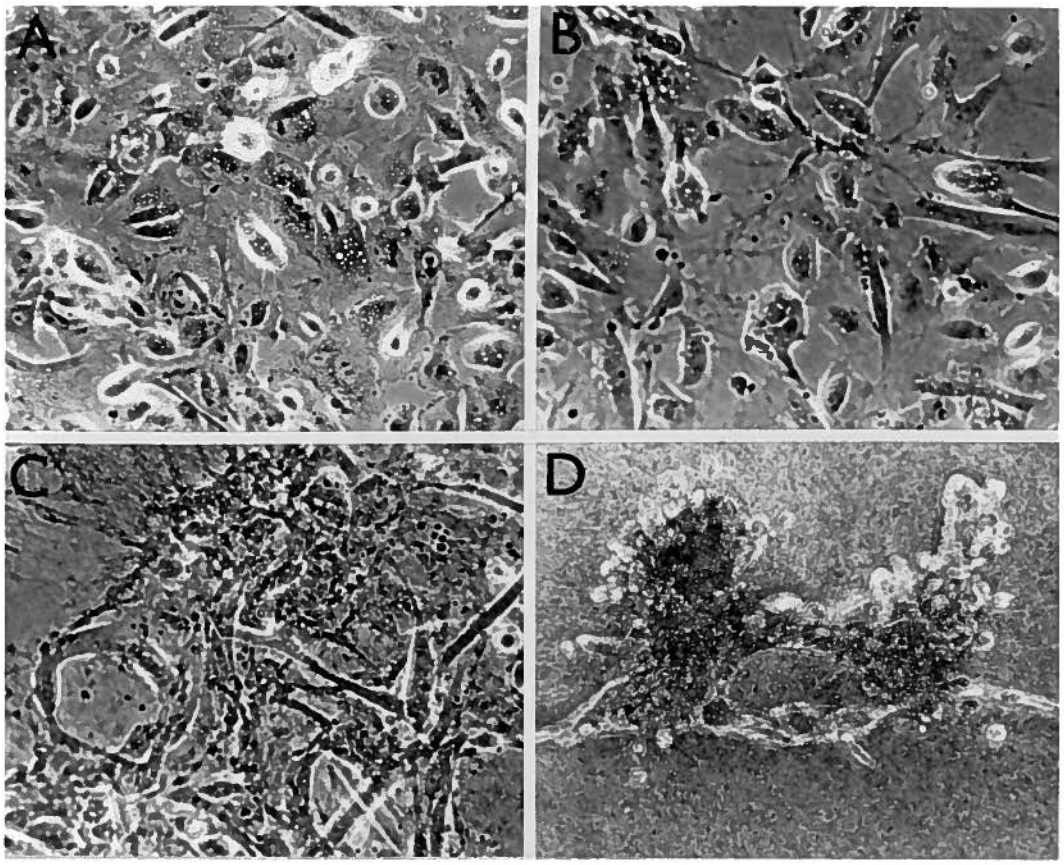

A
B
C
D

Figure 30. Invasion into Matrigel by HOSE Cells

A scanning electron micrograph demonstrates that HOSE cells maintained on Matrigel penetrate the matrix and become covered with it (A), X300. HOSE cells lyse and invade Matrigel and eventually adhere and spread onto the underlying plastic (area enclosed by three arrowheads in B). B, Phase microscopy, X100.

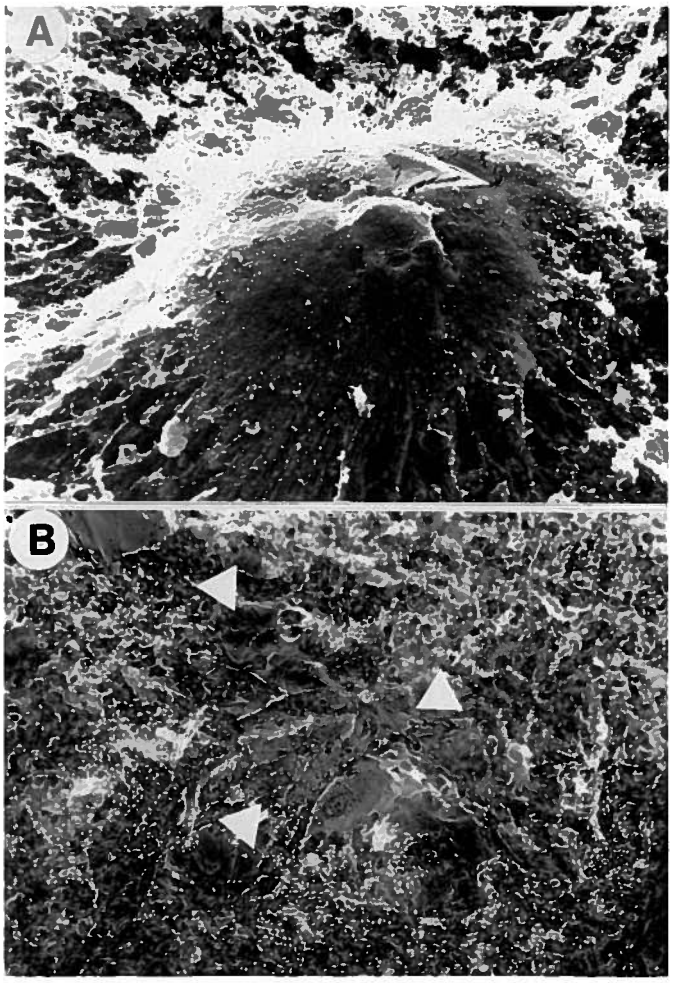
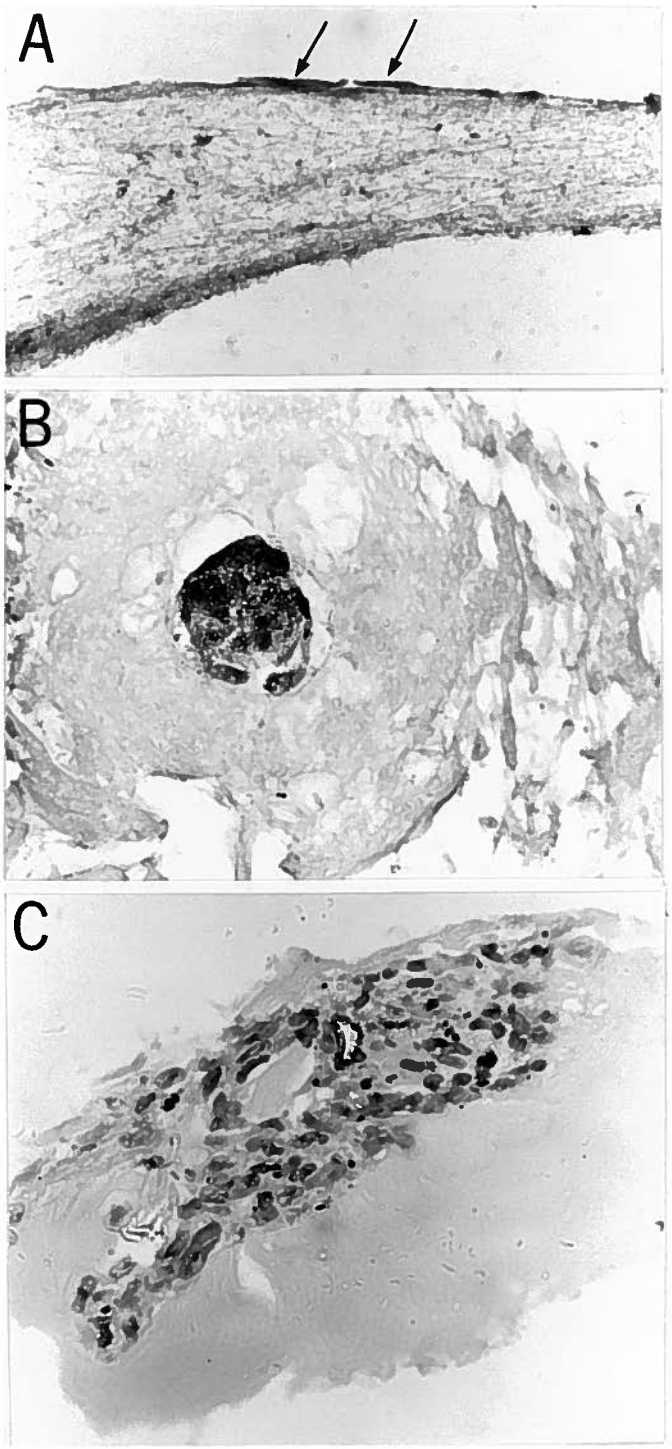

A
B

Figure 31. Cross-sections of HOSE on Collagen and Matrigel

Cross-sections of HOSE cells maintained on collagen gels indicate that the HOSE cells are flattened and remain on the apical surface of the gel (arrows in A) while HOSE cells invade Matrigel (B,C). In cross-section, HOSE cells in Matrigel appear as cell nests (B) or as cords of cells (C). The epithelial nature of HOSE is maintained as indicated by the retention of keratin (A & B). A & B, Immunocytochemically stained for keratin, X375. C, Hematoxylin and eosin, X250.


A
B
C

antibodies. Figure 32 illustrates a typical profile of integrins expressed by HOSE following 48 hours on plastic, collagen gels, fibrin clots, and Matrigel. The pattern of integrins was the same: 1.) among early and late passage HOSE cells; 2.) between cobblestone and atypical epithelial HOSE cells; 3.) within the same case, Van HOSE, tested at passage 2 and passage 4; and 4.) between HOSE cells and IOSEVan cells (data not shown). VLA-6 and β4, comprising a laminin receptor, were absent in all cases. With the exception of collagen gels, receptors for vitronectin (VNR), collagen, fibronectin, and laminin (VLA-2,-3, -5) were present in all cases, although VLA-2 was present in low amounts. Integrin expression was almost always greatest on plastic and least or absent on collagen gels.

To see if the diminished amount of integrins expressed on collagen at 48 hours was an indication of integrin receptor downregulation or merely artifactual, IOSEVan were plated on PL and COLL gels for 1-48 hours and immunoprecipated for VNR on three separate occasions. This integrin was chosen for time course study as it is one of the integrins that is consistently expressed at high levels on PL and low levels on COLL at 48 hours. If the downregulation was artefactual (i.e. due to the method of cell harvesting via collagenase treatment), then diminished levels of integrins would be present throughout 48 hours of culture on COLL. However, if the downregulation was real, then the cells, coming from parental stocks maintained on plastic and expressing high levels of integrins on their cell surfaces, should express high levels of integrins during the early intervals on COLL and then show a downregulation of receptors. Figure 33 shows that the downregulation of integrin expression on COLL does not appear to be an artefact. Levels of VNR remained relatively high on IOSEVan cells throughout 48 hours of culture on PL. On COLL, though, the level of VNR was high during the first 24 hours of culture a diminished expression clearly evident by 48 hours. While VNR expression appeared to increase during the first 24 hr on collagen gels, this upregulation of integrin expression was not found consistently.

(3.) Do Substrata Influence HOSE Cell Growth?

Growth curves indicated that the substrata could be divided into three groups: 1) those that supported intense HOSE cell growth (PL, PD); 2) those which supported little or no growth (COLL, PD+COLL, FB); 3) those where the cell number eventually decreased (EHS) (figure 34A).

Figure 32. Survey of HOSE Integrins

Immunoprecipitations of specific integrins from ^{125}I-labelled HOSE cells. Cells were labelled, extracted, and immunoprecipitated with the appropriate antibodies. Immunoprecipitates were analyzed by SDS-PAGE under non-reducing conditions and the bands were visualized by autoradiography. Lanes 1,5,9,13 are HOSE cells maintained on plastic; lanes 2,6,10,14 are HOSE cells maintained on collagen gels; lanes 3,7,11,15 are HOSE cells maintained on fibrin clots; lanes 4,8,12,16 are HOSE cells maintained on Matrigel. (A), lanes 1-4, HOSE cells immunoprecipitated with anti-human β_1 monoclonal antibody; lanes 5-8, HOSE cells immunoprecipitated with anti-human VLA-2 monoclonal antibody; lanes 9-12, HOSE cells immunoprecipitated with anti-human VLA-3 monoclonal antibody; lanes 13-16, HOSE cells immunoprecipitated with anti-human VLA-5 monoclonal antibody. (B), lanes 1-4, HOSE cells immunoprecipitated with anti-human vitronectin receptor monoclonal antibody; lanes 5-8, HOSE cells immunoprecipitated with anti-human β_4 antibody; lanes 9-12, HOSE cells immunoprecipitated with anti-human VLA-6 monoclonal antibody. Molecular weight standards are myosin (200KD), B-galactosidase (116KD), phosphorylase B (97KD), and albumin (67KD).

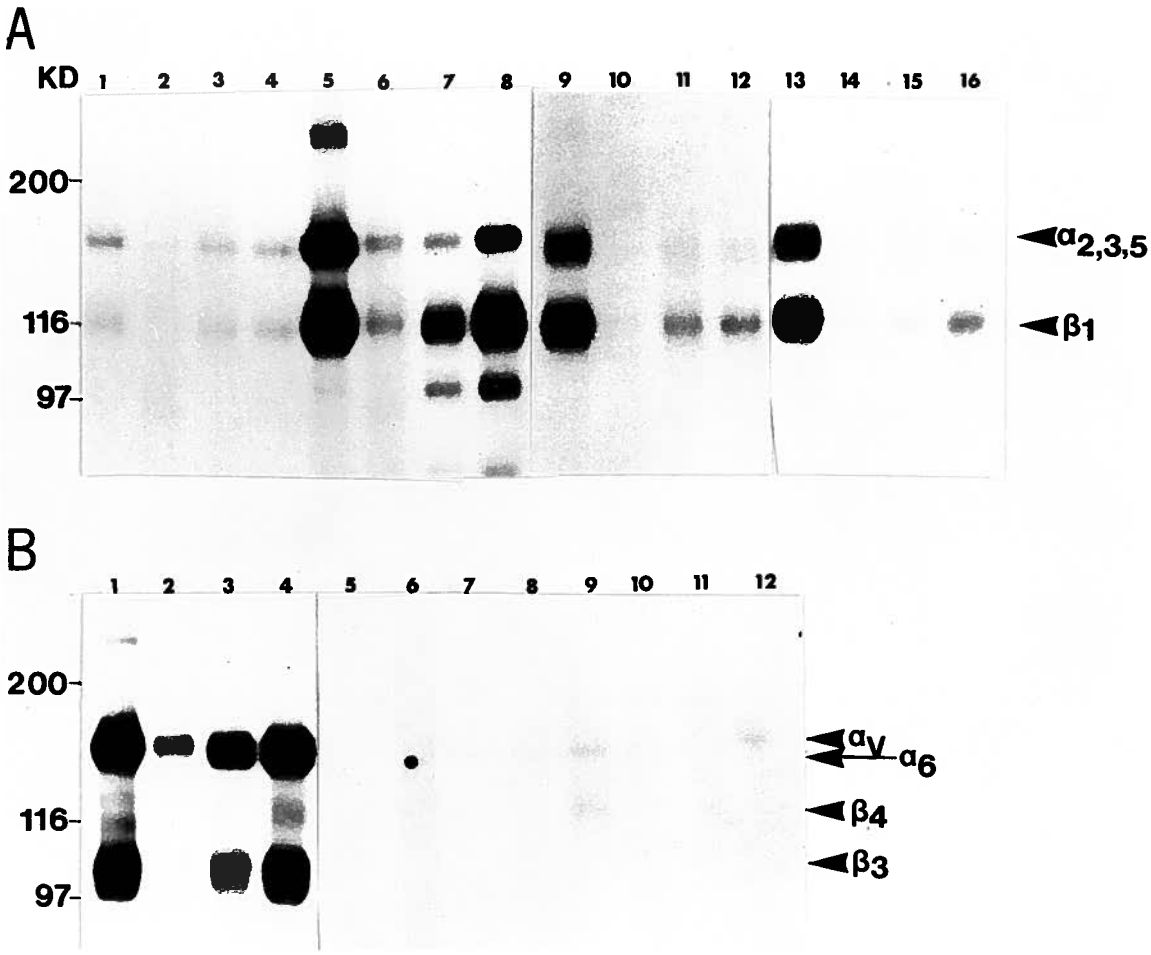

A
KD
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
200
116
97
α2,3,5
β1
B
1 2 3 4 5 6 7 8 9 10 11 12
200
116
97
αV
α6
β4
β3

Figure 33. Integrin Time Course

Immunoprecipitations of vitronectin receptor from I-^{125} labelled IOSEVan cells. Cells were labelled, extracted, and immunprecipitated with rabbit anti-vitronectin receptor antibody. Immunoprecipitates were analyzed by SDS-PAGE under non-reducing conditions and the bands were visualized by autoradiography. Lanes 1-4 are IOSEVan cells maintained on plastic and lanes 5-8 are IOSEVan cells maintained on collagen gels. Cells were immunoprecipitated with anti-vitronectin receptor antiserum following 1 hour (lanes 1 & 5), 4 hours (lanes 2 & 6), 12 hours (lanes 3 & 7), and 48 hours incubation on plastic and collagen gels. The molecular weight standards are the same as those described in figure 32.

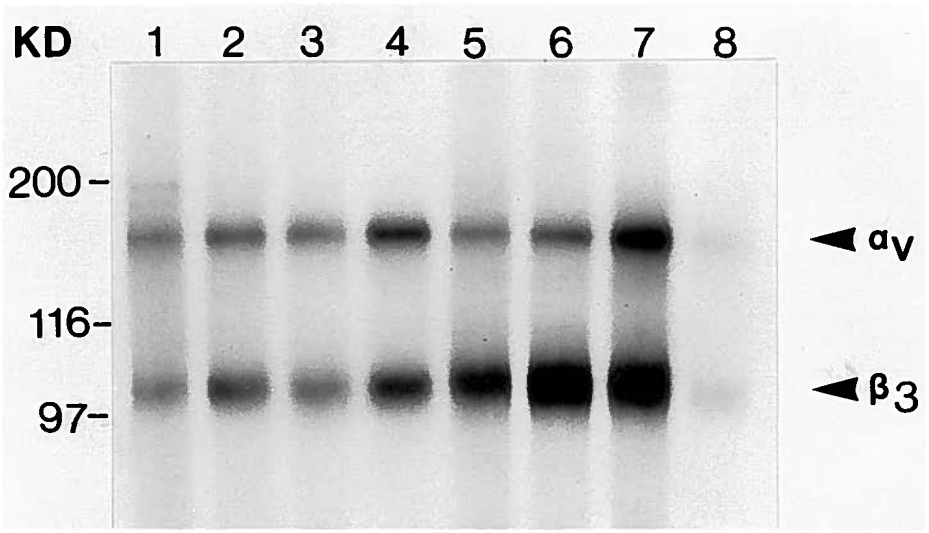
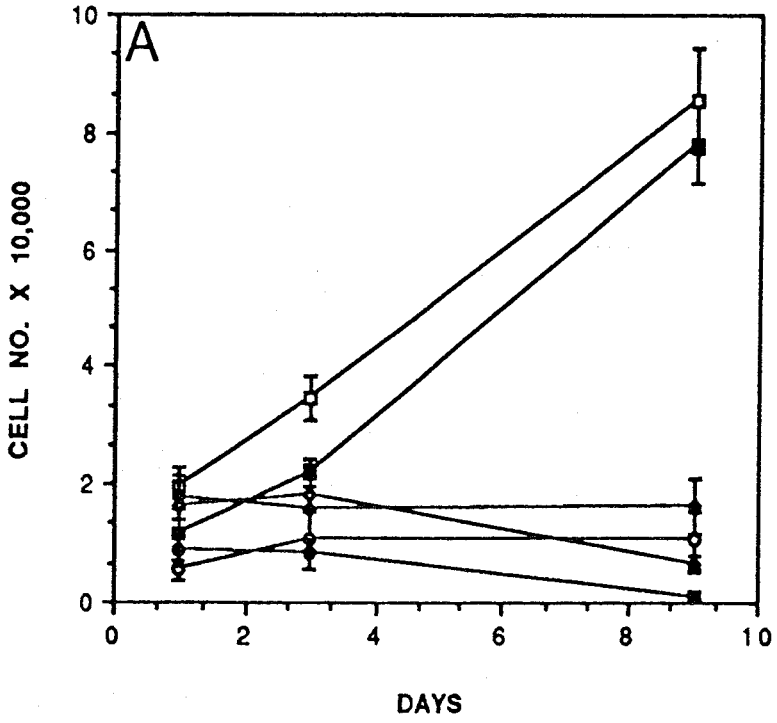
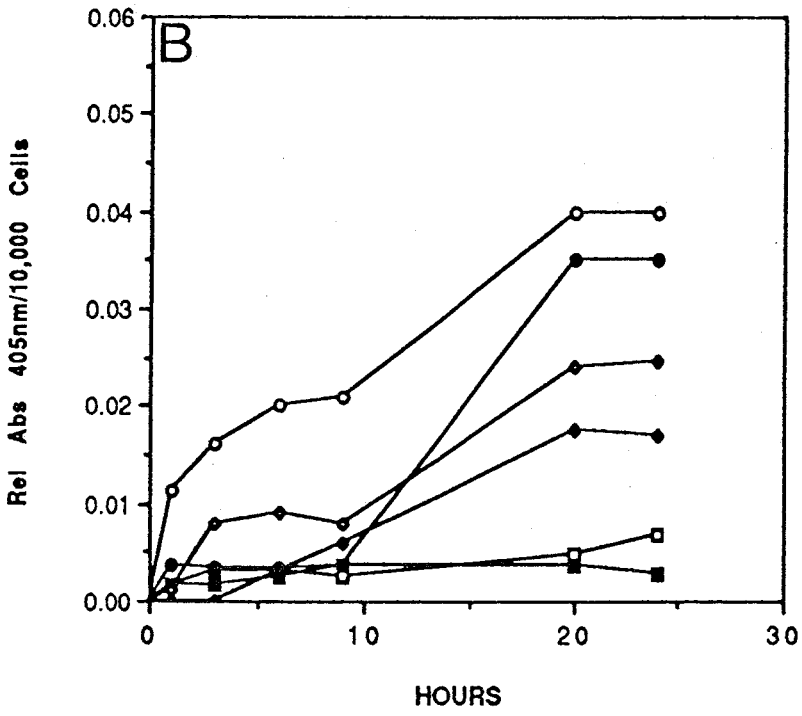

KD
1
2
3
4
5
6
7
8
200-
116-
97-
αV
β3

Figure 34. HOSE - Growth Curves and Chymotrypsin-like Activity

Growth curves (A) and chymotrypsin-like activity detected spectrophotometrically from conditioned media (B). HOSE cells were plated on plastic (open squares); placental matrix (closed squares); collagen gel (closed diamonds); collagen gel coated with placental matrix (open diamonds); fibrin clot (open circles); and Matrigel (closed circles). Cell growth is inversely related to protease production.


A
CELL NO. X 10,000
DAYS
0
2
4
6
8
10


B
Rel Abs 405nm/10,000 Cells
HOURS
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0
10
20
30

(4.) Do HOSE Cells Produce Proteases?

Since the morphological data suggested that HOSE cells invaded Matrigel, the cultures were examined to determine if these cells produced proteolytic enzymes which may degrade ECM and allow invasion. For most assays, HOSE cells were plated onto the various substrata in medium 199:105/15% FBS overnight and then replaced with reduced serum-containing medium (199:105/1% FBS, 199:105/0.5% FBS, 199:105/PC-1) for the remainder of the experiment. The conditioned medium was collected following two to three days incubation on the various substrata because by this time the different morphologies were clearly established while the cell numbers between the various matrices were still comparable. Analyses of conditioned culture medium from all substrata demonstrated that normal HOSE secrete proteolytic enzymes.

A chymotrypsin-like peptidase with phenylalanine specificity (figure 34B and table 6) and an elastase-like peptidase with alanine specificity (table 7) were detected spectrophotometrically. Chymotrypsin-like activity and elastase-like activities were inversely related to cell growth (figure 34 and tables 6 & 7). That is, while the greatest amount of HOSE cell growth was seen on plastic and placental-ECM, the least amount of chymotrypsin-like and elastase-like activities was seen on these substrata. Conversely, while Matrigel, fibrin clots, and collagen gels supported little or no cell growth, chymotrypsin-like and elastase-like activities were greatest on these substrata. Trypsin-like activity was not detected in any of the conditioned media. Chymotrypsin-like and elastase-like activities were still demonstrable 9 days after subculture and while the amounts were slightly less than protease activities detected at D3, the relationship of protease activities to different substrata remained the same (tables 8 & 9). Further, there was no difference in the pattern of chymotrypsin-like and elastase-like activities regardless of serum supplementation used (tables 6-9). These results suggest that HOSE cells constitutively produce chymotrypsin-like and elastase-like proteases although the amount of protease production is influenced by substratum. Only background amounts of protease activities were detected from conditioned media collected from substrata controls.

Major bands of gelatinolytic activity were consistently found in the conditioned medium of HOSE maintained on the various substrata. For the first 2 cases tested, the cells were plated in 199:105/15%FBS for 24 hrs which was then replaced with 199:105/DM and collected after an additional 24 hr. Substrata controls consisted of substrata-coated wells alone incubated for 24 hours in 199:105/DM having been

Table 6. Chymotrypsin-Like Activity in HOSE Cultures

	Change in Absorbance @ 405nm/hr per 10^4 Cells ($X10^{-4}$)*						
Cases:	#1	#2	#3	#4	#4	#5	#5
Medium Supplement:	1% FBS	1% FBS	1% FBS	0.5% FBS	PC-1	0.5% FBS	PC-1
Plastic	3.0	1.7	4.3	0.1	0.6	0.4	0.0
Collagen	7.0	23.7	30.4	8.0	8.7	6.7	13.0
Fibrin	17.4	25.3	60.4	9.6	5.6	16.8	12.6
Matrigel	14.5	49.5	77.5	71.4	126.6	933.9	53.0

* = values corrected for background in Tables 6-9

Table 7. Elastase-Like Activity in HOSE Cultures

Change in Absorbance @ 405nm/hr per 10^4 Cells ($x10^{-4}$)

Cases:	#1	#2	#3	#4	#5
Medium Supplement:	1% FBS	1% FBS	1% FBS	0.5% FBS	0.5% FBS
Plastic	0.85	2.0	0.0	0.4	0.6
Collagen	3.1	28.0	72.0	4.0	1.7
Fibrin	22.4	37.4	167.4	4.0	N.D.
Matrigel	36.6	167.3	172.1	146.8	69.5

N.D.= not done

Table 8. Chymotrypsin-Like Activity in Conditioned Medium From Day 9 HOSE Cultures

	Change in Absorbance @405nm/hr per 10^4 Cells ($x10^{-4}$)			
Cases:	#4	#4	#5	#5
Medium Supplement:	0.5% FBS	PC-1	0.5% FBS	PC-1
Plastic	1.1	1.2	0.0	1.1
Collagen	4.0	5.2	1.8	2.2
Fibrin	4.9	9.3	1.5	1.9
Matrigel	10.5	24.2	4.7	6.5

Table 9. Elastase-Like Activity in Conditioned Medium From Day 9 HOSE Cultures

	Change in Absorbance @405nm/hr per 10^4 Cells ($x10^{-4}$)			
Cases:	#4	#4	#5	#5
Medium Supplement:	0.5% FBS	PC-1	0.5% FBS	PC-1
Plastic	7.8	12.5	2.6	19.0
Collagen	15.2	12.5	2.9	20.1
Fibrin	13.9	25.5	3.4	25.8
Matrigel	31.3	155.7	56.0	49.1

previously incubated for 24 hours in 199:105/15% FBS for 24 hours. A gelatinolytic band at 30KD was expressed exclusively by HOSE cells and was absent in substrata controls (figure 35A). A broad band of lysis was also found at roughly 42KD in both conditioned media from cells and conditioned media from substrata controls, although there appeared to be a greater amount of gelatinase associated with the cell-derived conditioned medium (figure 35A). A clear doublet of lysis was also present at approximately 97KD, but was similar in conditioned medium from HOSE cells as well as from substrata controls (figure 35A) suggesting the carryover of serum proteases. Essentially all gelatinolytic activity was abolished following incubation of gels in 10 mM EDTA (figure 35B), indicating that these gelatinases are metalloproteases.

To distinguish further between gelatinases secreted by HOSE cells and those present in and carried over from serum, HOSE cells from one case, Cello, were washed extensively in 199:105/DM and plated on plastic, fibrin clots, collagen gels, Matrigel in 199:105/DM overnight. The following day the medium was replaced with fresh 199:105/DM, and after a further 24 hour of culture the conditioned medium was collected, concentrated, and subjected to gelatin zymography. HOSE cells plated and maintained in 199:105/DM attached and spread on plastic, collagen gels, and fibrin clots and were epithelial, spindle-shaped, and epithelial respectively in response to these substrata. However, cells plated in 199:105/DM failed to adhere to Matrigel and remained as floating rounded cells, so that the subsequent medium change at 24 hours resulted in removal of HOSE cells from Matrigel wells. It appears that some factor(s) present in serum is necessary for HOSE cell adhesion to Matrigel. Again, controls consisted of substrata-coated wells plated in DM and treated as those wells that had received cells. Similar to the results described above, gelatin zymography revealed that HOSE cells secrete a 30KD gelatinase as well as a 42 KD gelatinase that may be the same as that found in serum (figure 36A). However, as cells plated in 199:105/DM did not adhere to Matrigel, no gelatinase activity could be detected in Matrigel-coated wells. Substrata controls did not show any gelatinolytic activity (figure 36A), abrogating any endogenous gelatinase activities from within any substrata that might have leeched out into the conditioned medium. As with the other gelatin zymograms, the gelatinases produced by HOSE cells plated in 199:105/DM are metalloproteases as activity was abolished following incubation in 10 mM EDTA (figure 36 B).

Neither collagenase nor plasminogen activator activities were detected. During 2 days of culture HOSE cells secreted no detectable collagenase or proteases capable of

Figure 35. HOSE - Gelatin Zymography 1

Conditioned media collected from HOSE cells plated in 199:105/15%FBS for 24 hours and then maintained for 24 hours in 199:105/DM were analyzed by gelatin zymography. (A), conditioned media collected from HOSE cells maintained on: plastic (lane 1); collagen gel (lane 2); fibrin clots (lane 3); and Matrigel (lane 4). Conditioned media collected from substrata controls maintained without HOSE cells: plastic (lane 5); collagen gel (lane 6); fibrin clots (lane 7); and Matrigel (lane 8). (B), same as in A except that the gel was incubated in 10mM EDTA. HOSE cells maintained on the various substrata express a 97, 42 and 30 KD gelatinase while substrata controls express a 97 and 42 KD gelatinase. All gelatinolytic activity was abolished following incubation with EDTA, a divalent cation chelator, indicating that the gelatinases are metalloproteases.

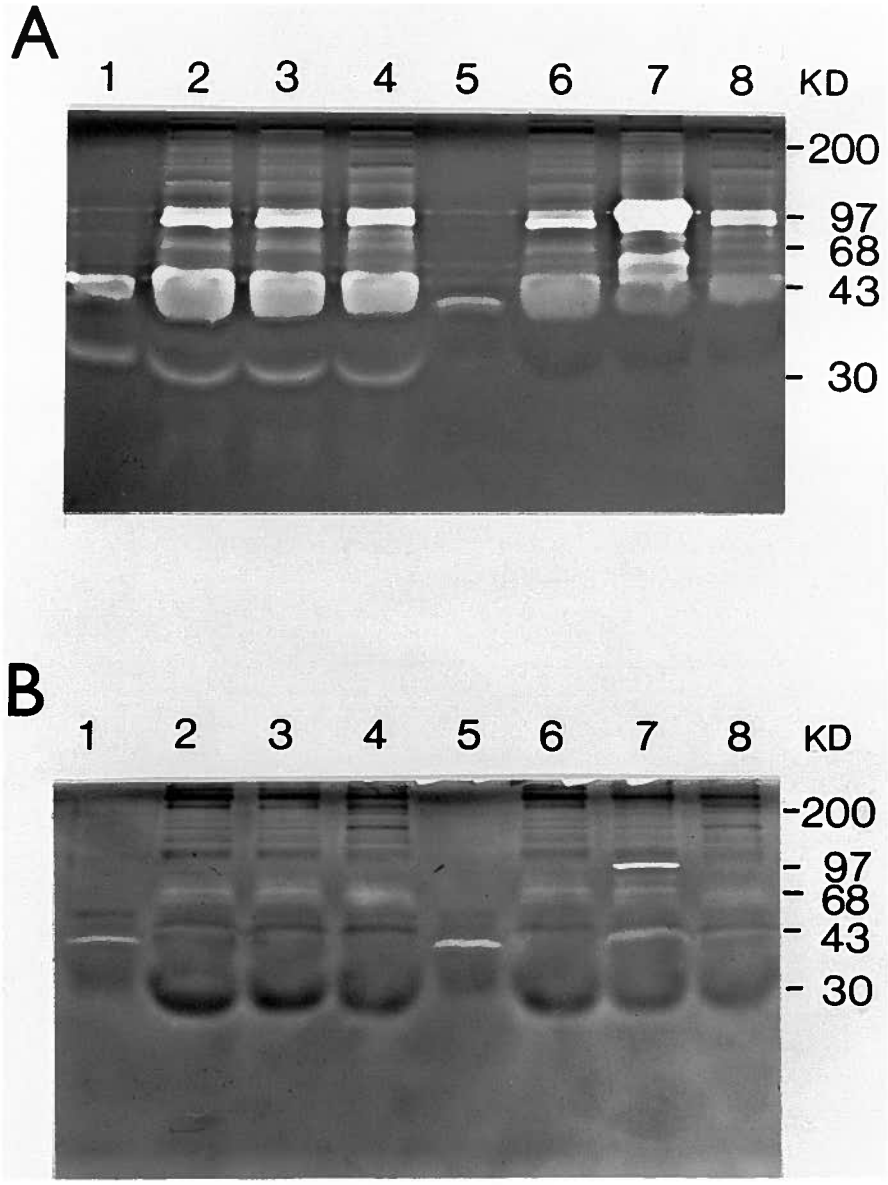
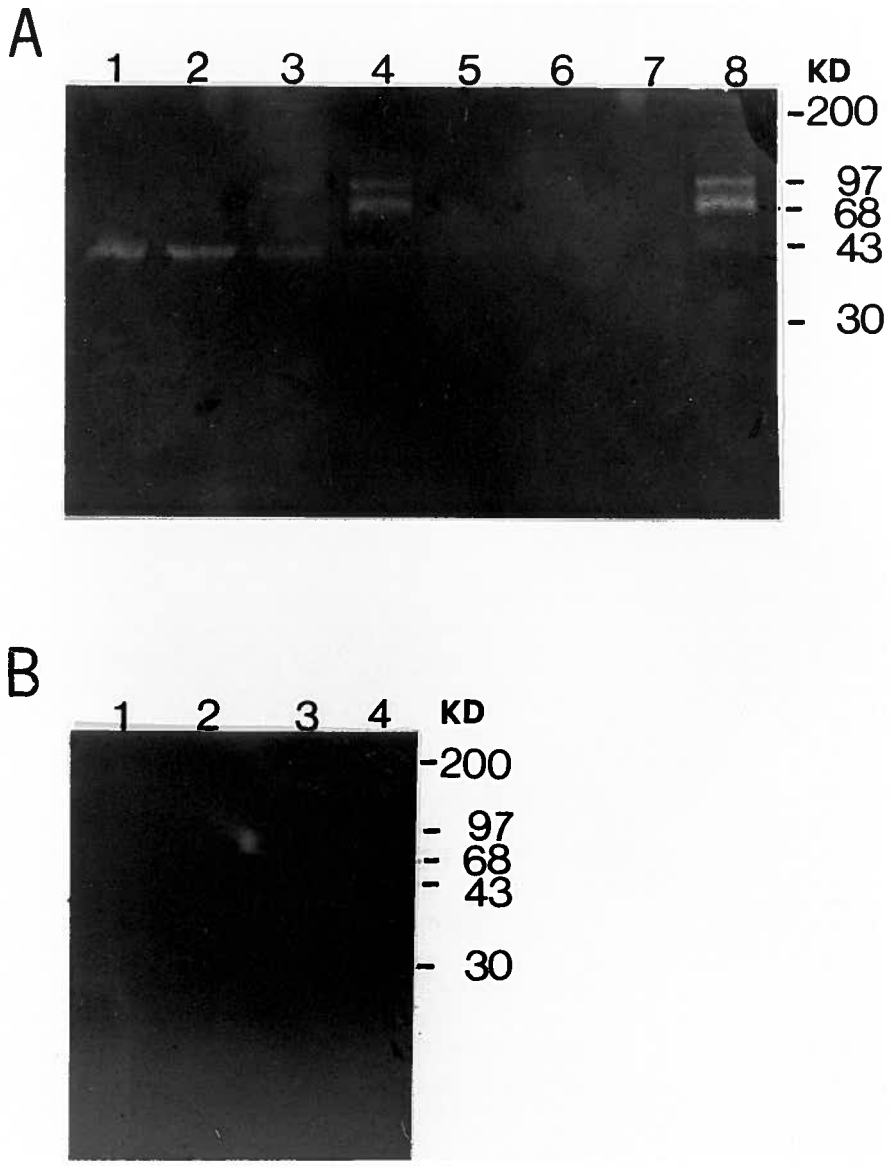

A
1 2 3 4 5 6 7 8 KD
200
97
68
43
30
B
1 2 3 4 5 6 7 8 KD
200
97
68
43
30

Figure 36. HOSE - Gelatin Zymography 2

Conditioned media collected from HOSE cells plated in 199:105/DM for 24 hours and then maintained for an additional 24 hours in fresh 199:105/DM were analyzed by gelatin zymography. (A), conditioned media collected from HOSE cells maintained on: plastic (lane 1); collagen gel (lane 2); fibrin clots (lane 3); and Matrigel (lane 4). Conditioned media collected from substrata controls maintained without HOSE cells: plastic (lane 5); fibrin clots (lane 6); collagen gel (lane 7); and Matrigel (lane 8). (B), lanes 1-4 same as in A, except that the gel was incubated in 10mM EDTA. When maintained in defined medium, HOSE cells, except those on Matrigel, express a 42 KD gelatinase not present in substrata controls. As in figure 35, gelatinase activity was abolished following treatment with EDTA.


A
1 2 3 4 5 6 7 8
KD
200
97
68
43
30
B
1 2 3 4
KD
200
97
68
43
30

degrading collagen type I regardless of the substratum on which the cells were maintained. Figure 37 shows that conditioned medium from HOSE cells maintained on plastic, placental-ECM, fibrin clots, collagen gels, collagen gels coated with placental-ECM, and Matrigel failed to degrade the α1 and α2 chains of native collagen type I.

HOSE cells secreted no detectable levels of plasminogen activator (PA) into their conditioned medium during 2 days of incubation on the various substrata while standards of human urokinase produced lysis in the agarose. The lack of PA detection may be due to the presence of plasminogen activator inhibitor (PAI-1) detected in both early and late passage HOSE cultures (figure 38). HOSE cells stained intensely for PAI-1, while serum controls did not stain. Further, LP-9 cells served as positive controls and also stained strongly for PAI-1 compared to their respective serum controls. As PAI-1 binds PA, it possibly made PA undetectble in the assays used in this study.

Figure 37. HOSE - ^{3}H-Collagen Type I Digestion

Conditioned media from HOSE cells maintained on various substrata were analyzed by SDS-PAGE and the bands visualized by autoradiography. Conditioned media collected from HOSE cells maintained on: plastic (lane 1); placental matrix (lane 3); collagen gel (lane 5); collagen gel coated with placental matrix (lane 7); fibrin clots (9); and Matrigel (lane 11). Conditioned media collected from substrata controls maintained without HOSE cells: plastic (lane 2); placental matrix (lane 4); collagen gel (lane 6); collagen gel coated with placental matrix (lane 8); fibrin clots (lane 10); Matrigel (lane 12). Lane 13, collagen type I standard. All HOSE cells failed to degrade collagen type I.

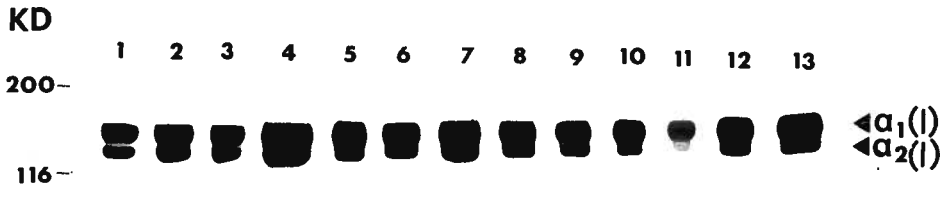

KD
1
2
3
4
5
6
7
8
9
10
11
12
13
200–
116–
α1(I)
α2(I)

Figure 38. Expression of Plasminogen Activator Inhibitor by HOSE Cells

HOSE cells (A,B), like LP-9 cells (C,D), stain uniformly for plasminogen activator inhibitor (PAI-1). HOSE cells failed to stain when the primary antibody was replaced with normal serum (E,F). A,C,E, Immunofluorescence microscopy. B,D,F, Phase microscopy of same fields shown in A,C,E respectively. X500.

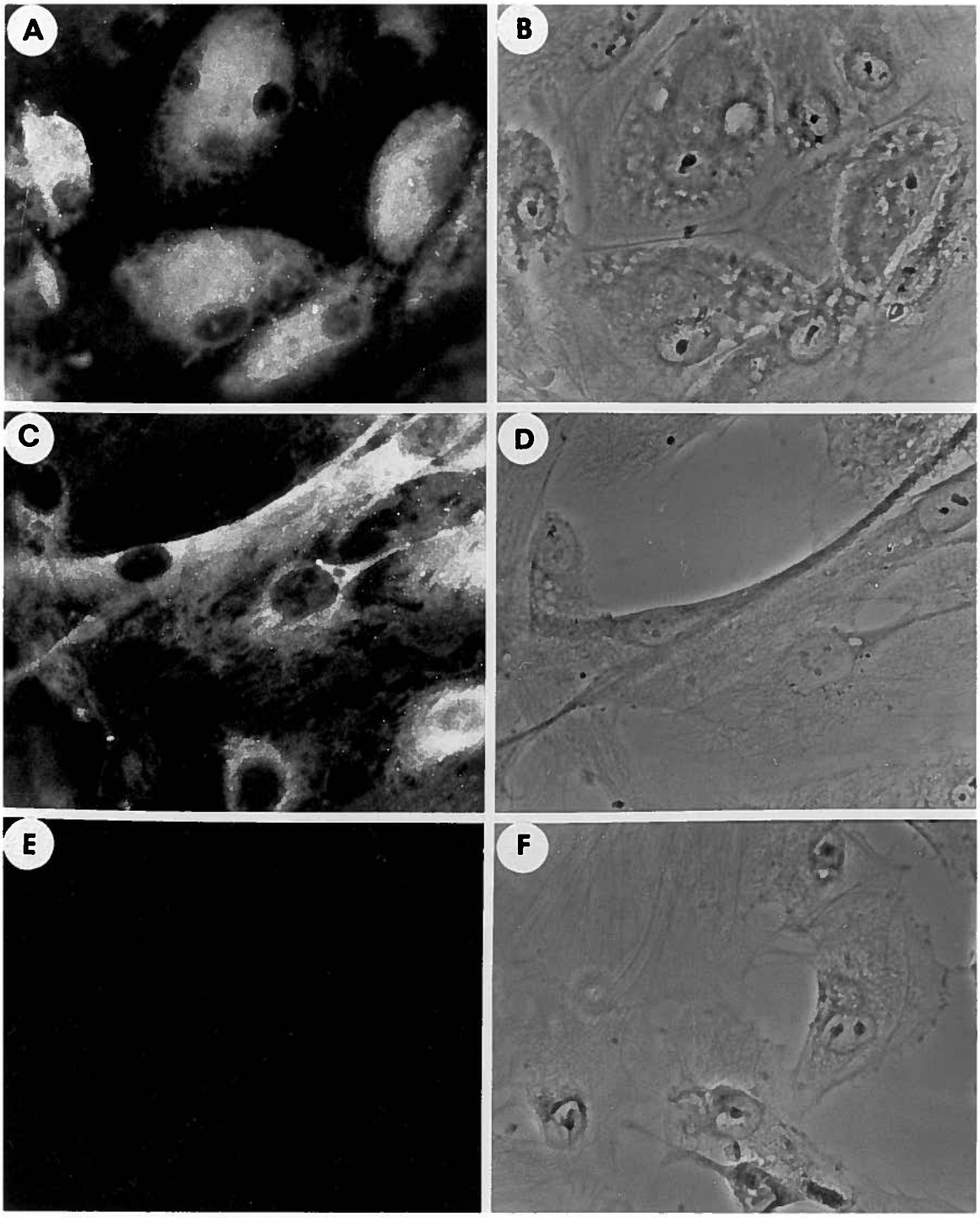

A
B
C
D
E
F

TABLE 10. Summary of Immunofluorescence Studies

Figure	Antigen	Test Material	Control for 1°Ab	Control Material
2	Keratin	HOSE cells: 1°+2°Ab, +ve	PBS + 2°Ab, -ve	Amniotic Cells: 1°+2 Ab, +/-ve Amniotic Cells: PBS + 2°Ab, -ve HUDF: 1°+2°Ab, -ve HUDF: PBS + 2°Ab, -ve
5	Laminin	ROSE 239 Cells: 1°+2°Ab, +ve	NRbS + 2°Ab, -ve	
5	Collagen Type I	ROSE 239 Cells: 1°+2°Ab, +ve HUDF: 1°+2°Ab, +ve	NShS + 2°Ab, -ve NShS + 2°Ab, -ve	
10	Keratin	HOSE Cells: 1°+2°Ab, +/-ve	PBS + 2°Ab, -ve	C4-I Cells: 1°+2°Ab, +ve C4-I Cells: PBS + 2°Ab, -ve HUDF: 1°+2°Ab, -ve HUDF: PBS + 2°Ab, -ve
12	Laminin	HOSE Cells: 1°+2°Ab, +ve	NRbS + 2°Ab, -ve	ROSE 239 Cells: 1°+2°Ab, +ve ROSE 239 Cells: NRbS + 2°Ab, -ve HUDF: 1°+2°Ab, -ve HUDF: NRbS + 2°Ab, -ve
13	Collagen Type IV	HOSE Cells: 1°+2°Ab, +ve	NRbS + 2°Ab, -ve	
14	Collagen Type I	HOSE Cells: 1°+2°Ab, +ve	NShS + 2°Ab, -ve	ROSE 239 Cells: 1°+2°Ab, +ve ROSE 239 Cells: NShS + 2°Ab, -ve
	Collagen Type III	HOSE Cells: 1°+2°Ab, +/-	NGS + 2°Ab, -ve	ROSE 239 Cells: 1°+2°Ab, +ve ROSE 239 Cells: NGS + 2°Ab, -ve
	Keratin	HOSE Cells: 1°+2°Ab, +ve	PBS + 2°Ab	C4-I Cells: 1°+2°Ab, +ve C4-I Cells: PBS + 2°Ab, -ve HUDF: 1°+2°Ab, -ve HUDF: PBS + 2°Ab, -ve

Table 10. Continued

19	Laminin	ROSE 199-ECM: 1°+2°Ab, +ve	NRbS + 2°Ab, -ve	ROSE 239 Cells: 1°+2°Ab, +ve ROSE 239 Cells: NRbS + 2°Ab, -ve HUDF: 1°+2°Ab, -ve HUDF: NRbS + 2°Ab, -ve
20	Actin	ROSE 199-ECM: NBD, -ve	PH, -ve NBD + PH, -ve	Rat Intestine: NBD, +ve Rat Intestine: PH, -ve Rat Intestine: NBD + PH, -ve
	Laminin	ROSE 199-ECM: 1°+2°Ab, +ve	NRbS + 2°Ab, -ve	Rat Intestine: 1°+2°Ab, +ve Rat Intestine: NRbS + 2°Ab, -ve
21	Collagen Type I	ROSE 199-ECM: 1°+2°Ab, +ve	NShS + 2°Ab, -ve	HUDF: 1°+2°Ab, +ve HUDF: NShS + 2°Ab, -ve
38	PAI-1	HOSE Cells: 1°+2°Ab, +ve	NRbS + 2°Ab, -ve	LP-9 Cells: 1°+2°Ab, +ve LP-9 Cells: NRbS + 2°Ab, -ve

+ve = positive
-ve = negative
+/-ve = postive and negative
1° = primary
2° = secondary

DISCUSSION

A. Tissue Culture Techniques

(1.) Scrape-Derived HOSE Cultures

In spite of its clinical importance, studies on the role of HOSE in ovarian carcinogensis have been severely limited by the lack of experimental systems. To adapt culture methods for HOSE from methods used to culture ovarian surface epithelium from rat and rabbit, two problems had to be overcome. First, the means to isolate the epithelium from the rest of the ovary had to be modified because in the human biopsy specimens it is present in very small amounts compared with, for example, the papillary ovarian surface epithelium of the rabbit ovary. Secondly, the culture medium used to propagate animal surface epithelium proved inadequate for the culture of its human counterpart (Auersperg et al. '84). Recent methods allowed for the isolation, propagation, and characterization of HOSE cells in culture, but the techniques used were laborious and the cultures were frequently contaminated with stromal and follicular cells. Furthermore, the potential of the cells to proliferate while retaining their normal differentiated form was limited (Auersperg et al. '84, Siemens & Auersperg '88). One of the tissue culture techniques developed in the course of this study was an improved method for the preparation and culture of HOSE cells from normal human ovaries. Instead of preparing explants, advantage was taken of the tenuous attachment of HOSE to its underlying connective tissue and sheets of HOSE cells were scraped off the biopsies. In addition to the improved yield, the scrape method also greatly increased the purity of the cultures. In contrast to procedures that use explants (Auersperg et al. '84) or enzymatic dissociation (Hamilton et al. '80) of the original tissue, the ovarian tissue below the surface epithelium remained undisturbed by the scrape method. As a result, contamination by stromal and follicular cells was rare. Occasional groups of contaminating fibroblasts could be removed with a rubber scraper. Histologic examination of biopsy specimens confirmed that scraping denuded 90 to 100% of the ovarian surface of HOSE with little disruption to underlying structures. Thus, normal surface-derived HOSE was selected over cyst and invagination-lined HOSE which is frequently atypical. In additon to the improved yield and greater purity of the cultures, the scrape method has the obvious advantages of being considerably faster and simpler than either explant culture or culture of enzymatically dissociated cells.

The ease with which HOSE is scraped off biopsy specimens suggests that previous reports on the desquamation of HOSE with increasing age (McKay et al. '61, Sauramo '52) are likely an artefact. Also, the tenuous attachment of HOSE to its underlying stroma has clinical and physiological implications as HOSE may be denuded during any gynecological surgery. Since adhesion formation is common following ovarian surgery, standard ovarian surgical techniques may promote adhesion formation by removing HOSE which is important for covering, protecting, and re-epithelializing the ovary. Lastly, there are contradictory reports on the effects of denuding the ovarian surface and subsequent ovulations. For example, Gillet ('91) showed that removal of rabbit ovarian surface epithelial cells reduced the number of conceptions compared to the undenuded contralateral ovary while Rawson & Espey ('77) demonstrated that follicles proceeded to ovulate even after the rabbit ovarian surface epithelium had been denuded. Therefore, although there is no direct evidence that HOSE contributes to ovulation, the possiblity that HOSE contributes to normal ovarian functions such as ovulation should not be discounted prematurely.

By previous methods, the best culture medium to maintain HOSE in its tissue-specific epithelial form was medium 199:202/15% FBS, but the cells grew slowly and could only be subcultured once or twice, approximately 6 population doublings. Addition of EGF/HC increased the growth rate and growth potential considerably, but caused modulation of the HOSE cells to an atypical, fibroblastic phenotype (Siemens & Auersperg '88). This modulation was initially reversible, but eventually became permanent. It interfered with cell identification and with the interpretation of experimental results. In the culture medium used at present, the substitution of a synthetic component, MCDB 105 (McKeehan et al. '78) for MCDB 202 has eliminated the need for EGF/HC to support rapid growth for up to 12 population doublings, but results in the modulation to a fibroblast-like phenotype with increasing passage in culture (Kruk et al. '90). MCDB 202 and MCDB 105 are both modifications of medium F12. Their ingredients are the same, but a number of components differ in relative concentrations. Several of these differences may have contributed to the improved growth of HOSE in MCDB 105-supplemented medium. Compared with MCDB 202, MCDB 105 has cysteine and calcium concentrations that are closer to the optimum for the growth of other epithelial cells (McKeehan et al. '84). Furthermore, the higher levels of glutamine and proline in MCDB 105 may enhance extracellular matrix synthesis and, consequently, contribute to the epithelial morphology of HOSE cells. Finally, the buffering capacity of MCDB 105

minimizes pH fluctuations in response to shifts between air and the incubation atmosphere and, thus, provides an improved microenvironment.

This simplified and more successful methodology for the culture of HOSE cells opened the way for the in depth investigations of HOSE described herein.

(2). Percoll Decontamination

The second tissue culture technique developed during the course of this study, stemmed from Percoll centrifugation experiments performed to separate HOSE cells from stromal fibroblasts and resulted in a method to remove mold infections from cultures. Microbial contaminations in tissue culture are usually of environmental origin and include bacteria, yeast, and molds. Most commonly, contaminated cultures are discarded, though they can, at least temporarily, be controlled with the use of antibiotics and fungicides. However, the potential exists for the development of drug resistant infections and cytotoxic effects on the cultured cells (Adams et al. '86, Dickman et al. '90, Freshney '83, Hanas & Simpson '86, Laska et al. '90, Ramsammy et al. '88, Van der Auwera & Meunier '89). Further, antibiotics such as streptomycin and gentamicin and fungicidal agents such as fungizone, can adversely affect cell metabolism, growth, morphology, differentiation, and protein synthesis (Adams et al. '86, Dickman et al. '90, Freshney '83, Laska et al. '90, Ramsammy et al. '88, Van der Auwera & Meunier '89). Percoll centrifugation provides an alternate means of removing fungal contaminants from cultures. Similar to the report by Behrens and Paronetto ('84) yeast infections could not be eliminated by Percoll gradient centrifugation in this study and Percoll centrifugation did not enhance decontamination of yeast-infected cultures when used in conjunction with fungizone. The difficulty in removing yeast infections may, in part, result from complex yeast-host cell interactions such as the internalization of yeast by host cells, which renders yeast inaccessible to decontamination (Merkel et al. '88).

In contrast, the Percoll method proved highly successful for the elimination of molds. Percoll is a gradient material composed of non-toxic polyvinylpyrrolidone-coated colloidial silica particles (Pertoft et al. '77, Pertoft & Laurent '77) and is widely used for the separation of different types of cells, subcellular particles, viruses, and bacteria (Kreamer et al. '86, Pertoft & Laurent '77, Raber & D'Ambrosio '86, Roskelley & Auersperg '90). The non-toxic nature of Percoll allowed cell bands, devoid of mold, to be recovered and plated directly, without washing, into dishes containing tissue culture medium. There did not appear to be any acute toxic effects on cells following Percoll treatment since viability was always

greater than 94%. By the present method, approximately 50% of the centrifuged cells were retrieved, but it is possible that recovery could be increased with improved cell band retrieval. Following centrifugation in Percoll, there were no differences in rates of growth, morphology, and capacity for differentiation, as shown by the production of extracellular matrix material. The maintenance of these characteristics among cultures is not unexpected as it has been shown elsewhere that centrifugation in Percoll does not affect such cellular parameters (Knecht et al. '89, Kreamer et al. '86. Pertoft et al. '77, Roskelley & Auersperg '90).

This method is now used routinely in our laboratory and, to date, several primary cultures from different sources have been successfully decontaminated of mold. The similar banding characteristics of a variety of cells suggest that Percoll decontamination can accommodate different cell types and may, therefore, be generally applicable.

B. HOSE Cells Cultured from Scraped Biopsies are a Relevant Model System

Having developed a simpler and more successful method to culture HOSE cells, it was important to establish the relevance of this model system as a means to study the biology of normal HOSE and its role in ovarian carcinogenesis. The greater the number of characteristics retained by HOSE cells in culture compared to their *in vivo* counterparts the greater would be the relevance. Initial characteristics of HOSE cells in culture included the presence of keratin, numerous microvilli, 17-β-hydroxysteroid dehydrogenase activity, and pleomorphism (Auersperg et al. '84) similar to HOSE *in vivo* (Blaustein & Lee '79, Czernobilsky '85, Czernobilsky et al. '84, Gillet et al. '91, Motta et al. '80, Van Blerkom & Motta '79). In this study the list of characteristics of cultured HOSE cells was expanded. Cultured HOSE cells were shown to express vimentin, keratin subtypes #7, 8, 18, 19, glycogen, lipid, variable amounts of mucin like their *in vivo* counterparts (Blaustein & Lee '79, Czernobilsky '85, Czernobilsky et al. '84, Viale et al. '88). The pleomorphism was analyzed and specific cell shape could be related to the presence of matrix. Cultured HOSE cells express the four keratin subtypes characteristic of simple epithelia (Moll et al. '82, Steinert & Parry '85, Steinert et al. '85) like HOSE *in vivo* (Czernobilsky '85, Czernobilsky et al. '84), but there was a gradual loss of keratin expression with increasing passage which is in agreement with other reports (Auersperg et al. '84) while the remaining markers were unaffected with

subsequent cell passaging. The keratins #8 and #19 appear to be prefentially lost with increasing passage in HOSE cultures. Many epithelial cell types co-express vimentin and keratin in culture (Ben-Ze'ev '84a,b,'86, Celis et al. '85, Rheinwald & O'Connell '85), however, co-expression of vimentin and keratin in normal epithelium *in vivo* has so far been reported only in mesothelium, HOSE, oviductal epithelium, and parietal endoderm (Czernobilsky '85, Lane et al. '83, Rheinwald & O'Connell '85, Viale et al. '88). Czernobilsky ('85) has suggested that the co-expression of vimentin and keratin in HOSE may reflect the mesodermal origin of this epithelium. The mesothelium, which is embryologically related to HOSE in that they both arise from the coelomic epithelium, is another mesodermally-derived epithelium which co-expresses vimentin and keratin in culture (Connell & Rheinwald '83). Further, the retention of lipid and glycogen by HOSE cells in culture mimics their *in vivo* counterparts (Blaustein '84, Blaustein & Lee '79). Lipid droplets were found distributed in varying amounts among cultured HOSE cells. The presence of lipid in cultured HOSE cells may indicate favourable nutritional culture conditions such that the cells store excess glucose and fatty acids in the insoluble, non-toxic forms of glycogen and triglycerides. Glycogen was clearly localized as abundant, amylase-sensitive, cytoplasmic granules. This is not surprising since cultured cells are exposed to an excess of glucose, a nutrient source in the culture medium, which is often stored by cells as glycogen (Freshney '83). The healthy status of these cells is further reflected by the presence of mitotic figures and large nuclei with prominent nucleoli indicative of ribosomal protein synthesis.

HOSE cells in culture demonstrate tremendous pleomorphism under different circumstances. HOSE outgrowths have been catagorized into one of three forms: (1.) compact, monolayered epithelial cells; (2.) flat epithelial cells; and (3.) atypical epithelial cells (Auersperg et al. '84). The compact and flat epithelial HOSE cells described in culture appear to correspond to the type A and type B cells respectively shown by Gillet et al. ('91) *in vivo* so that it would appear that the heterogeneity of HOSE cells *in vivo* (Gillet et al. '91) is maintained in culture. Interestingly, rat ovarian surface epithelial cells (Adams & Auersperg '81, Hamilton et al. '80) retain a homogeneous pavementlike appearance for long periods in culture and then either become stationary or progress to continuous lines (Adams & Auersperg '85) while HOSE cells acquire a heterogeneous appearance in culture and have, to date, never progressed to continuous cells lines. The present studies showed that the heterogeneous morphology was independent of the expression of two markers of epithelial differentiation, mucin and keratin, as these markers could be found in

HOSE cells expressing phenotypes ranging from epithelial to fibroblast-like. HOSE assumed various phenotypes ranging from flat epithelial to fibroblast-like to groups of rounded cells depending upon the kind of substratum they were plated upon. The relationship of these changes to events that occur *in vivo* remains to be investigated.

Except for the apparent loss of tight junctions the methods presented here permit the culture of normal HOSE cells which exhibit most of the characteristics of their *in vivo* counterparts, suggesting that cultured HOSE cells derived from scraped biopsy material are relevant for the study of normal HOSE biology and the role of HOSE in ovarian carcinogenesis.

Yet, it is important to note that even though the list of HOSE cell characteristics has expanded, there still does not exist a single, specific, unique marker for HOSE cells. For example, mammary epithelial cells in culture can be identified by their ability to produce milk components (Emerman et al. '77, Li et al. '87, Seely & Aggler, '91, Wicha et al. '82). Cultured HOSE cells share some features with other cell types, and not surprisingly most closely mimic mesothelial cells as HOSE itself is a modified mesothelium. Presently, there exists a battery of tests that can distinguish HOSE cells from other cell types which might arise from the ovarian biopsy and which might contaminate HOSE cultures. For example, if the cells are keratin positive, they are not fibroblasts or endothelial cells. Similarly, the presence of 17-β-hydroxysteroid dehydrogenase activity excludes the possibilty that the cells are mesothelial. Lastly, HOSE cells can be distinguished from granulosa cells because human granulosa cells contain small amounts of keratin subtype #7 (Czernobilsky '85, Czernobilsky et al. '84) in contrast to HOSE cells and granulosa cells contain a number of steroidogenic enzymes (Goldring et al. '86) that are absent in HOSE cells (Blaustein & Lee '79, Hoyer '80, Rembiszewska & Brynczak '85).

C. HOSE:Matrix Interrelationships

(1.) Ovarian Surface Epithelial-Derived Extracellular Matrix

Further characterization of cultured HOSE cells in this study showed that HOSE cells secrete extracellular matrix made of both basement membrane and stromal matrix components. Although this study only examined matrix synthesis by HOSE in culture, there is no reason to believe that the cells might not have the ability to express a similar phenotype *in vivo*. Cultured rat ovarian surface epithelial cells also secrete basement membrane and stromal matrix components (Auersperg et al. '91b). ECM isolated from ROSE 199 cultures in the present study also contains both

basement mambrane and stromal components. It is particularly interesting that ROSE 199 cells in crowded cultures form ridges and papillae, as well as multiple cell monolayers separated by layers of collagenous matrix (Adams & Auersperg '85). Thus, increasing numbers of proliferating, monolayered rat ovarian surface epithelial cells per unit area of plastic substratum are accommodated through a continuous increase in available ECM so that ECM derived from these cells may provide a means to circumvent the restraints of density-dependent growth in culture. In contrast to some other species (Nicosia & Johnson '84), papillary outgrowths of normal ovarian surface epithelium are scarce in the rat and human (Hamilton et al. '80, Siemens & Auersperg '88). Only a modest increase in these structures has been reported to occur at the site of follicular rupture and in postmenopausal women (Makabe et al. '80, Motta & Van Blerkom '80, Nicosia '83, '87). However, in a high proportion of serous ovarian tumours that are benign or of borderline malignancy, the HOSE is greatly amplified and forms papillae with stromal cores (Young et al. '89). Thus, *in vivo*, increased numbers of HOSE cells may be accommodated on the limiting ovarian surface through the formation of papillae. It is tempting to speculate that the autonomous deposition of an ECM by rat and human ovarian surface epithelial cells might, at least in part, provide the stromal cores for papillae that occur physiologically and pathologically. The capacity of ovarian cells to form collagenous matrix autonomously would render the cells less dependent on stroma contributed by normal connective tissue cells and might, therefore, be an important factor for ovarian neoplastic progression. These results suggest that the deposition of basement membrane and stromal matrix components may be a general feature of all mammalian ovarian surface epithelial cells.

The capability of ovarian surface epithelial cells to secrete stromal matrix is an unusal feature among epithelia and may be related to the location of this epithelium. Perhaps the ovarian surface epithelium, like all other epithelia, contributes to its underlying basement membrane, but may also contribute directly to the formation of either the specialized layer of dense collagenous material commonly referred to as the tunica albuginea or to elements of the ovarian stroma. Alternatively, the capacity of the ovarian surface epithelium to secrete stromal matrix may be related to its origin. The expression of fibroblastic characteristics by HOSE (morphological modulation between epithelial and fibroblastic phenotypes, depositon of stromal matrix) may be related to the close developmental relationship of ovarian stroma and surface epithelium, both of which are derived from the gonadal ridge and may be a new example of epithelial-mesenchymal transformation. The retention of a degree

of plasticity that permits cells to express stromal as well as epithelial characteristics would be in keeping with other properties of HOSE cells, such as their lability of keratin expression and their propensity to assume fibroblast-like shapes and growth patterns in culture (Siemens & Auersperg '88). Very similar phenotypic modulations occur in cultured mesothelial cells (Connell & Rheinwald '83). Various degrees of epithelial-mesenchymal interconversions have also been reported in a number of other epithelia of mesenchymal origin, including the developing kidney (Ekblom '84), and interestingly, regressing Mullerian duct (Trelstad et al. '82). The developing cornea and longterm cultures of rat intestinal epithelium represent other examples of epithelial cells that produce a stroma rich in connective-tissue forms of collagen (Hay '82, Sambuy & De Angelis '86).

A highly reproducible and abundant ECM was derived from an immortalized, non-tumorigenic rat ovarian surface epithelial cell line. The ECM was prepared and compared following three different preparative techniques, two chemical treatments and one physical treatment of ROSE 199 cultures. The ECM was complex in terms of both its component elements and morphology. The three forms of ECM appear similar with the exception that FT-derived ECMs showed the presence of residual nuclei. While none of the preparations demonstrated cytoplasmic debris following staining for actin, which is a standard method to ascertain cellular debris in ECM preparations (Aggler '88), residual nuclear material was present in all preparations as determined by DNA staining. Preliminary experiments suggest that improved washing of ROSE 199-ECMs and treatment with DNAse may eliminate these contaminants. ROSE 199-ECM consists of complex bundles of fibers, amorphous material, and striated collagen fibers. It is a unique ECM in that it contains both basment membrane and stromal elements. Unusual ECMs have been reported elsewhere. For example, Brauer & Keller ('89) reported on the isolation of basment membrane material from a murine teratocarcinoma cell line that lacked collagen type IV. Interestingly, collagen type IV is also lacking in ROSE 199-ECM. ROSE 199-ECM demonstrated biological activity by supporting cellular adhesion, spreading, and growth. ROSE 199-ECM, then, is a novel ovarian-derived biological substratum that may prove useful for the maintenance of cell growth or differentiation similar to such commonly used substrata as fibrin clots (Kadish et al. '79), collagen gels (Emerman et al. '77, Emerman & Pitelka '77, Lee et al. '84, Li et al. '87, Montesano '86, Wicha et al. '79), and Matrigel (Davis et al. '90, Hadley et al. '85, Kleinman et al. '86, Madison et al. '85). Other potential uses of ROSE 199-ECM include a reliable

source of matrix components and the abundance of collagen in this matrix which may prove useful for studies in collagen fibrillogenesis and matrix assembly.

(2.) Physical ECM Remodelling

Many cell types contract floating collagen gels (Emerman et al. '77, Emerman & Pitelka '77), but only a few cell types have been shown to remodel unrimmed collagen gels (Bell et al. '79, Schafer et al. '89). It is well established that fibroblasts contract unrimmed collagen gels (Bell et al. '79) and this process has served as a model for wound repair in culture (Buttle & Ehrlich '83). Recently, Schafer et al. ('89) reported on the ability of keratinocytes to contract unrimmed collagen gels and suggested that keratinocytes also play a role in wound repair. On the basis of the results of this study, HOSE cells can now be added to the list of non-fibroblastic cells capable of physically remodelling matrix as evidenced by the contraction of organoids. The physical ability of HOSE cells to contract ECM parallels the fibroblast model of wound repair and may be important for repair of the ovulatory defect. Interestingly, Schafer et al. ('89) noted that the degree of gel contraction increased with inceasing age of donor keratinocytes and the authors suggested that the changes in dermal collagen and the wrinkling of skin with age may be related to keratinocyte function. It is tempting to speculate something similar with HOSE cells. Perhaps the ovarian shrinkage observed with age is facilitated, in part, by the matrix remodelling capabilities of HOSE cells.

The morphological events associated with organoid contraction by HOSE cells appear to be the same to those described for collagen gel contraction by fibroblasts and suggests, perhaps, that contraction occurs by the same process in both systems. In the fibroblast model, gel contraction is analogous to squeezing water out of a sponge (Bell et al. '79). Gel contraction does not involve collagen degradation, but rather consolidation of collagen fibers (Bell et al. '79, Guidry & Grinnell '85). Morphologically, during contraction, collagen fibrils are bound individually and in clusters at the cell surface and are surrounded by cellular microvilli and blebs (Grinnell & Lamke '84). Subsequently, contraction of the collagen gels occurs in two steps. There is movement and re-arrangement of the collagen fibers by an actin dependent mechanical process (Buttle & Ehrlich '83, Guidry & Grinnell '85, '86). This is followed by changes in intermolecular bonds between collagen fibrils which lead to fibril stabilization by non-covalent collagen-collagen interactions (Buttle & Ehrlich '83, Guidry & Grinnell '85, '86). Grossly, contraction is seen as a consolidation of collagen fibers along the periphery of the contracting gel. A dense

cellular ring appears along the edge of the contracting gel and the cells along the edge align themselves in a circumferential pattern on the perimeter of the gel (Buttle & Ehrilch '83).

Contraction of collagen gels is thought to result from the tractional forces exerted by cells on collagen fibers (Nogawa & Nakanishi '87). The cells do not simply shorten in length like contracting muscle cells. Instead of pulling on the substratum by shortening themselves, the cells exert a shearing force tangential to their surface. This force is traction and should be distinguished from contraction which would involve shortening of the cell itself even though cellular traction is caused by cytoplasmic contractions. Because it is traction rather than simple contraction which distorts rubber membranes, collagen gels, and other flexible substrata, the cells actually elongate in the direction of the contractile force they exert rather than shorten (Harris et al. '80, Stopak & Harris '82).

All organoids in which the ROSE 199-ECMs were not attached to the collagen gel failed to contract. Similarly, HOSE cells plated onto unrimmed collagen gels failed to contract suggesting that HOSE cells alone on collagen gel may not be able to generate sufficient tractional force to contract matrix. If non-attached ROSE 199-ECMs were re-attached to the collagen gels, HOSE cells often then contracted the organoids. This indicates that one definite requirement for organoid contraction is that the ROSE 199-ECM had to be in direct contact with the collagen gel component of the organoid. The role of ROSE 199-ECM in organoid contraction is unknown. It is possible that interaction between the collagen gel and components of the ROSE 199-ECM (i.e. fibronectin) may result in matrix-driven translocation. Matrix-driven translocation is a biophysical process in which there is rapid movement of cells or inert particles from one region of a non-uniform collagen/fibronectin matrix to another (Newman et al. '85, '87). Matrix-driven translocation involves the interaction and movement of matrix components among each other so that particles are carried along with this matrix movement. In organoids a matrix-driven translocation-like process, generated by the interaction of collagen gel and ROSE 199-derived fibronectin or laminin may serve to distribute HOSE cells quickly around the organoid so that HOSE cells could then contract the organoid. Alternatively, matrix-driven translocation-like processes may add to the existing tractional forces exerted by HOSE cells to generate sufficient total force to contract the organoid. If matrix-driven translocation-like forces are present they could not be solely responsible for organoid contraciton as controls organoids (i.e. without cells) failed to contract. Further, interaction between collagen gel and ROSE 199-

ECM components may re-align or cross-link the existing matrices such that all the components are interlocked. Such matrix interactions might serve to enhance organoid contraction as the tractional forces exerted by HOSE cells could be transmitted throughout the re-organized interlocking matrix network.

For cellular processes such as migration, wound healing, and morphogenesis to occur, signals from the ECM to the cell, often via integrins, must be translated into events which re-organize the existing ECM. The induction of collagenase production by fibroblasts following binding to fibronectin via the fibronectin receptor (Werb et al. '89) illustrates an example by which integrins indirectly mediate matrix remodelling. Recently, reports that $\alpha 2/\beta_1$, a collagen receptor, directly mediates collagen gel contraction (Gullberg et al. '90, Schiro et al. '91) provide evidence that integrins can mediate matrix remodelling by cueing physical events. Cells lacking $\alpha 2$ or expressing mutant forms of this integrin failed to contract collagen gels. Yet, when $\alpha 2$ cDNA was transfected into cells lacking VLA-2, the cells expressed VLA-2 at their surfaces and acquired the ability to contract collagen gels. Interestingly, HOSE cells express VLA-2, so this integrin may mediate organoid contraction by mediating interactions between HOSE cells and collagen. It would be interesting to see if the HOSE cells which failed to contract organoids have insufficient or altered VLA-2 expression. Others suggest that collagen gel contraction requires the co-operative interaction between VLA-2 and a second integrin, presumably VLA-5, a fibronectin receptor (Clark '90). This is interesting as HOSE cells express both VLA-2 and VLA-5 and they, or other integrins, may function co-operatively to mediate organoid contraction.

While the exact mechanism of gel contraction is unknown, a number of factors have been reported to influence gel contraction. For example, cell number and concentration of collagen gel relate directly to the degree of gel contraction (Bell et al. '79, Ura et al. '91). Similarly, serum (Anderson et al. '90, Buttle & Ehrlich '83, Gullberg et al. '90), transforming growth factor-β (Montesano & Orci '88, Ura et al. '91), and platelet-derived growth factor (Anderson et al. '90, Gullberg et al. '90) increase the degree of gel contraction while factors such as heparin (Graham et al. '87, Guidry & Grinnell '87) decrease the degree of gel contraction. EGF and transforming growth factor-α appear to have no effect on gel contraction (Montesano & Orci '88, Ura et al. '91) which is in aggreement with the results in the present study. The addition of EGF/HC, which are mitogenic for HOSE cells, and reduced serum levels had no effect on the degree of organoid contraction. However, HOSE cells may be independent of the effects of some exogenous growth factors which influence

gel contraction because HOSE cells are capable of autonomous production of growth factors (Auersperg et al. '91a). Potential mechanisms by which growth factors such as platelet-derived growth factor or transforming growth factor-β influence gel contraction include increased synthesis or reorganization of actin or matrix receptors, and stimulation of cell migration (Heino et al. '89, Ignotz & Massague '86, Leof et al. '86). In the present study, the addition of 3T3 fibroblasts to HOSE organoids increased the degree of contraction. This is in aggreement with other reports which employed the co-culture of stromal and epithelial components to more accurately represent a reconstruction of an *in vivo* situation (Schafer et al. '89, Ura et al. '91).

The use of fibroblast mediated collagen gel contraction and HOSE mediated organoid contraction appear to be relevant models systems for wound healing (Bell et al. '79). However, other potential uses of collagen gel contraction as a model for many normal and abnormal cellular activities exist. These include: morphogenesis as exemplified by gland formation (Nogawa & Nakanishi '87); disease states such as scirrhous carcinoma of the stomach (Ura et al. '91); normal skin wrinkling associated with aging (Schafer et al. '89); and abnormal skin wrinkling associated with epidermolysis bullosa (Ehrlich et al. '83). Hopefully, HOSE organoids may provide a model system for the study of normal as well as abnormal ovarian conditions, such ovarian shrinkage with age and cyst formation.

(3.) Effect of Substrata on HOSE Morphology, Growth, Integrin Expression and Protease Production

The complexitity of the organoid system made it difficult to examine the relationships between HOSE cells and specific matrix components. To overcome this problem HOSE cells were maintained on simpler substrata. These substrata (plastic, collagen gels, fibrin clots, and Matrigel) had profound effects on HOSE cell morphology, growth, integrin expression, and protease production. These substrata induced morphological changes in HOSE cells that resemble their *in vivo* counterparts during various morphogenetic processes and reflect the dynamic nature of HOSE as well as its plasticity and heterogeneity both *in vivo* and *in vitro*. HOSE cells remained epithelial on both plastic and fibrin clots, however, while they were a cohesive monolayer varying from flat epithelial cells to compact epithelial cells on plastic, they assumed a more dispersed epithelial phenotype on fibrin clots. On collagen gels, HOSE cells assumed a spindle-shaped morphology, and they formed aggregates of rounded cells that joined other aggregates via branching structures on

Matrigel. HOSE cells proliferated rapidly on plastic only. Interestingly, in spite of the dramatic morphological changes in response to substrata, the cells remained keratin positive indicating that they were still epithelial cells.

In its normal resting state (non-proliferating and non-migratory), HOSE consists of a cohesive simple monolayer that varies from squamous to columnar (Blaustein '81a,'84, Blaustein & Lee '79, Clement '87, Nicosia '83, Papadaki & Beilby '71). Following ovulation, the continuity of HOSE is disrupted and it has been suggested that ovarian surface epithelial cells surrounding the ovulatory site proliferate and migrate over and into the ovulatory defect to contribute to wound repair (Bullough '42, Osterholzer et al. '85, Van Blerkom & Motta '79). Those cells which proliferate from the wound edge and migrate into the ovulatory defect are the source of type B cells according to Gillet et al. ('91). Once repair is completed, the continuity of HOSE is restored and HOSE returns to its resting state. There has been no detailed report that HOSE cells proliferate only at the edges of the ovulatory wound site and that they migrate over and into the ovulatory defect as dispersed epithelial cells. However, endothelial cells and other epithelial cells which grow as cohesive monolayers in culture, proliferate only at the wound edges when the continuity of the monolayer is disrupted. Following wounding, cells migrate from the wound edges to cover the defect (Kadish et al. '79, Wong & Gotlieb '88). Endothelial cells, mesodermally derived like HOSE, respond to fibrin clots in the same manner as HOSE cells. Fibrin clots stimulate a morphological change in endothelial cell monolayers so that cohesive epithelial monolayers become more dispersed (Kadish et al. '79). Fibrin clots also stimulate endothelial cell migration (Kadish et al. '79, Wong & Gotlieb '88). Therefore, fibrin clots do not stimulate endothelial cell growth, but provide a migratory cue and cause an alteration in cellular morphology. It is tempting to speculate that, *in vivo*, HOSE cells also proliferate only at the wound edges and then migrate onto the fibrin clot as dispersed epithelial cells, and that this behaviour is represented *in vitro* in the response of HOSE cells to fibrin clots. Fibrin clots, then, may provide similar migratory cues to HOSE cells as to endothelial cells.

Collagen gels induce a phenotypic modulation of HOSE cells to a spindle-shaped morphology, a loss of integrin expression, and an inhibition of proliferation while keratin expression is retained. These collagen gel-induced responses may represent an epithelial-mesenchymal transformation of HOSE cells similar to the epithelial-mesenchymal transformations that can be induced by suspending embryonic anterior lens epithelial cells (Greenburg & Hay '82,'86), adult thyroid follicular cells (Greenburg & Hay '88), or Madin-Darby canine kidney cells (Zuk & Hay '90, Zuk et

al. '89) within collagen gels. In particular, during the collagen-induced epithelial-mesenchymal transformation of thyroid follicular cells, the epithelial cells assumed a spindle-shaped morphology, lost the capacity to produce thyroglobulin and lost the expression of $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins while keratin expression was retained (Greenburg & Hay '88). Further, it was shown that transformation of thyroid epithelium to mesenchyme-like cells did not require cellular proliferation (Greenburg & Hay '88). There may be other explanations for the lack of HOSE cell proliferation on collagen gels. For example, many epithelial cells acquire a differentiated phenotype on rimmed collagen gels and do not proliferate on such collagen gels (Emerman et al. '77, Emerman & Pitelka '77). Alternatively, HOSE exposed to stromal matrix *in vivo* might be stimulated to proliferate. Disruption of matrix *in vivo* is often associated with the release of growth factors sequestered in the matrix (Gospodarowicz et al. '83, Roberts et al. '88, Vlodavsky et al. '87). Perhaps growth factors are sequestered from the culture medium by the collagen gel, thereby depriving HOSE cells of the necessary growth stimulatory signals.

Like HOSE cells on fibrin clots and collagen gels, HOSE cells on Matrigel did not grow. A lack of proliferation may reflect inadequate culture conditions or, more likely, deficient cell anchorage. However, if the cell aggregates formed on Matrigel are a reflection of *in vivo* morphogenesis as seen in sex cord formation (Gondos '75), or Mullerian duct formation (Josse & Picard '86, Vigier et al. '89), or, even possibly, invasiness as demonstrated by ovarian carcinoma cells (Blaustein '81a, Scully '77) these morphogenetic events may not normally be associated with proliferation. Indeed, at midgestation HOSE undergoes periods of intense mitotic activity, however, this appears to be limited to HOSE at the ovarian surface and not to HOSE cells within sex cords (Gondos '75). An important point demonstrated by the capacity of HOSE cells to penetrate Matrigel is that invasion appears to be part of the normal HOSE phenotype. Therefore, HOSE cells can be added to the increasing list of normal cells capable of invading Matrigel (Allen et al. '90, Fisher et al. '89, Noel et al. '91, Sanders & Prasad '89). This also emphasizes that invasion into Matrigel is not a characteristic restricted to malignant cells as is so often reported (Erkell & Schirrmacher '88, Starkey et al. '87). Care must be employed when using this criterion as a marker for malignancy. It also emphasizes the necessity for normal cellular counterparts when studying pathological and malignant disease states. The culture of normal HOSE, then, should not be excluded from studies utilizing ovarian epithelial cancer cells.

The different HOSE morphologies on the various substrata did not translate into the expression of different integrins at the cell surfaces. Instead, HOSE cells expressed the integrins VLA-2,-3,-5, and VNR, receptors for collagen, laminin, fibronectin, and vitronectin when maintained on plastic, fibrin clots, and Matrigel. However, all HOSE cells lacked the VLA-6 (α_6/β_4) integrin, the so-called epithelial integrin (Kajiji et al. '89, Tamura et al. '90). The lack of expresion of the epithelial integrin by HOSE cells may be attribulable to the mesodermal origin of this epithelium. More likely, the term epithelial integrin is a misnomer as non-epithelial cells, such as osteosarcoma cells (Dedhar & Saulnier '90), express this integrin. Interestingly, time course studies suggested that there is a downregulation of integrin receptors when HOSE cells are maintained on collagen gels. The mechanism of the downregulation remains unclear, but may be analogous to the EGF:receptor model (Cohen '87, Gill et al. '87). EGF-responsive cells often upregulate their receptors in the absence of ligand. When the ligand, EGF, is provided, the ligand-receptor complex is internalized. The net effect is to downregulate the number of receptors at the cell surface. Maybe, in the case of HOSE cells and integrins, the cells maintain a high level of integrins at their cell surface on plastic and fibrin because ligands for their receptors are unavailable. Although Matrigel contains ECM components, by virtue of its non-adherent effect on HOSE the cells on Matrigel are hardly in contact with the matrix and might again maintain a high level of integrins at their cell surfaces. Even when HOSE cells penetrate Matrigel, there always appears to be a zone, probably lytic in nature, separating HOSE cells from Matrigel (see figure 31). Therefore, like plastic and fibrin clots, HOSE cells in Matrigel may not be in contact with laminin or collagen, but perhaps it is only on collagen gels that HOSE cells are in contact with ECM. There may be other mechanisms involved as suggested by the literature (Dedhar & Saulnier '90, Guo et al. '91). It would be interesting to see if similar changes occur on fibrin clots probed for fibrin receptors (Du et al. '91, Philips et al. '91, Rouslahti '91). Alternatively, since integrin downregulation is seen only in spindle-shaped HOSE cells on collagen gel, perhaps integrin downregulation is associated with this specific morphology. Alterations in integrin expression, then, may be related to cell shape similarly to the effect of cell shape on protease production by epithelial cells, where the more rounded the epithelial periodontal ligament cells the greater the amount of proteases produced (Hong & Brunette '87). It would be interesting to compare, then, the integrin profiles between epithelial HOSE (i.e. routinely maintained on plastic) and spindle-shaped HOSE cells induced by other means than collagen, such as following treatment with

EGF (Siemens & Auersperg '88). While the expression of HOSE integrins *in vivo* is unkown, it is tempting to speculate that, at least in culture, VLA-2, -3, and -5, mediate HOSE cell-cell interactions as has been demonstrated between normal keratinocytes (Carter et al. '90, Larjava et al. '90) and several human tumour cell lines (Kaufmann et al. '89). Such integrin-mediated cell-cell interactions may provide cohesiveness between HOSE cells for the maintainence of monolayers.

This is the first demonstration of protease production by HOSE cells. HOSE cells produce a chymotrypsin-like peptidase, an elastase-like peptidase, and 30 KD and 42KD gelatinases which are metalloproteases. This has implications for normal ovarian functioning such as the initiation and repair of ovulation, and for normal development such as sex cord formation. Like invasion, protease production appears to be part of the normal HOSE phenotype and not limited to the malignant phenotype. However, the 72KD and/or the 94KD type IV collagenase, recognized to be important for normal events such as wound healing (Granda et al. '90) and tumour invasion (Liotta et al. '83) and most often associated with normal connective tissue cells (Emonard & Grimaud '90, Murphy et al. '89, Overall et al. '89) and malignant cells (Brown et al. '90, Davis & Martin '90, Emonard & Grimaud '90, Liotta et al. '79, Murphy et al. '89, Okada et al. '90, Zucker et al. '89) were not found associated with HOSE cells. The exact mechanism of regulation of protease production by HOSE cells remains unknown. However, it would be interesting to see if protease production by HOSE cells is under the same hormonal influences as proteases produced by other ovarian cell types such as the granulosa and thecal cells (Curry et al. '89, Dhanasekaran & Moudgal '88, Ny et al. '85, Reich et al. '85a,b, Tilly & Johnson '87).

PA-I is common among many cell types (Cicilia et al. '89, Ginsburg et al. '86, Levin '83, Nielsen et al. '82, Ny et al. '86, Pannekoek et al. '86, Takahashi et al. '91) and, so, it is not surprising that it is present in HOSE cells. Recently, Takahashi et al. ('91) showed that human omental mesothelial cells in culture release urokinase plasminogen activator (uPA) and plasminogen activator inhibitor (PAI-1), although they could not detect tissue type plasminogen activator (tPA). The inability to detect tPA, like the lack of plasminogen activator (PA) detection from HOSE cells in this study, may be due to the co-expression of large amounts of PAI-1 which is known to regulate PA activity by binding to PA (Blasi et al. '87, '90, Krukithof '88, Monge et al. '89, Seiffert et al. '90). Endothelial cells also produce PA and PAI and it is suggested that tightly regulated PA and PAI secretion serve to maintain the luminal surface of endothelial cells free of adherent material so that

blood flow is unimpeded (Collen '80, Van Hinsbergh et al. '87). Further, endothelia, mesothelia, and HOSE have nonadhesive surfaces and it has been suggested that PA and PAI secretion serves to prevent adhesion among organs coated with epithelia by preventing or reducing the adhesion of material to these epithelial surfaces (Buckman et al. '76). Possibly, then, these same functions of PA and PAI could be attributed to lubricative functions of HOSE cells. Recently, an interesting relationship was described between PAI-1 and vitronectin in that PAI-1 binds to vitronectin (Salonen et al. '89) in a uniform distribution on the substratum (Jensen et al. '90, Pollanen et al. '87). The components of the vitronectin:PAI-1 complex retain cell attachment-promoting activity and the capacity to inhibit plasminogen activator. This suggests that the complex regulates plasminogen activator-induced lysis by cells at the sites, such as focal contacts, where vitronectin promotes cellular adhesion and confines plasminogen activator activity to discrete sites of pericellular proteolysis (Jensen et al. '90, Pollanen et al. '87, Seiffert et al. '90). Perhaps the vitronectin receptor on HOSE cells co-distributes with PAI-1 expression to similarly limit plasminogen activator-mediated cellular proteolysis and maintain cellular adhesion.

In summary, the role of HOSE in the remodelling of the ovarian cortex appears to be broader than is generally assumed, involving synthetic, physical, and proteolytic functions. During the postovulatory phase, in particular, these cells may not only proliferate, migrate, and deposit a basement membrane, but may also play an active role in the rebuilding of the underlying connective tissues.

SUMMARY

The human ovarian ovarian surface epithelium undergoes dynamic morphological changes associated with development, ovulation, aging, and malignant transformation. Interactions between HOSE cells and ECM may influence these changes. In this study: 1) improved methodology to culture HOSE cells from biopsy material was developed; 2) cultured HOSE cells were further characterized; 3) an ovarian-derived ECM was isolated and characterized; and 4) the interactions between HOSE cells and various substrata were examined in an attempt to study the dynamic, pleomorphic, and morphogenetic nature of HOSE.

An improved methodology to culture HOSE cells from biopsy material was developed by changing the method of biopsy handling. By taking advantage of the tenuous attachment of HOSE to its underlying stroma, HOSE was scraped off the ovarian surface with a rubber policeman. Scraping generated epithelial fragments that settled in culture dishes and from which HOSE cells grew out as epithelial monolayers. Scraping is superior to the explantation method previously described in terms of speed, simplicity, and greater purity and yield of HOSE cells. An additional culture technique was developed to eliminate mold from mold-infected cultures. A simple one-step Percoll gradient centrifugation was developed, using the fungus *Cladosporium* as a prototype. Centrifugation in Percoll removed mold contamination and did not affect cell morphology, growth, or the ability of several cell types to produce ECM suggesting that Percoll decontamination may be a generally applicable technique for decontamination of mold infected cultures.

The list of HOSE characteristics in culture was expanded. HOSE cells maintained in culture continued to express a number of characteristics seen in their *in vivo* counterparts such as the co-expression of vimentin and keratin subtypes #7, 8, 18, and 19 and the expression of variable amounts of mucin and lipid. Unfortunately, to date, there is no unique marker for HOSE cells although it is possible to discriminate HOSE from contaminating cell types that might originate from biopsy material.

A predominantly fibrous ECM was derived from an immortalized, but non-tumorigenic rat ovarian surface epithelial cell line. This ROSE 199-ECM contained both basement membrane and stromal ECM components and supported the attachment, spreading, and growth of several cell lines. It was hoped that HOSE cells maintained on ROSE 199-ECM would assume a morphology similar to their *in vivo* counterparts, that is, a cuboidal monolayer, however, HOSE cells plated onto HOSE 199-ECM dispersed as single, cells throughout the ROSE 199-ECM.

In terms of HOSE-ECM interactions, the results from this study also suggest that HOSE plays an active role in ECM remodelling, that is, both the deposition and degradation of ECM. HOSE cells produce both basement membrane and stromal matrix components and this may be a common feature of ovarian surface epithelia. This study is the first report that HOSE cells secrete proteases. HOSE cells produce a chymotrypsin-like protease, an elastase-like protease, and 30KD and 42KD gelatinases which are metalloproteases. In addition to ECM remodelling via deposition of ECM material or enzymatic degradation of ECM, HOSE cells are also capable of physically remodelling ECM as evidenced by the ability of HOSE cells to contract specific mixtures of ECM into organoids. HOSE-ECM interactions are not unidirectional as substrata also influence HOSE morphology, growth, and protease production. These capabilities may be important for normal ovarian development and normal adult functions. While such proteolytic activities and invasiveness are characteristics of the malignant phenotype, they appear to be part of the normal HOSE phenotype. With regards to why HOSE is a preferred site of ovarian cancer development there may be many factors, but perhaps aberrations of these normal functions contribute to pathological states. HOSE cells in culture appear to be a relevant and a potentially valuable model system for the study of ovarian biology and carcinogenesis.

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