EPITHELIAL AND MESENCHYMAL CHARACTERISTICS OF THE OVARIAN SURFACE EPITHELIUM IN TWO AND THREE DIMENSIONAL CULTURE

by

HELEN GRACE DYCK

B.Sc., Queen's University, 1985

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

Department of Anatomy

We accept this thesis as conforming

to the required standard

UNIVERSITY OF BRITISH COLUMBIA

April, 1995

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Anatomy

The University of British Columbia
Vancouver, Canada

Date April 11, 1995

DE-6 (2/88)

ABSTRACT

The ovarian surface epithelium (OSE) is thought to give rise to 85% of ovarian cancers, predominantly in invaginating clefts and cysts in the ovarian stroma. The cells of the ovarian neoplasms express more complex epithelial characteristics than the cuboidal OSE on the surface. As well, ovarian cancer cells when cultured retain their epithelial characteristics whereas OSE expresses a dual epithelial-mesenchymal phenotype and gradually loses its epithelial characteristics. These observations raised several questions: 1) could cyst-like structures be developed in culture; 2) could improving culture conditions avoid the loss of epithelial characteristics by OSE; and 3) were variations in expression of epithelial and mesenchymal characteristics clinically significant.

This study used a rat OSE line (ROSE 199) to explore the use of two collagenous 3-D culture systems for the development of cysts: seeding cells in sponges and gels. The impact of collagen on the morphology of these cells was also assessed using collagen coats and seeding on gels. It became apparent that two sublines of ROSE 199 had developed that differed in the amount of matrix deposited and in the ability to migrate into the extracellular matrix (ECM); one subline exhibiting a predominantly epithelial response whereas the other showed characteristics of an epithelial-mesenchymal conversion response. The collagen sponge supported the formation of cyst-like structures and affected the organization, but not the types, of basic ECM components produced by the ROSE 199 High ECM subline. Therefore the sponge system is useful for developing cyst and cleft-like structures in culture, while the two sublines may be useful for determining the signals that trigger the mesenchymal response in OSE.

Co-culturing ROSE 199 with human OSE was used in an attempt to improve culture conditions that would maintain the original characteristics of the latter. ROSE 199, like human OSE in culture, produces both epithelial and mesenchymal ECM components.

However, unlike human OSE, it forms a cuboidal epithelium on a thick autologous matrix with underlying cellular layers. It was hoped that ROSE 199 could provide the signals that human OSE needed to maintain their epithelial characteristics. This did not occur. Instead, the human OSE cells migrated into the ROSE matrix assuming a mesenchymal morphology even when able to maintain epithelial colonies when grown alone. Therefore this system does not improve the retention of epithelial characteristics by human OSE.

Thirdly, the study examined whether neoplastic progression or genetic changes associated with a family history of ovarian cancer affected the expression of epithelial and mesenchymal characteristics. Normal OSE from women with (FH-OSE) and without (NFH-OSE) a family history of ovarian cancer, SV40 immortalized lines of each, and ovarian cancer lines were compared. The parameters examined were keratin and collagen III expression as cytodifferentiation markers, and three-dimensional morphogenesis. The results indicate there is a decrease in expression of mesenchymal characteristics with neoplastic progression while epithelial characteristics are maintained, and that FH-OSE cells are phenotypically different from NFH-OSE and are less prone to undergo epithelial-mesenchymal conversion in culture. This suggests that an increase in commitment to an epithelial phenotype and/or reduced responsiveness to signals in culture may be one of the earliest changes in the process of ovarian carcinogenesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

C4II.....a cervical carcinoma line used as a positive keratin control

ECM.....extracellular matrix

EDTA.....ethylene-diaminetetra-acetic acid

FAA.....formalin-acetic-alcohol, a fixative

FBS.....fetal bovine serum

FH-.....family history of ovarian cancer

FH-IOSE.....SV40 immortalized line of FH-OSE

FITC.....fluorescein isothiocyanate

GAGs.....glycosaminoglycans

H & E.....hematoxylin and eosin

huFib.....human dermal fibroblasts

huOSE.....human ovarian surface epithelium

NFH-.....no family history of ovarian cancer

NFH-IOSE...SV40 immortalized line of NFH-OSE

OSE.....ovarian surface epithelium

PAS.....periodic acid / schiff reagent, method for staining carbohydrate groups

PBS.....phosphate buffered saline

RITC.....rhodamine isothiocyanate

ROSE 199....a spontaneously immortalized rat ovarian surface epithelial cell line

ROSE 239....a spontaneously immortalized rat ovarian surface epithelial cell line

SV40.....simian virus 40

XGPRT.....xanthine-guanine phosphoribosyl transferase

ACKNOWLEDGMENTS

I would like to thank Dr. Nelly Auersperg for her encouragement, support and guidance throughout this research. I would also like to thank the other members of my research committee, Dr. Bruce Crawford and Dr. Joanne Emerman for their comments and critical evaluation of this work. I would like to thank Dr. Phil Reid, Linda Trueman, and Michael Iagallo for their help with the histochemistry methods, as well as processing and cutting. I would also like to thank Sarah Maines-Bandiera and Dr. Patricia Kruk for their instruction in tissue culture techniques and immunofluorescent staining.

This research was supported by grants from the Medical Research Council to Dr. N. Auersperg and fellowships from the B.C. Foundation for Non-Animal Research.

CHAPTER 1

INTRODUCTION

Background

Even though ovarian cancer is the leading cause of death from gynecological neoplasms among North American women, the etiology and early events in the development of this disease remain among the least understood of all major human malignancies. The importance of understanding the early events in ovarian carcinogenesis is highlighted by the fact that the five-year survival rate of women with ovarian carcinomas has remained at only 30-40%, largely because most of these cancers are at an advanced stage when first diagnosed (Piver et al., 1991; Perez et al., 1991). The majority of ovarian carcinomas are thought to be derived from the ovarian surface epithelium (OSE) (Young et al., 1989).

OSE in vivo

The OSE is a simple cuboidal epithelium that covers the surface of the ovary and is continuous with the mesothelium, a squamous epithelium that lines the peritoneal cavity. It is a mesodermally derived epithelium that originates from the coelomic mesothelium. In the adult, it has been shown to be composed of two cell types found in distinct zones (Gillet et al., 1991): one is cuboidal with a higher density of microvilli (type A), and the other is squamous with a larger diameter (type B). The type B cells are thought to arise during ovulatory repair (Gillet et al., 1991).

The cells appear relatively undifferentiated at the light microscopic level while ultrastructurally they are also fairly unremarkable. The apical surface is dome shaped and covered with numerous microvilli. The basolateral surfaces tend to be infolded and have dilated intercellular spaces. There are incomplete tight junctions, gap junctions and desmosomes connecting the cells. The nuclei are large and irregular in outline and sometimes lobulated, containing heterochromatin clumps and homogenous nucleioli. The cytoplasm of the cells contains numerous free ribosomes, a Golgi body on the apical side

of the nucleus, a little RER and SER and lipid droplets. There are numerous cytoplasmic vacuoles which contain a granular and finely stranded material similar to that found in the intercellular spaces and in fibroblast-like cells of the outer cortex (Papadaki and Beilby, 1971). They are separated from the underlying stroma by a basal lamina of varying thickness and a dense collagenous tunica albuginea, in which the collagen fibrils run parallel to the surface of the ovary.

Like the squamous mesothelial cells, OSE cells express both low molecular weight keratins and vimentin (Czernobilsky et al., 1985) and are capable of transcellular fluid transport. The OSE cells differ in that they contain large amounts of glycogen and are positive for 17-ß-hydroxysteroid dehydrogenase, and have more complex intercellular junctions, as well as a predominantly cuboidal shape (reviewed in Nicosia and Nicosia, 1988; and Blaustein, 1981a).

Inclusion cysts

The OSE can penetrate into the underlying stroma, forming cysts and clefts. In some mammalian species the formation of narrow clefts appears to be induced by hormonal stimulation and may play a role in oocyte maturation and ovulation (Harrison & Harrison Matthews, 1951). In humans, cysts and clefts are present from the fetal period onward (Blaustein, 1981b) though they are most prevalent in post-menopausal ovaries. The epithelium that lines the cysts and clefts is often columnar and synthesizes a number of proteins that are not produced by the OSE on the surface. Among these proteins are non-specific alkaline phosphatase, placental alkaline phosphatase, human milk fat globule antigen Ca19-9 (Nouwen et al., 1987), and placental lactogen (Blaustein et al., 1982) which are also expressed in serous cystadenomas of borderline malignancy. It is thought that the cysts and clefts are the main sites of carcinogenesis for the common epithelial cancers of the ovary. This association is particularly evident in serous neoplasms where there exist gradations between inclusion cysts and serous cystadenomas (Fenoglio et al., 1977; Blaustein et al., 1982).

Ovarian Cancer

In contrast to the unremarkable morphology of the OSE, the epithelia of ovarian neoplasms are more differentiated. They exhibit a variety of specialized features, including more abundant secretory organelles and cytoplasmic inclusions, and resemble epithelia of Müllerian duct origin, which form the basis of their classification (Table 1). A common epithelial carcinoma often consists of several types of epithelial cells, for example serous cystadeonmas may contain both ciliated cells and non-ciliated secretory cells resembling the ciliated cells and peg cells of the oviduct. The greatest variety of types of differentiation are found in the mucinous cystadenocarcinomas in which some cells look like small intestinal enterocytes with brush borders, others are packed with similar mucins to the goblet cells, and a smaller subset share ultrastructural and antigen features with pyloric gastric cells or peptide hormones with enteroendocrine cells (Tenti et al., 1992). Some of the differentiation features are not present in the more malignant tumors, such as cilia on cells of serous cystadenocarcinomas, and often the columnar cells give way to round or oval cells, but the majority of intracellular features remain (Fenoglio, 1980, Young et al., 1989).

tumor classification:	epithelium resembles:
serous, benign and malignant	tubal
endometroid, benign and malignant	endometrial
clear cell	variable
mucinous, benign	endocervical
mucinous, malignant	intestinal
Brenner	transitional

Table 1. Classification of the common epithelial tumors of the ovary

Incidence and risks

The common epithelial tumors account for 2/3 of all ovarian neoplasms, while the malignant forms account for 80-90% of ovarian cancer in the Western world. The borderline and invasive serous tumors account for 35% to 40% of all ovarian cancers followed by endometroid (15%), clear cell (5%), and mucinous. Brenner tumors are rarely

malignant (Young et al., 1989). Most of these cancers occur between the ages of 40 to 80+, the average age being 59 years (Lynch et al., 1993).

One in 70 women will develop ovarian cancer (1.4%) and about 5% of women with ovarian cancer report a first or second degree relative with ovarian cancer. However, probably fewer than 3% of these women fall into the category of hereditary cancer syndromes (Kerlikowske et al., 1992). Hereditary cancer syndromes fall into three groups: 1) breast-ovarian cancer syndrome which involves families with clustering of ovarian and/or breast cancer, 2) site-specific ovarian cancer, with clustering of only ovarian cancer and 3) Lynch syndrome II which involves nonpolyposis colorectal cancer, endometrial carcinoma, and ovarian carcinoma (Lynch et al., 1993). Pedigree analysis suggests that the syndromes have an autosomal dominant pattern of inheritance which would give a woman as high as a 50% lifetime probability of developing ovarian cancer (Piver et al., 1991). A genetic marker, the BRCA1 gene, has been established for the breast-ovarian syndrome group and, in its mutated forms, confers a risk of close to 100% for either breast or ovarian cancer, though the cumulative risk for ovarian cancer is lower than breast (44% vs. 87% by age 70) (Ford et al., 1994). The probability of developing ovarian cancer in women who report one or two first or second degree relatives with ovarian cancer who do not fall into a syndrome category is 5-7% (Kerlikowske et al., '92).

The two most identifiable non-hereditary risk factors for ovarian cancer are the absence of use of oral contraceptives and nulliparity (reviewed in Piver et al., 1991). Conversely, the use of oral contraceptives reduces the risk, even in women with a family history of ovarian cancer (Gross and Schlesselman, 1994). This has led to the development of the theory that incessant ovulation is a major factor in ovarian carcinogenesis (Fathalla, 1971). The presumed effects of ovulation include a greater potential to form inclusion cysts (Radisavljevic, 1977) and an increase in mutational hits due to a rapidly dividing cell population (Godwin et al., 1993). Also there is, presumably, increased exposure to growth and differentiation factors involved with wound healing.

Models for studying ovarian carcinogenesis

Animal models

A number of studies have been done using animal models both in vivo and in vitro to try to understand the process of ovarian carcinogenesis. These include the impact of ovulation on OSE proliferation in rabbits which are reflex ovulators (Osterholzer et al., 1985), and the effects of corpus luteum extract on rabbit OSE in culture (Setrakin et al., 1989). Cultured rat OSE has been used to study cytogenetic changes arising from constant proliferation (Godwin, et al., 1992) and the effects of oncogenes such as ras (Adams and Auersperg, 1981; Hoffman et al., 1993). However, it is unclear to what extent the behavior of OSE from other species mimics that of human OSE. There are differences between species as to the extent which OSE covers the surface of the ovary. For example, in horses the ovary is only half covered whereas in armadillos it is patchy; rabbits, in contrast, form an abundance of papillary structures. OSE invaginations or crypts also appear to have species dependent functions. The formation of these crypts is markedly associated with oocytes in marsupials and insectivores and directly related to the ovulatory cycle in seals. However, they are not present in a number of species; though in some, like ferrets, they can be induced by gonadotropins and estrogens (Harrison and Harrison Matthews, 1951). The relevance of animal models is further brought into question by the fact that ovarian cancers are rare in all species except the domestic chicken (reviewed in Fathalla, 1971). Older hens appear to develop oviductal and ovarian tumors, almost exclusively, when kept free of oncoviral contaminants (Fredrickson, 1987).

Human OSE in culture

The loose attachment of OSE to the tunica albuginea of the human ovary has made it possible to obtain fairly pure cultures of human OSE (Kruk et al., 1990). The cells are grown routinely in Medium 199:MCDB105 (1:1) with 15% FBS. Reduced serum and serum-free medium supplements have also been developed but whereas cells are

maintained in these preparations, they do not proliferate as readily (Elliott and Auersperg, 1992). A number of studies have looked at the production of growth factors by human OSE and have shown that these cells in culture will synthesize TGF-β (Berchuck et al., 1992), amphiregulin (Johnson et al., 1991), and IL-1, IL-6 and a number of colony stimulating factors (Ziltner et al., 1993).

Early passages of human OSE have an epithelial cobblestone morphology composed of cuboidal cells with microvilli on their apical surface and a poorly defined basal lamina. Most of the cells, however, are flattened and are polarized insofar as they contain microvilli on their apical surfaces. The cells adhere and grow on plastic, but they lose the cobblestone pattern with subsequent passaging, assuming a fibroblast-like morphology and reducing cell contact to a few widely spaced desmosomes (Auersperg et al., 1984).

Expression of the epithelial markers keratin and cytovillin have been shown to diminish with passaging, while expression of vimentin and extracellular matrix (ECM) components remained constant (Auersperg et al., 1994). Human OSE cells express both stromal ECM components and basement membrane components (i.e., collagens I and III as well as laminin and collagen IV) and contain the enzyme prolyl-4-hydroxylase required for stabilization of the triple helix conformation in collagen (Kruk et al., 1994; Auersperg et al., 1994).

Human OSE cells have been cultured on a variety of substrata to maintain and induce a more cuboidal phenotype, including fibrin clots, collagen gels, and Matrigel (Kruk et al., 1994). These experiments revealed some interesting responses of cultured OSE to exogenous ECMs. In contrast to the cohesive monolayers on plastic, fibrin clots enhanced cell dispersion and collagen gels the conversion to an fibroblastic phenotype, whereas the cells invaded Matrigel as multicellular aggregates. It is not too surprising, therefore, that the level of protease production was greatest on Matrigel and lowest on plastic. Proliferation was also substratum dependent and inversely related to protease secretion. A slightly different pattern was seen in examining integrin expression where it

was again highest on plastic but lowest on collagen gels. All substrata maintained expression of keratin. OSE, when seeded on top of the collagen gel, did not contract it (Kruk and Auersperg, 1992) but a few cells did invade this matrix (Kruk et al., 1994). When the human OSE cells were cultured on a combination of a rat OSE matrix (ROSE 199 ECM) placed on a collagen gel, they contracted both structures and cells penetrated into the matrix (Kruk and Auersperg, 1992). These results demonstrate that not only is the behavior of OSE regulated by the ECM but also that OSE has an ability to restructure the ECM thorough synthesis, lysis and physical contraction.

Objectives

Among OSE in vivo there appears to be an increase in epithelial characteristics in the cells which line inclusion cysts and clefts, which are often columnar, acquire cilia, and express markers in common with cystadenomas. It has been suggested that maybe the micro-environment, produced by inclusion cysts and crypts, leads to the expression of metaplastic Müllerian phenotypes as well as the epithelial markers (Blaustein et al., 1982; also reviewed in Nicosia and Nicosia, 1988). The growth of cells on planar substrata is not conducive to the formation of cysts or clefts, and though the OSE contraction of ROSE 199/ collagen gel organoids led to the formation of cleft-like structures, their formation was not consistent (Kruk, 1992). Part of the following set of experiments were designed to test the suitability of various three-dimensional supports for the formation of cyst-like structures and to test the impact of collagen preparations and three dimensional culture on tissue organization.

There are three widely used methods for providing three-dimensional support and formation of cyst-like structures. Two of these involve growing cells within gels of either basement membrane components (Matrigel) or collagen type I. For example, oviductal cells will form spheres and tubes when plated within Matrigel (Joshi, 1991), whereas endothelial cells will form tubes (Madri et al., 1988) and thyroid cells will form follicles

(Greenberg and Hay, 1988) in collagen gels. However, unlike other epithelial cells which differentiated on Matrigel, human OSE cells remained as solid cell aggregates and lysed the matrix. Therefore plating cells within this substratum was not further tested in our system. The third method is to grow cells within a collagen sponge. This method has proved advantageous for maintaining the tissue organization of tumors in long term culture, including ovarian tumors (Freeman and Hoffman, 1986). It has also been used extensively for studying events in wound repair (Doillon et al., 1987) and the differentiation of mesenchymal cells, such as osteoblasts (Casser-Bette et al., 1990).

The second part of the work described here was devoted to devising a culture model in which human OSE cells could maintain a cuboidal phenotype. Unlike human OSE cells which assume a mesenchymal morphology with passaging, spontaneously immortalized rat OSE lines will maintain a cuboidal phenotype. The formation of cuboidal to columnar cells was particularly of interest in the ROSE 199 line which produces an abundant ECM composed of both stromal and basement membrane components. The provision of ROSE 199 matrix alone, however, did not maintain an epithelial human OSE phenotype (Kruk, 1992); instead, the cells became fibroblastic and invaded the matrix. This raised the question whether the method for removing the ROSE 199 cells from the matrix also removed critical ECM components and whether co-culturing ROSE cells with human OSE would provide a better system than the matrix alone. Co-culturing of stromal cells with epithelial cells has been used successfully by a number of people to maintain or induce differentiation (Vachon et al., 1993; Senoo et al., 1989). Therefore, it was decided to examine the effects of co-culturing with ROSE lines on the morphology of human OSE cells.

The observations to date indicate that human OSE gradually loses a number of its epithelial characteristics in culture while maintaining those characteristics, e.g., ECM production and matrix contractility, which would enable it to be an active participant in stromal repair. This is in contrast to the type of metaplasia seen in ovarian neoplasms

where the tendency is to acquire complex epithelial characteristics. To determine whether there was any clinical significance to this observation, the expression of keratin and collagen III and three-dimensional morphology were examined in normal OSE from women with and without a family history of ovarian cancer, in SV40 immortalized lines of each, and in ovarian cancer lines to see if these groups showed differences in expression of epithelial and mesenchymal characteristics.

It was hoped that this work would improve our understanding about the expression of epithelial and mesenchymal characteristics by OSE in relation to the process of ovarian carcinogenesis and further refine culture models for studying this process.

CHAPTER 2

GENERAL MATERIALS AND METHODS

I. Tissue culture

I.1 Culture conditions and Media

Cultures were incubated at 37°C either in a humidified incubator with 5% CO_2:95% air or in sealed flasks. Depending on the cell type (see below), the synthetic media used were Medium 199:MCDB 105 (1:1) (Sigma, St. Louis, MO) and Waymouth's medium MB 752/1 (Gibco, now Canadian Life Technologies, Burlington Ont.).

I.2 Supplements:

Serum supplements

Standard culture conditions included the addition of fetal bovine serum (FBS) to the medium with the concentration dependent on cell type (see below).

Replacements for FBS were:

1) Omni serum, (ABi, Columbia, Maryland), an undefined calf serum supplemented with the following growth factors in unknown quantities: insulin, EGF, Brain Derived Growth Factor, transferrin, vitamins and trace elements.

2) PC-1, consisting of: Pederson's fetuin (Ventrex Labs, Portland ME; 500 μg/ml), and insulin 15 μg/ml, transferrin 20 μg/ml, lipoic acid 0.1 μg/ml, phosphatidylcholine 200 ng/ml, and ethanolamine 0.33 μg/ml (Sigma) (Elliott and Auersperg, 1992). Pederson's fetuin contains 0.05% undefined serum proteins. PC-1 was added daily to cultures.

Ascorbate-2-phosphate

Ascorbic acid oxidizes quickly under normal media storage (4°C) and tissue culture (37°C) conditions requiring daily addition to cultures even though both Waymouth 752/1 and FBS contain ascorbic acid. The magnesium salt of ascorbate 2-phosphate, in contrast, is stable for up to 7 days at 37°C and up to 30 days at 5°C, with similar collagen

stimulating levels as ascorbic acid (Hata and Senoo, 1989). Medium supplementation with 0.5 mM ascorbate 2-phosphate (WAKO Bioproducts) was carried out on parallel cultures of ROSE 199 on plastic and on all cell types grown in Spongostan.

Antibiotics

Antibiotics used were either 1) 50 μg/ml gentamicin (GIBCO) or 2) 50 units/ml of penicillin and 50 μg/ml streptomycin (GIBCO).

I.3 Subculture

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 and spun at 100g in a clinical centrifuge for 5 min.

I.4 Preparation of substrata

Plastic and Liners:

Cells were plated in a 0.5 ml suspension directly into culture wells. In some experiments 60 mm dishes with a removable plastic liner were used to obtain cross-sections without removing the cells from the plastic substratum.

Collagen coats:

Rat tail tendon-derived collagen gel solution (courtesy of Dr. J.T. Emerman) was plated into each well and allowed to stand for 10 min. before removing excess solution. The wells were then allowed to dry overnight before plating the cells.

Collagen gels:

Collagen gels were prepared by the method of Emerman and Pitelka (1977). In brief, 0.45 ml of collagen gel solution was added to each well and 0.1 ml of a 2:1 mixture of Medium 199 (10x) and 0.34 N NaOH was added and stirred to ensure complete mixing. Cell suspensions were added to the gel either before or after polymerization.

Spongostan:

A dehydrated collagenous sponge, Spongostan, was obtained from Health Designs Industries, (Rochester, NY). Pieces either 1) 10 x 7.5 x 1 mm or 2) 5 x 3.5 x 1 mm in size were cut and rehydrated using ddH_2O and 1-2 changes of media before being placed in wells and inoculated with 0.1 ml of cell suspension. The sponges and cells were incubated for 2 hours prior to the addition of 2 ml of media, to allow the cells to adhere to the sponge.

II. Sources of Cells:

Rat OSE lines:

Two spontaneously immortalized rat OSE lines, ROSE 199 and ROSE 239, were developed in the Auersperg lab by Anne Adams (1985). ROSE 239 is an monolayered epithelial line which produces only a small amount of ECM. ROSE 199 in contrast is a multilayered epithelial line which produces abundant ECM composed of both basement membrane and stromal components (Auersperg et al., 1991). Stock cultures were grown in Waymouth's medium MB 752/1 supplemented with 2.5% Omni serum.

Human OSE

Fragments of OSE were detached from the surface of grossly normal ovaries with a rubber scraper and cultured (Kruk et al., 1990). These ovaries were obtained from women either with a strong family history of ovarian/breast cancer (FH), as defined by two or more first degree relatives with ovarian/breast cancer (Lynch and Lynch 1992), or from the general population where there was no family history (NFH). Four of the FH patients were found to be linkage positive to the BRCA-1 locus, and these individuals underwent prophylactic oophorectomies; the rest of the specimens were obtained from women undergoing surgery for non-malignant gynecological diseases. FH-OSE were obtained courtesy of Dr. H. Lynch (Creighton University School of Medicine, Omaha, NE) and Dr.

T. Hamilton (Fox Chase Centre, Phil. PA). Both FH-OSE and NFH-OSE were cultured in Medium 199:MCDB 105 (1:1) with 15% FBS.

SV40 T immortalized lines:

Previously immortalized non-tumorigenic lines of NFH-OSE, (NFH-IOSE) and FH-OSE (FH-IOSE) were produced by transfection with early SV40 genes using calcium phosphate or lipofectin (Gibco) (Maines-Bandiera et al., 1992). Stock cultures were grown in Medium 199:MCDB 105 (1:1) with 2% FBS.

Ovarian Cancer lines:

Eight different ovarian cancer cell lines were used. NIH:OVCAR-2 used in p.14, NIH:OVCAR-3, NIH:OVCAR-4 (p.11), NIH:OVCAR-5 (p.99), NIH:OVCAR-8 (p.16), NIH:OVCAR-10, (p.13,14) and Sk-OV3 were obtained from Dr. T. Hamilton, and CaOv3 from Dr. J. Fogh, (Fogh and Trempe, 1975).Stock cultures were grown in Medium 199:MCDB 105 (1:1) with 2% FBS.

Control cells

The control cells used were human dermal fibroblasts (huFib) for collagen III, and a cervical carcinoma line, C4II (Auersperg et al., 1989), for keratin. Human dermal fibroblasts were also used in co-culture experiments.

IV. Fixation and Processing for Histology:

Fixatives

The fixatives used were 10% formalin in phosphate buffered saline or formalin-acetic-alcohol (FAA: 10 ml 40% formalin, 5 ml glacial acetic acid, 85 ml absolute EtOH) for samples which were to be embedded and sectioned. Methanol (-20°C) was used as a fixative for immunofluorescence.

Embedding media:

Paraffin: A rapid processing protocol was used due to the small size of the samples.

70% EtOH 1 x 20 min.
95% EtOH 2 x 20 min.
100% EtOH 3 x 20 min.
Xylene: 2 x 20 min.
Paraffin: (60° vacuum) 1 x 45 min. and 1 x 60 min.
5 μm sections were cut and stained.

JB4: A JB4 kit was obtained from Polysciences, Inc. (Warrington, PA). Samples were either dehydrated using the same alcohol series as above prior to incubating in a 50:50 mixture of catalyzed solution A and absolute EtOH for 1 hour, and overnight in 100% solution A prior to embedding in the mixed JB4 solution, or placed directly into the catalyzed solution A overnight prior to embedding. There did not seem to be a distinguishable difference in morphology with either method. 2 μm sections were cut and stained with Hematoxylin and Eosin (H&E).

Processing of cultures grown on different substrata

Plastic and Collagen Coats: A variety of methods were explored for ease of preparation and preservation of morphology. Gentle scraping or pipetting prior to fixation was often sufficient to detach cultures of ROSE 199. Other samples were embedded in situ in JB4 and removed from the dish before complete polymerization, cut into strips and re-imbedded in JB4.

Plastic liners: Cells were fixed in situ and the plastic liner cut into strips and embedded in paraffin and JB4. Unfortunately upon rehydration of the JB4 sections the strip of plastic liner would tear from the surrounding JB4 matrix. Therefore this embedding medium was not suitable. It was also difficult to obtain good paraffin sections due to rippling of the liner. ROSE 199 cells grown on the liners appeared to deposit matrix more slowly then on plastic, otherwise the results supported those obtained from scraped cultures, therefore this method was not pursued further.

Collagen gel and Spongostan: These cultures were fixed in situ and removed prior to embedding in either paraffin or JB4.

V. General Immunofluorescence Methods

Cultures to be examined by immunofluorescence methods were cultured on glass coverslips. The cultures were rinsed with serum free media 3 x 5 min. prior to fixation. In general, cultures were fixed prior to the addition of antibodies and control serum; exceptions are noted in the pertinent experiments. After fixation, cultures were rinsed 3 x 5 min. in PBS and incubated 20 min. in goat serum to block non-specific staining of the secondary antibody. Experimental cultures were blotted and the primary antibody added and then incubated for 1 hr. at room temperature, unless otherwise noted. Duplicate cultures did not receive primary antibody and served as a specificity control for the second antibody. All cultures were then rinsed 3 x 5 min. in PBS and incubated for 1 hr. at room temperature with the secondary antibody to which was attached the immunofluorescent tag. They were then rinsed 3 x 5 min. in PBS and mounted onto slides with Gelvatol pH 6.5 (O'Guin et al., 1985). They were examined with an Axiophot photo microscope (Carl Zeiss, Oberkochen, Germany) and photographed using Kodak T-MAX 400 film (pushed to 800) with a 40X neofluor objective. Frozen sections of tissue were treated in the same way as cultures on coverslips.

CHAPTER 3

EPITHELIAL / MESENCHYMAL MODULATIONS OF RAT OVARIAN SURFACE EPITHELIUM

Introduction

Cysts and clefts lined by OSE in the ovarian stroma appear to be the prime target sites of ovarian carcinogenesis. The reasons for this are unclear but proximity to the ovarian stroma and/or a confined microenvironment have been suggested as possible factors. In order to investigate the role of these structures in ovarian carcinogenesis it would be helpful to develop a culture system in which some of these factors could be examined. The three-dimensional systems that were chosen to explore the potential of developing cyst-like structures in culture were culturing cells inside collagen gels and the collagenous sponge, Spongostan. The impact of collagen on the morphology of the cells was further assessed by growing the cells on two other types of collagen preparations: collagen coated dishes and on top of collagen gels. The effect of three-dimensional sponge culture was determined by examining the resulting cellular organization and by histochemical analysis. The cell line chosen to explore these systems was the spontaneously immortalized rat OSE line, ROSE 199.

Primary and low passage cultures of rat OSE form epithelial monolayers that are keratin positive and vimentin negative while able to produce collagens I, III, and laminin (Adams and Auersperg, 1981; Hornby et al., 1992; Auersperg et al., 1991). Two spontaneously immortalized lines were derived from primary rat OSE cultures by Anne Adams (1985). When the line ROSE 199 was formed, it behaved in a similar fashion to primary rat OSE forming monolayered epithelial colonies. When it reached passage 19 a change occurred and crowded cultures multilayered and formed ridged structures (Adams and Auersperg, 1985). ROSE 239 was a similar line developed at the same time, however it did not acquire the ability to multilayer but remained as a monolayer even when crowded. The genetic changes involved in the immortalization are unknown but they

conferred an extended life span in culture (Adams and Auersperg, 1985), a decrease in population doubling time (Hoffman et al., 1993), and an increase in cell density (Auersperg et al., 1991). The multilayered, ridged structure of ROSE 199 is partly responsible for the increase in cell density seen in this line, doubling that of ROSE 239 which remains monolayered (7-8 x 10^5 cells/cm^2 vs. 3-4 x 10^5 cells/cm^2) (Auersperg et al., 1991). With time, both ROSE 199 and ROSE 239 showed a reduction in the proportion of cells which express keratin, while acquiring vimentin in all cells (Hornby et al., 1992). In the case of ROSE 199 keratin expression is strongest in the cells overlying the ridges, whereas surrounding epithelial colonies were negative, suggesting that the ECM in these regions enhanced the expression of keratin (Hornby et al., 1992). Both lines retained the capacity of primary rat OSE to synthesize collagens I and III. However ROSE 199 acquired a density dependent rate of synthesis, with little collagen production during rapid growth, but reaching primary rat OSE production levels post confluence (Auersperg et al., 1991). Electron microscopy revealed that the collagen is deposited in the ROSE 199 ECM as short banded fibrils (Adams & Auersperg 1985). The lines also retain the capacity to synthesis and secrete laminin (Auersperg et al., 1991), and ROSE 199, at least, a well defined but discontinuous basal lamina (Adams & Auersperg 1985). Thus the two lines, as characterized to date, retain the ability to express some but not all the epithelial/ mesenchymal characteristics of primary rat OSE.

A cuboidal phenotype coupled with abundant matrix production made the ROSE 199 cells an ideal line to study the impact of various exogenous collagen substrata on the three-dimensional organization and expression of epithelial and mesenchymal phenotypes of OSE cells. Collagen substrata can be prepared in various ways resulting in dry collagen coats, collagen gels, floating collagen gels and collagenous sponges. These in turn have differing impacts on cellular differentiation and morphology. Seeding cells on top of collagen gels result in epithelial colonies whereas seeding cells within gels can lead to formation of three-dimensional structures (Madri et al., 1988, Chambard et al., 1981).

Fibroblast cells within collagen gels display tissue specific organization of cells and have been used to asses the contractile ability of fibroblasts, a property that is important in the process of wound healing (Doane and Birk, 1991; Bell et al., 1979). Floating collagen gels have been found to increase differentiation in a number of epithelial cells (Emerman and Pitelka, 1977; Michalopoulos and Pitot, 1975). Collagen gels contain bioactive collagen unlike Spongostan which has been heat sterilized, a process that denatures collagen (Kleinman et al., 1987). This difference was expected to have an impact on cellular behavior. However, the three-dimensional support provided by denatured collagen sponges facilitate production of specialized ECM by a number of mesenchymal cells: for example, osteoblast-like cells form a bone-like tissue and express high levels of osteocalacin and alkaline phosphatase when grown on collagen sponge but not on plastic (Casser-Bette et al., 1990).

In view of the evidence that three-dimensional support as provided by the collagen sponge can affect the production of components of the ECM as well as its organization, it was decided to compare the production of matrix by ROSE 199 on plastic and in Spongostan over the course of several weeks. Histochemical methods were used to give a broad picture of changes in the principal components of ECM. ECM is composed of three basic classes of polymers: collagens, proteoglycans and glycoproteins, all which contain varying degrees of carbohydrate chains linked to small or large protein cores. One of the most widely used methods for the detection of carbohydrate groups is the periodic acid / Schiff reagent reaction (PAS), reviewed by Reid and Park (1990). The allochrome procedure devised by Lillie (1965) allows one to view carbohydrates and collagens simultaneously by combining the PAS method with a variation of a van-Giesson method for collagen. The presence of reticulin, which contains collagen III, can be distinguished from larger fibers of collagen I using silver reticulin methods. Sialic acids present on laminin, a component of basement membranes, can be distinguished from other carbohydrates using a selective oxidation procedure (Volz et al., 1987), whereas

proteoglycans can be distinguished by their anionic groups, mainly sulfate esters and carboxyl groups, by using a cationic dye such as Alcian Blue at pH 1.0 and 2.5. This combination of histochemical methods thus allowed one to examine collagen organization, and distinguishing between collagen I and III, as well as detecting variations in carbohydrate content and the presence of anionic groups in the ECM.

During my experiments it became apparent that two sublines of ROSE 199 had developed over the course of culturing these cells. These sublines differed in the amounts of ECM produced on plastic and the tendency of the cells to become mesenchymal. They have been designated ROSE 199 High ECM and ROSE 199 Low ECM. Two cloned lines, developed by serial dilution and single cell plating, were used to confirm the nature of the sublines. ROSE 199 cl. G11 was derived from the High ECM subline and ROSE 199 cl. E11/A4 from the Low ECM subline.

Materials and Methods

Impact of collagenous substrata on morphology in two and three-dimensions

ROSE 199 High ECM and ROSE 199 Low ECM and two cloned lines, G11 (from High ECM) and E11/A4 (from Low ECM) were grown on plastic, collagen coats, and collagen gels, and in Spongostan. The two sublines, but not the two clones were also seeded within polymerizing collagen gels. Cells were grown for four weeks in standard medium (see chapter 2) and antibiotics. Plastic and Spongostan cultures were also grown with and without 0.5 mM ascorbate 2-phosphate. Cultures were monitored using phase microscopy. Quadruplicate samples were run in each experiment and 3 to 4 experiments were run for each subline.

Samples were formalin fixed and processed as described in the general methods. The majority of the samples were embedded in JB4. Sections were stained with H & E. Cultures were examined for cellular morphology, multilayering potential, invasion and contraction of 3-D collagen structures, and formation of cyst-like structures.

Impact of three-dimensional culturing on ROSE 199 ECM development

Cell culture:

The ROSE 199 High ECM subline was grown in 10 x 10 x 3 mm pieces of Spongostan and on plastic control wells in standard medium. The cells were plated at approximately 5 x 10^5 cells per well and the medium was changed daily. Cultures were terminated at 2.5, 3, 4, and 5 weeks.

Preparation of blocks:

At each time point two Spongostan samples and one plastic control sample were removed from their wells and were fixed for 5 hours in FAA. Two additional 5 week samples were fixed in formalin. The samples were then processed according to the following program: 100% EtOH 1 x 15 min. & 1 x 30 min.; xylene 3 x 15 min.; paraffin (60°C) 2 x 20 min. They were then embedded in paraffin and 5μm sections were cut.

The following control blocks were used: rat liver, colon, kidney, duodenum and stomach, and human lymph node. These had been formalin fixed, except liver which was fixed in FAA, and processed according to the usual methods.

Histological procedures: The following procedures were carried out on each block:

Hematoxylin and eosin.

Performed using Gill's III hematoxylin as a general morphological stain.

Allochrome

Performed using a 1% periodic acid solution and two oxidation times, 15 min, and 30 min., followed by Pararosaniline schiff reagent (certified biological stain lot no. 792591B; Fisher Scientific Co, Fairtown, NJ) prepared by the method of Barger and Delameter (1948) for either 15 or 30 min., followed by a mixture of 0.01% Chicago Blue 6B (C.I.) in saturated aqueous picric acid.

The control procedures for carbohydrate staining were the following. Amylase digestion: salivary amylase for 30 min. at 37°C to remove glycogen. Acetylation:

pyridine: acetic anhydride 1:1 mixture 60°C for 1 hour to block periodic reactive groups, except C=C, by esterification. Saponification: 0.5% KOH in 75% EtOH for 30 min. at room temperature to restore the alcohols which had been blocked by esterification. Schiff alone: 30 min. to see if there were any schiff positive substances.

The expected results were that collagen I and III would stain blue, glycogen and other carbohydrates pink, and collagen IV purple due to its close association with carbohydrate components.

Selective oxidation/ Schiff reaction for detecting sialic acids

The selective oxidation/ schiff procedure was performed using the method of Volz (1987) to see if there were any detectable sialic acids present in the ECM. Sections were oxidized for 1 hour at 4°C in 0.44 mM periodic acid and stained with Schiff reagent for 1 hour. The control procedures were acetylation and saponification as described in the allochrome method above. The expected results were that sialic acids would stain pink.

Silver stain for reticulin fibers

The silver stain for reticulin was performed using the method of Gordon & Sweets (Bancroft and Cook, 1984). In brief, sections were brought to water and oxidized using acidic potassium permanganate for 5 min., rinsed, bleached with oxalic acid, and treated with iron alum for 5 min. and washed well. A solution of ammoniacal silver was applied for 15 sec. Tthe sections were rinsed well in water and reduced in a weak solution of 40% formalin in water for 30 sec. A 5% solution of sodium thiosulfate was then applied for 5 min. and counter stained with nuclear fast red. The usual toning with 0.2% yellow gold chloride solution was omitted to improve the contrast of collagen I with the background. The expected results were collagen I yellow, reticulin black branching fibers, basement membranes black.

Alcian Blue pH1.0 and pH 2.5/ Nuclear fast red (NFR)

Sections were stained with Alcian Blue 8GX at pH 1.0 and 2.5 for 30 min. and counterstained with Nuclear Fast red (1 sec.) for the detection of anionic groups (Kiernan,

1990). Usually at pH 1.0 these are sulfate esters and at pH 2.5 these will include any sulfate esters and carboxylic acids.

Results

Impact of collagenous substrata in two and three dimensions

The two sublines, ROSE 199 High ECM and ROSE 199 Low ECM, were found to have distinct behaviours on the substrata studied. The differences in their responses were confirmed by the behavior of their respective cloned lines.

When first seeded on plastic or plastic liners, both sublines, as well as ROSE 239, formed colonies of flattened epithelial cells with single elongated cells in the surrounding spaces. Once confluence was reached (24 hrs) both ROSE 199 Low ECM and ROSE 239 remained as epithelial sheets whereas ROSE 199 High ECM acquired ridges. When sectioned it was seen that both sublines formed multilayered structures consisting of an ECM band surrounded on either side by cell layers. This band presumably represents a layer of ECM parallel to the surface of the culture plate, as it was always present though of varying thickness. In contrast, ROSE 239 formed a single epithelial layer with no detectable matrix, similar to primary rat OSE cells. The ROSE 199 Low ECM subline formed a cuboidal to columnar epithelium on top of the ECM band and a predominantly monolayered flattened epithelial layer on the bottom (Fig. 1a). This was comparable to that described by Adams (1985) which was shown to produce a discontinuous basal lamina by both cell layers. In the ROSE 199 High ECM subline, the top cell layer was also cuboidal to columnar, the acellular ECM band was twice as wide and the underlying cells were predominantly fibroblastic and were often surrounded by a looser ECM (Fig. 2a). The addition of ascorbate-2-phosphate to the culture medium enhanced the production of ECM for both sublines, resulting in wider acellular bands which would divide and result in nests of cells situated between two acellular bands. Another difference that was observed upon the addition of ascorbate was stratification of the top layer which occurred in a

number of areas in both sublines, though that layer remained predominantly a single cuboidal to columnar cell layer (Fig. 1b & 2b).

On collagen coated dishes both sublines formed more compact colonies which took 2-3 days longer to reach confluence then their plastic dish counter parts. However, once confluence was reached, they looked identical to those on plastic. In sections, ROSE 199 High ECM formed similar multilayered structures as when grown on plastic (Fig. 2c). ROSE 199 Low ECM, however, formed a single cuboidal layer with no evidence of the multilayered structure that formed when grown on plastic (Fig. 1c).

Cells seeded on top of collagen gels resulted in epithelial islands of cells which took 2 - 3 weeks to reach confluence. ROSE 199 High ECM formed a cuboidal layer of cells on the surface of the gel and penetrated into the gel as cords of cells or single fibroblastic cells (Fig. 2d-f). These cells were able to contract the gel and detach it from the plastic well. None of the cords of cells appeared to contain a lumen nor were any cysts-like structures formed by the invading cells, regardless of whether the gels were floating or attached. In contrast, ROSE 199 Low ECM remained as a single cuboidal layer on the surface of the gel (Fig. 1d).

When the cells were seeded inside the polymerizing collagen gel, either singly or as small clumps, they remained as small cell groups. Larger cellular aggregates would form balls with elongated cells attached to them. Some of the cases also exhibited gel contraction. The ROSE 199 High ECM cells proliferated better in this environment than the ROSE 199 Low ECM cells. Cross sections revealed that cells remained as single cells, as solid aggregates or surrounded dense clumps of ECM material but did not form hollow structures (Fig. 3 a-f).

In Spongostan, ROSE 199 High ECM filled the spaces with cells, similar in appearance to embryonic mesenchyme cells, suspended in ECM. Flattened epithelial cells lined the surfaces and there were very few, if any, of the cuboidal to columnar cells seen on the other substrata. Serial sectioning revealed that the cells also formed cyst-like

structures lined by squamous cells. The addition of ascorbate-2-phosphate to these cultures did not appear to affect ROSE 199 High ECM (Fig. 4a-d). In contrast, ROSE 199 Low ECM lined the sponge spicules with flattened cells and produced a similar band of ECM as when grown on plastic (Fig. 4 e, g). The E11/A4 clone produced less ECM than the uncloned subline. There were very few cells found in the matrix produced by ROSE 199 Low ECM. Ascorbate-2-phosphate enhanced the deposition of ECM in this subline and increased the number of cyst-like structures lined by cuboidal cells seen per section (Fig. 4 f, h). In contrast to the ROSE 199 sublines, ROSE 239 covered the sponge spicules with flattened to cuboidal cells with very little ECM (Fig. 4i). Addition of ascorbate-2-phosphate did not affect this phenotype (Fig. 4j).

The results from this portion of the experiments indicate that there are two sublines of ROSE 199 which differ in the amount of matrix they produce, in their ability to modulate to mesenchymal cells which can migrate into ECM, and in their responses to various collagen substrata and to ascorbate. The capacity of ROSE 199 High ECM to modulate between epithelial and mesenchymal forms was confirmed by using a cloned line which also exhibited a similar capacity and by subculturing crowded cultures sparsely which resulted in epithelial colonies until the cells again became crowded.

The bioactive collagen found in the coats and gels decreased the production of ECM and the formation of multilayered structures, with a greater influence on the Low ECM subline than the High ECM subline. The inert collagen found in Spongostan, in contrast, affected the organization of the ECM and diminished the proportion of cuboidal cells, with a greater effect on the High ECM line. Addition of ascorbate increased the production of ECM on plastic for both sublines, but only that of the Low ECM subline on Spongostan. Cyst-like structures were only seen in Spongostan, suggesting that this was the best substratum, among those tested, for developing this type of three-dimensional organization in OSE.

Figure 1. ROSE 199 Low ECM morphology on planar substrata.

Cells were grown on: (a, b) plastic, without (a) and with (b) ascorbate 2-phosphate, (c) collagen coated dishes, and (d) on top of collagen gels. Stained with H & E x 270.

Figure 2. ROSE 199 High ECM morphology on planar substrata.

Cells were grown on: (a, b) plastic, without (a) and with (b) ascorbate 2-phosphate, (c) collagen coated dishes, and (d-f) on top of collagen gels. Sections stained with H & E, a, b, c, d, f: X 270, e: X 70.

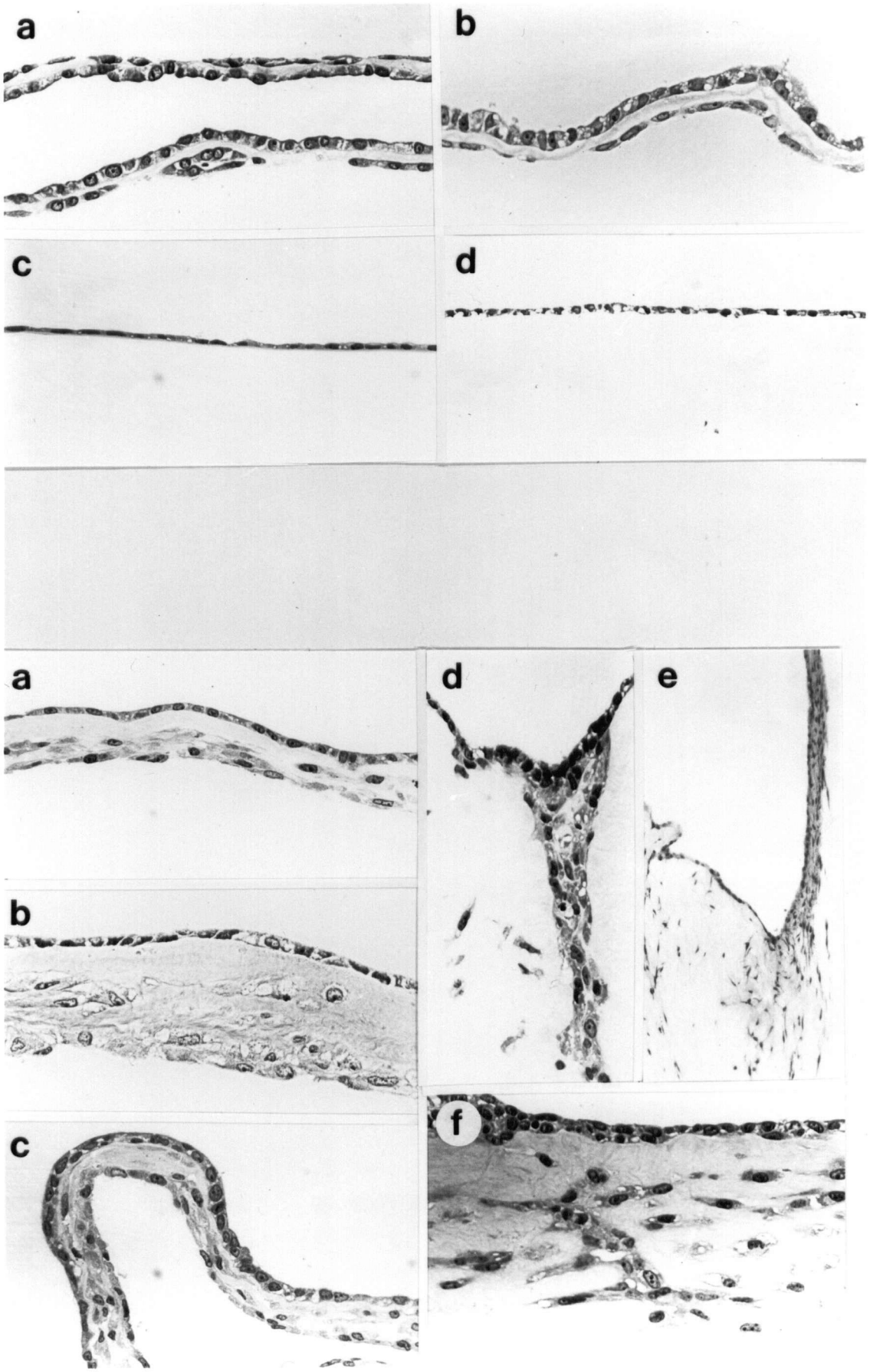

a
b
c
d
a
d
e
b
c
f

Figure 3. ROSE 199 subline morphology within collagen gels.

a-d) ROSE 199 Low ECM and e, f) ROSE 199 High ECM. Sections stained with H&E, X 270.

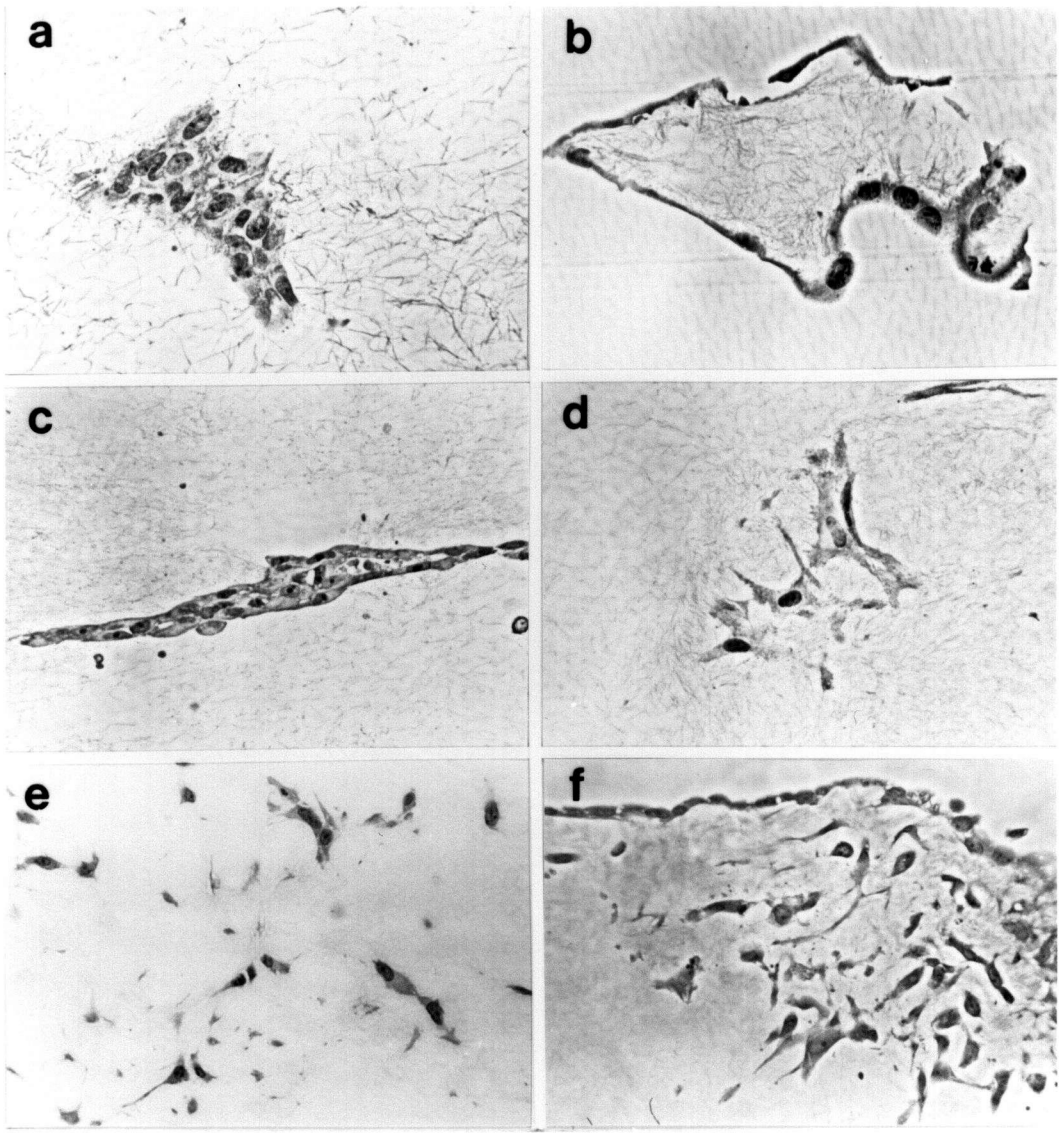
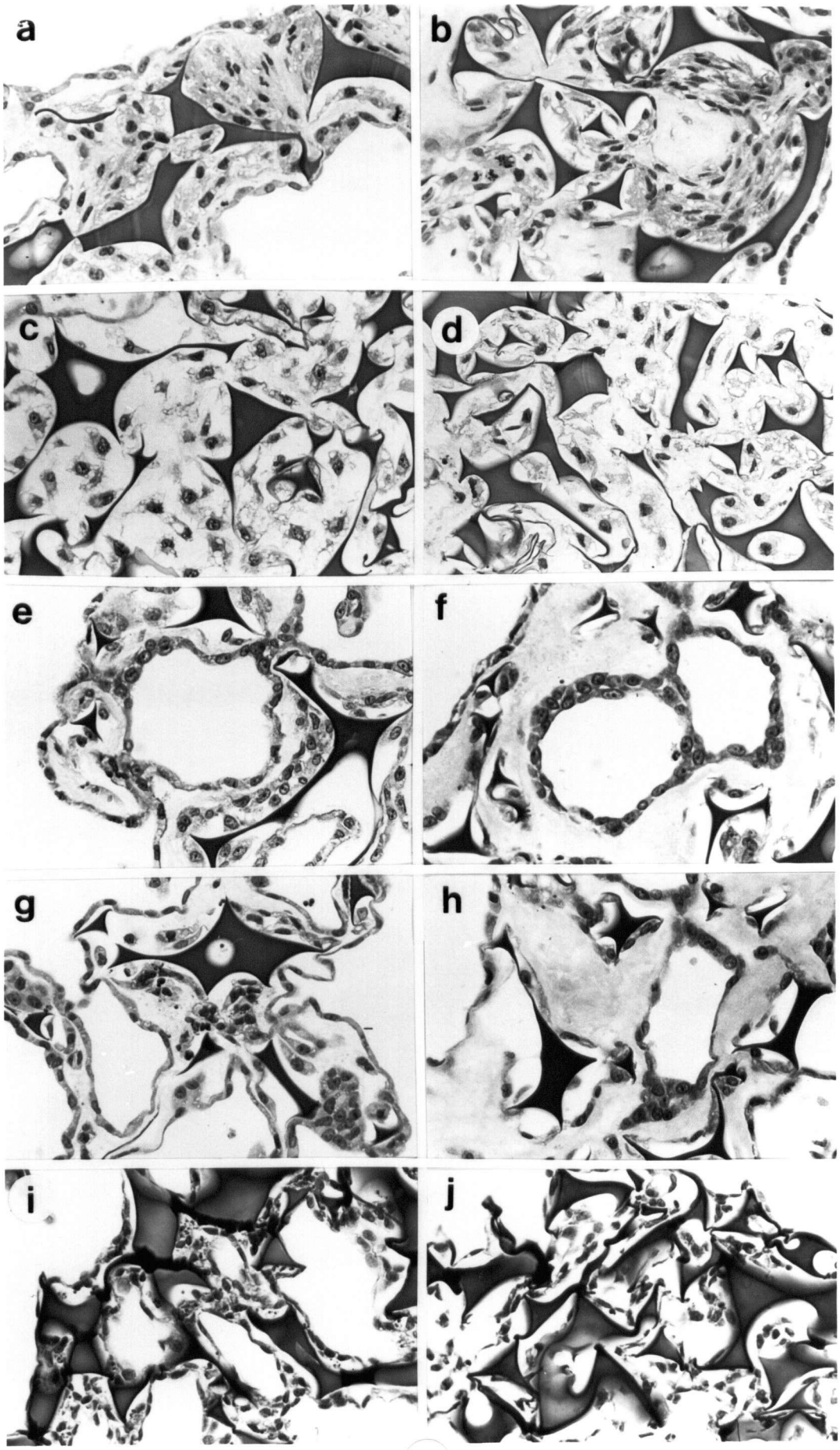

a
b
c
d
e
f

Figure 4. Morphology of ROSE lines in Spongostan and impact of ascorbate -2 phosphate.

a - d) ROSE 199 High ECM without (a, b) and with (c, d) ascorbate 2-phosphate. e-h) ROSE 199 Low ECM without (e, f) and with(g, h) ascorbate 2-phosphate. i, j) ROSE 239 without (i) and with ascorbate (j). Sections stained with H&E X 270.


a
b
c
d
e
f
g
h
i
j

Impact of three-dimensional culturing on ROSE 199 ECM development

Cultures

Plating 5 x10^5 cells per 16 mm well resulted in the cells becoming confluent within 24 hours on plastic. The same number of cells inoculated within the sponge resulted in a number of cells which did not adhere to the sponge but eventually adhered to the plastic underneath or remained in suspension. Coating the plastic with agar, though inhibiting the attachment of cells to the plastic, did not increase the number of cells which adhered to the sponge. The cells appeared to grow more rapidly around the perimeter of the sponge and gradually penetrated into the central area.

Distribution of cells and ECM

Phase microscopy of the cultures grown on plastic did not reveal any differences between the cultures at 2.5, 3, 4, and 5 weeks. Sections of scraped cultures from each time point revealed the characteristic pattern of ROSE 199 High ECM: a cuboidal to columnar simple epithelium on a ECM layer with underlying fibroblastic cells in a looser ECM. The width of the ECM layer (seen as a band) and the underlying cells varied within each sample but there were no noticeable differences between the time points. In some areas, the 3, 4 and 5 week samples contained a dense band of ECM with no underlying cells, which exhibited a different staining pattern from the other ECM band (e.g. basophilia and sialic acids).

The Spongostan cultures, in contrast, showed a distinct time dependent distribution of cells and ECM (Fig. 5a). The cells filled 1/4 of the sponge at 2.5 weeks, mainly around the perimeter, and gradually filled the sponge until at 5 weeks it was completely full. The distribution of visible ECM lagged behind that of the cells in the 2.5 and 3 week samples but by 4 and 5 weeks equaled the cellular distribution. Acidophilic vesicles, both cytoplasmic and extracellular, were most numerous at weeks 3 and 4 but had disappeared by week 5.

Distribution of Collagen

Collagen was detected using the silver stain for reticulin and the Chicago Blue stain in the allochrome method. Comparison with control tissues revealed that neither stained basement membranes (collagen IV), contrary to what was outlined in the methods (Kiernan, 1990), whereas the reticulin stain was able to distinguish between large collagen fibers (type 1) and reticulin (containing type III). The collagenous matrix of Spongostan did not stain in the reticulin method but did stain in the allochrome procedure, though the sponge spicules were a darker blue than the collagen in the ECM.

In the samples grown on plastic, collagen I and reticulin fibers were located in the acellular ECM band but not in the looser ECM found between the fibroblastic cells or between cells of the cuboidal layer. There was what appeared to be a slight increase in the amount of reticulin fibers with time. Collagen, a principal component of the ECM, was found as a band of variable thickness within the acellular ECM band.

In the cultures grown in Spongostan, reticulin fibers were found surrounding cells and anchoring them to the sponge spicules, as well as in acellular parts of the ECM. They were equally distributed throughout the ECM at the various time points; thus there was an increase in staining with time. Collagen I fibers were also present as singly or in clumps. The changes in the distribution of the clumps and fibers with time are depicted in Fig. 5a.

The reticulin stain also stained granular material in the cytoplasm of ROSE 199 cells on both plastic and Spongostan. In the samples grown on plastic the cells that stained were predominantly in the cuboidal layer whereas those in Spongostan were grouped together in discrete areas. None of the control tissues exhibited a similar cytoplasmic staining which suggests that it was staining reticulin precursors. The location of these granules in the cuboidal layer of the plastic cultures further suggests that these cells may be responsible for the production of reticulin in the ECM.

Carbohydrate staining:

The intensity of PAS staining was rated on a scale of 0 to 4, (0 negative and 4 strong) and is represented on the bar graphs as different intensity of shading (Fig. 5 b, c). The intensity of staining was affected by oxidation time and time in culture. The control tissues and procedures stained as expected with one exception. The saponification procedure increased the number of reactive groups in the ROSE 199 ECM and the connective tissue and basement membranes of the control tissues as well as the cytoplasm of the proximal convoluted tubule of the kidney.

The intracellular PAS staining was strongest in the 30 minute allochrome procedure (Fig. 5b) where it was found as a discrete granular material. Staining was abolished after pre-treatment with amylase indicating that the granules were probably glycogen. There was an increase in the percentage of cells which contained granules with time (Fig. 5b). There was no detectable intracellular labeling after amylase digestion except for a very small amount of sialic acids.

The pre-treatment with amylase removed the intensely stained glycogen granules; allowing the more weakly stained carbohydrate moieties present in the ECM to be distinguished. In those samples grown on plastic the carbohydrates were present in close association with the collagen and appeared as pink or purple lines. The staining found in the ECM of the Spongostan samples was slightly weaker than that on plastic and the distribution lagged behind the amount of ECM present at the different time points (Fig. 5c). The saponification procedure increased the intensity and distribution of the PAS staining present in the ECM to the extent that all the ECM stained in the Spongostan samples, though it was still confined to lines and bands in the plastic ones. This procedure demonstrates the presence of o-acylated neutral sugars.

There were very few sialic acids present in the ECM of cultures grown on Spongostan, though the acidophilic vesicles stained more strongly. On plastic there was a

larger amount of sialic acids both around cells and in the ECM band: the levels were similar to those of the basement membrane in the glomerulus of the kidney. The most distinctive presence of sialic acid groups was in the unusual, dense basophilic band, found in the weeks 3,4, and 5 samples, though the significance of this band is unknown.

Alcian blue staining at pH 1.0 and 2.5

The intensity of alcian blue staining was also ranked from 0 to 4 (0 negative and 4 strong). The results for cultures grown on Spongostan and plastic are presented in Fig. 5d & e, respectively. For the most part the staining of the ROSE 199 ECM was stronger at pH 1.0 than at pH 2.5. This was unexpected because usually the sulfate groups which stain at pH 1.0 will also stain at pH 2.5. This does not appear to be the case in this set of samples. Others have also reported a similar decrease in staining at pH 2.5 of sulfated polysaccharides which stained at pH 1.0, (Cole et al., 1985), though there is no explanation to date for this phenomenon.

There were sulfate groups present in the ROSE 199 ECM cultured on plastic in all the time points examined (Fig. 5e). In addition there was a small amount of sulfate groups present on the apical surface of the cuboidal cells in weeks 4 and 5. There were no anionic groups detected in ROSE 199 ECM cultured in Spongostan until week 5 when sulfate groups were detected (Fig. 5d). The presence of carboxyl groups was not demonstrated as there was diminished staining at pH 2.5 in all the cultures.

Growing ROSE 199 High ECM in Spongostan was found to affect predominantly the organization of the collagen fibers in the matrix and the morphology of its cells. It took longer for the cells to fill the sponge and there was a corresponding delay in the deposition of matrix components. On plastic all the basic components were present in the first time period examined (2.5 weeks) whereas the sulfated proteoglycans were not detected in the sponge cultures until the fifth week, and sialic acids did not reach the levels found on plastic at any time point.

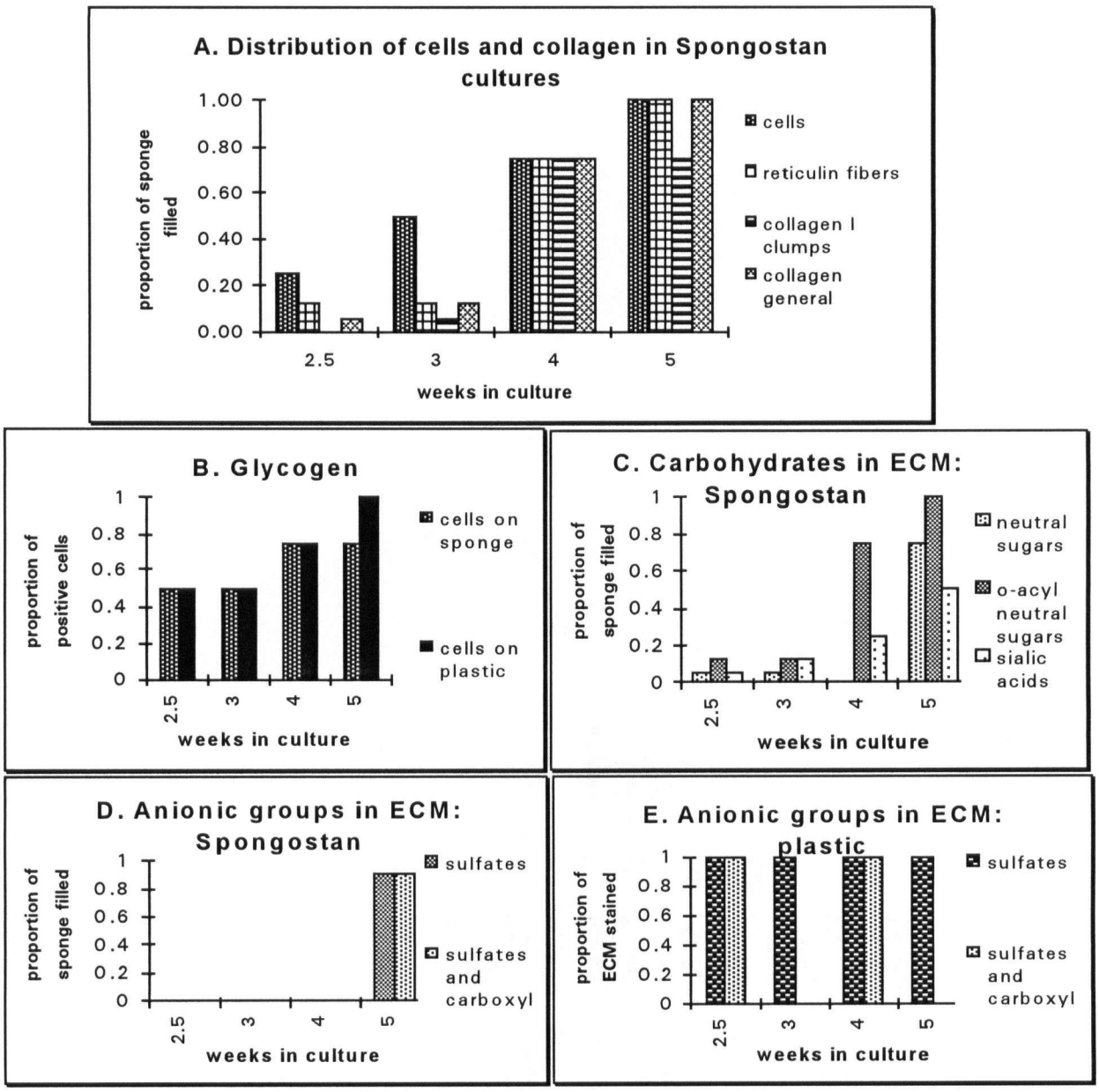


Figure 5. Distribution of ECM components produced by ROSE 199 High ECM on Spongostan and plastic. In graphs B-E, darker bars represent not only different groups but also more intense staining. A) Cells and collagen as detected by reticulin ▦, ▤ and allochrome ▩ methods. B) Proportion of cells with glycogen on Spongostan and plastic. C) Distribution of classes of carbohydrate groups in the ECM produced on Spongostan. D) Distribution of anionic groups in ECM produced as detected by Alcian Blue pH 1.0 (sulfates) and pH 2.5 (sulfates and carboxyls) on Spongostan and E) on plastic.

Discussion

The results of this study indicate that culturing of ROSE 199 has led to the development of a subline, ROSE 199 High ECM, which produces a thicker ECM layer when grown on plastic, than ROSE 199 Low ECM, and incorporates cells with a mesenchymal morphology into its ECM. This difference between the sublines is reflected in their respective responses to the various collagen substrata where they demonstrate two distinct patterns. The ROSE 199 Low ECM subline exemplifies a predominantly epithelial response whereas the ROSE 199 High ECM subline demonstrates elements of an epithelial-mesenchymal conversion response. The typical epithelial response to plating on collagen gels or coats appears to be a retention of a cuboidal epithelial morphology (Michalopoulos and Pitot, 1975, Ghosh et al., 1991); a pattern repeated by the ROSE 199 Low ECM line. The ROSE 199 High ECM subline also maintained a cuboidal morphology when on the surface of the gel but also gave rise to numerous cells which were able to penetrate into the gel, some remaining as cords of cells, while the majority were found as single cells scattered throughout the gel. There are varying reports in the literature as to the ability of the epithelial cells which have been plated on the surface of gels to penetrate into the gel. Emerman and Pitelka (1977) reported that mammary epithelial cells will penetrate into the gel whereas Michalopoulos and Pitot (1975) found that hepatocytes do not. Endothelial cells depend on whether they are from large vessels or microvessels as to ability to penetrate into matrix, a characteristic that parallels their ability to undergo epithelial-mesenchymal conversion in response to wounding (Madri et al., 1991). The acquisition of the ability to migrate into a collagen gel is used as an end point in studies of epithelial-mesenchymal conversion in the endothelium of the developing heart (Runyan et al., 1992). The results from these various studies when taken together suggest that the variations found in the ability of epithelial cells to migrate into the matrix may be a reflection of either contaminating mesenchymal cells or the presence of cell types with the

ability to undergo an epithelial-mesenchymal conversion. In the ROSE 199 system, the use of cloned lines rules out the possibility of a contaminating cell type supporting the hypothesis that the cells of the High ECM subline have acquired the ability to modulate between epithelial and mesenchymal phenotypes whereas the Low ECM subline did not. As a result, it is perhaps not too surprising that the behavior of the High ECM subline when plated inside a gel is a predominantly mesenchymal one, with cells separating and becoming migratory whereas the Low ECM subline grew primarily as cell aggregates. Neither subline formed distinctive cyst-like structures as has been reported for MDCK cells (Zuk et al., 1989) or thyroid cells (Greenberg and Hay, 1988).

Though the mechanisms behind epithelial-mesenchymal conversion remain to be elucidated, the process involves a number of steps, including loss of cell-cell adhesion, reorganization of cell-matrix adhesion molecules and cytoskeleton, and motility through ECM (Hay, 1991). The classes of molecules involved in these steps include down-regulation of cell-cell adhesion molecules, such as E-cadherin, which has been shown to be expressed by ROSE 199 cells (Hoffman et al., 1993) and is down-regulated both in embryonic epithelial-mesenchymal conversions (Takeichi, 1988; Burdsal et al., 1993) and in ROSE 199 cells transfected with c-H-ras which assume a predominantly mesenchymal morphology (Hoffman et al., 1993). Another set of molecules that play various roles in the steps of epithelial-mesenchymal conversion are GAGs and proteoglycans which affect cell adhesion, proliferation and migration (Wight et al., 1992). For example, suppression of the proteoglycan, syndecan, in S115 mouse mammary tumor cells induces a mesenchymal morphology (Leppä et al., 1991). Hyaluronate and chondroitin sulfate are likely to be present as they increase cell migration through matrix as exemplified by the increase in fibroblast migration in three-dimensional collagen gels (Docherty et al., 1989). Increased expression of proteases are also found during the epithelial-mesenchymal conversion response. For example, urokinase expression is found to be increased in the developing avian somite and in endocardial cushion cells during epithelial-mesenchymal conversion

and appears to influence the motile ability of these cells (McGuire and Alexander, 1992 & 1993). The combinations of molecules and signals involved are probably unique to each set of conversions responses. However, these examples suggest possible types of components that would need to be present to achieve a full epithelial-mesenchymal conversion. The expression of these various classes of molecules, in turn, is regulated by a variety of factors, including components of the ECM and growth factors such as TGF-β that also influence the production of ECM components.

The behavior of ROSE 199 is intriguing because it suggests that the production of mesenchymal markers (for example: collagen I and III) is quite distinct from the assumption of the mesenchymal morphology. This is unusual as most epithelial cells, even in culture, do not produce the stromal collagens (Hay, 1991). In the majority of epithelial cells that do, such as microvascular endothelial cells (Lipton, 1992), mesothelial cells (Davila and Crouch, 1993) and lens epithelia (Hay, 1990) the cells have assumed a mesenchymal morphology concurrently or prior to the expression of these markers. Exceptions do exist and at least one other rat epithelial line has been reported to form multilayered structures similar to ROSE 199 Low ECM when grown on plastic (Sambuy and De Angelis, 1986). In addition, the expression of mesenchymal morphology does not always lead to the expression of mesenchymal markers as exemplified by the behavior of MDCK cells in collagen gels (Zuk et al., 1989). The most common scenario in culture is that epithelial cells dedifferentiate and loose the expression of their epithelial markers but do not acquire the expression of mesenchymal markers (Kleinman et al., 1987). In the case of ROSE 199, however, we have a cell line which remains epithelial while producing large quantities of collagens (both type I and III, also in Auersperg et al., 1991), while the expression of a mesenchymal morphology was only acquired by one subline. Another unusual feature of the epithelial-mesenchymal modulations of ROSE 199 High ECM is that the mesenchymal cells which arise from the epithelial cells are able to revert to an

epithelial morphology when passaged and seeded sparsely, an event which rarely, if ever, occurs in other systems (Hay, 1991).

The acquisition of cells with mesenchymal morphology and the ability to penetrate and migrate into ECM can be used to explain, in part, the differing behaviours of the two sublines. It can account for the differences seen in the cultures grown on collagen gels and the presence of cells in the ECM of ROSE 199 High ECM on plastic and Spongostan. It may also account for the striking difference in responsiveness to thin collagen coats. The presence of migratory cells that could penetrate the collagen coat would account for the persistence of the multilayering in ROSE 199 High ECM and not in ROSE 199 Low ECM. The persistence of matrix production by ROSE 199 High ECM, however, is not as easily accounted for, as one could also expect an inhibitory effect on matrix production as seen on the thicker collagen gels. The lack of responsiveness to the thin collagen coat may be a result of degradation of the collagen by proteolytic enzymes or contraction of the collagen coat allowing for greater exposure to the underlying plastic, as a similar response appears to occur when ROSE 199 was cultured on thin coats of basement membrane matrix, Matrigel (data not shown). Another possibility is that the cells that form the underlying layers are capable of forming the ECM layer in response to exposure to plastic, though the reticulin staining demonstrated intracellular reticulin only in the cuboidal cells in those cultures grown on plastic. However, the contribution to the formation of the ECM layer by the underlying cells that are in contact with the plastic can not be discounted and their absence may also contribute to the decrease in ECM production by ROSE 199 Low ECM grown on collagen coats.

Exogenous collagen gels have been shown to down-regulate the synthesis of ECM components for both epithelial cells (Streuli and Bissell, 1990) and fibroblasts (Mauch et al., 1988), but down-regulation of synthesis was accompanied by an increase in the deposition of these products into the gel matrix adjacent to the cells. In these two examples the ability of the cells to contract the gel seems to play an important role in the

regulatory process, as diminution in synthesis of ECM components was seen in the mammary epithelial cells only when the gels were floated (Streuli and Bissell, 1990) and in fibroblasts when they were seeded inside the gel and had contracted the gel into a dense matrix (Mauch et al., 1988; Berthod et al., 1993). When fibroblasts were grown in a lyophilized collagen sponge they also decreased their collagen synthesis and deposition when compared to post-confluent cultures on plastic (Doillon et al., 1987), suggesting that the bioactivity of the collagen may be responsible for the difference in matrix deposition by ROSE 199 on gels and in the inert collagen of Spongostan. The addition of GAGs to bioactive (i.e., not denatured) collagen sponges stimulated the synthesis and deposition of collagen by fibroblasts compared to plastic and sponges composed only of collagen but decreased synthesis when incorporated into gels (Doillon, et al., 1987; Berthod et al., 1993). These studies highlight the importance of both ECM components and their organization in the regulation of matrix production. Differences in composition and organization may explain why, in ROSE 199 Low ECM, the ECM deposition is decreased by a thin layer of exogenous collagen whereas endogenous collagens found in the ROSE 199 Low ECM matrix permit the production of a thicker, more compact matrix, which can be further increased by addition of ascorbate.

Production of ECM is enhanced by ascorbic acid (Franceschi, 1992, Kao et al., 1990). This is due in part to its role as a co-factor in the hydroxylation of proline and lysine which is required for helix formation and thus secretion of collagen fibrils (Franceschi, 1992). There is also evidence that it stimulates the transcription of the procollagen α1 (I) gene and increases the life span of the procollagen α1 (I) mRNA (Chojkier et al., 1989). Though the exact mechanism behind these later events is still unclear there is evidence that points to a role for ascorbic acid induced lipid peroxidation products as stimulators (Chojkier et al., 1989; Geesin et al., 1991), as well as the increase in collagen deposition in the ECM (Grinnell et al., 1989; Franceschi, 1992). Ascorbic acid has also been shown to stimulate the production of GAGs (Kao et al., 1990) and

fibronectin as well as basement membrane components such as laminin (Yue et al., 1990) and collagen IV (Ono et al., 1990), and thus can influence cell morphology and differentiation.

Both sublines responded to the addition of ascorbate 2-phosphate when grown on plastic by increasing the thickness of the acellular ECM band, whereas only the Low ECM subline appeared to increase its ECM deposition when grown in the collagen sponge. The cause of the differential ascorbate effect on matrix deposition in the High ECM subline is unclear. It may be that the mesenchymal-type cells are less responsive to the increased levels of ascorbate as these are the cells which predominate in the sponge cultures whereas the epithelial cells predominate in plastic cultures. It is also possible that the loose, irregular organization of the collagen fibrils around the cells in the sponge cultures provides a stronger inhibitory feedback for further matrix production then the linearly arranged collagen bundles of the plastic cultures.

The differences in matrix organization between plastic and Spongostan cultures of the High ECM subline were examined in more detail in the time course study. Matrix deposition appeared to be dependent on cell density, as has been demonstrated for collagen synthesis on plastic (Auersperg et al., 1991). Collagen fibrils in Spongostan, however, were more haphazard in their orientation; perhaps as a result of a more disorganized cellular orientation, as these two factors have been shown to be linked in fibroblasts (Grinnell et al., 1989). It was interesting that in Spongostan the reticulin fibers were often arranged around cells as if to anchor them to the sponge spicules, in contrast to the cultures on plastic where reticulin fibers were parallel to the cell layer and did not appear to be in close association with the cells. The two other important distinctions were the delays in the incorporation of sulfate groups and sialic acids into the ECM as the sulfate groups were not detected in the ECM on Spongostan cultures until the fifth week, whereas sialic acids were not present at any time point in the ECM on Spongostan, though both were present in all weeks on plastic cultures. These two groups are found mainly in

sulfated proteoglycans and in glycoproteins, respectively (Kiernan, 1990). The delay in the incorporation of these components might explain the decrease in expression of the cuboidal to columnar phenotype in Spongostan cultures, as both play important roles in cellular differentiation (Wight et al., 1992; von der Mark et al., 1992). This is particularly the case for the glycoprotein, laminin, which contains abundant sialic acids (Fujiwara et al., 1988) and is responsible, for example, for the conversion of embryonic mesenchyme to kidney epithelium (Ekblom, 1989). One would need to explore this hypothesis further using more accurate methods of identification such as antibody labeling or in-situ hybridization. In contrast to matrix components, glycogen accumulation did not appear to be dependent on ECM deposition, but on time in culture, as both Spongostan and plastic cultures showed a time dependent increase in the percentage of cells with glycogen.

Both sublines appeared to be capable of forming cyst-like structures in Spongostan, in contrast to their behavior in collagen gels. This appeared to be dependent on the degree of matrix production as ROSE 239 cells, which produced little matrix, did not form these structures while the number of these structures increased in the Low ECM subline when ECM production was enhanced by ascorbate. The addition of ascorbate also increased the number of cuboidal cells which lined the cysts in the Low ECM subline. The reasons for this phenomenon are unclear, but they point to the importance of supplementing media with ascorbate in the maintenance of ROSE 199 epithelial morphology.

This work raises a number of interesting questions regarding the signals that regulate epithelial-mesenchymal interconversion and matrix production. It supports the hypothesis that the expression of stromal components and a mesenchymal morphology are not necessarily linked, and suggests that a complete modulation can be reversible. It suggests that ROSE 199 ECM production is regulated similarly to that of fibroblasts (e.g., down regulated by exogenous collagen gel and up regulated by ascorbate supplementation). It also demonstrates that Spongostan, but not collagen gels, is a suitable three-dimensional support for the formation of cyst-like structures by ROSE 199 cells of

both sublines, and thus potentially useful for studying this type of micro-environment on OSE cells. The phenotypic differences between the sublines may serve as a useful model for distinguishing between the influence of "stromal" cells vs. matrix components alone, though further characterization of these cells and their respective matrices would need to be carried out to determine the applicability of this system in a wider context.

CHAPTER 4

CO-CULTURE OF ROSE LINES AND HUMAN OSE

Introduction

Stromal interactions play an important role in epithelial differentiation and organogenesis and include: interactions with ECM components (Kleinman et al., 1987), release of paracrine factors by stromal cells, and direct cell-cell interactions (reviewed by Donjacour and Cunha, 1991). The correct deposition of basement membranes, which is important for the development of cell polarity, has been shown to be enhanced in culture by floating collagen gels (Streuli and Bissell, 1990) and interactions with mesenchyme (Vachon et al., 1993). The use of co-cultures or feeder layers provides a culture system in which these interactions can occur permitting the differentiation of epithelial cells (Vachon et al., 1993; Senoo et al., 1989; Boutin et al., 1992). This study was designed to see if the ROSE 199 line, which forms an abundant ECM underlying the columnar epithelium, could provide the signals needed to form a cuboidal epithelium in human OSE (huOSE).

ROSE 199 cells reach confluence more quickly than huOSE, therefore a method was needed to remove the ROSE cells before they crowded out the huOSE cells. The removal of ROSE 199 cells using ammonium hydroxide, sodium deoxycolate or repeated freeze-thaws produced a cell-free matrix which did not maintain an epithelial phenotype in huOSE, instead the cells became fibroblastic and invaded the matrix (Kruk, 1992). Therefore, a method was needed to remove the ROSE cells, or stop their proliferation, when in actual co-culture, without affecting the huOSE. Most selection methods currently used involve transfection of the population to be kept with a resistant gene. The transfection method, however, requires a population of rapidly growing cells which would deplete the useful life span of huOSE for co-culturing. It was decided to use a transfection method that would introduce an enzyme into ROSE 199 which would render the cells susceptible to the cytotoxic effects of a chemical which does not affect normal cells. The enzyme chosen was xanthine-guanine phosphoribosyl transferase (XGPRT) (Mulligan and

Berg 1981), which can be back selected using 2-thioxanthine, a cytotoxic substrate analogue (Gilbert and Harris, 1988). The enzyme is involved in the salvage pathway of purine nucleotide synthesis and converts xanthine to XMP which is then converted to GMP and is present in bacterial but not mammalian species. The de novo synthesis of GMP results when the intermediate IMP is converted to XMP and then to GMP, a process that can be blocked at two levels (see Fig. 6). One is at formation of IMP from precursors which is blocked by aminopterin, and the other is at the conversion of IMP to XMP which is blocked by mycophenolic acid. Mammalian cells transfected with E.coli XGPRT enzyme and grown in media containing aminopterin and mycophenolic acid along with adenine and xanthine will grow well, whereas those without the enzyme will not (Mulligan and Berg, 1981). The addition of 2-thioxanthine to cells which contain the E.coli XGPRT enzyme will produce cytotoxic effects whereas the addition to normal cells without the enzyme allows them to continue to proliferate (Gilbert and Harris, 1988). Thus adding the enzyme to ROSE 199 cells, selecting for the cells containing the enzyme using mycophenolic acid and aminopterin, then culturing those cells with huOSE and back selecting by adding 2-thioxanthine should halt the proliferation of ROSE 199 and not huOSE.

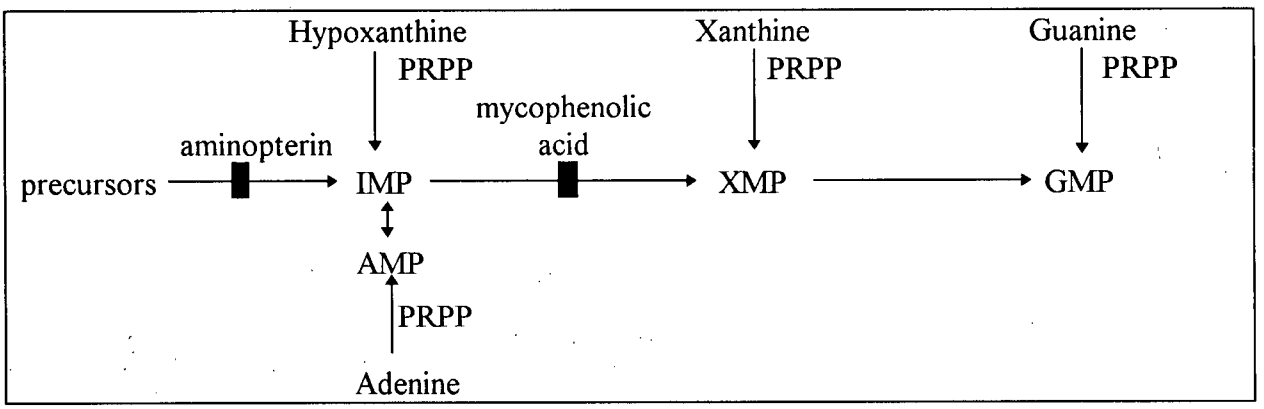


Figure 6. Pathways of purine nucleotide synthesis and sites of inhibition by aminopterin and mycophenolic acid. PRPP, phosphoribosyl pyrophosphate.

Because both populations of cells involved in the co-culture are capable of forming epithelial cells, an identification method was needed to distinguish the two cell populations. OSE cells do not have a large number of species specific antigens available for distinguishing the two species; therefore various methods were tested for the labeling of cells. These included the use of vital dyes such as the fluorescent carbocyanine dye $DiI_{18(3)}$ (Honig and Hume 1986) used to study long term cultures of neurons, and the nuclear fluorescent label Hoechst 33258. In addition, a rat cell antibody was raised in rabbits to label ROSE 199, and the species specific 5B5 antibody to human prolyl 4-hydroxylase (Höyhtyä et al., 1984) was used to label the huOSE. The SV40 immortalized line IOSE-80 contained the SV40 large T antigen which served as an effective marker for these cells.

The cells were grown in a variety of culture conditions to see if there were changes in the way they interacted with one another. Thus they were grown on planar substrata, plastic and collagen coats; in suspension, using rotary flasks; and in the three-dimensional matrix, Spongostan.

Materials and Methods

Culture conditions

Cells were co-cultured on plastic, in suspension, and in Spongostan. The rat lines used included ROSE 199, both sublines and clones, and ROSE 239, and the human lines, IOSE-80 (Van) and NFH-OSE. Media used were 199:105/15% FBS for co-cultures with NFH-OSE, and either 199:105/5% FBS or Waymouth's medium MB 752/1/2.5% Omni serum with IOSE-80. Stock cultures were dissociated using trypsin with and without EDTA (Chapter 2). The presence of EDTA in the dissociation process did not appear to influence the re-aggregation of any of the cell types, over the time spans used in this study.

Plastic or Collagen coated plastic: Cells were plated at ratios of 1:1 or 2:1 (human:rat), seeded densely or sparsely and grown for periods of 1 to 2 weeks. Human

cells were either added when plating the ROSE cells or after ROSE cells were confluent. Control cultures included each type of cell alone and the co-culture of IOSE-80 with human dermal fibroblasts (huFib), seeded at the same time or after huFib was confluent.

Suspension: Cells were used at ratios of 1:1 or 1:2 (human:rat) and at concentrations of approximately 500 x 10^3 cells/ml in 5 ml of media in a 50 ml Teflon flask. Flasks were placed on a rotary shaker platform at 40-90 rpm for 12 to 24 hours (Steinburg, 1975; Takeichi, 1977). Due to the large number of cells required only IOSE-80 were used as human cells in these experiments.

Spongostan: Cells were seeded at ratios of 1:1 or 1:2 (human:rat) simultaneously and allowed to grow for 1 to 4 weeks. Controls were sponges of each cell type alone.

Identification Methods

Labeling methods tested to distinguish between cell types in co-culture included vital dyes and cell specific antibodies.

Vital Dyes: Two vital dyes were tested as markers for the two cell types: Hoechst 33258 (Sigma) which intercalates with DNA and thus labels nuclei and $DiI_{18(3)}$ (Molecular Probes, Eugene, OR) which labels lipids, principally those in the plasma membrane. The cells were incubated with their respective dyes before trypsinization and plating. Hoechst 33258 was used at a concentration of 4 μg/ml and incubated for 30 min. at 37 °C. $DiI_{18(3)}$ was used at 5 μg/ml to 10 μg/ml and incubated for 1 hour 37°C. Both types of cultures were then washed 5 X 10 min., trypsinized and mixed. The use of vital dyes for identification was unsuccessful. Hoechst 33258 did not label nuclei for longer than 24 hours and $DiI_{18(3)}$ was released into the media for at least 72 hours post incubation which allowed the other cell type to also become labeled. This prolonged release of $DiI_{18(3)}$ was unexpected as this dye has been used in long term (several weeks) cultures of neurons without the label spreading to other cells (Honig and Hume, 1986).

Antibodies: The antigen markers used to distinguish the two cell types were 1) SV40 T ag (Ab-2) Oncogene Science (Uniondale, NY), 2) 5B5 obtained from Dako, Corp. (Carpinteria, CA), a species specific antibody to human prolyl 4-hydroxylase for NFH-OSE and 3) A-1, an antibody raised against surface antigens on rat cells as part of this study. A-1 was produced by immunizing rabbits with freshly dissociated rat spleen cells to produce a polyclonal antibody capable of both identifying rat cells and selecting against them using complement.

A-1 could be used successfully to distinguish between rat and human cells if the antibody was added before fixation. However, prior fixation with methanol and permeabilization abolished cell type specific staining, while fixation with formalin reduced staining on ROSE 199 cells and did not allow for labeling of the IOSE-80 cells with the antibody to SV40 T antigen. A-1 could be used successfully to label the surface areas of cultures, however the limitation of using unfixed cultures impeded penetration into the deeper layers of multilayered cultures. SV40 T ag was successful for identifying IOSE-80, whereas 5B5 was more limited in its use of identifying NFH-OSE because of variability of expression among cells within cases. The method of choice for distinguishing the cells in co-culture was using A-1 for ROSE 199 and SV40 T ag for IOSE-80.

Unfixed co-cultures of ROSE 199 and IOSE-80 were incubated with A-1 at 37°C for 1/2 hour (coverslips and cell aggregates) or overnight (Spongostan). They were washed 3 x 10 minutes in serum free medium and then fixed briefly, 1-2 minutes, in -20°C methanol, washed again and incubated for 1 hour R.T. (coverslips and cell aggregates) or overnight at 4°C (Spongostan) with SV40 T ag. After another series of washings the cultures were incubated with Goat anti-Mouse IgG - FITC and Goat anti Rabbit IgG RITC for 1 hour R.T. (coverslips and cell aggregates) or overnight at 4°C (Spongostan).

Staining cultures with H & E allowed one to distinguish ROSE 199 cells from IOSE-80 cells based on size of nuclei and staining properties. ROSE 199 cells stained

more intensely with both eosin and hematoxylin, whereas IOSE-80 had larger nuclei and a cytoplasm which blended in with the surrounding ECM.

Visualization:

Coverslips were mounted in Gelvatol and examined using a Zeiss Axiophot. Cell aggregates were suspended in PBS in cloning cylinders stuck onto coverslips using silicone and examined using the inverted fluorescent microscope. Some cultures were scraped and stained with H & E. Spongostan cultures were placed on coverslips and examined using the confocal microscope. Alternatively, the Spongostan cultures were embedded in JB4 and sectioned at 2 μm and examined using the Zeiss Axiophot. Each method for examining Spongostan cultures had its limitations. The confocal microscope was useful for obtaining three-dimensional organization of the cells whereas the JB4 sections were useful for examining cell type relationships.

Selection method

The pSV2gpt plasmid (ATCC cat. # 37145) containing E.coli XGPRT, was obtained, purified and transfected, using lipofectin, into ROSE 199 High ECM and Low ECM sublines. Cells that expressed the enzyme were selected using a modification of the method of Mulligan and Berg (1981), specifically using Waymouth 752/1 medium with 10% FBS, and xanthine 250 μg/ml, thymidine 10 μg/ml, and mycophenolic acid 5 μg/ml (Sigma). Gilbert and Harris (1988) developed a method for back-selecting using 2-thioxanthine which is cytotoxic to cells that contain this enzyme. Media used in back-selecting were Medium 199:MCDB 105/10% and 5% FBS, Waymouth/5% FBS, or DMEM/5% FBS with concentrations of 2-thioxanthine (Sigma) ranging from 0.5 mg/ml to 2 mg/ml.

Cultures were obtained which expressed the xanthine-guanine phosphoribosyl-transferase as demonstrated by their resistance to the mycophenolic acid selection medium which killed untransfected cells. Back-selection with 2-thioxanthine, however, was

unsuccessful. There was either little or no effect (0.5 mg/ml & 1 mg/ml) on cell growth or the cytotoxic effect was similar in both transfected and untransfected lines (2 mg/ml). Thus this method could not be used to selectively kill ROSE 199 cells in mixed cultures.

Results

Co-culturing the various sublines of ROSE 199, as well as ROSE 239, with either low passage or immortalized NFH-OSE resulted in cell segregation between the different cell types.

Planar surfaces

When seeded together on plastic, the ROSE lines formed round epithelial colonies whereas both the low passage and immortalized huOSE formed ridges of elongated cells surrounding them, although the same huOSE cells, when grown alone formed epithelial monolayers. Examination of cultures grown on coverslips indicated that there were no huOSE cells in the round epithelial colony areas (Fig. 7 a, b) and that the larger IOSE-80 nuclei appeared to be beneath the smaller ROSE 199 nuclei. Cross-sections through scraped layers suggested that the ridges were covered by the smaller, darker, epithelial ROSE cells while the huOSE cells were located in the ECM layer (Fig. 7 c, d). Plating the cells on collagen coated dishes resulted in broader ridges, though otherwise the cultures appeared similar to those on plastic. The pattern did not vary with different seeding ratios of the two cell types or with different ROSE or huOSE lines. Seeding IOSE-80 with huFib cells resulted in a cell layer which lacked the epithelial/ridge pattern but in which all the cells had a similar fibroblastic morphology with no epithelial cells on the surface (Fig. 7 e, f).

When the ROSE lines were plated first and allowed to reach confluence before the addition of IOSE-80, very few of the IOSE-80 cells adhered to the underlying layer. Instead, they formed either single cells or floating balls of cells similar to those found in the cell suspension experiments. Occasionally these balls would adhere to the ridges

formed by the ROSE lines. Cross-sectioning of scraped cultures revealed that the IOSE-80 were located in the ECM layer and had a fibroblastic morphology whereas the cells which lined the surface were ROSE cells. If the ROSE lines were not 100% confluent before seeding IOSE-80, then there would be areas which resembled the pattern obtained when seeding together. Plating huFib first and allowing the cells to become confluent before adding IOSE-80 was not noticeably different from plating the cells together. Thus, on planar substrata, neither co-cultures nor feeder layers of ROSE lines nor huFib resulted in epithelial huOSE.

Suspension cultures

Cells were grown in rotating flasks to see if the cells would adhere to one another if they were in suspension. The different cell types exhibited different degrees of cellular aggregation under the conditions tested. ROSE 239 remained as single cells unless the rotation was slowed to the point where the cells settled to the bottom (40 rpm), indicating little cell-cell adhesion among ROSE 239 cells when in suspension (Fig. 8 a). ROSE 199 sublines and clones formed small cell aggregates (5-30 cells) with the ROSE 199 High ECM subline forming slightly larger clumps. These cell aggregates were usually spherical with irregular borders (Fig. 8 b). The IOSE-80 line, in contrast, formed large cellular aggregates of tightly compacted cells (several hundred to thousand cells) even at high speeds (90 rpm) (Fig. 8 c). There were a number of structures which appeared as if two or more spheres were fusing, and in two experiments there were long worm like structures. Cross-section of the cellular aggregates (both ROSE 199 and IOSE-80) revealed solid structures (Fig. 8 d). When placed together in the same flask, the cells of each type behaved as if alone and there were no differences when examined at 13, 24 or 48 hours.

Plating of the cell aggregates on collagen coated plastic resulted in similar patterns to those described for plating of mixed single cell suspensions.

Thus growing cells in suspension, which abolishes cell-substratum adhesions and maximizes cell-cell adhesions, did not promote cellular aggregations between the two cell types.

Spongostan

In Spongostan the IOSE-80 grew faster than ROSE 199 and filled the sponge more completely, unlike the growth on plastic where the epithelial colonies of ROSE lines covered a greater surface area then the IOSE-80 ridges. In the areas of the sponge in which only one cell type was found, the cells behaved much the same way as when cultured alone. ROSE 199 formed epithelial cell layers and produced ECM whereas IOSE-80 filled the spaces in the sponge with aggregates of elongated cells with little ECM. In the areas where both cell types were found, either on the surface or in the sponge interior, ROSE 199 was located on the surface of a cell-matrix aggregate and IOSE-80 was located within the ECM (Fig. 9). This does not rule out the possibility that ROSE 199 cells are also located within the matrix due to the limitations of the penetration of the rat cell label. Most importantly, however, these results demonstrate that the IOSE-80 cells were not located on the surface of mixed cell aggregates in 3-dimensional culture, but penetrated into the ROSE 199 matrix, confirming the results obtained on planar substrata.

Figure 7. IOSE-80 co-culture on planar substrata with ROSE 199 and human fibroblasts. a, b) Co-culture with ROSE 199: IOSE- 80 labeled with antibody to SV40 large T antigen, a) immunofluorescence and b) phase microscopy (X 270). Sections through c) IOSE-80 co-cultured with ROSE 199 Low ECM cl. E11/A4, arrow: larger IOSE-80 nuclei, arrow head: smaller, darker staining ROSE 199, epithelial cells on top resemble the ROSE 199 cell pattern found alone. d) ROSE 199 ECM cl. E11/A4 grown alone. e, f) IOSE-80 co-cultured with human dermal fibroblasts e) in section and f) under phase microscopy; both cell types have a similar fibroblastic appearance and there are no cuboidal cells on the surface. c-e stained with H & E X 270; f) X 70.

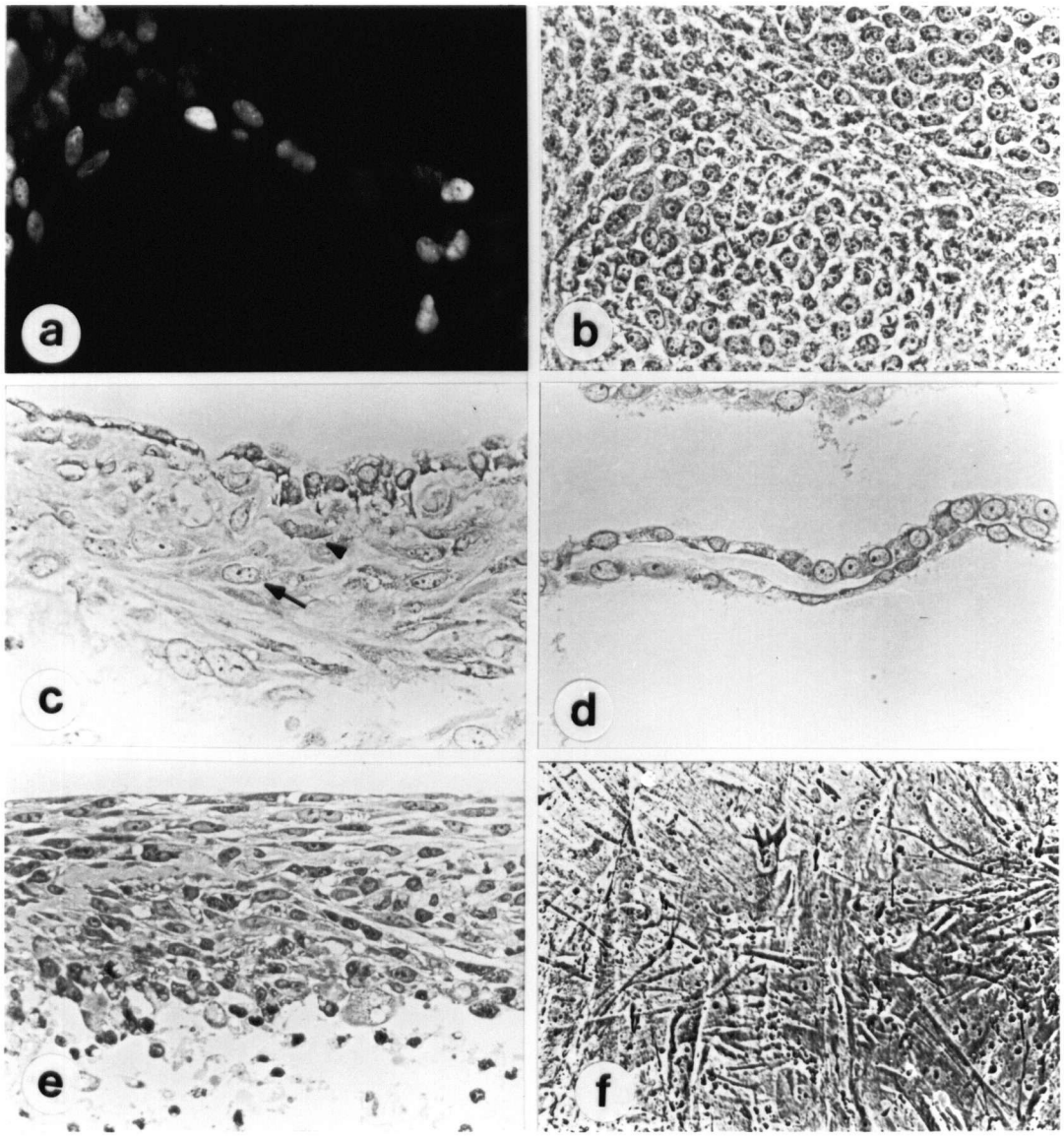
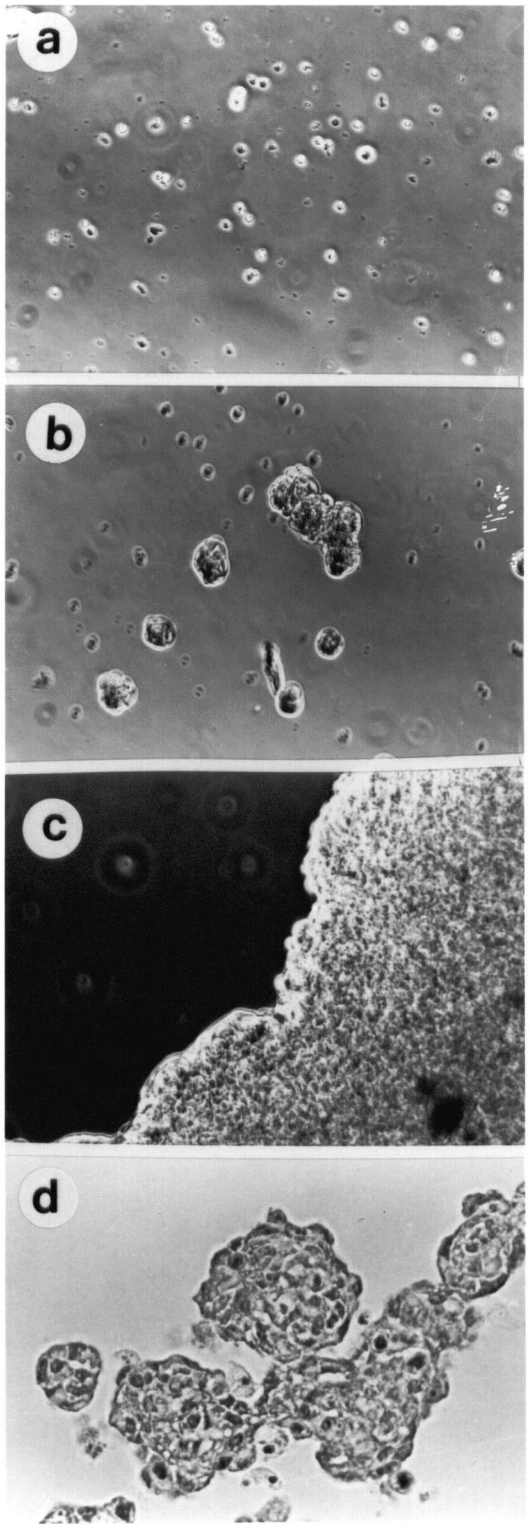

a
b
c
d
e
f

Figure 8. IOSE-80 and ROSE lines in suspension cultures.

a) ROSE 239, b) ROSE 199 High ECM, c) IOSE-80, and d) section through IOSE-80 cell aggregate. a-c) phase microscopy X 70; d) section stained with Toluidine Blue x 270.


a
b
c
d

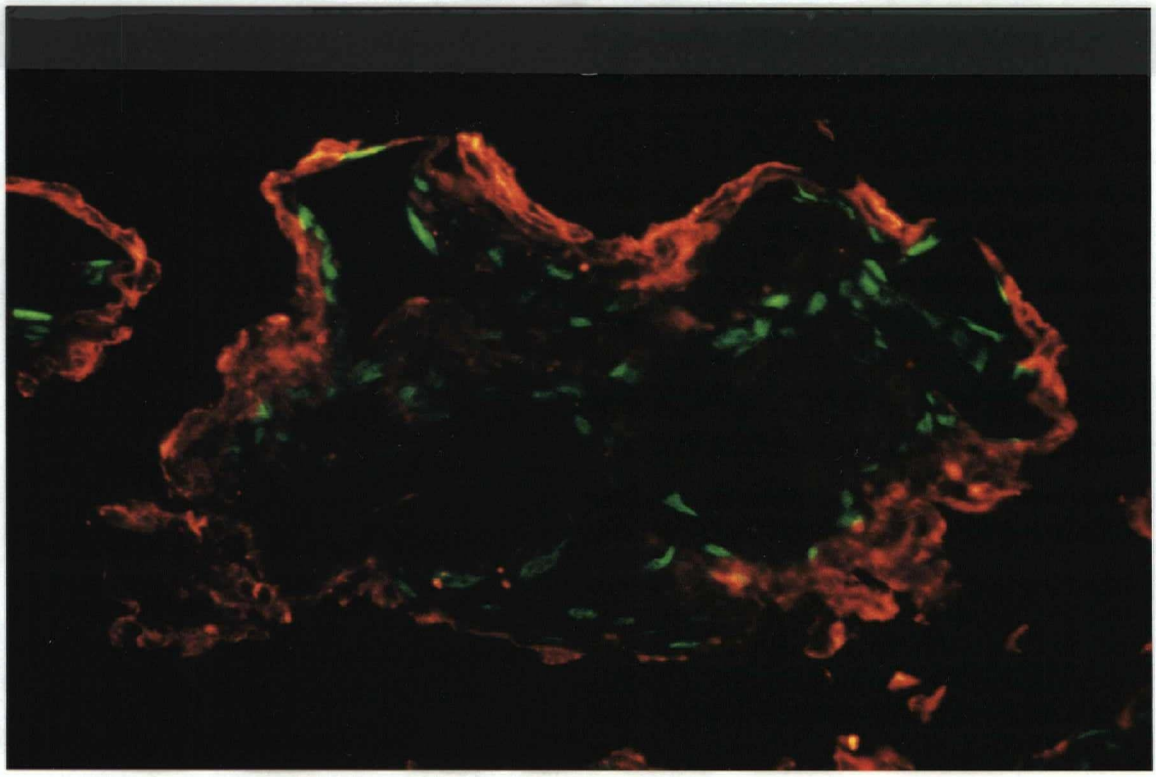


Figure 9. IOSE-80 and ROSE 199 High ECM co-cultured in Spongostan.

IOSE-80 labeled with FITC (antibody to SV40 large T antigen) and ROSE 199 labeled with RITC (antibody A-1 against rat cell surface antigens).

Immunofluorescence microscopy x 535.

Discussion

The co-culturing of ROSE lines, both ROSE 199 and ROSE 239, with huOSE resulted in the assumption of a mesenchymal morphology by the huOSE, and in the case of the ROSE 199 lines, migration of huOSE cells into the matrix, similar to that seen with ROSE 199 matrix alone (Kruk, 1992).

The cell populations did not intermingle, even in conditions which encourage cell-cell adhesion such as rotary cell culture (Steinberg, 1975). This is probably a result of different cell-cell adhesion molecules between the different cell population (Steinberg and Garrod, 1975). For example, ROSE 199 has been demonstrated to express E-cadherin (Hoffman et al., 1993), whereas this molecule appears to be absent on cultured huOSE (Auersperg et al., 1994).

The two cell populations showed different degrees of cell-cell and cell-substrate adhesion. This was suggested by the large flat epithelial colonies formed by ROSE lines on plastic and collagen coats which excluded the huOSE (both low passage and immortalized lines), whereas the huOSE cells were confined to narrow ridge areas surrounding them. When cell-substratum adhesion was enhanced by providing a collagen coat, the ridges of huOSE were broader occupying a greater surface area. In contrast, cell-cell adhesion appeared to be greater in IOSE-80 then in ROSE lines as they were able to maintain cohesive cell aggregates under rotational conditions that prevented the aggregation of other cells. This is similar to the two patterns observed for the sorting out of embryonic liver and limb-bud cells in the experiments of Steinberg and Garrod (1975).

The response of huOSE when plated on confluent layers of ROSE 199 mimics the interactions between cultured human ovarian tumor cells and human mesothelial cells, where the tumor cells adhered to the mesothelium only when the underlying ECM layer was exposed (Niedbala et al., 1985). The high frequency of metastasis to the peritoneal cavity in ovarian carcinogenesis, however, suggests that adhesion does occur in vivo. It is

tempting to speculate that the increase in collagen secretion into the peritoneal ascites that accompanies ovarian carcinomas (Zhu, 1993), may enhance the adhesion of the cancer cells.

The results of this study indicate that instead of providing the signals that huOSE need to form a cuboidal phenotype, the ROSE 199 provided signals which resulted in a conversion to a mesenchymal phenotype, not only in NFH-OSE which will assume a mesenchymal morphology on plastic in our culture conditions, but in IOSE-80(Van) which maintains an epithelial morphology on plastic (Maines-Bandiera et al., 1992). The use of fibroblast control cultures on plastic indicates that the human dermal fibroblasts were also unable to provide the appropriate signals, suggesting that the failure of ROSE lines to maintain an epithelial phenotype in huOSE was not necessarily due to species variations.

CHAPTER 5

EPITHELIAL AND MESENCHYMAL CHARACTERISTICS OF HUMAN OSE: CHANGES WITH NEOPLASTIC PROGRESSION.

Introduction

The process of ovarian carcinogenesis poses a number of interesting problems. First of all, ovarian carcinomas remain clinically silent until they reach an advanced stage, with few consistently good markers for detecting their presence through non-invasive means. Second, there appears to be a shift in differentiation among ovarian carcinomas towards more specialized epithelial phenotypes than the original OSE cells possess. Third, a few families are genetically predisposed to developing ovarian cancer. However, very little work has been done with the OSE of these women. This present study was undertaken to determine whether overtly normal OSE from women with a known increased risk of developing ovarian cancer expresses preneoplastic phenotypic changes that could be explored as potential predictive markers.

The work done to date with OSE from women with no family history of ovarian cancer (NFH-OSE), show that these cells are highly responsive to environmental influences. Under standard culture conditions they modulate from an epithelial to a mesenchymal form with time and passages in culture. In vivo, similar epithelio-mesenchymal conversions have been demonstrated in two cell types that are closely related to OSE, viz. pleural mesothelial cells responding to injury and repair (Davila and Crouch, 1993), and the cells of the developing Müllerian duct during regression in response to Müllerian inhibiting substance (Trelstad et al., 1982). In contrast to normal OSE, immortalized OSE and especially ovarian carcinoma cells maintain their epithelial characteristics in prolonged culture (Maines-Bandiera et al., 1992; Auersperg et al., 1994; Hamilton et al., 1983; Fogh and Trempe, 1975). A recent study on the expression of the epithelial marker CA125 in normal OSE from women with a strong family history of

ovarian cancer (FH-OSE) suggests that there is a similar trend in this group as well (Auersperg et al., 1995). Furthermore, preliminary data suggest that the expression of mesenchymal characteristics in culture by OSE diminishes with neoplastic progression (Dyck and Auersperg, 1994). Thus, in the course of carcinogenesis, OSE cells become more firmly committed to an epithelial phenotype and less apt to undergo epithelio-mesenchymal conversion in response to the environmental signals encountered in culture.

In the present study, we examined OSE cultures from women with and without family histories of ovarian cancer to test the hypothesis that a reduced capacity for epithelio-mesenchymal conversion by OSE cells may represent a (pre)neoplastic change. The parameters that were chosen to define cytodifferentiation were expression of keratin as an epithelial marker and collagen type III as a mesenchymal marker. Keratin was chosen as it diminished with passage in NFH-OSE, but was maintained in both immortalized NFH-OSE and the two cancer lines tested (Auersperg et al., 1994). As well, the three-dimensional tissue organization was examined by growing them in the collagenous matrix, Spongostan. Epithelial morphogenesis was defined by the capacity of cells to form epithelial structures, and mesenchymal morphogenesis by a dispersed, fibroblast-like cell distribution, secretion of extracellular matrix, and by sponge contraction which is a behavior characteristic of fibroblasts during wound healing and when cultured in collagen gels (Bell et al., 1979). The expression of these differentiation markers by OSE was defined in the intact ovary, in early passages in culture, and following immortalization with SV40, and was compared to ovarian carcinoma cell lines. The results suggest that OSE from women with a family history of ovarian cancer has a reduced capacity to undergo epithelio-mesenchymal conversion compared to the OSE of other women.

Material and Methods

Double staining for keratins and collagen III propeptide

Twelve cases of NFH-OSE were used in primary culture or passages (p.) 1 to 11 and five cases of FH-OSE were used in p.4 and two were repeated in p.5 and 6 respectively. Three NFH-IOSE lines were used in p.7-9 and six FH-IOSE lines in p.6-13. Two cases were used which did not fall into the above categories; one was a case without a family history of ovarian cancer but with contralateral ovarian cancer (OSE-114 used in p.2 and 3) and the other case was an immortalized line (IOSE-53 (Heff) tested in p.9) who only had one first degree relative with cancer of the ovary or colon, but three second degree relatives with cancer of the ovary, breast, and colon respectively. Ovarian Cancer lines used were Sk-OV3, CaOv3, NIH:OVCAR-2 (p.14), NIH:OVCAR-3, NIH:OVCAR-4 (p.11), NIH:OVCAR-5 (p.99), NIH:OVCAR-8 (p.16), NIH:OVCAR-10, (p.13,14). Control cells were human dermal fibroblasts for collagen III, and a cervical carcinoma line, C4II (Auersperg et al., 1989), for keratin. Senescing cells were defined as cultures which did not reach confluence in several weeks. The cells were cultured in their standard media and serum concentrations, as defined in Chapter 2. One NFH-IOSE line and an ovarian cancer line were also cultured in media containing 0.5 mM ascorbate 2-phosphate.

Cells were grown on glass coverslips. They were then fixed in methanol for 15 min. at -20°C and processed as described previously (Auersperg et al., 1994). The cells were incubated at room temperature, for one hour, with a mixture of both the monoclonal anti-keratin antibodies, AE-1 and AE-3 (gift of Dr. T. T. Sun, New York University, New York) (Cooper et al., 1985), and the polyclonal antibody to collagen III amino terminal propeptide, rabbit anti-PIIINP (gift of Dr. Liela Risteli, University of Oulu, Oulu, Finland) (Risteli et al., 1988). They were then incubated with a secondary antibody cocktail of fluorscein isothiocyanate (FITC) labeled goat anti-mouse IgG (Jackson, West Grove, PA) and rhodamine (RITC) labeled goat anti-rabbit IgG (Cappel, West Chester, PA), also for

one hour. To demonstrate the staining of normal OSE on the ovary, 5-μm frozen sections of ovaries were treated similarly.

The intracellular staining of the ovarian cells was similar in pattern and intensity distribution to the control cells. The keratin stained in the typical filamentous array pattern in all cells. Collagen III was localized intracellularly within what appeared to be vesicles and 85% of the fibroblasts were positive. Extracellular collagen III, when present, stained in a fine fibrillar pattern. The number of positive cells for each marker was scored out of 1000 cells per case. Primary cultures exhibited regional variation; therefore 3 groups of 1000 were counted and averaged for these cases. Statistical analysis was carried out using an one way ANOVA and Tukey's test for multiple comparisons.

Spongostan cultures: Three-dimensional organization.

Five cases of NFH-OSE were used in passages 2-8 (three in p.2, one in p.4,5,8 and one in p.5,6) and three cases of FH-OSE in p.4. Two NFH-IOSE lines were used in p.7-9 and three FH-IOSE lines in p.6-13. OSE-114 was used in p.2 and IOSE-53 in p.6-9. The ovarian cancer lines used were Sk-OV3, CaOv3, NIH:OVCAR-3, NIH:OVCAR-4, NIH:OVCAR-5, NIH:OVCAR-8, and NIH:OVCAR-10. The cells were cultured in their standard media and serum concentrations (Chapter 2). The NFH-IOSE lines were also cultured in serum-free PC-1 supplemented medium (Chapter 2) (Elliott and Auersperg, 1992). All were cultured with and without 0.5 mM Ascorbate-2-phosphate and with antibiotics. However, neither PC-1 nor ascorbate-2-phosphate affected proliferation, contraction of sponge, or morphology in any of the human lines.

Spongostan was cut into pieces either 5 x 3.5 x 1 mm or 10 x 7.5 x 1 mm in size and rehydrated using ddH_2O and 1-2 changes of media before being placed in wells and inoculated with 0.1 ml of cell suspension (approximately 10^4 cells in the small size or 10^6 cells in the larger sponges). Two or four pieces were used for each case and type of media. The sponges and cells were incubated for 2 hours prior to the addition of medium, to

allow the cells to adhere to the sponge. Proliferation was estimated by the time required to fill the sponge. The NFH-OSE and FH-OSE were grown for 4 weeks, at which point the NFH-OSE cells were visible throughout the Spongostan. FH-OSE cultures never filled the sponges, even when maintained up to 6 weeks. NFH-IOSE and FH-IOSE lines were grown for 2-3 weeks, when their cells were visible throughout the Spongostan. The cancer lines exhibited varying growth rates. The majority filled the sponges in 3-4 weeks whereas some required 6-9 weeks. All sponges were fixed in 10% formalin and embedded in paraffin. 5 μm sections were stained with hematoxylin and eosin. To evaluate sponge contraction, the surface area of the sponges was compared prior to addition of cells and prior to fixation.

Results

Low Passage culture

In contrast to cryostat sections of 5 ovaries, where OSE stained for only keratin, and the stroma was positive for collagen III, the four primary cultures of NFH-OSE expressed both keratin (87-99% of cells) and collagen III (80-93% of cells) (Fig. 10), indicating that the culture conditions triggered collagen III production. This pattern was maintained in the case used in p.1 which corresponded with one of the ovaries tested. As previously demonstrated, the number of cells expressing keratin diminished to 2-14% with passaging in culture and concurrently the cells shift from an epithelial to a more atypical, mesenchymal morphology (Auersperg et al., 1994). Collagen III expression remained high in p.3-11 (66-95%) (Fig. 10).

In contrast to NFH-OSE, cultures of FH-OSE in p.4 retained a high proportion of keratin positive cells (87-100%), low numbers of collagen III positive cells (28-58%) and a predominantly epithelial morphology (Fig. 10). This phenotype persisted even in cells examined in later passage (p. 5-6), which showed signs of senescence. Two of the cases

tested were linkage positive for 17q where the BRCA1 gene is located, but this did not appear to influence the results.

In three-dimensional sponge culture, the NFH-OSE cells proliferated rapidly. They formed a stratified layer of squamous cells on the outermost sponge surface. In the spaces within the sponge they became spindle-shaped and were separated from one another by a loose ECM (Fig. 11 a). Over a period of 3-4 weeks they contracted the sponge to approximately half its original surface area. This process was likely responsible for the formation of cleft-like structures that were found penetrating from the surface and lined by flat cells (Fig. 11 a). This mesenchymal tissue organization occurred independently of passage number (p2-8) and of morphology on plastic.

FH-OSE grew very slowly in the three-dimensional sponge and often remained as rounded single cells adhering to the collagen sponge. They adhered tightly to the sponge spicules, frequently as single rounded cells, and sometimes aggregated to form epithelial nests of cells and short rows of cuboidal cells (Fig. 11 b). They did not produce a detectable ECM and were not found suspended in the spaces between sponge spicules. The FH-OSE did not reduce the overall dimensions of the sponges and did not form surface invaginations, though some localized contraction occurred in regions of high cell density.

Effects of Immortalization

The three NFH-IOSE lines had widely divergent patterns of keratin and collagen III expression. NFH-IOSE-80 (Van) had a high proportion of both keratin (100%) and collagen III (93%) staining cells that were epithelial in morphology. Addition of ascorbate 2-phosphate did not change the staining pattern or morphology of this line. NFH-IOSE-29 (Mar) cells were keratin negative and 99% were collagen III positive with an atypical morphology predominating, whereas 100% of NFH-IOSE-120 (Helm) cells were 100% keratin positive and 30% collagen III positive with an epithelial morphology. The FH-

IOSE also showed variations in patterns of expression with a slight increase in the number of cells expressing collagen III while the number of cells expressing keratin decreased (Fig. 10). All but one of the six immortalized FH-OSE lines remained morphologically epithelial. Two of the cases were matching right and left ovaries and presented similar results. The two cases tested that were 17q linkage positive had the fewest numbers of cells which expressed keratin, and one assumed an atypical morphology upon immortalization.

The two NFH-IOSE lines (80 & 29) grew well in the three-dimensional sponge, filling the spaces with tightly packed cells that were elongated or rounded and with little ECM, and covered the outer surface of the sponge with a stratified layer of squamous cells (Fig. 11 c). They contracted the sponge to 1/2 its volume within two weeks. These cultures needed to be monitored closely because the cells in the interior of the sponge tended to die. Both these lines were very similar in the three-dimensional sponge even though they have different morphologies on plastic, epithelial and atypical respectively, and different patterns of keratin and collagen III expression.

The three FH-IOSE lines also proliferated rapidly in the sponge, filling it within three weeks, and produced a small amount of matrix. Unlike the NFH-IOSE or FH-OSE, they formed a lacy network of loosely packed cells and a number of round and oval structures lined by squamous to cuboidal epithelium (Fig. 11 d). These structures resembled cysts but were thought to be formed from the passive support of the sponge structure and did not appear to contain secreted substances. The three lines were indistinguishable in their morphology. The FH-IOSE did not contract the sponge.

Unusual cases

The two lines used which did not fit into the above categories presented some interesting results. In the OSE-114 case, from a normal ovary that had ovarian cancer in the contralateral ovary, the percentage of cells expressing keratin was 83% and 85%, in

p.2 and 3, respectively, while the percentages that were positive for collagen III were 33% and 50%. These cells also retained a predominantly epithelial morphology. This follows the pattern found in FH-OSE. In Spongostan, however, OSE-114 grew well and behaved as the other NFH-OSE cases, and was indistinguishable from them in sections.

The other case, IOSE-53, with a minor family history, expressed keratin in 39% of cells and collagen III in 38% of cells (tested 3x in p.6). This was a dramatic decrease in the expression of keratin for this line compared to the results obtained by others (Auersperg et al., 1994; Maines-Bandiera, 1992). The expression of collagen was similar to that of FH-IOSE. The IOSE-53 line did not grow well in Spongostan. Sectioning revealed that most of the cells were dead with only a few scattered living cells. This occurred despite repeated trials and terminating the cultures at various time points (1-6 weeks), suggesting that these cells might be undergoing senescence.

Ovarian cancer lines

Most of the cancer lines tested had 100% keratin positive cells (exceptions: SK-OV-3, 65% and NIH:OVCAR-10, 0%) (Fig. 10). Collagen III was expressed in few cells and was usually faint. NIH:OVCAR-10 had the most cells expressing collagen III with 10%, NIH:OVCAR-2 had 4% and NIH:OVCAR-4 had less than 1%, the rest were negative (Fig. 10). The morphologies of these lines were epithelial except for SK-OV-3 in which a number of the cells were atypical. Addition of ascorbate-2 phosphate to NIH:OVCAR-3 did not stimulate collagen III production in these cells.

The ovarian cancer lines were all very epithelial in the sponge, though there was variability in their ability to proliferate and fill the sponge and in the patterns they assumed; each line displaying a characteristic growth pattern and organization (Fig.12). Most lines consisted of cuboidal to columnar cells that lined the sponge spicules (Fig.11 e, f, & 12 c, d, e), some remained as rounded cells that clustered in masses on the spicules (NIH:OVCAR-10) (Fig. 12 a), whereas others formed round cell masses (Caov-3) (Fig.12

b). The organization of these cells in three-dimensions could not be predicted by their morphology on plastic, and resembled sections of ovarian carcinomas (especially Fig. 12 d, e, f). Several lines appeared to form cyst-like (Fig. 11.f & 12.c). None of the cancer lines contracted the sponge.

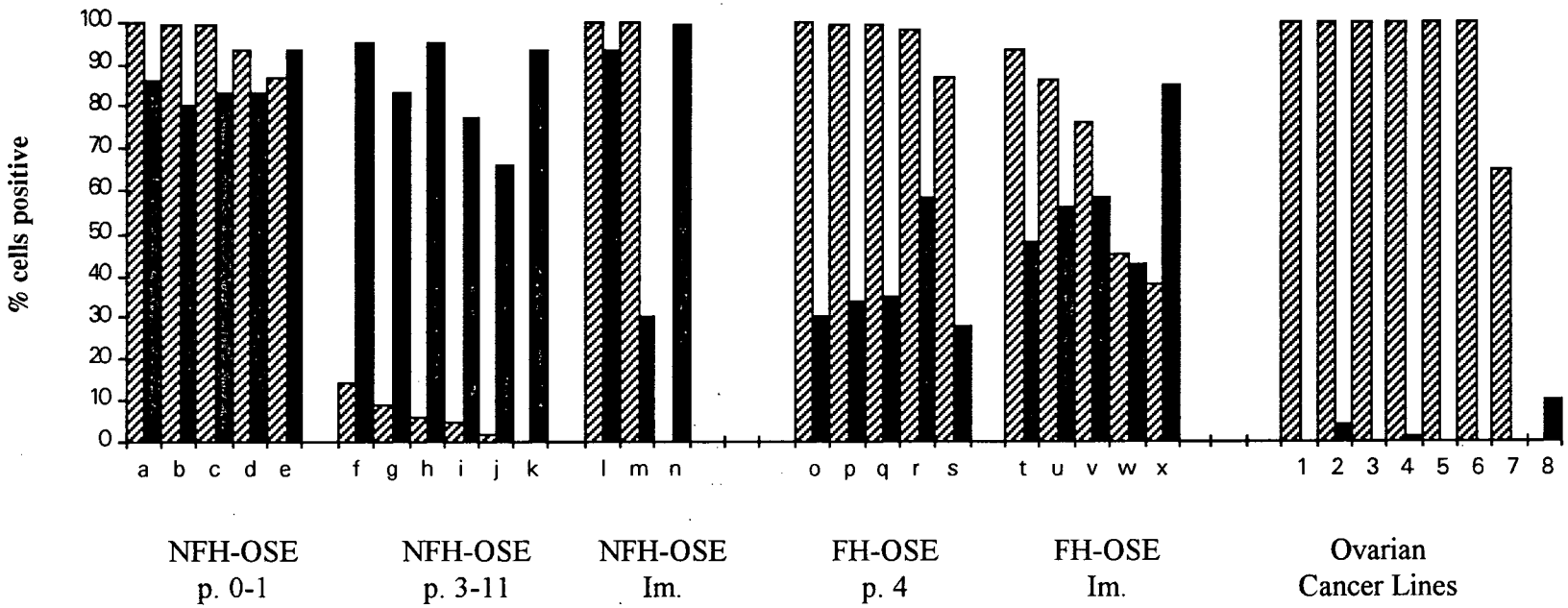


Figure 10. Expression of keratin and collagen III. The percentage of cells expressing keratin ▨ and collagen III ▮, cases arranged by decreasing keratin expression within each cell type. There is a trend for reduced keratin expression in the Im. FH-OSE group, but only the p.3-11 NFH-OSE group is significantly lower than the other four groups ($p < 0.01$). Collagen III expression is higher in both p.0-1 and p.3-11 NFH-OSE than in the FH-OSE ($p<0.01$). The cancer lines are significantly lower than all other groups ($p<0.01$). Cases used were NFH-OSE: a) OSE-131 p.1, b) OSE-134 p.0, c) OSE-137 p.0, d) OSE-135 p.0, e) OSE-152 p.0, f) OSE-17 p.4, g) OSE-133 p.3, h) OSE-97 p.4, i) OSE-31 p.5, j) OSE-34 p.11, k) OSE-10 p.6. Immortalized NFH-OSE: l) IOSE-80 p.7, m) IOSE-120 p.7, n) IOSE-29 p9. FH-OSE: o) HO-104 p.4, p) HO-106 p.4, q) HO-117 p.4, r) HO-114 p.4, s) HO-121 p.4. Immortalized FH-OSE: t) HIO-102 p.6, u) HIO-118 p.9 v) HIO-117 p.10, x) HIO-113 p.13, w) HIO-108 p.7. Ovarian cancer cell lines: 1) CaOv3, 2) OVCAR-2 p.14, 3) OVCAR-3, 4) OVCAR-4 p.11, 5) OVCAR-5 p.99, 6) OVCAR-8 p.16, 7) SKOv3, 8) OVCAR-10 p.13.

Figure 11. Morphology of three-dimensional cultures.

a) NFH-OSE (OSE-97 p.5), arrow points to lumen of cleft; b) FH-OSE (HO-117 p.4); c) Immortalized NFH-OSE (IOSE-80) p.7; d) Immortalized FH-OSE (HIO-121p.8); e) ovarian cancer line SK-OV-3 showing columnar cells; f) ovarian cancer line NIH:OVCAR-5 showing cyst-like structures. Light microscopy, hematoxylin & eosin, asterisks on sponge spicules, X 270.

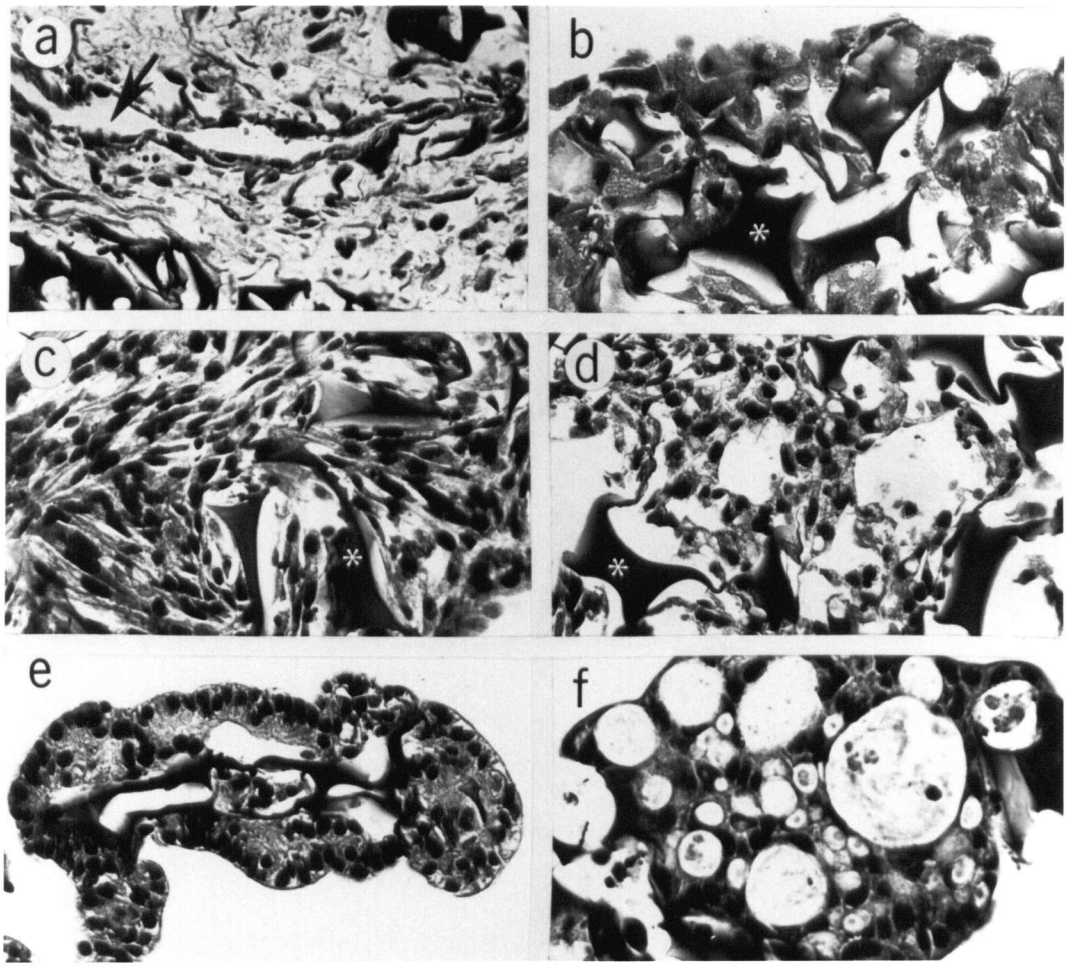
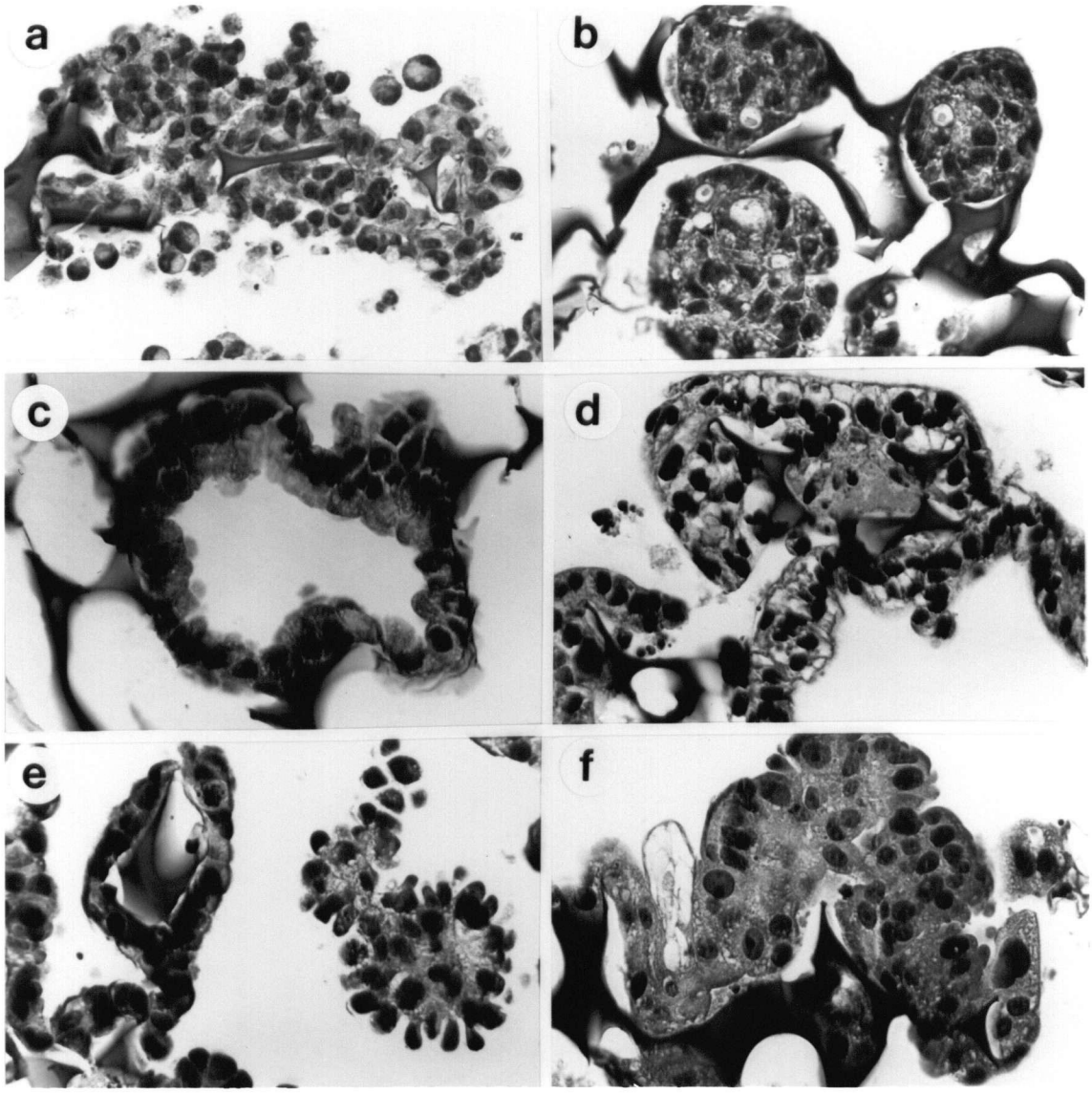

a
b
c
d
e
f

Figure 12. Morphology of ovarian cancer lines grown in Spongostan.

The various cancer lines showed cell line specific morphology when grown in Spongostan. a) NIH:OVCAR-10, b) Caov-3, c) NIH:OVCAR-8, d) SK-OV-3, e) NIH:OVCAR-4, and f) NIH:OVCAR-3. H&E stained sections X 300.


a
b
c
d
e
f

PREDOMINANT PHENOTYPE OF OSE IN VIVO AND IN CULTURE: INFLUENCE OF FAMILY HISTORY AND NEOPLASTIC PROGRESSION

		2-DIMENSIONAL CULTURE[a]					
	IN VIVO	NFH-OSE		FH-OSE	NFH-OSE	FH-OSE	OVARIAN
		p.0-1	p.3-11	p.4-6	IM	IM	CARCINOMAS[b]
KERATIN	100	96	7	97	66	67	83
COLLAGEN III	0	85	85	35	71	58	2
MORPHOLOGY	ep	ep	mes	ep	ep	ep	ep

	3-DIMENSIONAL CULTURE				
	NFH-OSE	FH-OSE	NFH-OSE	FH-OSE	OVARIAN
	p.3-11	p.4-6	IM	IM	CARCINOMAS[b]
GROWTH PATTERN					
- predominant morphology	mes	ep	variable	ep	ep
- distribution within spaces	dispersed	lining surfaces	closely packed	loosely packed	cysts, spheres
- morphology of surface cells	squamous	cuboidal	squamous	squamous	cuboidal and columnar
- proliferation[c]	rapid	slow	rapid	rapid	rapid
ECM PRODUCTION	much	no	little	little	no
CONTRACTION[d]	50	0	50	0	0

Table 2. ep, epithelial; mes, mesenchymal (predominant cell shape); NFH, no family history of ovarian cancer; FH, with a family history of ovarian cancer; IM, Simian virus 40 immortalized cells, p., passage.

[a] The numbers represent the average percentage of cells expressing a characteristic

[b] based on 8 ovarian cancer lines

[c] based on time required to fill the sponge (see text)

[d] % reduction of sponge surface area from original size

Discussion

This study supports others which have shown that neoplastic progression in OSE cells is accompanied by an increased commitment to an epithelial phenotype and/or reduced responsiveness to the signals which induce epithelio-mesenchymal conversion upon explantation into culture (Auersperg et al., 1994; Maines-Bandiera et al., 1992; Hamilton et al., 1985). Importantly, it also suggests that similar changes may occur in overtly normal OSE from women with a family history of ovarian cancer, and that therefore increased commitment to an epithelial phenotype and reduced responsiveness to environmental signals might represent predisposing factors or very early changes in ovarian carcinogenesis.

Though sample sizes were small, significant differences in the percentage of cells expressing the epithelial and mesenchymal cytodifferentiation markers keratin and collagen III and differences in the expression of epithelial characteristics at the level of three dimensional morphogenesis were found between NFH-OSE, FH-OSE and ovarian cancer cell lines, suggesting that the genetic changes that distinguish these three groups are associated with different capacities for phenotypic modulation (Table 2). Together, they indicate that FH-OSE cells respond differently to the culture environment than NFH-OSE cells, since FH-OSE cultures retain a predominantly epithelial phenotype with only a small percentage of cells with mesenchymal characteristics, whereas the NFH-OSE cells, upon explantation to culture, express a dual phenotype and gradually lose their epithelial characteristics. This hypothesis supports our previous observation that expression of CA125 and epithelial morphology on plastic tends to be maintained longer in FH-OSE than in NFH-OSE (Auersperg et al., 1995).

Introduction of the SV40 large T viral gene into normal human cells will usually increase the life span of the cells, though not indefinitely, by binding to the growth regulatory proteins p53 and Rb. Because the large T gene can interfere with growth regulation without causing the cells to become tumorigenic, it has been used as a model

for studying the step-wise progression of carcinogenesis. In OSE, immortalization introduced this characteristic of neoplastic progression, viz. increased proliferation, allowing the FH-IOSE to fill the sponge, but it did not induce a shift in the NFH-OSE phenotype to that of FH-OSE cells nor of FH-OSE to the cancer lines. The expression patterns of the cytodifferentiation markers were variable between cases in both NFH-IOSE and FH-IOSE and therefore these groups were not significantly different from their respective unimmortalized groups. The trends in the two groups, however differed in that NFH-IOSE retained keratin, as found previously (Maines-Bandiera et al., 1992), whereas in the FH-IOSE the expression of keratin diminished, while collagen increased slightly.

The unusual case IOSE-53, with a minor family history, showed a marked decline in keratin expression from earlier studies (Maines-Bandiera et al., 1992), as well as an inability to grow in the sponge. This decrease in growth potential suggests that these cells are reaching the end of the extended life span conferred by SV40 large T, and are entering a period of crisis, comparable to normal senescence (Rubelj and Pereira-Smith, 1994), from which a few cells may emerge and grow indefinitely (true immortalization). The decrease in keratin and collagen III expression could therefore be attributed to normal senescence, comparable to the p.11 NFH-OSE which had the lowest expression of collagen III in that group (66%). However, senescent FH-OSE (p.5-6) retained keratin expression in the majority of their cells. Therefore it is unclear whether the low percentage of collagen III expression in IOSE-53 is due to senescence or to its minimal family history.

The culture conditions which induced expression of mesenchymal characteristics in NFH-OSE had the least influence on the ovarian cancer lines. These lines not only retained the epithelial form of cytodifferentiation, but in addition formed polarized epithelia and cysts in three dimensional culture. Their resemblance to actual ovarian carcinomas, though lacking stroma, demonstrates that Spongostan provides an adequate environment for these cells to differentiate. They exhibited the autonomy characteristic of cancer cells, since such complex epithelial structures were formed without the provision of the connective tissue-

derived basement membrane components required by most epithelial cells. Our findings complement in vivo comparisons of epithelial differentiation markers in OSE and ovarian cancers. Ovarian cancers express an epithelial cell type 200-kd glycoprotein, human milk fat globule antigen and high molecular weight keratins (Van Niekerk et al., 1993) and form complex epithelial histologic structures (Young et al., 1989), whereas OSE is negative for these epithelial markers; vimentin, in contrast, is uniformly present in OSE but more heterogeneous among carcinomas (Van Niekerk et al., 1993). The similarities between our findings and the in vivo characteristics of OSE and of ovarian cancers, respectively, suggest that the differences in differentiation observed here may be significant in the process of ovarian carcinogenesis.

The potential use of these characteristics as markers for susceptibility is also suggested by the pattern found in OSE-114, from a normal ovary with contralateral ovarian cancer, which expressed a cytodifferentiation pattern similar to FH-OSE, while exhibiting a growth pattern and three-dimensional morphogenesis similar to NFH-OSE. The decrease in intracellular expression of collagen III in the ovarian cancer lines was, however, unexpected as the serum levels of the aminoterminal propeptide of collagen III increase in advanced ovarian cancer, and are used as a marker to predict changes in the clinical behavior of these tumors (Risteli et al., 1992). Further investigation by Risteli's group revealed that the increase in serum levels is due to increased levels secreted into the peritoneal cavity and not from blood vessels draining the tumor, and that 41% of the procollagen synthesized and released into the peritoneal ascites fluid was collagen III vs. collagen I (Zhu et al., 1993). Thus the decrease in expression in collagen III in the epithelium of ovarian carcinomas would need to be confirmed using freshly isolated cells, though the passage numbers of some of the cancer lines were similar to the immortalized lines. It would be interesting to examine the levels of pro-collagen I to see if there is an overall decrease in mesenchymal characteristics with neoplastic progression or whether it is specifically collagen III levels which decrease. Differential regulation of collagen I and

III synthesis has been demonstrated for several factors including 1) TGF-β, which enhanced production of collagen I on its own, but that of collagen III only in the presence of ascorbate (Appling et al., 1989); and 2) estrogen supplements in post-menopausal women which increased collagen III production in relation to collagen I in skin (Savvas et al., 1993). Both these factors could potentially play important roles in OSE as TGF-β is produced by both ovarian carcinoma lines and normal OSE, though only OSE can activate it (Berchuck et al., 1992), while the changes in estrogen levels with menopause may decrease the capacity of OSE to produce collagen III.

Many cell types react to explantation into culture by mimicking a regenerative (repair) response. Thus, the conversion to a mesenchymal form may be reflecting the normal regenerative response of OSE cells in vivo. Modulation from epithelial to mesenchymal phenotypes has been found to occur during different stages of development and, more recently, during the process of wound healing. It is a property found in other mesodermally derived epithelial cells such as capillary endothelial cells and pleural mesothelium during wound healing (Lipton et al., 1992: Davila and Crouch, 1993). Davila and Crouch (1993) found co-expression of keratin with vimentin and collagen I propeptide in the reactive mesothelial cells of patients with pleuritis, both on the regenerating lining and in the submesothelial regions. Thus the expression of collagen III in response to the present culture conditions may be paralleling the response of OSE to wound healing. The assumption of a fibroblastic morphology at ovulation may also be part of the wound healing response and could facilitate migration into the fibrin clot that overlies the ovulatory defect and perhaps even the underlying stroma, similar to the keratin positive cells found in the sub-mesothelial region by Davila and Crouch (1993). This may account for keratin positive cells found in the ovarian stroma by Van Niekerk (1993). These epithelial-mesenchymal characteristics of OSE reflect its close developmental relationship to ovarian stromal fibroblasts and suggest that OSE may be relatively uncommitted and closer to a stem cell form than other coelomic epithelial derivatives.

The ability to contract Spongostan appears to mimic another feature of wound repair, contraction of the extracellular matrix. This contractile ability of OSE has been demonstrated previously in the collagen gel/ROSE 199 organoids (Kruk and Auersperg, 1992). Our results suggest that this property also diminishes with neoplastic progression and is greatly reduced in FH-IOSE. Thus, the cell types examined in this study may differ in their response to wound healing signals in vivo.

The underlying mechanisms responsible for the expression of mesenchymal characteristics in OSE are unknown. However, Lipton's work with capillary endothelial cells suggest some interesting possibilities (1992). These cells normally express an epithelial phenotype in vivo but switch to a mesenchymal phenotype when they become reactive in response to wounding. The retention of the epithelial phenotype is dependent on the presence of cAMP, whereas Ca^{+2} in the absence of cAMP mediates the switch to the mesenchymal phenotype. In addition, histamine alone will induce a stable transitional phenotype with a mesenchymal morphology, which upon the addition of cAMP will revert back to an epithelial morphology, whereas the removal of histamine (and cAMP) will irreversibly switch on the mesenchymal program (Lipton et al., 1992). The decreased ability of FH-OSE to express the mesenchymal characteristics found in NFH-OSE might reflect an interference with one of these signaling pathways. This interference could lead to differing responses both to factors released after ovulation as well as factors that are present at different stages of follicular development. This may account for the "protective" effect of periods of anovulation in ovarian carcinogenesis.

Further work needs to be done to confirm these results with a larger number of cases and to determine whether the changes in expression of epithelial and mesenchymal characteristics are a by-product of the genetic changes associated with ovarian carcinogenesis or whether the loss of expression of mesenchymal characteristics leads to a cell type that is more prone to undergo neoplastic progression.

CHAPTER 6

GENERAL DISCUSSION

The experiments described in the preceding chapters supported the work of others on the dual expression of epithelial and mesenchymal characteristics in cultured OSE. They also, importantly, demonstrate that a significant difference in the degree of expression of these characteristics exists among various populations of OSE derived cells, reflecting differing responses to the culture environment for not only ovarian cancer cells, but also for normal OSE from women with a family history of ovarian cancer. Differences in expression of epithelial and mesenchymal characteristics are also shown between two sublines of the rat OSE line ROSE 199, highlighting the separation of the production of mesenchymal products from the assumption of a mesenchymal morphology.

The variety of patterns formed by the ovarian cancer lines in the sponge emphasizes the importance of three-dimensional organization on the differentiation of cells in culture, as has been shown for an ovarian cancer line grown in micro-carrier beads (Becker et al., 1993) and many other cell types. The collagenous sponge also proved successful in allowing cells to grow in close proximity, with potentially higher concentrations of secreted products in the interior of the sponge then when grown on a planar surface. However the formation of lumina lined by cuboidal or columnar cells, as found in the inclusion cysts or clefts on the ovary, was limited to those cells which expressed less of a mesenchymal phenotype in culture, primarily the ovarian cancer lines, and to a lesser extent the FH-IOSE, and ROSE 199 sublines.

The behavior of the ROSE 199 sublines in response to the various substrata emphasized the differences between these lines and huOSE in culture. Unlike the huOSE

which when plated on collagen gels assumed a mesenchymal morphology (Kruk et al., 1994), the ROSE 199 sublines retained the expression of an cuboidal morphology. The ability of one of the sublines to also modulate to a mesenchymal morphology may serve as a model to elucidate the signals needed to trigger the mesenchymal phenotype in huOSE. However, it may be that these cells require very different stimuli to undergo the conversion, as the normal culture conditions induce this response in NFH-OSE, but not as completely or irreversibly in the ROSE 199 High ECM subline. The difference in responsiveness to culture conditions between ROSE 199 and huOSE was further illustrated by their behavior when co-cultured, in that the huOSE demonstrated a very strong tendency to become mesenchymal and reside in the matrix, whereas ROSE 199 lines preferred the surface covering, epithelial location. Thus it appears that ROSE 199 does not produce either a matrix or paracrine factors sufficient to prevent the assumption of the mesenchymal phenotype by NFH-OSE, instead this phenotype is enhanced in co-cultures. The differences between the ROSE 199 sublines and huOSE could be due to species differences or to the changes brought about in the process of spontaneous immortalization of ROSE 199. Their presence points to the potential limitations of using the ROSE cells for studying human ovarian carcinogenesis.

In vitro models are also limited by the proliferative potential of huOSE cells. This can be overcome to a certain extent by the transfection of viral genes such as the SV40 large T gene. However, immortalization also appears to introduce more variability in expression patterns of cytodifferentiation patterns, such as keratin and collagen III. This variation, introduced by the selection by transfection of a small percentage of the original population, would need to be verified using a greater number of cases.

The dual expression of epithelial and mesenchymal characteristics both in vivo, as seen by the concurrent expression of vimentin and keratin (Czernobilsky et al., 1985), and in primary culture, with continued dual expression of the intermediate filaments and both basement membrane and stromal ECM components (Auersperg et al., 1994), indicates a

retention of mesenchymal characteristics by this epithelium of mesodermal origin. The retention in culture of the expression of ECM components when coupled with the finding that induced expression of these components by other epithelia of mesodermal origin, such as pleural mesothelium and microvascular endothelium, occurs during wound healing (Davila and Crouch, 1993; Lipton et al., 1992) suggests that similar changes may be involved in OSE during wound healing and stromal repair after ovulation.

An increase in collagen synthesis, as demonstrated using ascorbate in ROSE 199 lines on plastic, can lead to the stratification of cells on the surface suggesting that matrix may have been deposited on the luminal surface of these cells. This coupled with the fact that NFH-IOSE (like the ovarian cancer cells described by Niedbala, 1985) would only attach in areas where there was exposed ROSE 199 ECM suggests that the increased production of collagens stimulated by ovarian carcinomas could enable widespread peritoneal metastasis by improving adhesion of cancer cells.

The three-dimensional morphogenesis of NFH-OSE and their fibroblastic behavior on collagen gels (Kruk et al., 1994) and ROSE 199 matrix (Kruk, 1992, and co-culture experiments in this thesis) demonstrates that these cells are, in theory, capable of penetrating into the stromal compartment of the ovary as fibroblastic cells. This ability to penetrate stroma, coupled with the ability to contract Spongostan and ROSE 199/collagen organoids (Kruk and Auersperg, 1992), suggests that NFH-OSE may play an active role in the formation of clefts and cysts, as opposed to passively being trapped through loss of stromal mass in the post-menopausal ovary. In theory, epithelial cells that become mesenchymal lose cell-cell adhesions prior to migrating through the stroma (Hay, 1990). If, however, both phenotypes are expressed at the same time in OSE, one could potentially have cells which migrate into the stroma while still adhering to the surrounding cells forming crypts, a process which appears to occur cyclically in some mammals (Harrison and Harrison Matthews, 1951). Thus, in humans, the invagination of OSE could be

triggered by factors released at the time of ovulation without necessarily being associated with the ovulatory defect.

The loss of epithelial characteristics by NFH-OSE in response to culture has been well documented. In vivo, however, there is very little evidence demonstrating the loss of epithelial characteristics by NFH-OSE. Ovulation may induce the more squamous phenotype of the B type cells found by Gillet (1991), and isolated keratin positive cells have been found in the stroma by Van Niekerk (1993), which potentially suggest modulations to a simpler epithelium and acquisition of migratory characteristics respectively. What is perhaps more striking in vivo, is the gain of the more differentiated Müllerian epithelial characteristics, perhaps with an accompanying loss of mesenchymal characteristics, as seen in ovarian carcinomas in vivo and in vitro. Müllerian differentiation is not confined to the OSE, being found in other parts of the peritoneal mesothelium, but it is most common on or near the ovary (Lauchlan, 1972). The variety of forms into which OSE can differentiate are striking and include: 1) all types of mature Müllerian duct tissue, 2) urinary bladder, and 3) gastro-intestinal cells. The lesions often contain cells which followed a slightly different differentiation pathway; thus mucinous cystadenomas can contain serous cell types and vice-versa. The degree of mimicry is perhaps greatest in the benign conditions of endosalpingiosis and endometriosis which show similar responses to hormonal cycling as the tubal and uterine epithelium respectively (Lauchlan, 1972).

It is unclear whether the steps in ovarian carcinogenesis may lead through these stable, highly differentiated benign lesions or whether the cells undergoing malignant transformation experience interference at differing points in the epithelial differentiation pathways. What these experiments have demonstrated is that in women with an increased risk of developing cancer due to hereditary factors, the OSE expresses less of a mesenchymal phenotype and more of an epithelial one. This phenotypic difference in women with a family history of ovarian cancer, is one of the first reported differences between these two groups of OSE. It is supported by our previous observation that higher

expression of CA125 and an epithelial morphology on plastic are more typical of FH-OSE than NFH-OSE (Auersperg et al., 1995). These differences in responsiveness to the culture environment, which include expression of keratin and collagen III, morphology in two- and three-dimensions, and contraction of Spongostan, suggest that these cells may respond differently to wound healing signals and may retain more of an epithelial response pattern rather then a mesenchymal one. It would be interesting to determine whether these women have an increase in the number of inclusion cysts or Müllerian differentiation compared to women with no family history of ovarian cancer.

In summary, the work with OSE to date indicates that though these cells are able to express a dual epithelial/mesenchymal phenotype they can also become predominantly fibroblastic, as they do during culture, or predominantly epithelial, as they do during neoplasia. The next important step in using the OSE culture model will be to determine what signals control the different differentiation pathways and to see if the predominantly epithelial phenotype can be triggered in culture, or prevented in vivo.

REFERENCES

Adams, A.T. and Auersperg, N. (1981) Transformation of cultured rat ovarian surface epithelial cells by Kirsten murine sarcoma virus. Cancer Res 41:2063-2072.

Adams, A.T., and Auersperg, N. (1985) A cell line ROSE 199, derived from normal rat ovarian surface epithelium. Exp Cell Biol *53*:181-188.

Appling, W.D., O'Brien, W.R., Johnston, D.A., and Duvic, M. (1989) Synergistic enhancement of type I and III collagen production in cultured fibroblasts by transforming growth factor ß and ascorbate. FEBS Letters 250:541-544.

Auersperg, N., Siemens, C.H., Myrdal, S.E. (1984) Human ovarian surface epithelium in primary culture. In Vitro 20:743-75.

Auersperg, N., Kruk, P. A., MacLaren, I. A. Watt, F. M., and Myrdal S. E. (1989) Heterogeneous expression of keratin, involucrin, and extracellular matrix among subpopulations of a poorly differentiated human cervical carcinoma: possible relationships to pattern of invasion. Cancer Res. *49:*3007-14.

Auersperg, N, MacLaren, I.A., and Kruk, P.A., (1991) Ovarian surface epithelium: autonomous production of connective tissue-type extracellular matrix. Biol Reprod 44:717-724.

Auersperg, N., Maines-Bandiera, S. L., Dyck H. G., and Kruk, P. A. (1994) Characterization of cultured human ovarian surface epithelial cells: phenotypic plasticity and premalignant changes. Lab. Invest. 71:510-518.

Bancroft, J., and Cook, H., (1984) *Manual of Histological Techniques*. London, Churchill Livingston.

Barger, D.J. and Delameter, E.D. (1948) The use of thionyl chloride in the preparation of Schiff's reagent. Science 108:121-122.

Becker, J.L. Prewett, T.L., Spaulding, G.F. and Goodwin, T.J. (1993) Three-dimensional growth and differentiation of ovarian tumor cell line in high aspect rotating-wall vessel: morphologic and embryologic considerations. J Cell Biochem 51:283-289.

Bell, E., Ivarsson, B., and Merril, C. (1979) Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential *in vitro.* Proc. Natl. Acad. Sci. USA *76:*1274-78.

Berchuck, A., Rodriguez, G., Olt, G., Whitaker, R., Boente, M.P., Arrick, B.A., Clarke-Pearson, D.L, Bast, R.C. (1992) Regulation of growth of normal ovarian epithelial cells and ovarian cancer lines by transforming growth factor β. Am J Obstet Gynecol 166:2:676-684.

Berthod, F., Hayek, D., Damour, O., and Collombel, C. (1993) Collagen synthesis by fibroblasts cultured within a collagen sponge. Biomaterials 14:749-754.

Blaustein, A. (1981a) Surface (germinal) epithelium land related ovarian neoplasms. Pathol Annual 16:247-294.

Blaustein, A. (1981b) Surface and inclusion cysts in fetal ovaries. Gynecol Oncol 12:222-233.

Blaustein, A., Kaganowicz, A., and Wells, J., (1982) Tumor markers in inclusion cysts of the ovary. Cancer 49:722-726.

Boutin, E.L., Battle, E., Cunha, G.R. (1992) The germ layer origin of mouse vaginal epithelium restricts its responsiveness to mesenchyme inductors: uterine induction. Differentiation 49:101-107.

Burdsal, C.A., Damsky, C.H., and Pedersen, R.A. (1993) The role of E-cadherin and integrins in mesoderm differentiation and migration at the mammalian primitive streak. Development 118: 829-844.

Casser-Bette, M., Murray, A.B., Closs, E.I., and Schmidt, J. (1990) Bone formation by osteoblast-like cells in a three dimensional cell culture., Calcif Tissue Int 46:46-56.

Chambard, M., Gambrion, J., Mauchamp, J., (1981) Influence of collagen gel on the orientation of epithelial cell polarity: follicle formation from isolated thyroid cells and preformed monolayer. J Cell Biol 91:157-166

Chan, D., Lamande, S.R., Cole, W.G., and Bateman, J.F. (1990) Regulation of procollagen synthesis and processing during ascorbate induced extracellular matrix accumulation in vitro. Biochem J 269: 175-181.

Chojkier, M., Houglam, K., Solis-Herruzo, J., and Brenner, D.A. (1989). Stimulation of collagen gene expression by ascorbic acid in cultured human fibroblasts. J Biol Chem 264:(28): 16957-16962.

Cole, K., Park, C.M., Reid, P.E. Sheath, R.G. (1985) Comparative studies of the cell wall of sexual and asexual atropurpurea (rhodophyta). I. Histochemistry of polysaccharides. J. Phycol 21:585-592.

Cooper D, Schermer A, Sun T. (1985). Classification of human epithelia and their neoplasms using monoclonal antibodies to keratins: strategies, applications and limitations. Lab Invest 52:243-56.

Czernobilsky, B., Moll, R., Levy, R., and Franke, W.W. (1985) Co-expression of cytokeratin and vimentin filaments in mesothelial, granulosa and rete ovarii cells of the human ovary. Eur. J. Cell Biol., *37:* 175-190.

Davila R. M., and Crouch E. C. (1993) Role of mesothelial and submesothelial stromal cells in matrix remodeling following pleural injury. Am. J. Pathol., *142:* 547-555.

Doane, K.J., and Birk, D.E., (1991) Fibroblasts retain their tissue phenotype when grown in three-dimensional collagen gels. Exp Cell Res 195:432-442.

Docherty, K., Forrester, J.V., Lackie, J.M., and Gregory, D.W. (1989) Glycosaminoglycans facilitate the movement of fibroblasts through three-dimensional collagen matrices., J Cell Sci 92:263-270.

Doillon, C.J., Silver, F.H., and Berg, R.A. (1987) Fibroblast growth on a porous collagen sponge containing hyaluronic acid and fibronectin. Biomaterials 8:195-200.

Donjacour, A.A. and Cunha, G.R. (1991) Stromal regulation of epithelial function. Cancer Treat & Res 53:335-64.

Dyck, H. G. and Auersperg, N. The expression of mesenchymal characteristics by human ovarian surface epithelial cells diminishes with neoplastic progression. Proc. AACR 85th annual meeting. *35:*162, 1994.

Ekblom, P. (1989) Developmentally regulated conversion of mesenchyme to epithelium. FASEB J 3:2141-2150.

Elliott, W.M., and Auersperg, N. (1992) Growth of normal human ovarian surface epithelial cells in reduced serum and serum-free media. In Vitro Cell Dev Biol 29A:9-18.

Emerman, J.T., and Pitelka, D.R., (1977) Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. In Vitro 13:316-328.

Fathalla, M. F. (1971) Incessant ovulation - a factor in ovarian neoplasia? Lancet 1971-2:163.

Fenoglio, C.M., Castadot, M.J., Ferenczy, A., Cottral, G.A., Richart, R.M. (1977) Serous tumors of the ovary. I ultrastructural and histochemical studies of the epithelium of benign serous neoplasms, serous cystadenomas and serous cysteadenofibromas. Gynecol Oncol 5:203-218.

Fenoglio, C.M. (1980) Overview Article: Ultrastructural features of the common epithelial tumors of the ovary. Ultrastructural Pathology 1:419-444.

Fogh, J. and Trempe, G. (1975). New tumor cell lines. In: Human Tumor Cells in Vitro. Ed. Fogh J. Plenum Publishing Corp. New York. p1115-46.

Ford, D., Easton, D.F., Bishop, D.T., Narod S.A. Goldgar, D.E. and the Breast Cancer Linkage Consortium (1994) Risks of cancer in BRCA1-mutation carriers. Lancet 343:692-95.

Franceschi, RT. (1992). The role of ascorbic acid in mesenchymal differentiation. Nutr Rev *50* (3):65-70.

Fredrickson, T.N. (1987) Ovarian tumors of the hen. Environ Health Perspect 73:35-51.

Freeman, A.E and Hoffman R.M. (1986) In vivo-like growth of human tumors in vitro. Proc Natl Acad Sci USA 83:2694-2698.

Fujiwara, S., Shinkai, H., Deutzmann, R., Paulsson, M., and Timpl, R. (1988) Structure and distribution of N-linked oligosaccharide chains on various domains of mouse tumor laminin. Biochem J 252:453-461.

Geesin, J.C., Hendricks, L.J., Falkenstein, P.A., Gordon, J.S., and Berg, R.A. (1991) Regulation of collagen synthesis by ascorbic acid: characterization of the role of ascorbate-stimulated lipid peroxidation. Arch Biochem Biophysics *290* (1): 127-132.

Ghosh, D., Danielson, K.G., Alston, J.T., and Heyner, S. (1991) Functional differentiation of mouse uterine epithelial cells grown on collagen gels or reconstituted basement membranes., In Vitro Cell Dev Biol 27A:713-719.

Gilbert, P.X. and Harris, H. (1988) The role of ras oncogene in the formation of tumors. J Cell Sci 90:433-446.

Gillet W.R., Mitchell A., and Hurst P.R. (1991) A scanning electron microscopic study of human ovarian surface epithleium: characterization of two cell types. Human Reprod. *6*:645-650.

Godwin, A.K., Testa, J.R., Handel, L.M., Liu, Z., Vanderveer, L.A., Tracey, P.A. and Hamilton, T.C. (1992) Spontaneous Transformation of Rat Ovarian Surface Epithelial Cells: Association with Cytogenetic Changes and Implications of Repeated Ovulation in the Etiology of Ovarian Cancer. J Natl Cancer Inst 84:592-601.

Godwin, A.K., Testa, J.R. and Hamilton, T.C. (1993) The biology of ovarian cancer development. Cancer 71:530-6.

Greenberg, G., and Hay, E.D. (1988) Cytoskeleton and thyroglobulin expression change during conversion of thyroid epithelium to mesenchyme-like cells. Development 102: 605-622.

Grinnell, F., Fukamizu, H., Pawelek, P., and Nakagawa S. (1989) Collagen processing, crosslinking, and fibril bundle assembly in matrix produced by fibroblasts in long term cultures supplemented with ascorbic acid. Exp Cell Res 181:483-491.

Gross, T.P. and Schlesselman J.J. (1994) The estimated effects of oral contraceptive use on the cumulative risk of epithelial ovarian cancer. Obstet Gynecol 83:419-24.

Hamilton, T.C., Young, R.C., McKoy, W.M., Grotzinger, K.R. Green, J.A., Chu, E.W., Whang-Peng, J., Rogan A.M., Green, W.R., and Ozols, R.F., (1983). Characterization of a human ovarian carcinoma line (NIH:OVCAR-3) with androgen and estrogen receptors. Cancer Res *43*:5379-5389.

Harrison R.J. and Harrison Matthews L. (1951) Sub-surface crypts in the cortex of the mammalian ovary. Proc. Zool. Soc. Lond. *120*:699-714.

Hata, R., and Senoo, H., (1989). L-Ascorbic acid 2-Phosphate stimulates collagen accumulation, cell proliferation, and formation of a three-dimensional tissue-like substance by skin fibroblasts. J Cell Physiol *138*:8-16.

Hay, E.D. (1990) Role of cell-matrix contacts in cell migration and epithelial mesenchymal transformation. Cell Diff Dev 32;3:367-75.

Hay, E.D. (1991) Collagen and other matrix glycoproteins in embryogenesis. *In:* Cell Biology of Extracellular Matrix, 2nd Ed. Hay E.D. ed. Plenum Press, New York.

Hoffman, A.G., Burghardt, R.C., Tilley, R., and Auersperg, N., (1993) An *in vitro* model of ovarian epithelial carcinogenesis: changes in cell-cell communication and adhesion occurring during neoplastic progression. Int. J. Cancer *54*:828-838.

Honig, M.G. and Hume, R.I. (1986) Fluorescent carbocyanine dyes allow living neurons of identified origin to be studies in long-term culture. J Cell Biol 103:171-187.

Hornby, A.E., Pan, J., and Auersperg, N., (1992) Intermediate filaments in rat ovarian surface epithelial cells: changes with neoplastic progression in culture. Biochem Cell Biol 70:16-25.

Höyhtyä, M., Mylllylä, R., Piuva, J., Kivirikko, K.I., and Tryggvason, K. (1984) Monoclonal antibodies to human prolyl 4-hydroxylase. Eur J Biochem 141:477-482.

Johnson, G.R., Saeki, T., Auersperg, N., et al., (1991) Response to and expression of amphiregulin by ovarian carcinoma and normal ovarian surface epithelial cells: nuclear localization of endogenous amphiregulin. Biochem Biophys Res Commun 180: 4481-8.

Joshi, M.S. (1991) Growth and differentiation of the cultured secretory cells of the cow oviduct on reconstituted basement membrane. J Exp Zoo 260:229-238.

Kao, J., Huey, G., Kao, R., and Stern, R., (1990). Ascorbic acid stimulates production of glycosaminoglycans in cultured fibroblasts. Exp & Mol Path *53*:1-10.

Kerlikowske, K., Brown, J.S., and Grady, D.G. (1992) Should women with familial ovarian cancer undergo prophylactic oophorectomy? Obstet. Gynecol. *80:*700-7.

Kiernan, J.A. (1990) *Histological and Histochemical Methods: Theory and Practice*. 2nd Ed., Pergamon Press, Toronto, Canada.

Kleinman, H.K., Graf, J., Iwamoto, Y., Kitten, G.T., Ogle, R.C., Saski, M., Yamada, Y., Martin, G.R., and Luckenbill-Edds, L., (1987) Role of basement membranes in cell differentiation. Annals NY Acad Sci *513*:134-145.

Kruk, PA. (1992) Human Ovarian surface epithelial cells in culture: Characterization and matrix interrelationships. Ph.D. Thesis, University of British Columbia.

Kruk, P. A., and Auersperg, N. (1992) Human ovarian epithelial cells are capable of physically restructuring extracellular matrix. Am. J. Obstet. Gynecol. *167:*1437-43,

Kruk, P. A., Maines-Bandiera, S. L., and Auersperg, N. (1990). A simplified method to culture human ovarian surface epithelium. Lab. Invest. *63:*132-36,

Kruk, P.A., Uitto, V-J., Firth, J.D. Dedhar, S., and Auersperg, N. (1994) Reciprocal interactions between human ovarian surface epithelial cells and adjacent extracellular matrix. Exp Cell Res 215:97-108.

Lang L.M., Gailey C, Myrdal SE, Dedhar S, Auersperg N. (1992). Divergence in patterns of invasion among subpopulations derived from a human carcinoma clone: roles of intercellular contacts and of cell-substratum adhesion. Tumor Biol *13*:364-77.

Lauchlan SC. (1972) The secondary müllerian system., Obstet Gynecol Surv 27:133-146.

Leppä, S., Härkönen, P., and Jalkanen, M. (1991) Steroid-induced epithelial-fibroblastic conversion is associated with syndecan suppression in S115 mouse mammary tumor cells. Cell Regulation 2:1-11.

Lillie, R.D., (1965) *Histopathological Technique and Practical Histochemistry.* 3rd Ed. McGraw Hill.

Lipton, B. H., Bensch K. G., and Karasek M. A. (1992) Histamine-modulated transdifferentiation of dermal microvascular endothelial cells. Exp. Cell Res. 199: 279-291.

Lynch, H.T. and Lynch, J.F. (1992) Hereditary ovarian carcinoma. Hemat Oncol Clinics of North America 6:783-811.

Lynch, H. T., Watson, P., Lynch, J. F., Conway, T. A., and Fili, M. (1993) Hereditary ovarian cancer: heterogeneity in age at onset. Cancer *71:*573-81.

Madri, J.A., Pratt, B.M., and Tucker, A.M., (1988). Phenotypic modulation of endothelial cells by transforming growth factor-B depends upon the composition and organization of the extracellular matrix. J Cell Biol *106*:1375-1384.

Madri JA, Bell L, Marx M, Merwin J.R., Basson C, and Prinz C., (1991). Effects of soluble factors and extracellular matrix components on vascular cell behavior in vitro and in vivo: models of de-endothelialization and repair. J Cell Biochem 45:123-130.

Maines-Bandiera, S. L., Kruk, P.A., and Auersperg, N. (1992) Simian virus 40-transformed human ovarian surface epithelial cells escape normal growth controls but retain morphogenic responses to extracellular matrix. Am. J. Obstet. Gynecol., *167:*729-735.

Mauch, C., Hatamochi, A., Scharffetter, K., and Krieg, T., (1988). Regulation of collagen synthesis in fibroblasts within a three-dimensional collagen gel. Exp Cell Res *178*:493-503.

McGuire, P.G. and Alexander, S.M. (1992) Urokinase expression during the epithelial-mesenchymal transformation of the avian somite. Dev Dynamics 194: 193-197

McGuire, P.G. and Alexander, S.M. (1993) Inhibition of urokinase synthesis and cells surface binding alters the motile behavior of embryonic endocardial-derived mesenchymal cells in culture. Development 118: 931-937.

Michalopoulos, G., and Pitot, H.C., (1975). Primary culture of parenchymal liver cells on collagen membranes. Exp. Cell Res. 94:70-78.

Mulligan, R.C. and Berg, P. (1981) Selection for animal cells that express the E.coli gene coding for xanthine-guanine phosphoribosyltransferase. Proc Natl Acad Sci USA 78:2072-2076.

Nicosia, S.V., and Nicosia, R.F. (1988) Neoplasms of the ovarian mesothelium. Pathology of Human Neoplasms Ed. H.A. Azar Raven Press, NY.

Niedbala, M.J., Crikard, K., and Bernaki, R.J. (1985) Interactions of human ovarian tumor cells with human mesothelial cells grown on extracellular matrix. An in vitro model system for studying tumor cell adhesion and invasion. Expl Cell Res 160:499-513.

Nouwen, E.J., Hendrix, P.G., Eekdekens, M.K., and de Broe, M.E., (1987) Tumor marker in the human ovary and its neoplasms: a comparative immunohistochemical study. Am J Pathol *126*:230-242.

O' Guin WM, Scherner A, and Sun T-T. (1985). Immunofluorescence staining of keratin filaments in cultured epithelial cells. J Tissue Cult Meth *9*:123-128.

Ono, M., Aratani, Y., Kitagawa, I., Kitagawa, Y. (1990) Ascorbic acid phosphate stimulates type IV collagen synthesis and accelerates adipose conversion of 3T3-L1 cells, Exp Cell Res 187:309-314.

Osterholzer, H.O., Johnson, J.H., Nicosia, S.V. (1985) An auto-radiographic study of rabbit ovarian surface epithelium before and after ovulation. Biol of Reprod 33:729-738.

Papadaki, L., and Beilby, J.O.W., (1971) The fine structure of the surface epithelium of the human ovary. J. Cell Sci. *8:*445-465.

Perez, R.P. Godwin, A.K. Hamilton, T.C., and Ozols, R.F., (1991) Ovarian cancer biology. Seminars in Oncology *18*:186-204.

Piver, M.S., Baker, T.R., Piedmont, M., and Sandeki, A.M., (1991) Epidemiology and etiology of ovarian cancer. Seminars in Oncology *18*:177-185.

Radisavljevic, S.V. (1977) The pathogenesis of ovarian inclusion cysts and cystomas. Obst Gynecol 49:4:424-429.

Reid, P.E. and Park, C.M., (1990). *Carbohydrate Histochemistry of Epithelial Glycoproteins,* Stuttgart, New York. Fischer.

Risteli, J., Niemi, S., Trivedi, P., Mäentausta, O. Mowat, A. P., Risteli, L. (1988) Rapid equilibrium radioimmunoassay for the amino-terminal propeptide of human type III procollagen. Clin. Chem. *34:*715-18.

Risteli, L., Risteli, J., Puistola, U., Tomas, C., Zhu, G.-G., Kauppila, A. (1992) Aminoterminal propeptide of type III procollagen in ovarian cancer. Acta Obstet Gynecol Scand 71 suppl:99-103.

Roe, C.J., Whitehead, R.H., Walker, R.G., Dowling, J.P., Birchal, I I., and Kincaid-Smith, P.S., (1991). Morphology of renal parenchymal cultures in collagen gels. Pathology *23*:25-29.

Rubelj, I. and Pereira-Smith O.M. (1994) SV40-transformed human cells in crisis exhibit changes that occur in normal cellular senescence. Exp. Cell Res 211:82-89.

Runyan, R.B., Potts J.D., and Weeks, D.L., 1992. TGF-β3 mediated tissue interaction during embryonic heart development. Molecular Reprod & Dev *32*:152-159.

Sambuy, Y., and De Angelis, I. (1986) Formation of organoid structures and extracellular matrix production in an intestinal epithelial cell line during long term in vitro culture. Cell Differentiation 19:139-147.

Savvas, M., Bishop, J., Laurent, G., Watson, N., and Studd, J. (1993) Type III collagen content in the skin of postmenopausal women receiving oestradiol and testosterone implants. Br J Obstet Gynecol 100:154-156.

Senoo, H., Tsukada, Y., Sato, T. and Hata, R. (1989) Co-culture of fibroblasts and hepatic parenchymal cells induces metabolic changes and formation of a three dimensional structure. Cell Biol Intl Rep 13:197-206.

Setrakin, S., Saunders, B.O., and Nicosia, S.V. (1989) Growth stimulation of ovarian and extraovarian mesothelia by corpus luteum extract. In Vitro 25: 42a

Siemens, C. H., and Auersperg, N. Serial propagation of human ovarian surface epithelium in tissue culture. J. Cell. Physiol. *134:*347-356, 1988.

Steinberg, M.S. (1975) Adhesion-guided multicellular assembly: a commentary upon postulates, real and imagined, of the differential adhesion hypothesis, with special attention to computer simulations of cell sorting. J Theor Biol 55:431-443.

Steinberg, M.S. and Garrod, D.R. (1975) Observations on the sorting-out of embryonic cells in monolayer culture. J Cell Sci 18:385-403.

Streuli, C.H. and Bissell, M.J. (1990) Expression of extracellular matrix components is regulated by substratum. J Cell Biol 110: 1405-1415.

Takeichi, M. (1977) Functional correlation between cell adhesive properties and some cell surface proteins. J Cell Biol 75:464-474.

Takeichi, M. (1988) The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. Development 102:639-55.

Tenti, P., Aguzzi, A., Riva, C., Usellini, L., Zappatore, M., Bara, J., Samloff, M., and Solcia, E. (1992) Ovarian mucinous tumors frequently express markers of gastric, intestinal, and pancreatobiliary epithelial cells. Cancer 69:2131-2141.

Trelstad, R.L., Hayashi, A., Hayashi, K., and Donahoe, P.K. (1982) The epithelial-mesenchymal interface of the male rat müllerian duct: loss of basement membrane integrity and ductal regression. Dev Biol *92*:27-40.

Vachon, P.H., Durand, J. and Beaulieu, J-F. (1993) Basement membrane formation and redistribution of the β1 integrins in a human intestinal co-culture system. Anatomical Rec 236:567-76.

Van Blerkom, J. and Motta, P. The ovary and ovulation. *In:* The cellular basis of mammalian reproduction, p53-65. Baltimore: Urban & Scharzenberg, 1979.

Van Niekerk, C. C., Ramaekers, F. C. S., Hanselaar, A. G. J. M., Aldeweireldt, J., and Poels, L. G. Changes in expression of differentiation markers between normal ovarian cells and derived tumors. Am. J. Pathol. *142:* 157-177, 1993.

Volz, D., Reid, P.E., Park, C.M., Owen, D.A., and Dunn, W.L. (1987) A new histochemical method for the selective periodate oxidation of total tissue sialic acids., Histochem J 19:311-318.

von der Mark, K., von der Mark, H., and Goodman, S. (1992) Cellular responses to extracellular matrix. Kidney Intl 41:632-640.

Wight, T.N., Kinsella, M.G., and Qwarnstrom, E.E. (1992) The role of proteoglycans in cell adhesion, migration and proliferation. Curr Op Cell Biol 4:793-801.

Yang, J. Flynn, D., Larson, L. et al., (1982) Growth in primary culture of mouse submandibular epithelial cells embedded in collagen gels. In Vitro 18:435-442.

Young, R. H., Clement, P. B., and Scully, R. E. (1989) The ovary. *In:* S. S. Sternberg (ed.), Diagnostic Surgical Pathology, pp. 1655- 1734. New York: Raven Press.

Yue, B.Y.J.T., Higginbotham E.J., and Chang, I.L. (1990) Ascorbic acid modulates the production of fibronectin and laminin by cells from an eye tissue-trabecular meshwork. Exp Cell Res 187:65-68.

Zhu, G.-G., Risteli, J., Puistola, U., Kauppila, A., and Risteli, L. (1993) Progressive ovarian carcinoma induces synthesis of type I and type III procollagens in the tumor tissue and peritoneal cavity. Cancer Res 53:5028-5032.

Ziltner, H.T., Maines-Bandiera, S., Schrader, J.W., and Auersperg, N. (1993) Secretion of bioactive interleukin-1, interleukin-6 and colony-stimulating factors by human ovarian surface epithelium, Biol of Reprod 49:635-41.

Zuk, A., Matlin, K.S., and Hay, E.D., (1989) Type-1 collagen gel induces Madin-Darby Canine Kidney cells to become fusiform in shape and lose apical-basal polarity. J Cell Biol 108:903-919.