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Development of a syngeneic mouse model for events related to ovarian cancer

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Mouse ovarian surface epithelial cells (MOSEC) were obtained from virgin, mature mice by mild trypsinization and were repeatedly passaged *in vitro*. Early passage cells (<20 passages) exhibited a cobblestone morphology and contact inhibition of growth. After ~20 passages *in vitro*, cobblestone morphology and contact inhibition of growth was lost. Tumor forming potential was determined by s.c. and i.p. injection of early and late passage cells into athymic and syngeneic C57BL6 mice. Subcutaneous tumors formed in ~4 months and were present only at the injection site. Intraperitoneal injection of late passage MOSEC into athymic and syngeneic mice resulted in growth of tumor implants throughout the abdominal cavity, and production of hemorrhagic ascitic fluid. Early passage MOSEC did not form tumors *in vivo*. Histopathologic analysis of tumors revealed a highly malignant neoplasm containing both carcinomatous and sarcomatous components. Late passage MOSEC expressed cytokeratin and did not produce ovarian steroids in response to gonadotropin stimulation *in vitro*. Ten clonal lines were established from late passage MOSEC. Each clone formed multiple peritoneal tumors and ascitic fluid after i.p. injection into C57BL6 mice. Three cell lines examined cytogenetically were polyploid with near-tetraploid modal chromosome numbers. Common clonal chromosome gains and losses included +5, +15, +19 and –X, –3, –4. One cell line had a clonal translocation between chromosomes 15 and 18 and another had a small marker chromosome; common structural abnormalities were not observed. These data describe the development of a mouse model for the study of events related to ovarian cancer in humans. The ability of the MOSEC to form extensive tumors within the peritoneal cavity, similar to those seen in women with Stage III and IV cancer, and the ability of the MOSEC to produce tumors in mice with intact immune systems, makes this model unique for investigations of molecular and immune interactions in ovarian cancer development.

Introduction

Ovarian cancer is the most frequent cause of death from gynecologic malignancy. More than 26 000 new cases of ovarian cancer were diagnosed in 1996 (1). Most women are diagnosed with late stage disease that requires aggressive surgical and chemotherapeutic strategies. Although significant advancement has occurred in both surgical and chemotherapeutic techniques, the 5 year survival rate for ovarian cancer has remained unchanged at <30% (2). Because early stages of ovarian cancer go unnoticed, little is known of the establishment and progression of the early stages of the disease. Laboratory models addressing this aspect of ovarian cancer are few. Success in developing new treatment modalities has occurred, at least in part, through use of tumor transplants into athymic mice. Transplantation of human tumors, xenographs and human tumor cell lines both s.c. and i.p. has provided useful insights into clinical treatment (3–8). Our interest in understanding early events in the establishment and progression of ovarian cancer led us to the development of a mouse model for the study of ovarian cancer. This model is based on information provided by other models utilizing human (9) and rat (10,11) ovarian surface epithelial cells and tumor cell lines (3–5). A recent set of studies demonstrated the ability of rat ovarian surface epithelial cells to undergo transformation after multiple passage *in vitro* (10,11). Those transformed cells formed tumors in athymic mice. The hypothesis that multiple passage of ovarian surface epithelial cells *in vitro* might induce transformation supports the theory of incessant ovulation and the development of ovarian cancer (1,10–12). If increased follicular rupture followed by epithelial proliferation to repair the rupture site was a risk factor for cancer then inducing 'repair', or increased cellular proliferation *in vitro* might also lead to transformation of the epithelial cells (10,11). It was our mission to establish a model of ovarian cancer in mice with intact immune systems. The ability of ovarian surface epithelial cells to form tumors in normal immune-intact animals would provide a model in which immune interactions in the establishment, progression and treatment of ovarian cancer could be investigated. In addition, establishment of a mouse model would ultimately allow for the application of gene manipulation strategies.

Abbreviations: HBSS, Hank's balanced salt solution; MOSEC, mouse ovarian surface epithelial cells.

Materials and methods

Animals

All mice were housed on a 12–12 h light–dark schedule and provided food and water *ad libitum*. C57BL6 and athymic nu/nu virgin female mice 8 weeks of age were obtained from Harlan (Indianapolis, IN). All animal procedures were approved by the Institutional Animal Care and Use Committee.

Isolation and culture of ovarian epithelial cells

Ovaries were removed from five adult female C57BL6 mice using aseptic techniques under a laminar flow hood and placed in a culture dish containing Hank's balanced salt solution (HBSS) at 4°C. Ovaries were rinsed with HBSS and placed in a 15 ml conical culture tube containing 10 ml HBSS with 0.2% trypsin. The ovaries were incubated in the trypsin solution at 37°C in a humidified atmosphere of 5% CO_2 and air for 30 min. Care was taken not to agitate the ovaries and the tube containing the ovaries was placed in a horizontal position with the ovaries distributed over the length of the tube. After 30 min the media containing epithelial cells was transferred to a fresh tube and 5 ml complete medium (DMEM supplemented with 4% FBS, 100

U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml sodium selenite) was added. The epithelial cells (approximately 100 000 per 10 ovaries) were pelleted by centrifugation (1000 *g* for 10 min at 22°C), resuspended in 2 ml complete medium and placed in a single well of a 6-well culture dish. All cells were subcultured with trypsin (0.2%) at near confluence. Early passage cells (pass 1 through 20) were subcultured at 2–3 week intervals and split at ratios of 1:3. Late passage cells (greater than pass 20) were subcultured at 1–2 week intervals and split at ratios of 1:10. Murine epidermal growth factor (EGF; Sigma, 2 ng/ml) was in the medium during the early passage growth. Late passage cells, >20 passages were cloned by limiting dilution. Ten cell lines were established. This procedure was carried out more than four times with results similar to those described in this study indicating reproducibility of this method.

Western analysis

The expression of cytokeratin was assessed by western analysis. Early and late passage mouse ovarian surface epithelial cells (MOSEC) grown to near confluence in a T75 flask were washed twice with cold phosphate-buffered saline. RIPA buffer (1 ml; 50 mM Tris–HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin) was added and the cells were scraped and collected. The proteins were loaded (50 µg), separated on 10% polyacrylamide gels and transferred to PVDF membranes using standard procedures. Western analysis was carried out as reported previously (13) with a monoclonal pan cytokeratin antibody (1:1000; Sigma C1801).

Immunofluorescence

Intact ovaries and ovaries subjected to trypsinization for epithelial cell removal were fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin and sectioned at 10 µm. Following deparaffinization, tissue sections were boiled in citric acid solution (0.01 M citric acid solution containing 0.01 M sodium citrate pH 6.0) for 10 min to unmask antigenic sites. MOSEC were seeded into 8-well chamber slides (10 000 cells/well/200 µl media) and incubated for 24 h. Media were removed and the cells were fixed in ice-cold acetone for 5 min. Immunohistochemistry on tissue sections and cultured cells was carried out as reported previously (14) using pan anti-cytokeratin (1:100; Sigma) and fluorescein-conjugated goat anti-mouse IgG (1:150; Jackson Immunoresearch Laboratory, West Grove, PA). Controls included replacement of the primary antibody with normal IgG at the same concentration and resulted in no immunostaining.

In vivo tumorigenicity

Female athymic and C57BL6 mice were used for assessment of tumor formation. Early and late passage MOSEC and clonal lines prepared from late passage MOSEC were injected either i.p. and/or s.c. (5×10^6 cells in 0.2 ml DMEM containing no additives). Animals were evaluated weekly for tumor growth and/or ascites accumulation. Intraperitoneal tumor formation was monitored by the accumulation of ascites fluid. Approximately 10–24 days after the development of visible ascites (detected by abdominal swelling) the animals were killed. At the time of death, ascites fluid was collected from the peritoneal cavity using a 10 cc syringe fitted with a 22 g needle. The peritoneal and thoracic cavities were opened with a single midline incision. Tumor location and sizes were noted and amount of tumor on 11 tissues and organs was scored on a 0, +, ++, +++ scale: 0, no tumor evident by gross examination; +, tumors grossly evident on one organ or tissue; ++, moderate tumor formation on more than one organ or tissue; +++, extensive tumor formation on several organs or tissues. Tumor implants and surrounding tissues were collected from multiple sites, fixed in 4% paraformaldehyde and embedded in paraffin for routine histological analysis, or fixed in glutaraldehyde and processed for electron microscopy. For routine histology, tissue sections were stained with hematoxylin and eosin. Control mice were injected with DMEM either s.c. or i.p.

Chromosome analysis

Chromosome analysis was performed using standard culturing, harvesting and G-banding staining methods (15). Briefly, cultures were harvested for cytogenetic analysis by colcemid exposure followed by hypotonic treatment and fixation in methonal:acetic acid (3:1). Modal chromosome numbers were determined by examining 20–30 G-banded metaphases. Complete analysis and karyotyping was performed on five or more metaphases in each of three cell lines.

Steroid analysis

Late passage MOSEC were seeded in 96-well plates (20 000 cells/well/0.2 ml media) with LH (ovine LH-S20, 50 ng/ml), FSH (ovine FSH-S16, 50 ng/ml), LH + FSH, 10^{-6} M androstenedione or FSH + androstenedione. Media were collected after 48 h, then fresh media and treatments were added for an additional 48 h. Accumulation of progesterone, androstenedione and estradiol in culture medium was measured by radioimmunoassay as described previously (16). Steroids are generally produced by ovarian theca and granulosa cells *in vitro* and are routinely measured using these techniques (16).

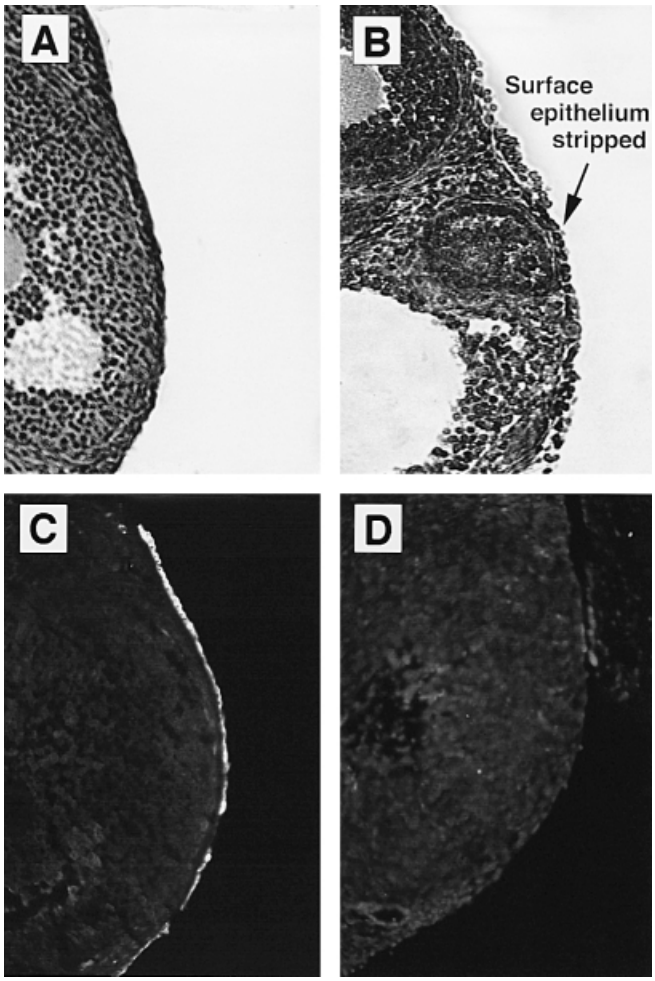

Fig. 1. Histological appearance of ovaries prior to and after trypsinization to remove surface epithelial cells. (A and C), intact ovaries; (B and D), ovaries following trypsinization. (**A** and **B**) are hematoxylin and eosin stained ovarian sections demonstrating the loss of the surface epithelial cells without perturbation of the remaining ovarian structures. (**C** and **D**) are immunofluorescence with anti-cytokeratin antibody demonstrating the loss of the cytokeratin-positive epithelial cells after trypsin treatment.

Results

Isolation of epithelial cells

Ovarian surface epithelial cells were obtained by gentle trypsinization of mouse ovaries. Mild trypsinization resulted in removal of the surface epithelium without disturbing the underlying stromal tissue (Figure 1A and B). Figure 1A and B reveals that the single layer of epithelial cells has been removed by trypsin leaving the underlying basement membrane intact. Immunofluorescence using a cytokeratin antibody demonstrated the presence of a single layer of fluorescent cells on the surface of the intact ovary whereas the ovaries treated with trypsin did not exhibit this fluorescence (Figure 1C and D).

Generation of the transformed phenotype in vitro

Following isolation, the MOSEC were maintained in culture by repeated passaging at a ratio of 1:3 using 0.2% trypsin. Freshly isolated cells had a very slow rate of growth and therefore EGF (2 ng/ml) was added to the cultures to stimulate

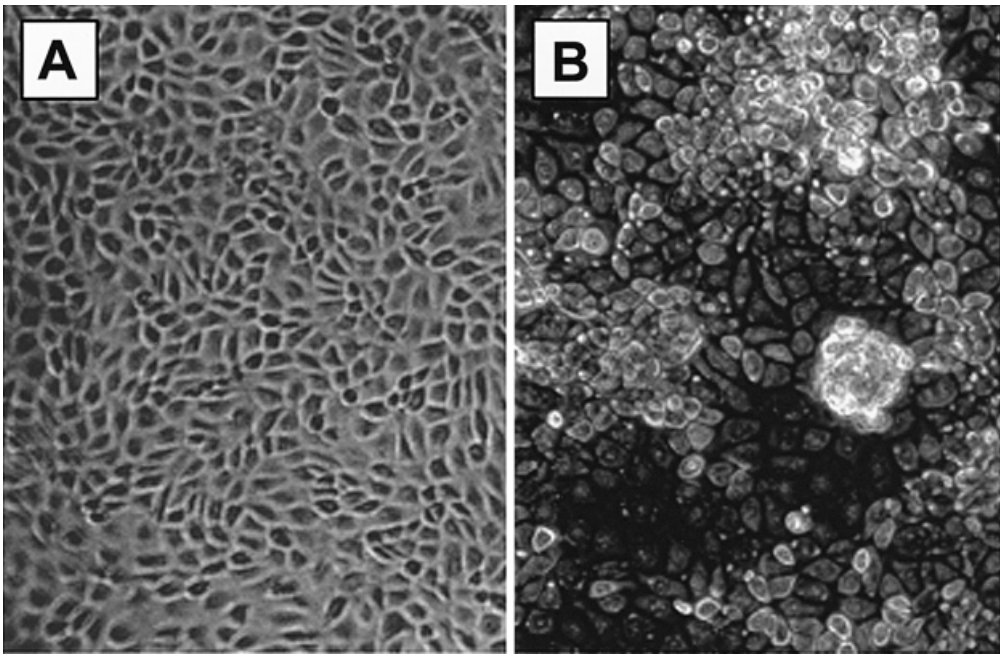

Fig. 2. Morphology of MOSEC *in vitro*. (**A**) Early passage cells exhibited typical cobblestone morphology and contact inhibition of growth. (**B**) Late passage cells lost the cobblestone morphology and no longer exhibited contact inhibition of growth.

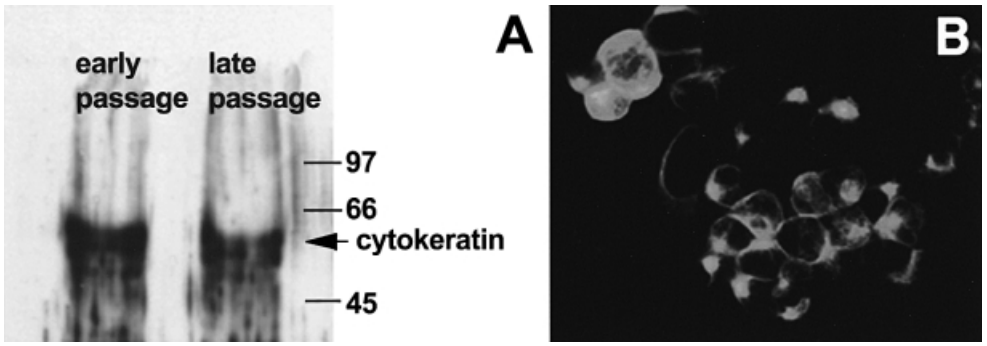

Fig. 3. Cytokeratin expression by MOSEC. (**A**) Western analysis of protein from early and late passage MOSEC using anti-cytokeratin antibodies. Cytokeratin of ~58 kDa was present in both early and late passage cells. (**B**) Immunofluorescent localization of cytokeratin in late passage MOSEC.

proliferation. Early passage cells split at a ratio of 1:3 required 2–3 weeks to reach confluency. These early passage cells exhibited 'cobblestone' morphology, typical of epithelial cells (Figure 2A) and contact inhibition of growth. After greater than 20 passages *in vitro* cobblestone morphology was no longer apparent and contact inhibition of growth was lost as evidenced by the growth of multiple layers of cells (Figure 2B).

General characterization of MOSEC in vitro

Expression of the epithelial cytoskeletal protein cytokeratin was examined. As shown in Figure 1C, the surface epithelial cells of the intact ovary expressed cytokeratin. Western analysis of both early and late passage cells and immunohistochemical analysis of late passage cells indicated that MOSEC express cytokeratin of ~58 kDa corresponding to cytokeratin types 5 and 6 (Figure 3).

The potential for steroid production by the late passage cells was assessed. Neither LH, FSH, androstenedione nor FSH in combination with androstenedione induced accumulation of steroids during a 96 h culture period (data not shown).

In vivo tumorigenicity: late passage MOSEC

The initial tumor forming capacity of late passage MOSEC was assessed by injection of the cells into immunocompromised, athymic mice. Cells were injected either s.c. or i.p. The s.c. growth of solid tumors was evident 4 months following injection of 5 million cells. Tumor formation remained confined to the area of s.c. injection; gross inspection revealed no tumors in the peritoneal or thoracic cavities after s.c. injection of late passage MOSEC. Injection of late passage cells into the peritoneal cavity of athymic mice resulted in the formation of ascitic fluid and multiple tumor implants. The formation of ascitic fluid was rapid in mice injected with late passage MOSEC; ~10 ml of hemorrhagic fluid was collected at the time of death, an average of 33 days after injection. At this time extensive tumor growth was observed throughout the peritoneal cavity. Multiple small tumors between 0.1 and 1 cm in diameter were present on the omentum, bowel, diaphragm, peritoneal wall and on the surface of all abdominal organs including the kidneys, pancreas, stomach and spleen (Figure 4). Invasion of the tumor cells into organs was observed only in the pancreas where tumorigenic cells were observed within pancreatic tissue. Tumors from injection of late passage MOSEC were not seen outside the peritoneal cavity. Histological inspection of the lungs, heart and brain revealed no metastases.

The ability of tumors to form in syngeneic, immunocompe-

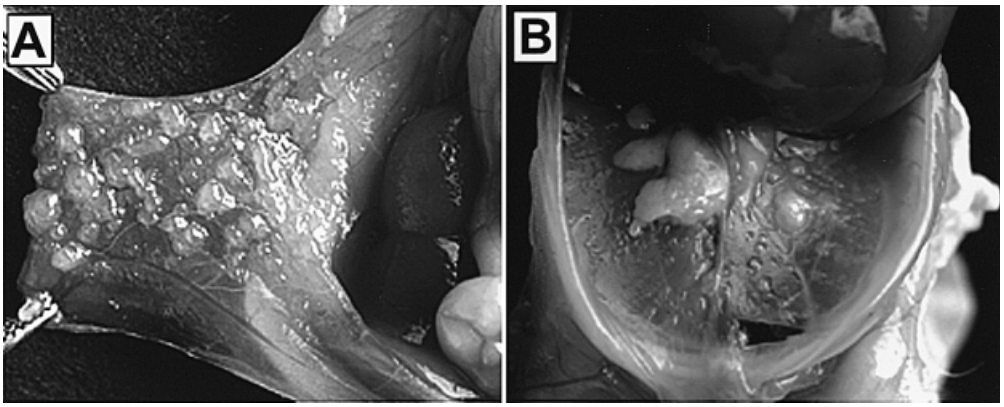

Fig. 4. Tumor formation following injection of late passage MOSEC into athymic mice. Multiple tumors were present throughout the peritoneal cavity, covering the body wall (**A**), diaphragm (**B**), and all peritoneal organs. This sample was collected 30 days after the i.p. injection of 5×10^6 late passage MOSEC. Similar tumor formation was observed in C57BL6 mice.

tent mice was determined by s.c. and i.p. injection of late passage MOSEC into C57BL6 mice. Similar to the observations in athymic mice, tumor formation following s.c. injection occurred in ~4 months. Again tumors were localized to the site of cell injection. Intraperitoneal injection of MOSEC into syngeneic mice resulted in the formation of ascites and multiple tumors, similar to those seen in athymic mice. Mice were killed within 10–14 days of the onset of accumulation of ascitic fluid, which occurred between 22 and 48 days after injection of the cells. Again ~10 ml of ascites fluid was collected at this time. The distribution and size of tumors was similar to that seen in the athymic mice.

Histological appearance of tumors

Histologically the tumors were composed of sheets of highly anaplastic malignant appearing cells with a biphasic growth pattern: a carcinomatous component with attempts of glandular formation (Figure 5A and B) intermixed with a sarcomatous component composed of fascicles of spindle shaped anaplastic cells (Figure 5C). The cells had large hyperchromatic vesicular nuclei with prominent nucleoli. Occasional tumor giant cells were also noted (Figure 5D). Many mitotic figures were present throughout the tumor. At the ultrastructural level, the epithelial nature of the cells was confirmed by the presence of multiple poorly formed desmosomes along the cell surface and by the presence of intracytoplasmic lumina and microvilli. In addition, the cells contained abundant rough endoplasmic reticulum and polyribosomes (Figure 5E and F). The histological and ultrastructural features of tumors formed following s.c. injection of cells were similar to those described for i.p. tumors.

In vivo tumorigenicity: early passage MOSEC

Early passage cells (5 million) were injected i.p. into C57BL6 mice. Six months after injection the animals appeared healthy. Ascites fluid was not present and upon inspection no tumors were found in the abdominal cavity, on the abdominal mesenteries or adherent to any organs. Thus, early passage cells did not form tumors.

Cloning MOSEC and in vivo tumorigenicity

Late passage MOSEC were cloned by limiting dilution and 10 clonal cell lines were established based on morphology of the cells. The *in vivo* tumorigenicity of each clonal line was determined by i.p. injection of 5×10^6 cells into C57BL6 mice. As described in Table I, all clonal lines resulted in the formation of peritoneal tumors. Time to death, based on ascites formation, and overall tumor load varied with the clonal lines (Table I). However, all clones developed i.p. tumors and ascites. Regression analysis indicated that tumor load did not have a direct correlation with the ascites formation and time to death. Occasional metastases through the diaphragm and in the lung were noted although the frequency was low (four of 55 mice) and was not limited to a single clone.

Karyotype of MOSEC clonal lines

Chromosome analysis was performed on three clonal cell lines. The number of chromosomes per cell ranged from 32 to 145 with modal numbers varying between 70 to 75. The majority of cells in each cell line had a near-tetraploid chromosome number. A few cells were near-diploid or near-octaploid. One clonal structural abnormality consisting of a translocation between chromosomes 15 and 18 was present in one cell line; a clonal small marker chromosome was identified in another cell line; and the third cell line had no clonal structural abnormalities. Each cell line had multiple numeric abnormalities. Consistent numeric anomalies observed in all three cell lines included loss of chromosomes X, 3 and 4, and gain of chromosomes 5, 15 and 19. Chromosome 19 was present in the highest number in all three cell lines (Figure 6).

Discussion

These studies describe a mouse model for potential use in the study of events related to ovarian cancer. Epithelial cells isolated from the surface of mouse ovaries grew in culture. Initial growth was very slow but was accelerated by the addition of EGF to the culture media. Subsequent studies of ovarian epithelial cells isolated in other experiments revealed variation in the rate of early passage cell growth. In some experiments EGF was omitted. In the absence of EGF morphological transformation *in vitro* was also observed. Our initial hypothesis was that multiple passage *in vitro* would mimic the proliferation of epithelial cells normally occurring after ovulation. It has been hypothesized that the amount of proliferation by these cells is related to transformation and that incessant ovulation is a risk factor in ovarian cancer due to the increased proliferation of the surface epithelium (12). The cellular events that support this hypothesis are not understood; however, several investigators have explored the growth of ovarian surface epithelial cells *in vitro* and *in vivo* (17–20). A correlation between cellular proliferation and transformation was shown for epithelial cells isolated from rat ovaries (10). After multiple passage of rat cells *in vitro*, a transformed phenotype

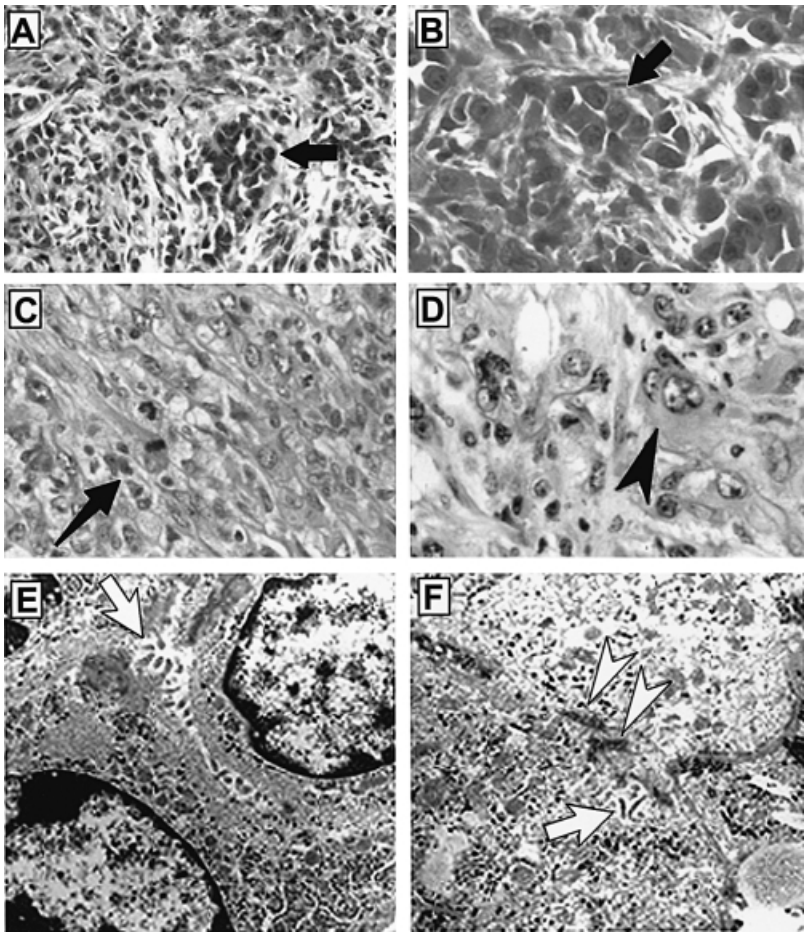

Fig. 5. Histological and ultrastructural appearance of tumors. MOSEC were injected into C57BL6 mice (5×10^6 cells i.p.) and tumors were collected 30 days later. The tumors shown are representative from the body wall, diaphragm and omentum. (A–D) Hematoxylin and eosin stained; (E and F) electron micrographs. Tumors contained areas of carcinomatous glandular formation (arrows, **A** and **B**), sarcomatous spindle shaped anaplastic cells (arrow, **C**) and occasional giant cells (arrowhead, **D**). Electron microscopy revealed the presence of microvilli (arrows, **E** and **F**) and desmosomes (arrowheads, F).

Table I. Ten cell lines were cloned from late passage MOSEC

Clonal lines	Tumor loads	Days to death
IC5	1.5 ± 0.2	105 ± 6
ID3	1.2 ± 0.2	95 ± 10
ID8	2.1 ± 0.3	114 ± 3
ID9	0.8 ± 0.3	99 ± 5
IF5	1.4 ± 0.2	182 ± 3
IG10	0.7 ± 0.1	72 ± 2
2C6	1.7 ± 0.2	133 ± 7
2C12	0.7 ± 0.2	101 ± 8
3E3	1.0 ± 0.2	125 ± 6
3B11	1.1 ± 0.2	99 ± 7

Each cell line was injected i.p. into C57BL6 mice (5×10^6 cells, $n = 6$). Animals were killed (days to death) and tumor load was measured and scored on a 0, +, ++, +++ scale for 11 different abdominal organs and tissues as discribed in Materials and methods. Tumor load represents the score means ± SE of all animals injected with a given clone.

was apparent (loss of contact inhibition of growth and cluster formation in agar) and tumors formed after injection into athymic mice (10). An additional recent study demonstrated tumor formation in nude mice following transformation of mouse ovarian surface epithelial cells with SV40 large T-antigen (21). Our results for mouse ovarian surface epithelial cells extended the findings in rodents to include a syngeneic model. Early passage mouse ovarian surface epithelial cells exhibited classical morphology of epithelial cells growing in a cobblestone array. As the cells reached confluence, cellular proliferation stopped. However, with multiple passages and continued cell division, the morphology of the cells changed, contact inhibition of growth was lost and the cells piled as proliferation continued. These morphological and growth characteristics *in vitro* are well known to represent those of transformed cells.

Events leading to transformation of MOSEC probably occurred during culture of the epithelial cells *in vitro*. The spontaneous transformation of the cells may provide a synchronous *in vitro* model to study the expression of specific genes during transformation. The questions as to what point in the culture do the cells become malignant and what genes are altered leading to that transformation are of extreme interest.

In addition, the ability to maintain MOSEC and the clonal cell lines in culture will provide opportunity for genetic manipulation. Recent studies using human ovarian cancer cell lines have assessed gene therapy strategies for ovarian cancer by addressing modes of gene transfer, introduction of specific genes to sensitize cells to chemotherapeutics, and methods for enhanced cellular transduction by gene targeting (6–8). These studies all utilize human cell lines and assess various endpoints, both *in vitro* and *in vivo* in athymic mice (6–8). Use of the present murine model in a similar manner will provide additional insights as to the potential for these types of gene

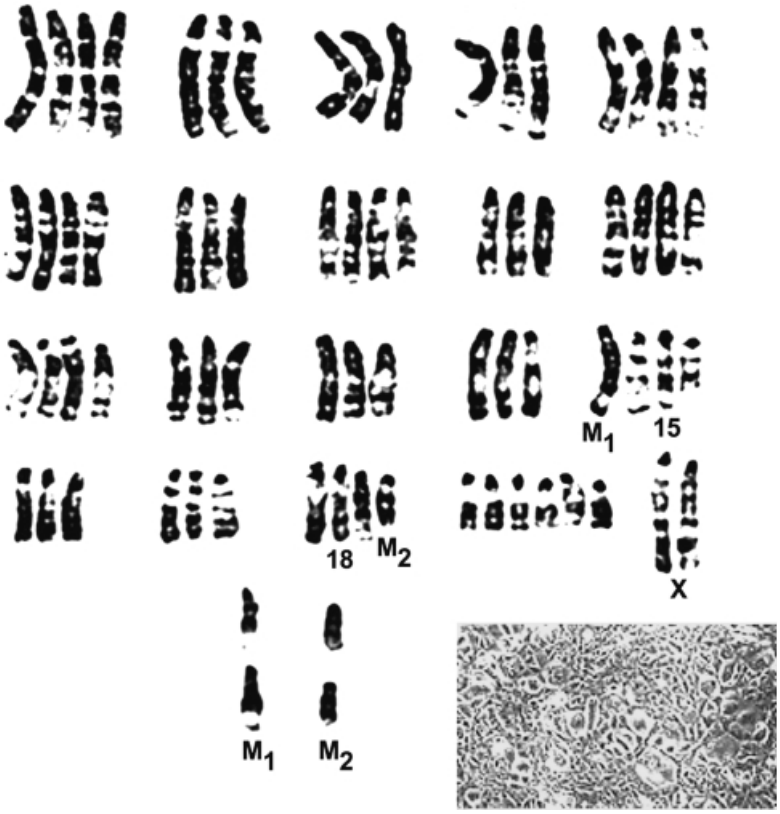

Fig. 6. Representative karyotype of late passage MOSEC clone ID8. Near-tetraploid cell (70 chromosomes) has a loss of multiple chromosomes and a gain of chromosome 19 relative to the tetraploidy (four copies of each chromosome). In addition, a translocation between chromosome 15 (M1) and chromosome 18 (M2) is present. Marker chromosomes (M1 and M2) involved in the translocation from two additional cells are arranged at the bottom rows. Inset: ID8 cells grown *in vitro*.

therapies. Use of these techniques together with other gene manipulation techniques now commonly used in the mouse will greatly extend the use of this model.

Through the development of clonal cell lines several insights were gained. The development of the cancer occurred over a longer period of time in the clonal cell lines compared with the parent, late passage cells; although the endpoints were similar. The slower development of disease using the cell lines may be more useful in studies designed to detect and manipulate molecular processes fueling the disease. In contrast, a more rapid progression of the disease is desirable for the examination of treatments and in this case use of the late passage cells (non clonal) would be applicable.

Chromosomal analysis of three cloned cell lines demonstrated polyploidy, aneusomy (gain or loss) of multiple chromosomes and rare structural abnormalities. The presence of chromosomal aberration in cancers has been known for some time. A progressive increase in ploidy has been noted in human ovarian cancer comparing the primary site, metastatic site and cells from ascites fluids (22). Structural and numerical chromosomal abnormalities in ovarian cancers have been described (23–26). The results of the chromosome analysis in three cell lines indicated that gain and loss of whole chromosomes and polyploidization are common occurrences in this model. These changes are often observed in human carcinomas, including epithelial ovarian carcinoma. A notable difference in the chromosome make-up of this mouse model and that of primary human ovarian carcinomas is the paucity of structural abnormalities in the mouse cell lines compared with the presence of multiple structural abnormalities in most human ovarian cancers (25). This difference, however, is most likely due to analysis of primary, high grade, advanced stage human ovarian tumors in contrast to the early stages of transformation within the mouse model. The karyotypic stability of the cloned cell lines has not been assessed; thus, with continued passage *in vitro* or with tumor growth *in vivo* additional chromosomal abnormalities may occur. Further analysis of the expression of specific genes in these cell lines is currently under investigation.

Probably the most important new aspect of this model is the ability of these cells to grow in mice with intact immune systems. Currently, all models of ovarian cancer use mice with non-functioning immune systems. Although these models provide important information, the interaction of the immune system in the development, progression and the treatment of ovarian cancer may prove to be critical in understanding the disease. For instance, the incidence of ovarian cancer is greatest in older women (27). With advancing age the immune system undergoes characteristic changes, usually resulting in decreased immune competence, termed immunosenescence (28,29). This mouse model of ovarian cancer can be used to investigate the role of the immune system in the establishment and progression of the disease.

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