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# GLAND FORMATION FROM HUMAN ENDOMETRIAL EPITHELIAL CELLS IN VITRO

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## SUMMARY

We have developed methods for the culture of human endometrial glandular epithelia in vitro. The culture medium is serum-free and is used in combination with Matrigel, an extracellular matrix material applied as a coating on cell culture plates. Cell growth begins as a monolayer, but the cells subsequently form glandular or organoid structures. The glands are composed of polar columnar cells facing a central lumen, which is enclosed by the apical surfaces of cells displaying numerous microvilli and sealed by tight junction complexes. The ability to study in vitro the complex process of glandular morphogenesis represents an important new tool in cell biology which may be used to investigate growth regulation, hormone production and dependency, and cellular recognition and interactions. Ultimately, these characteristics may be applied to study the alterations of glandular epithelia associated with neoplasia.

*Key words:* endometrium; epithelial cells; organogenesis.

## INTRODUCTION

The lack of suitable cell culture systems has inhibited research on epithelial cells ranging from the study of differentiation to carcinogenesis. To overcome this problem, we have developed a system for the cultivation in vitro of glandular epithelium from human endometrium.

The human endometrium is comprised of glands lined by columnar epithelia which are surrounded by nonepithelial, hormone-responsive stromal cells. Monolayer cultures of both cell types have been established in tissue culture (1,2,5-7,10,15,18), and the cells have been characterized with respect to histochemistry (16) and structural elements (12). Stromal cells typically propagate well in culture (15-18). However, most previous cultures of glandular epithelial cells have been short-lived, refractory to subculture, and the cells were flat and squamoid rather than columnar as found in vivo (15-18). The endometrial glandular epithelium has also been cultured as free-floating vesicles which reorient to form organoid structures in collagen gels (6). Our method allows long-term culture and passage of the endometrial epithelial cells. Furthermore, the epithelial cells are columnar, and the cellular interactions lead to the formation of glandular structures which closely approximate those formed in vivo.

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## MATERIALS AND METHODS

RPMI 1640 (#430-1800) L-glutamine (#810-1051), and nonessential amino acids (#320-1140) were from GIBCO Laboratories, Grand Island, NY; 17-beta-estradiol (E-8875), hydrocortisone (H-4001), prostaglandin $F_{2\alpha}$ (P-7652), putrescine (P-7505), glutathione (reduced) (G-4251), and dl-alpha-tocopherol (T-3251) were from Sigma Chemical Co., St. Louis, MO; ITS+ [insulin, transferrin, selenium, bovine serum albumin (BSA), linoleic acid] (40352), Matrigel (40234) and dispase (40235) were from Collaborative Research Inc., Bedford MA; and the 35 × 10-mm tissue culture dishes (3001) were from Falcon, Oxnard, CA. RL95-2 cells are from the American Type Culture Collection, Rockville, MD.

Growth medium was composed of RPMI 1640 supplemented with 10% conditioned medium from RL95-2 human endometrial carcinoma cells (19), 4 m*M* glutamine, 100 n*M* hydrocortisone, 10 n*M* estrogen, 28 n*M* prostaglandin $F_{2\alpha}$, 100 $\mu M$ putrescine, 40 $\mu M$ glutathione, 23 n*M* tocopherol, and ITS+. Incubation was at 37° C in a NAPCO model 3100 three-gas incubator in an atmosphere of 10% $CO_2$:13% $O_2$:77% $N_2$.

Conditioned medium was prepared from RL95-2 cells by passaging a confluent culture at a 1:8 split ratio in RPMI 1640 medium containing 1% fetal bovine serum. After 24 h the medium was changed to RPMI 1640 supplemented with ITS+. This latter serum-free, conditioned medium was collected after 72 h, filter

sterilized with a 0.22-μm filter to exclude microorganisms and RL95-2 cells, and stored at −20° C until use.

Hydrocortisone, prostaglandin $E_{2\alpha}$, and estrogen were added to the media to create a better approximation of in vivo proliferative conditions. Glutathione and tocopherol were added to lower the oxidative potential of medium. Putrescine, insulin, transferrin, selenium, and BSA were standard additives to serum-free media. Conditioned medium from RL-95 cells was added to enhance growth of the glands by virtue of growth factors produced by transformed cells. The only absolute requirement noted for growth was insulin.

Endometrial tissue was obtained with consent from patients undergoing hysterectomies at North Carolina Memorial Hospital. Specimens obtained in the proliferative phase of the cell cycle from young donors (less than 50 yr old) proliferated best in culture. The data presented have been reproduced with more than 10 specimens. After pathological examination, specimens of normal endometrial tissue were transported to the tissue culture laboratory, where, before culture, the glands were separated from the surrounding stromal cells by collagenase treatment followed by differential centrifugation in a modification of published methods (13,16). The tissue was minced and then treated with 2 ml of a solution of 2% collagenase for 3 to 4 h. The tissue was further dissociated by repetitive pipetting and diluted with 10 ml of growth medium. This mixture was centrifuged at 400 rpm for 2 min. The supernatant contained the stromal cells, and the pellet contained the gland fragments. The

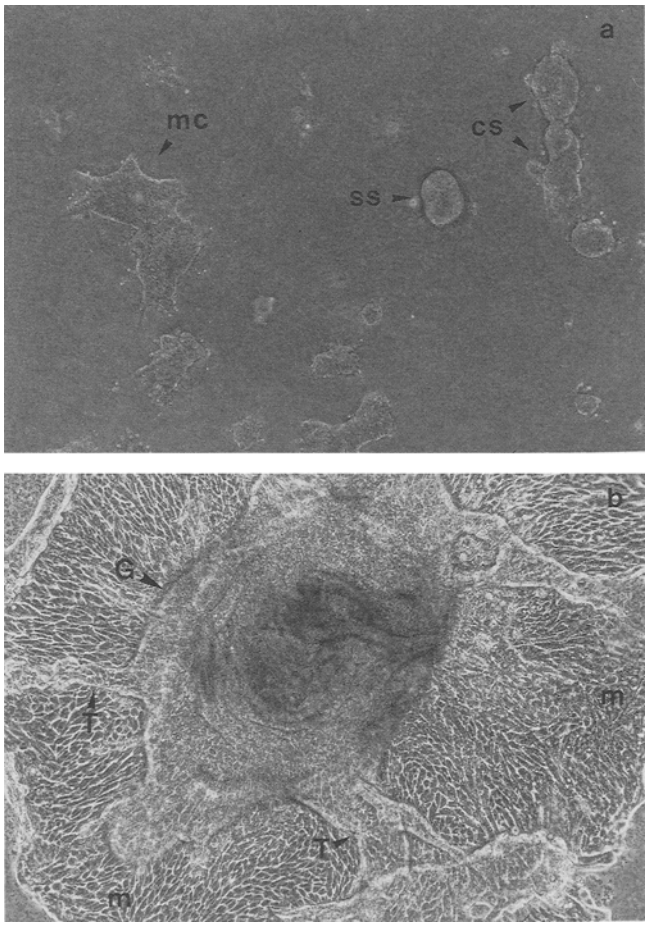


FIG. 1. Colony outgrowth and organogenesis in vitro occur from primary cultures of human endometrial gland fragments. Cultures are depicted 1 wk after establishment as gland fragments (*a*). ×100. Initially the gland fragments retain a spherical or cylindrical structure (*ss* and *cs*, respectively). These structures soon collapse as growth begins as a monolayer (*mc*). A large organoid which formed after 6 wk in culture is shown in (*b*). ×100. The large glandular structure (*G*) is located centrally, over the monolayer (*m*), and seems to be connected to other parts of the colony by smaller tubules (*T*).

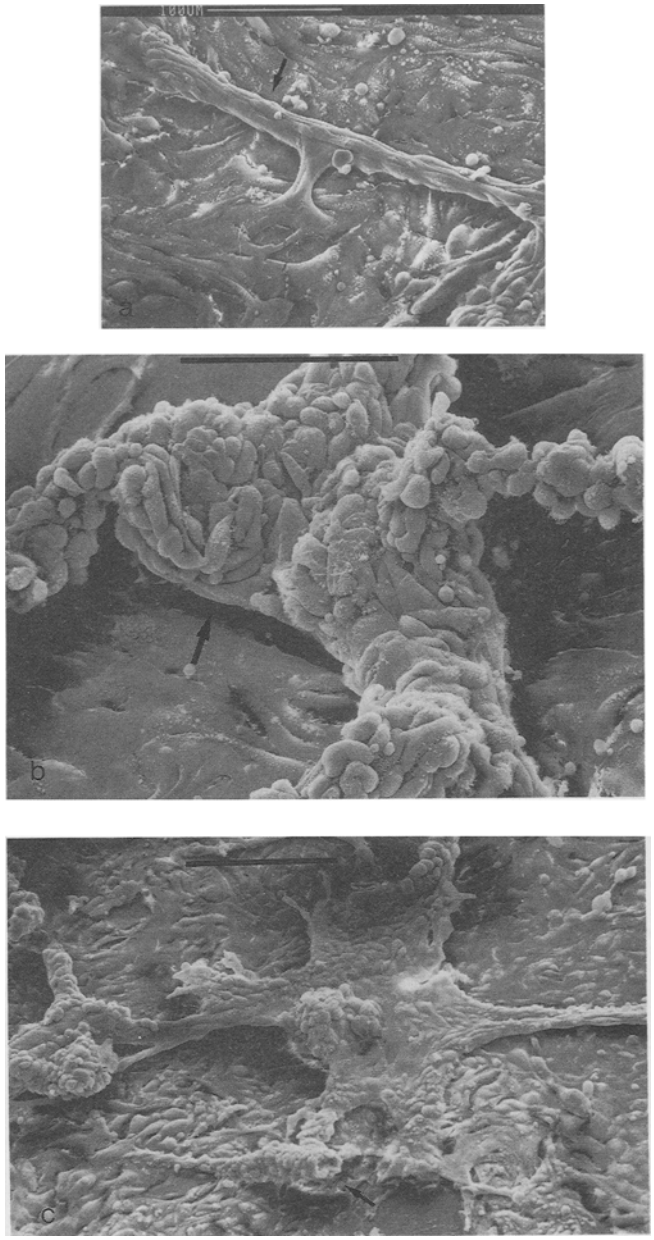


FIG. 2. Scanning electron microscopy illustrates the stages in organogenesis in vitro (*a–c*). *Bar* represents 100 μm in (*a*) and 200 μm in (*b*) and (*c*). Small tubular structures shown by the *arrow* in (*a*) typically form at 2 to 3 wk. Mid-sized organoids indicated by the *arrow* in (*b*) typically form at 4 to 5 wk, and the large organoids in the center of (*c*) typically form after 6 to 8 wk in culture. *Arrow* in the foreground indicates a structure that has broken, revealing what seems to be a central lumen.

centrifugation cycle was repeated. Gland fragments were then placed in culture on Matrigel, an extracellular matrix product (8). The tissue culture dishes were precoated with a thin layer of Matrigel by spreading 250 μl on a 35 × 10-mm dish at 4° C. The gel was then hardened by incubation at 37° C for 30 min. Gland fragments were suspended in a 1:1 mixture of growth medium and Matrigel at 4° C and gently spread over the hardened gel at 250 μl/dish. The gel was hardened at 37° C for 2 h, and 2 ml of medium/dish was added. The medium was changed twice weekly.

Cultures were passaged by removing the medium, and the gel was digested with dispase, 0.5 ml/dish. After digestion of the gel, cells were washed twice by centrifugation and replated as above. Cultures were not screened routinely for mycoplasma, nor was chromosomal analysis performed.

## Results

Upon primary culture on the Matrigel extracellular matrix, gland fragments first attach as spherical or cylindrical fragments, and within 7 to 10 d flatten and spread as small monolayer colonies (Fig. 1 *a*). After several weeks in culture the colonies enlarge and eventually form large organoid structures (Fig. 1 *b*) in approximately 50% of the colonies. Glandular structures

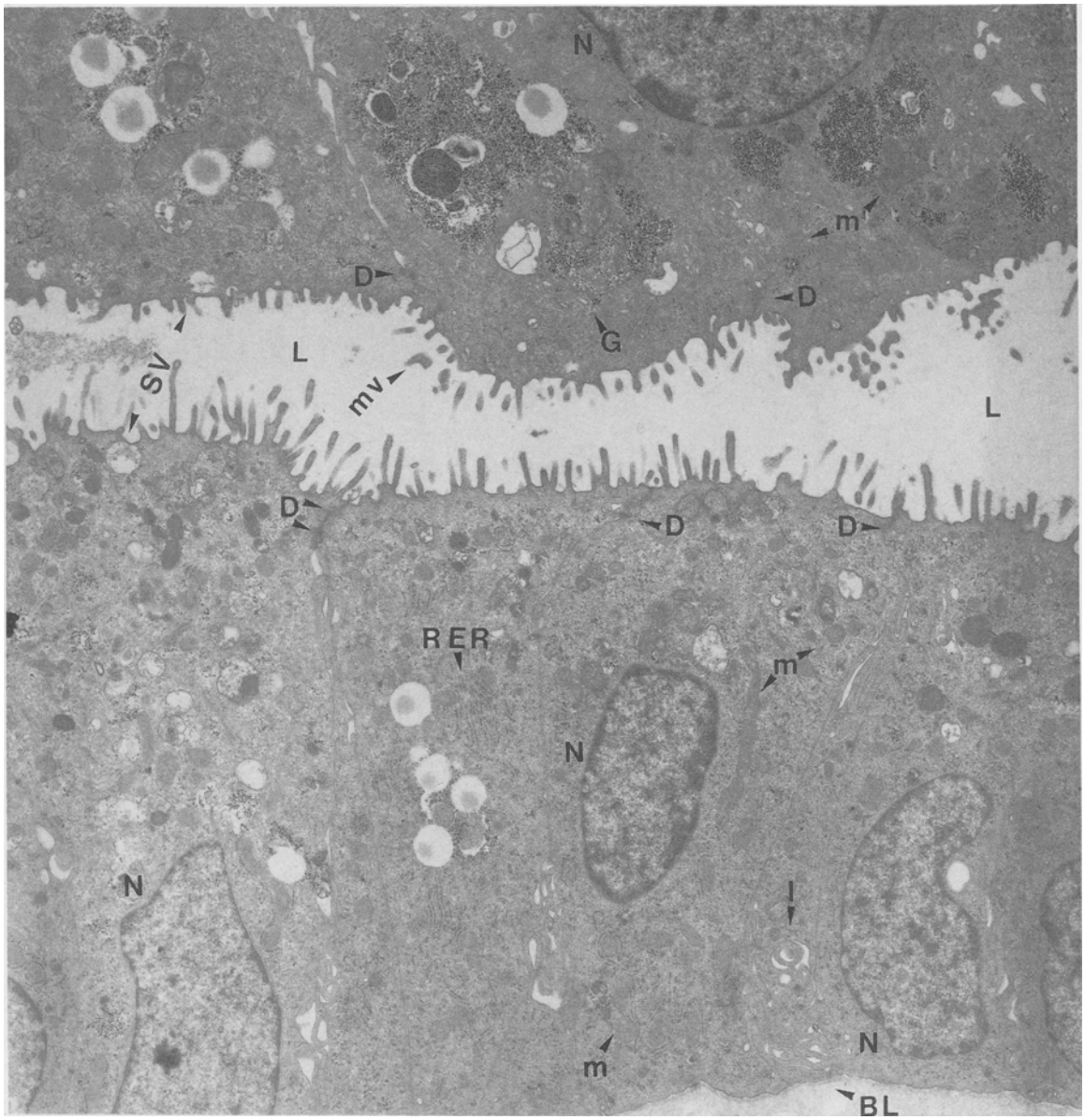


FIG. 3. A cross section of an organoid structure viewed by transmission electron microscopy. Organoids in Matrigel were embedded in Epon 812 resin, sectioned, and double stained (9) before microscopy. ×8600. Two rows of cells face a central lumen (*L*) which is lined by numerous microvilli (*mv*). Nuclei (*N*) are located basolaterally. Individual cells are columnar, and cell boundaries are sealed by desmosomes (*D*) at the apical surface and by a highly interdigitated plasma membrane (*I*) at the basal surface. Other structures indicated are secretory vesicles (*SV*), rough endoplasmic reticulum (*RER*), mitochondria (*m*), the Golgi apparatus (*G*), and a basal lamina (*BL*).

typically form above the monolayer colonies, usually in a central location, and often seem to be connected to other areas of the monolayer by smaller tubular structures. Cells may be enzymatically dispersed, passaged onto fresh Matrigel, and the process of glandular morphogenesis repeated. The scanning electron micrographs shown in Fig. 2 illustrate the sequence of events in glandular morphogenesis. Beginning with the formation of small tubules (Fig. 2 *a*), organogenesis proceeds to the formation of mid-sized organoids (Fig. 2 *b*) and culminates in large glandular structures (Fig. 2 c). Figure 2 *c, arrow* indicates a structure that seems to be a cylindrical gland fragment which has broken, revealing a central lumen. Growth is most vigorous during the first 6 to 8 wk. Cultures can be maintained routinely for as long as 6 mo.

Organoids that develop in vitro are comprised of columnar epithelia lining a central lumen (Fig. 3). This pattern of cellular organization recapitulates the structure of the glands in vivo. Individual cells are highly polar, showing indications of a basement membrane at their basal surface, and contain numerous microvilli and tight junctions at the apical surface. The nuclei have a typically basolateral location. A high degree of interdigitation between cells occurs, especially toward the basal cell surface. Further ultrastructural analysis reveals details consistent with a high degree of macromolecular synthesis. Mitochondria and rough endoplasmic reticulum are abundant; the former often seem to be encircled by the latter. The Golgi apparatus is visible, often in the process of budding vesicles, and secretory vesicles are abundant. Some secretory vesicles are seen in the process of fusing with the plasma membrane. The large number of mitochondria, the highly developed rough endoplasmic reticulum, the active Golgi apparatus, and secretory vesicles are indicative of active secretory function. Growth on Matrigel contrasted sharply with growth on the bare plastic surfaces of tissue culture plates. As reported previously (16,17) the epithelial cells grown on plastic are flat and squamoid rather than columnar, as are the cells grown on Matrigel. The cells grown on plastic are also typically short-lived, degenerating rapidly after 2 to 3 wk in culture. The extracellular matrix consistently provides a period of growth which lasts for 6 to 12 wk, followed by a maintenance period of an additional 3 mo.

## DISCUSSION

The methods we describe provide a model system which, with modifications appropriate to the hormonal and growth factor requirements of particular cell types, may be applied to the study of the function and regulation of other types of highly differentiated epithelial cells in vitro. The use of serum-free, chemically defined medium permits better controlled studies of hormonal influences on cellular function.

The columnar shape of the cells with the observation of microvilli, desmosomes, and a basal lamina provides clear evidence of the epithelial origin on these cells. The cells also stain positively for cytokeratin (data not shown).

Rat-tail collagen gels have been used to study the morphogenesis of mammary glandlike structures for mouse cell lines (13,14) and primary cultures of human mammary epithelia (20,21). These collagen gels reorient vesicles of endometrial glandular epithelia into the proper glandular orientation in which a central lumen is lined by the apical cell surfaces (6). These studies differed from this report in that the glandular morphogenesis or reorientation occurred in floating but not anchored gels. The observed differences may be due to different cell types, mammary vs. endometrial, or in the composition of the extracellular matrix. Both these experiments and our own indicate that an extracellular matrix is necessary for the development of organoid structures with proper orientation. Furthermore, a comparison of results indicates that the composition of the extracellular matrix is of critical importance in regulating organogenesis and differentiated cell function. Matrigel is an extract of basement membrane which contains laminin and type IV collagen as major components, and heparin sulfate proteoglycan, and entactin as minor components (8). Cells grown on this material demonstrate enhanced levels of differentiated functions (4,8,11). Human amnion basement membrane has also been used as a substratum for axon extension in vitro and in vivo (3). The use of these or other basement membrane materials in combination with serum-free media provides a heretofore unattainable opportunity to study complex biological processes in epithelial cells in vitro under conditions that are closer to those found in vivo.

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