Mesenchymal-Epithelial Interactions in an *In Vitro* Model of Neonatal Mouse Uterus

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Abstract. **Stromal factors have been implicated in epithelial growth of the fetal and neonatal mouse uterus, as well as in uterine epithelial proliferation in the adult. In the neonate, uterine growth is independent but responsive to estrogen, while epithelial proliferation in the adult uterus is hormonally regulated. A co-culture model was developed to study interactions between uterine epithelium and stroma. Uteri of neonatal mouse were enzymatically separated into epithelial and mesenchymal cell fractions, and these were cultured for 5 days on Millipore well inserts, either separately or in co-culture on opposite sides of the insert membrane. When epithelial cells were grown alone, inclusion of serum in the culture medium tripled the number of cells present after 3–5 days compared with serum-free medium. Co-culture in the presence or absence of serum resulted in a 5-fold increase in epithelial cell number after 5 days. Addition of estrogen had no significant effect on epithelial cell number regardless of the presence of mesenchyme. Epithelial cultures grown in medium conditioned by mesenchymal cells exhibited an intermediate increase in cell number. We therefore conclude that uterine mesenchyme from neonatal mouse produces a diffusible factor that enhances the growth of the overlying epithelium; however, whether the mesenchyme has a role in estrogen-stimulated epithelial proliferation has not been definitively ascertained.** [P.S.E.B.M. 1997, Vol 214]

In the neonatal mouse, the uterus is composed of a simple columnar epithelium and an undifferentiated mesenchyme which develops into myometrium and stroma during the first several days after birth (1). Stromal factors have been implicated in the fetal and postnatal epithelial growth of the uterus (2, 3). Furthermore, evidence has been accumulating that implies a role for the stroma in epithelial proliferation in the adult uterus. This epithelial growth is regulated by changes in the hormonal milieu that take place during the estrous cycle. During the follicular phase of the cycle prior to ovulation, the epithelium proliferates in response to rising levels of estrogen. However, attempts to elicit a proliferative response to estrogen in isolated epithelial cultures have met with failure. Cooke and co-workers (4) addressed the possibilities that culture conditions selected for a population of epithelial cells unresponsive to estrogen or caused their irreversible dedifferentiation. They cultured uterine epithelial cells from mice on collagen, then recombined the cultures with uterine mesenchyme and implanted them under the kidney capsules of ovariectomized adult hosts. Recombinants from hosts injected with estrogen exhibited greater epithelial proliferation from recombinants from oil-injected hosts, demonstrating that the cultured epithelial cells had retained their ability to proliferate in response to estrogen (4).

Because epithelial cells of the adult uterus express estrogen receptor (ER), it had been assumed that estrogen-induced epithelial proliferation was a direct result of the binding of estrogen to its receptor in these cells. However, Bigsby and Cunha used a neonatal BALB/c mouse model in which ER is present in uterine mesenchyme but undetect-

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able in the epithelium. They demonstrated that the epithelium nevertheless proliferated in response to estrogen injection (5). In another strain of mouse, CD-1, Yamashita *et al.* found that the epithelium contained ER-positive and ER-negative cells at 4 days of age. Estrogen induced expression of an estrogen-responsive protein, lactoferrin, in the ER-positive cells but not in the ER-negative cells. On the other hand, estrogen induced proliferation in both ER-positive and ER-negative epithelial cells, suggesting that this response is an indirect action of estrogen (6). There has only been a single report of estrogen-induced proliferation of uterine epithelial cells *in vitro,* and this study indicated not only that there was a requirement for the presence of stromal cells in the culture, but that physical contact between the two cell types was necessary for estrogen responsiveness (7). Studies of this nature led to the proposal that estrogen-induced epithelial proliferation in the uterus is mediated by its underlying stroma.

This report describes an *in vitro* model for use in studying interactions between uterine epithelium and stroma. It is a modification of a culture system described by Glasser *et al.* in which uterine epithelial cells from immature rats were grown on Millicell HA filters impregnated with EHS-tumor matrix (8). Effects of uterine stroma on the epithelial cultures were then investigated by the use of conditioned medium or by placing the Millicell inserts into wells containing stromal cultures (9). In our system, neonatal mouse epithelium lacking ER was co-cultured with ER-positive mesenchymal cells on opposite surfaces of insert membranes. In addition, a preliminary investigation was undertaken in hopes of providing evidence for the hypothesis that estrogen-induced epithelial proliferation is an indirect effect which is stromally mediated. Diffusible mesenchymal factors were found to result in an increase in epithelial cell number, but there was no additional increase when estrogen was added to the system.

Materials and Methods

Uterine horns from 2- to 3-day-old ICR mice (Harlan Sprague-Dawley, Indianapolis, IN) were trimmed of fat and mesentery, cut into three or four pieces, and incubated in 1% trypsin (Difco 1:250) for 1.5 hr at 4° followed by 1% soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) for 0.5 hr at room temperature. As described previously (10), the epithelium was then extruded as an intact tube by drawing each uterine piece into the tip of a narrow-bore pipette. The epithelial pieces were further dissociated into small aggregates of cells by a second round of trypsinization. A single-cell suspension of mesenchyme was obtained by vigorous shaking of the remaining uterine pieces in a solution of 0.05% trypsin and 0.05% collagenase A (Boehringer-Mannheim Biochemicals, Indianapolis, IN) in a 37°C water bath. The method of isolating the two cell types was tested by staining the individual cultures with antibodies against cytokeratin (Dako, Carpinteria, CA), a marker for epithelial cells, and desmin (Boehringer-Mannheim), a marker for cultured stromal cells. Both cell types were also tested for the presence of ER by antibody staining with H222 (kindly provided by Abbott Laboratories, Diagnostic Division, Abbott Park, IL, and by Geoffrey Greene, Ben May Laboratories for Cancer Research, University of Chicago, Chicago, IL).

The two cell types were cultured on Millicell HA tissue culture plate inserts (Millipore Corporation, Bedford, MA). The insert membranes were pre-coated with 5–10 μg fibronectin (Sigma), and cell types were plated either on separate inserts or on opposite sides of the same insert membrane. Mesenchymal cells were first grown in 25 cm^2 Falcon tissue culture flasks (Becton Dickinson, Oxnard, CA) for 5 days and then passaged onto the bottoms of the membranes at a density of 100,000 cells per membrane. Two days later, epithelial cells were seeded directly onto the tops of the insert membranes, using the equivalent of two uterine horns per insert. Plating medium consisted of Dulbecco's modified Eagle's medium/Ham's F-12 medium 1:1 (Gibco, Grand Island, NY) supplemented with 5% charcoal-stripped fetal bovine serum (csFBS) (Gibco), 10 μg/ml insulin (Sigma), 5 μg/ml transferrin (Sigma), 0.5 μg/ml penicillin-streptomycin (Gibco). After 24 hr of culture, cells were washed with phosphate-buffered saline (PBS) and changed to serum-free medium. The culture medium was changed every 2 days. 17β-estradiol (Sigma) added to cultures was at a final concentration of 10 n*M*.

In one experiment, epithelial cells were grown on transwell filters for 24 hr in plating medium and then changed to medium conditioned by mesenchymal cells. The conditioned medium was prepared by growing mesenchymal cells on transwell filters for 2 days in plating medium, then an additional 24 hr in serum-free medium, after which fresh serum-free medium was added to cultures and collected every 48 hr.

At the end of each experiment, the cells were fixed on the filter inserts for 10 min in 100% ethanol and stained for 15 min with 5 μg/ml Hoechst dye #33258. The fluorescent nuclei were videotaped and counted using an Image-1 analysis system. Sixty fields with an area of 0.06 mm^2 each were counted for each sample and data converted into number of epithelial cells per square milimeter. Data were assessed for significant differences by analysis of variance (ANOVA) and analysis of covariance.

Results

Immunocytochemical studies confirmed that our method of isolation produced pure cultures of epithelial and mesenchymal cells. Stained epithelial cultures were positive for cytokeratins (Fig. 1a), while mesenchymal cultures were positive for desmin (Fig. 1c) but negative for cytokeratin (not shown). Use of ER antibody resulted in positive nuclear staining of cultured mesenchymal cells, while epithelial cultures exhibited no staining (Fig. 2).

To determine the appropriate time for treatments, two preliminary studies were done to investigate the pattern of epithelial growth. Cells were fixed and counted 24 hr after plating and every 2 days thereafter. By Day 3 of culture,

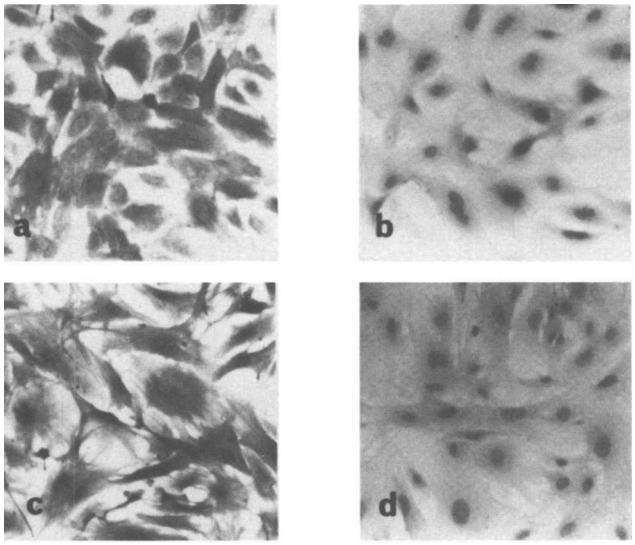

Figure 1. Immunohistochemical staining of cultured uterine cells for intermediate filaments. Uterine epithelial or mesenchymal cells were plated in 5% csFBS and 2 days later changed to serum-free medium for an additional 2 days. Cells were fixed for 10 min in 100% ethanol and stained for keratin or desmin using the avidin-biotinylated peroxidase method. (a) Cultured uterine epithelial cells showing positive staining for keratin. (b) Epithelial cells with primary antibody omitted. (c) Cultured uterine mesenchymal cells showing positive staining for desmin. (d) Mesenchymal cells without primary antibody. Magnification: ×270.

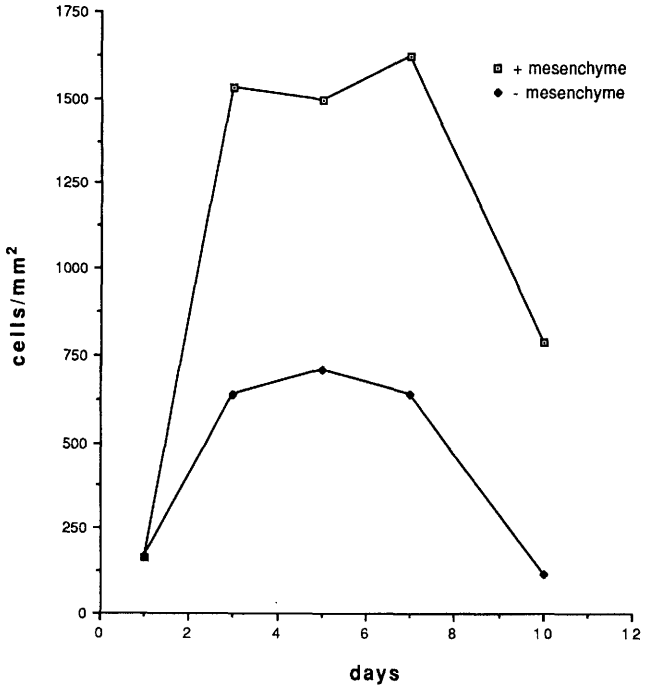

Figure 3. Pattern of growth of cultured uterine cells. Uterine epithelial cells were cultured with (open squares) or without (closed diamonds) mesenchyme on transwell filters. Samples were counted every 2 days beginning 24 hr after plating. Each point is the average number of cells from two wells (data are representative of two similar experiments).

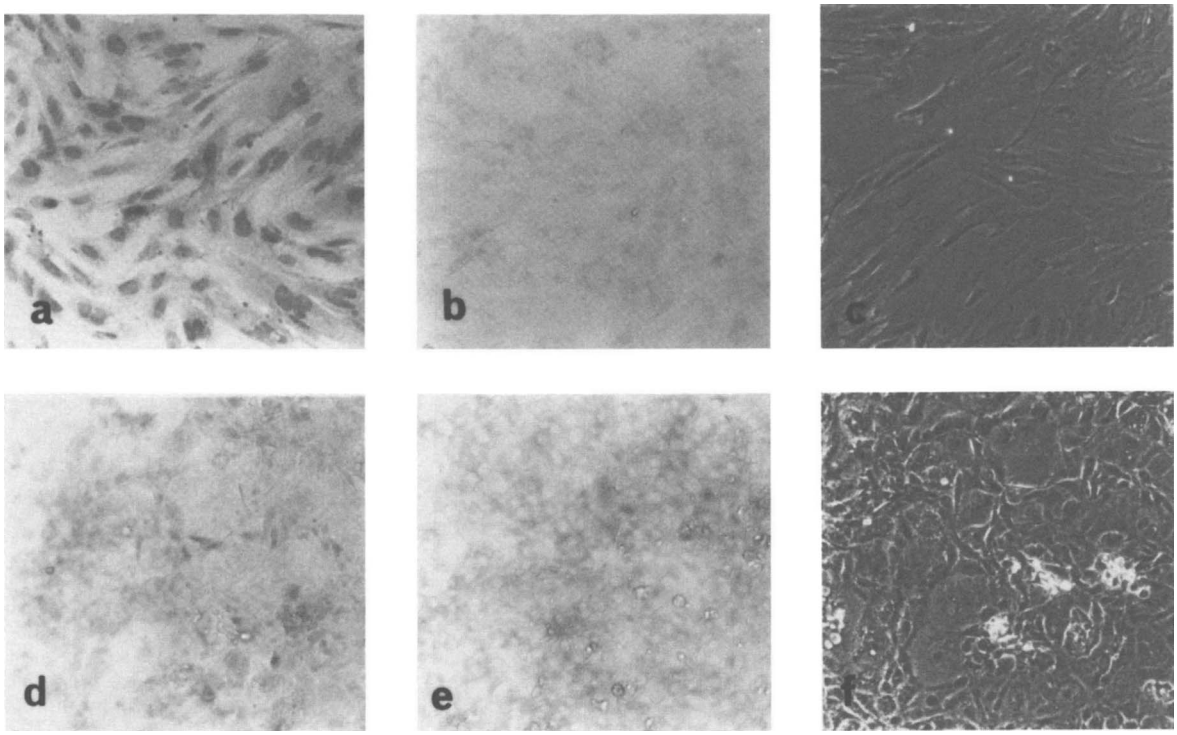

Figure 2. Immunohistochemical staining of cultured uterine cells for ER. Uterine epithelial or mesenchymal cells were plated in 5% csFBS and 2 days later changed to serum-free medium for an additional 2 days. Cells were fixed for 10 min in paraformaldehyde and immunostained using the H222 primary antibody. (a) Cultured uterine mesenchymal cells showing positive nuclear staining for ER. (b) Mesenchymal cells with primary antibody omitted. (c) Mesenchymal cells shown in Panel b with phase contrast. (d) Cultured uterine epithelial cells lacking nuclear staining for ER. (e) Epithelial cells without primary antibody. (f) Epithelial cells shown in Panel e with phase contrast. Magnification ×135.

epithelium grown either in the presence or absence of mesenchyme reached a plateau phase which lasted approximately 4 days. After 7 day of culture, cell number declined rapidly. Combined data from the plateau phase showed that the number of epithelial cells in co-culture was more than twice as great as in cultures with no mesenchyme present (Fig. 3). ANOVA showed this difference to be statistically significant, with means of 1550 ± 94 vs 660 ± 83 ($P < 0.0001$).

When epithelial cells were grown alone, serum was required for proliferation. Inclusion of serum in the culture medium resulted in a final cell number after 5 days that was 2.7 times higher than that of cultures grown in serum-free medium. Co-culture, even in the absence of serum, resulted in up to a 5-fold increase in epithelial cell number after 5 days compared with epithelial cells grown alone. There was no additional increase in cell number when serum was included in the co-cultures (Fig. 4).

To test the stimulatory effects of estrogen on quiescent epithelium, cells were treated with estrogen for 2 days beginning on Day 5 of culture or for 5 days beginning on Day 2 of culture. Estrogen had no effect on epithelial cell number regardless of whether the cells were grown alone or in the presence of mesenchyme (Fig. 5).

To determine whether mesenchymal stimulation of the epithelium was through cell-cell contact across the filter or if it was *via* a diffusible factor, epithelial cells were plated on filters and grown in medium conditioned by mesenchymal cells. The presence of conditioned medium led to an increase in cell number that was approximately 70% of that produced by transfilter co-culture (Fig. 6).

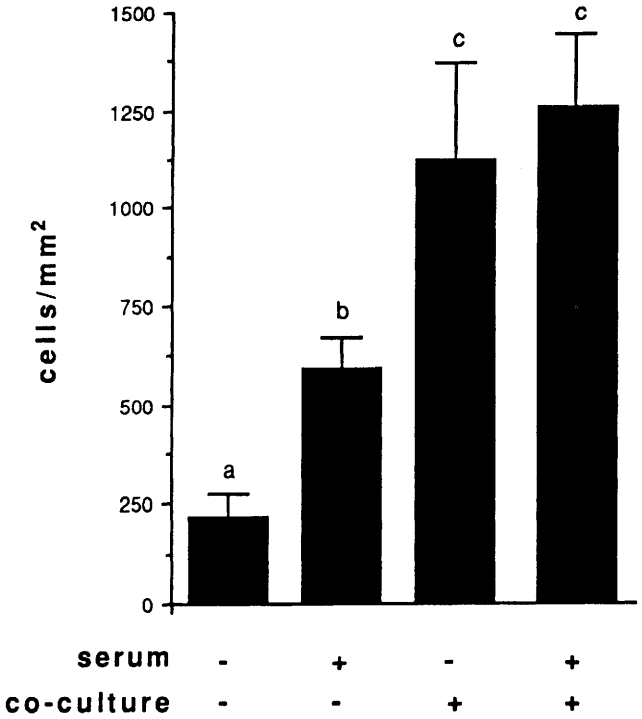

Figure 4. Effect of serum or co-culture on uterine epithelial cell number. Epithelial cells were cultured for 5 days on transwell filters with or without serum and in the presence or absence of mesenchyme. Data are expressed as mean ± SD ($n = 3$). Bars with no common superscripts are significantly different ($P < 0.05$).

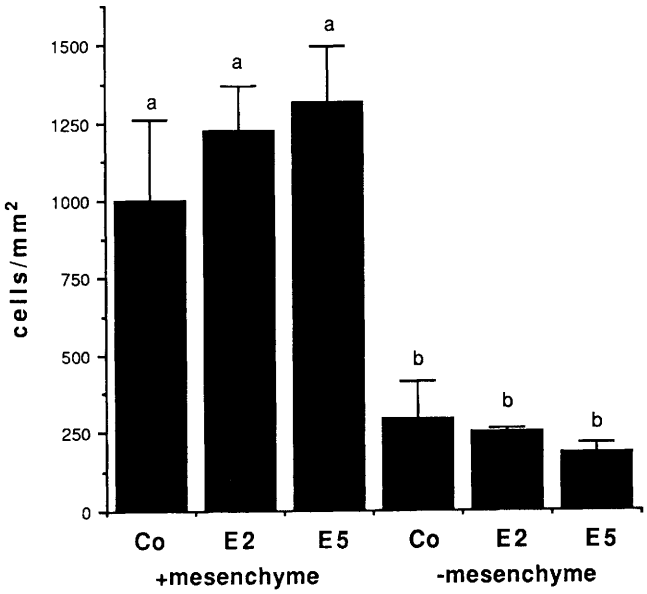

Figure 5. Effect of estrogen on cultured uterine epithelial cell number. Epithelial cells were cultured in serum-free medium with or without mesenchyme on transwell filters for 7 days. Estrogen (1×10^{-8} M) was added for 2 days (E2) during Days 5–7 or for 5 days (E5) during Days 2–7. Data represent mean ± SD ($n = 3$). Bars with no common superscripts are significantly different ($P < 0.05$).

Discussion

In this co-culture model, uterine epithelial cells grown in the presence of mesenchyme attained a final cell number that was up to five times greater than that of epithelial cells grown alone. The increased number of cells cannot be attributed to enhanced plating efficiency, because there was no difference in cell numbers between the two groups 24 hr after plating.

Although epithelial cultures responded to serum growth factors, the number of epithelial cells in co-cultures was not further increased by inclusion of serum in the medium, suggesting that the cells were already maximally stimulated. Furthermore, since epithelial cell number was also increased by serum-free medium conditioned by mesenchyme, it appears that the mesenchyme is secreting a factor that either stimulates proliferation or prevents cell death. The latter possibility, however, seems unlikely, since co-culture did not extend the period of epithelial cell survival once maximal density was attained.

Numerous studies have reported the requirement for growth factors in uterine epithelial cell cultures. Tomooka cultured uterine epithelium from immature mice on collagen gels and found that growth in serum-free medium could not be sustained for more than 3 days unless EGF was included in the medium (11). Uchima, using uterine epithelium from adult mice cultured in a collagen gel matrix, reported that both insulin and EGF stimulated proliferation and that, while deletion of EGF resulted in a slight decrease in proliferation, deletion of insulin completely abolished any growth (12). Our study is the first to demonstrate that uter-

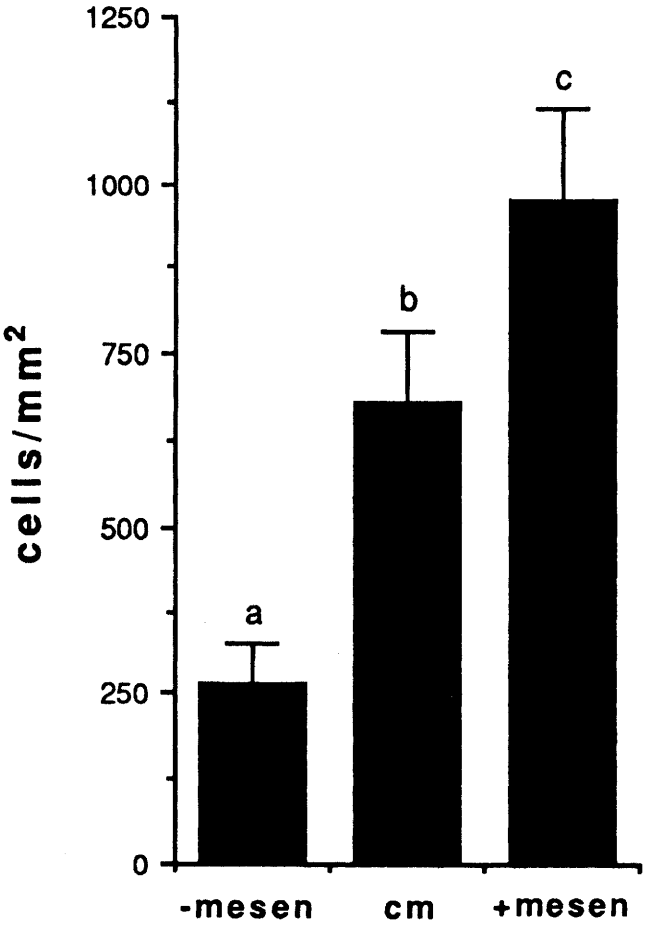

Figure 6. Effect of mesenchymal factor(s) on cultured uterine epithelial cells. Epithelial cells were grown for 7 days on transwell filters in serum-free medium or in medium conditioned by mesenchymal cells (cm). Data are expressed as means ± SD ($n = 3$). Bars with no common superscripts are significantly different ($P < 0.05$).

ine mesenchyme produces a factor in culture that affects the net growth of epithelial cells. The identity of this mesenchymally produced factor remains to be determined.

The stimulatory effect of estrogen on the uterine epithelium has been known for quite some time and has been extensively studied *in vivo*. However, demonstrating this effect *in vitro* has proven more difficult. In isolated epithelial cultures, estrogen has failed to elicit a proliferative response even when the presence of a functional ER system was demonstrated. Uchima *et al.*, using epithelial cells isolated from 40-day-old ovariectomized mice, reported that estrogen caused an increase in nuclear retention of ER, as well as an increase in progesterone receptor expression (12). Both of these estrogen responses are considered to be indicative of functional ER. However, estrogen actually reduced proliferation of epithelial cells in that study.

In Inaba's report of estrogen-induced epithelial proliferation *in vitro*, he concluded that contact with the stroma was a requirement (7). However, quantifying individual cell types in a mixed culture is problematic. Because the DNA content of pure cultures of stromal cells treated with estrogen was no different from that of untreated cultures, Inaba attributed the increased DNA content of estrogen-treated mixed cultures to epithelial proliferation. However, he did not test the possibility that stromal growth might have been stimulated by the presence of epithelial cells. Although we have not determined the extent of physical contact between the two cell types in our model, others have shown that cells do send processes through these types of filters (13–15). Despite the potential for mesenchymal/epithelial contact, however, our data indicated that estrogen had no effect on final epithelial cell number. The lack of a response to estrogen may be due to the epithelial cells already being maximally stimulated by the presence of stromal factors. The model we have developed should be a useful system for further characterization of stromal-epithelial interactions.

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