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Restoration of normal morphology and estrogen responsiveness in cultured vaginal and uterine epithelia transplanted with stroma

(tissue recombinants/epithelial–stromal interactions/primary epithelial cell cultures)

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ABSTRACT **We have investigated the capacity of vaginal and uterine epithelia (VE and UE) to reexpress normal morphology and hormone responsiveness following cell culture. VE and UE from adult ovariectomized mice were grown in a collagen gel matrix with serum-free medium for 7–10 days. Proliferation of these cells occurs in the absence of 17β-estradiol and is not stimulated by 17β-estradiol; the VE usually does not keratinize or stratify *in vitro*. Cultured VE and UE were recombined with homologous vaginal or uterine stroma (VS and US, respectively) and these recombinants were grown under the renal capsule of female hosts for 4 weeks. The epithelium of the VS + VE recombinants cycled, proliferated and stratified in response to estrogen and mucified normally in response to progesterone. The UE that was grown *in vivo* with cultured US also showed normal morphology and estrogen responsiveness. These changes in the UE and VE were not simply a result of return to the *in vivo* environment, as epithelia alone in collagen gels transplanted under the renal capsule did not survive. These results indicate that both VE and UE, which are not estrogen-dependent *in vitro*, reexpress their estrogen dependency and normal morphology when recombined with homologous stroma and grown *in vivo*. Thus, the changes these cells show when grown in culture are the result of altered conditions *in vitro* rather than an irreversible alteration in the cells themselves or the selection of specific subpopulations that are not mitogenically or morphologically responsive to estrogen under any condition.**

Cell culture has provided a powerful tool for studying many aspects of cell growth and function under controlled conditions. However, the utility of culture systems has been limited because of discrepancies in cell behavior *in vitro* and *in vivo* and the possibility that cells may lose their identity and capacity for normal function *in vitro*. Epithelial cells in culture exhibit marked changes in shape, protein-synthetic patterns, hormone responsiveness, and function compared with intact epithelia *in vivo* (1–3). Paradoxically, hormones that are primary regulators of cellular proliferation *in vivo* may have no growth-promoting effect on their target cells *in vitro*. This phenomenon is illustrated by the repeated observations that estrogen, the principal mitogen for female reproductive tract epithelium *in vivo* (4), is not mitogenic for isolated reproductive tract (or mammary gland) epithelia *in vitro* (5–10). For example, vaginal epithelial (VE) and uterine epithelial (UE) cultures from adult ovariectomized (Ovx) mice exhibited 4- to 8-fold and 3-fold increases, respectively, in DNA content when cultured for 10 days in collagen gel with serum-free medium in the absence of estrogen (5, 8). The addition of estrogen to the medium did not stimulate proliferation of these cells and, at higher concentrations, was actually inhibitory (5, 8). In addition, the VE did not usually stratify or keratinize *in vitro*.

The lack of estrogen mitogenicity *in vitro* raises the possibility that the cultured VE and UE have irreversibly dedifferentiated. Alternatively, cultured VE and UE may retain their identity and ability to function normally, but these features are not expressed owing to inadequacies of the culture system. It is also possible that *in vitro* culture leads to a permanent loss of estrogen responsiveness in these cell populations. To address these questions, we have examined the ability of cultured VE and UE to reexpress normal morphology and hormone responsiveness (proliferation) when recombined with homologous stroma and transplanted *in vivo*.

Our results indicate that cultured UE and VE recombined with homologous stroma and grown *in vivo* exhibit normal morphology (e.g., the VE stratifies and cycles) and that the epithelia reexpress the estrogen dependency for proliferation, which cannot be demonstrated *in vitro*.

MATERIALS AND METHODS

Epithelial Cell Isolation and Culture. Vaginae and uteri were dissected from ≈40-day-old BALB/cCrgl mice (Cancer Research Laboratory, University of California, Berkeley, CA) 11–12 days after ovariectomy. The vaginae were enzymatically dissociated by the procedure described previously (5), with some modification. Briefly, transversely sectioned vaginae were incubated in medium 199 containing 0.1% collagenase (CLS III, Cooper Biomedical, Freehold, NJ) and 5 mg of bovine serum albumin fraction V (Sigma) per ml for 2 hr at 37°C in a shaking water bath. Uteri were cut in half midsagittally and incubated in medium 199 containing 5 mg of trypsin (Difco, 1:250) per ml for 14 min at 37°C in a shaking water bath. Bovine serum albumin (5 mg/ml) was added to inactivate the trypsin, and the luminal epithelium was separated and minced as described (9). Epithelia were centrifuged on a preformed isopycnic Percoll density gradient (7, 11). The isolated epithelial cells were cultured for 7–10 days in a serum-free complete medium as described (5, 8, 9). All cultures were incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2 unless otherwise indicated.

Stromal Isolation and Culture. BALB/c mice were obtained from Bantin-Kingman (Fremont, CA) or the Cancer Research Laboratory colony.

The stroma used in these experiments either was freshly isolated or had been cultured for 4 weeks. The cultured stroma can support all aspects of epithelial cytodifferentiation, growth, and hormone responsiveness and thus is equivalent to fresh stroma in its ability to interact normally with

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Abbreviations: VE, vaginal epithelium; VS, vaginal stroma; US, uterine stroma; UE, uterine epithelium; Ovx, ovariectomized.

homologous fresh epithelium (12). The procedures for preparation of stroma either for recombination or for culture and later recombination have been described (12–14). Briefly, vaginae and uteri from 2-day-old mice were enzymatically dissociated in 1% trypsin (Difco Laboratories) for 90 min at 4°C. The VE was then separated from the stroma manually, whereas UE and uterine stroma (US) were separated by gently drawing the uterine pieces into a reduced-bore Pasteur pipette, resulting in the extrusion of the epithelium (13, 14). The stroma obtained in this manner then was used for recombination with cultured epithelium or was placed into culture. To prepare the stroma for culture, stromal explants were plated onto 35-mm culture dishes (Falcon) containing a rat tail collagen gel that had been coated previously with an extracellular matrix derived from bovine corneal endothelial cells (15). One milliliter of a rat tail collagen solution was then added to each dish. Following gelation of the collagen, 2 ml of a medium containing Dulbecco's modified Eagle's medium/Ham's F-12 medium, 1:1 (vol/vol), supplemented with 15% fetal bovine serum, 50 μg of gentamicin per ml, and 0.25 μg of Fungizone per ml was added (12). Media, serum, and antibiotics were obtained from the University of California, San Francisco Cell Culture Facility. The culture medium was changed every 4 days over the 4-week culture period.

Tissue Recombination and Transplantation. Following 7–10 days of culture, epithelium for recombination was obtained by incubating the epithelium-containing collagen gels with 1% collagenase for 30–45 min at 37°C. To make recombinations of cultured stroma and cultured epithelium, isolated epithelial colonies were removed, washed in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution, and then transferred with a drawn-out Pasteur pipette onto the top of the collagen containing the cultured stromal cells. Recombinations of fresh stroma + cultured epithelium were prepared similarly by placing pieces of stroma on a solidified agar medium and then adding epithelium onto the stroma (14). The recombinants were allowed to adhere overnight in a humidified incubator. Homologous recombinations consisting of cultured vaginal stroma (VS) + cultured VE and cultured US + cultured UE were prepared to determine if these epithelia would express normal morphology and, in the case of VE, cyclicity of epithelial differentiation, when recombined with stroma and grown *in vivo*. Heterologous recombinations of fresh 2-day-old VS + cultured UE and 2-day-old US + cultured VE were prepared to determine if either of these epithelia could be instructively induced by heterologous stroma.

The recombinants were grafted under the renal capsule of intact female hosts as described (14). To assess whether the stromal cultures and fresh stroma were free of epithelial contamination, gels containing only US or VS and pieces of US or VS were grafted. After 4 weeks of growth, the transplants were prepared for histological examination and stained with hematoxylin/eosin (14).

Epithelium in the collagen gel matrix was transplanted to examine the effects of the *in vivo* environment on the growth and morphology of the isolated cultured epithelial cells. Pieces of collagen gel (≈5 mm in diameter) containing cultured UE or VE that had been grown *in vitro* for 10 days were excised from the collagen gel. The epithelium-containing gels were grafted under the renal capsule as described above. Hosts bearing these transplants were killed 2, 6, or 10 days following transplantation, and the grafts were removed and processed for histological examination as above.

Hormone Responsiveness. To determine if the epithelia of the recombinants transplanted *in vivo* were dependent upon estrogen for proliferation, hosts were Ovx 3 weeks following grafting and 1 week later were injected with 100 ng of 17β-estradiol (Sigma) in 0.1 ml of corn oil or an equal volume of corn oil alone. Eighteen hours later, the grafts were removed, cut into 1-mm^3 pieces, and incubated in Dulbecco's modified Eagle's H-21 medium containing 5 μCi of [^{3}H]thymidine (specific activity = 80 Ci/mmol; 1 Ci = 37 GBq; Amersham) per ml for 4 hr. The tissues were then fixed and processed for autoradiographic examination as described.

To test the ability of the recombined cultured VE to mucify normally in response to progesterone, fresh VS from 2-day-old neonates was recombined with cultured VE and grown in an intact female host for 10 days, at which time the hosts were Ovx. Five days later, 17β-estradiol and progesterone (12.5 ng and 62.5 μg, respectively) in 0.1 ml of corn oil were injected s.c. daily for 3 days (16, 17). The hosts were then killed and the transplants were processed and stained with hematoxylin/eosin as above or were stained with periodic acid/Schiff reagent to determine if mucus was present in the epithelium (18).

RESULTS

Following 10 days of culture, collagen gels containing VE or UE contained numerous epithelial colonies (Fig. 1*A*). The

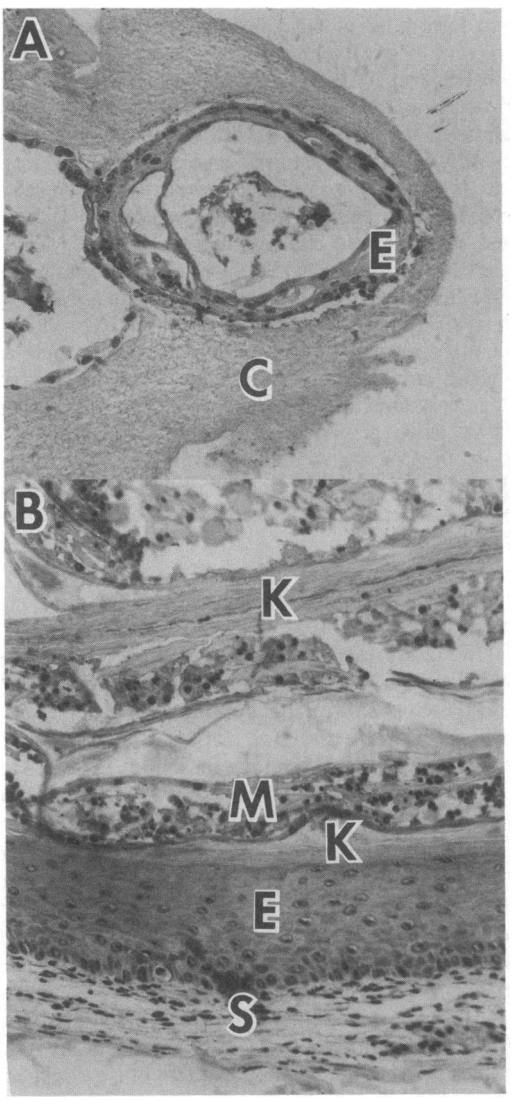

FIG. 1. (*A*) VE (E) grown in collagen gel (C) for 10 days. The VE was two or three cell layers thick and was not keratinized. (*B*) A tissue recombinant composed of cultured VS (S) + cultured VE (E) that had been grown in an intact female host for 4 weeks. Note that the epithelium of this recombinant is stratified, and alternating sloughed layers of mucified (M) and keratinized (K) cells lie above the epithelium, indicating that normal cycling has occurred. (×290.)

epithelium of cultured VS + cultured VE recombinants (n = 13) grown for 4 weeks in intact female hosts was stratified, and alternating layers of sloughed keratinized and mucified cells were visible above the epithelium (Fig. 1*B*), indicating that the VE in the recombinant had cycled in concert with the host VE. The epithelium in the cultured US + cultured UE recombinants (Fig. 2; n = 9) was also morphologically normal but consisted only of luminal epithelium without glands.

Epithelium was never observed in transplants of fresh 2-day-old VS or US (n = 19) or of collagen gels containing cultured VS or US (n = 12; data not shown). Thus, the original stromal cultures and fresh stroma were free from epithelial contamination.

When epithelium-containing gels were transplanted and grown *in vivo* (n = 4 in all cases), epithelial colonies were identifiable after 2 days, and some host fibroblast invasion of the collagen was noted. After 6 and 10 days *in vivo*, VE could not be definitely identified, and the fibroblastic invasion from the graft site was progressively more extensive (data not shown).

The trophic effect of 17β-estradiol was examined in cultured VS + cultured VE and cultured US + cultured UE recombinants that had been grown in Ovx hosts. In 17β-estradiol-injected hosts, the epithelium of the cultured VS + cultured VE recombinants was stratified and showed intense labeling in the basal cells indicative of rapid cell proliferation (Fig. 3*A*; n = 8). Conversely, the VE in these recombinants grown in oil-injected Ovx hosts was atrophic, consisting of two or three cell layers, and little labeling was present (Fig. 3*B*; n = 4). The epithelium in cultured US + cultured UE recombinants in 17β-estradiol-injected hosts was also heavily labeled (Fig. 4*A*; n = 7), whereas that in oil-injected Ovx controls showed little labeling (Fig. 4*B*; n = 5). Hence, cultured UE and VE are responsive to 17β-estradiol in the homotypic tissue recombinants grown *in vivo*.

The epithelium of fresh VS + cultured VE recombinants (n = 6) mucified normally in response to progesterone (Fig. 5).

The cultured epithelium could not be instructively induced. The epithelium of cultured VS + cultured UE recombinants retained its simple columnar uterine-like appearance; the epithelium of US + VE recombinants consisted of stratified squamous epithelium four or five cell layers thick, and there was no evidence of cycling (data not shown).

DISCUSSION

VE and UE *in vitro* show several differences from their normal counterparts *in situ*, such as the lack of a mitogenic response to 17β-estradiol and generally, in the case of the VE, a lack of stratification and keratinization. This lack of 17β-estradiol mitogenicity *in vitro* has been noted by several investigators using UE, VE, and mammary epithelium (5–10). In addition, neoplastic cells derived from estrogen-dependent mammary, uterine, and other tumors do not proliferate in response to estrogen *in vitro* (19–21), although human mammary tumor cell lines in which estrogen has direct stimulatory effects on cell proliferation have been reported (22).

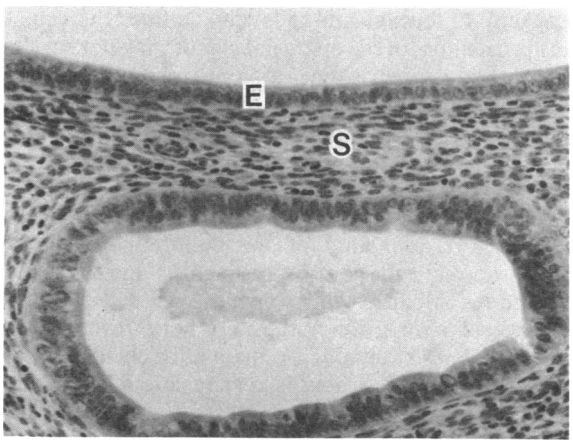

FIG. 2. A tissue recombinant composed of cultured US (S) + cultured UE (E) grown as in Fig. 1. The epithelium of this recombinant has the simple columnar morphology typical of normal uterine luminal epithelium, but no uterine glands are present. (×290.)

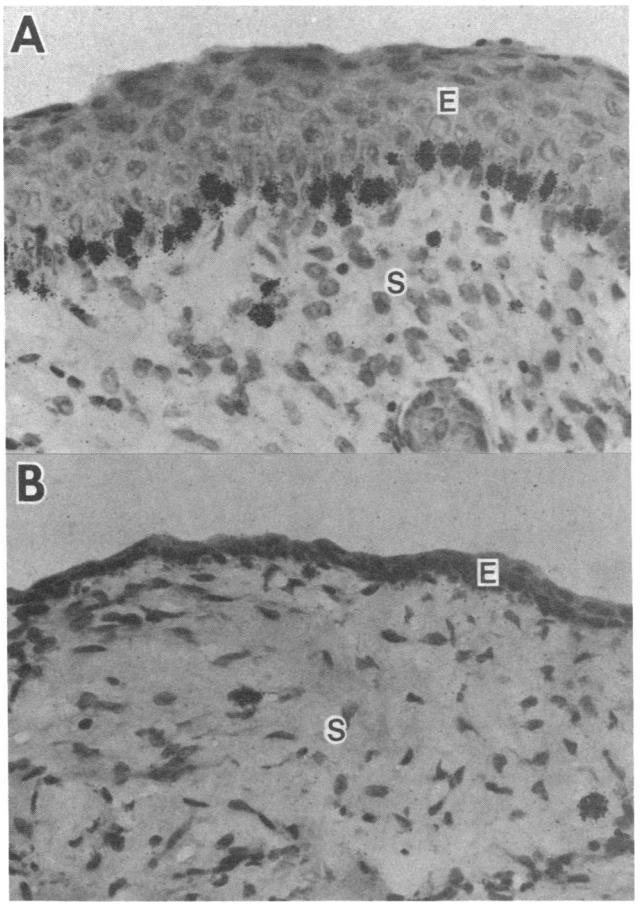

FIG. 3. [^{3}H]Thymidine labeling of cultured VS (S) + VE (E) recombinants in Ovx hosts. The hosts had been injected with either 100 ng of 17β-estradiol in oil (*A*) or oil alone (*B*) 18 hr previously. The epithelium of recombinants in 17β-estradiol-injected hosts (*A*) was stratified and heavily labeled, whereas that in oil-injected hosts was atrophic (*B*), consisting of only two or three cell layers that were sparsely labeled. (×350.)

Several possible explanations for the lack of estrogen mitogenicity *in vitro* have been proposed. Absence or abnormalities of the estrogen receptors (ER) of cultured epithelia could preclude a proliferative response to 17β-estradiol. However, both of these epithelia showed decreased cytosolic ER content, increased nuclear ER, and increased cytosolic progesterin receptor content in response to 17β-estradiol exposure *in vitro*, indicating that the cells have functional ER (8, 23).

Cunha *et al.* (24) have emphasized the importance of stroma in normal vaginal and uterine growth and hormone responsiveness and have postulated that estrogens may cause epithelial growth by acting indirectly via stroma-derived growth regulators. Therefore, isolated epithelia *in vitro* may not proliferate in response to 17β-estradiol owing to the lack of homologous stroma. Analogously, Sirbasku and co-workers (25, 26) suggest that estrogen does not directly stimulate growth *in vivo* but instead acts on tissues such as the uterus, kidney, and pituitary to induce secretion of growth factors

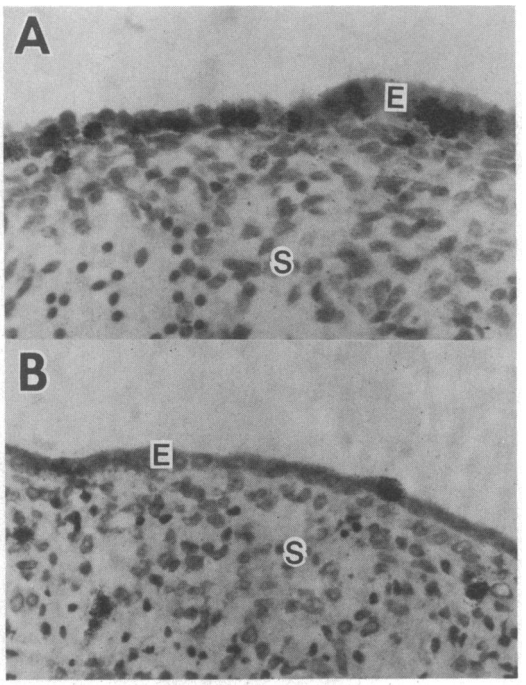

FIG. 4. [^{3}H]Thymidine labeling of cultured US + cultured UE recombinants grown in Ovx hosts injected with 17β-estradiol (*A*) or oil (*B*) as in Fig. 3. The epithelium of recombinants in 17β-estradiol-injected hosts (*A*) was heavily labeled, whereas few epithelial cells were labeled in recombinants grown in oil-injected hosts (*B*). (×360.)

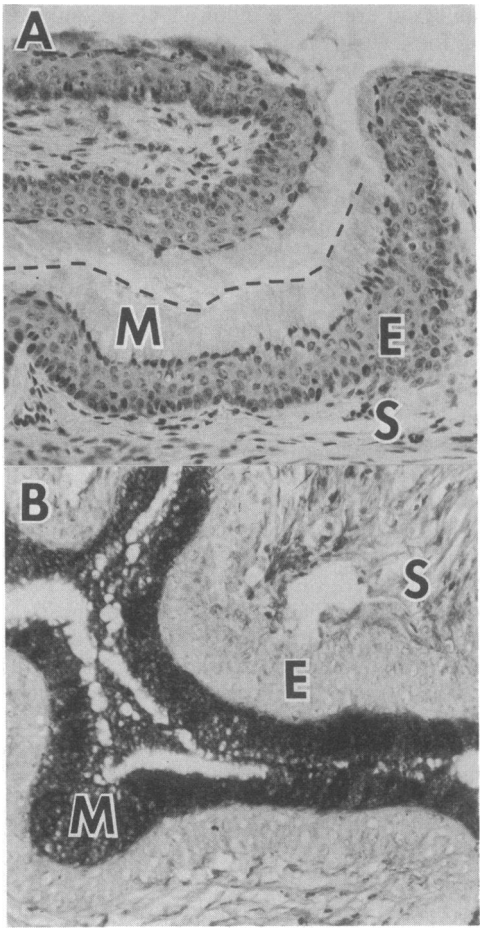

FIG. 5. Tissue recombinants composed of fresh VS (S) + cultured VE (E) grown in Ovx hosts injected with 17β-estradiol and progesterone. The recombinants were stained with hematoxylin/eosin (*A*) or periodic acid/Schiff reagent (*B*). (*A*) The epithelium of the recombinants was stratified but not keratinized, and the luminal layer of epithelium (M) was mucified and its cells were tall columnar with pyknotic nuclei. The apical border of this cell layer is indicated by a broken line. (*B*) The luminal layer of epithelium (M) stained intensely with periodic acid/Schiff reagent, indicating the presence of mucus. (×180.)

that act in concert with other autocrine or paracrine factors to produce growth of estrogen-target tissues. Sonnenschein and Soto have proposed that there are normally circulating inhibitors of estrogen-target cell proliferation and that estrogen stimulates cell proliferation indirectly by repressing this inhibitor (27).

The estrogen-free culture conditions could favor the selective proliferation of subpopulations of epithelial cells that are not estrogen-dependent or -responsive with respect to proliferation. Such culture conditions could also result in the irreversible transformation of the epithelial cells into cells that are no longer estrogen-dependent.

Our results indicate that cultured UE and VE recombined with homologous stroma and grown *in vivo* clearly express dependency on estrogen for proliferation, a characteristic not expressed *in vitro*. In addition, the epithelia are morphologically normal and the VE shows all of the characteristics of normal VE *in situ* (i.e., keratinization, mucification, and cycling). This suggests that the epithelial cells have not irreversibly dedifferentiated nor has the original epithelial population undergone transformation or selection for a subpopulation that is estrogen-independent. The epithelia (UE and VE) retain their capacity for normal morphogenesis and estrogen responsiveness following culture. Thus, the deficiencies these cells show when grown in culture are the results of altered conditions *in vitro* and not of an irreversible alteration in the cells themselves nor of selection of specific subpopulations that are not mitotically responsive to estrogen.

These results are comparable to data obtained with estrogen-dependent tumor cells. Like the cultured VE and UE, these tumor cells are estrogen-dependent for proliferation *in vivo*, but estrogen is not mitogenic *in vitro*. However, if these cultured cells are transplanted *in vivo*, they form tumors and again are estrogen-dependent for growth (28, 29). Our results indicate that this same phenomenon can be demonstrated with normal untransformed cells.

Juvenile and adult mouse UE *in situ* consists of luminal and glandular populations, but in our US + cultured UE recombinants epithelial glands are not present. The reason for the lack of gland formation is not clear: the epithelium may lose the capacity to form glands following culture, the cells that are the progenitors of the epithelial glands may not survive in culture, or the epithelial separation procedure may result in a population of only luminal cells.

Cultured VE or UE in collagen gels transplanted *in vivo* did not proliferate, and the VE did not keratinize or stratify. Hence, simply returning the cultured epithelia to an *in vivo* environment is not sufficient to restore normal morphology and function. The ability of the cultured epithelium to reexpress normal estrogen responsiveness and morphology only when recombined with stroma *in vivo* underscores the importance of stroma for normal hormone responsiveness and morphogenesis, although it does not conclusively indicate that the lack of stroma is responsible for the lack of 17β-estradiol mitogenicity on isolated epithelia *in vitro*. In general, however, our results are in agreement with previous reports that normal epithelial function in the reproductive tract is dependent on an interaction with stroma and that isolated epithelia will not grow and develop normally *in vivo* (25).

High doses of progesterone combined with low doses of 17β-estradiol induce mucification in the VE of Ovx mice (17, 18). The epithelium of our VS + cultured VE recombinants in Ovx hosts given this hormonal treatment showed all of the normal histological characteristics of mucification—i.e., the epithelium was stratified but not keratinized and the apical layer of VE consisted of tall columnar cells with hyperchromatic nuclei and periodic acid/Schiff reagent-positive mucins in the cytoplasm. The mucification response to progesterone is a unique feature of VE, and the ability of the cultured epithelium in the recombinants to respond in this manner further indicates its normality and ability to perform the functions associated with VE *in situ*.

Neonatal mouse UE and VE can be instructively induced by VS and US, respectively. However, this induction is age-dependent; neither UE or VE from adult mice can be instructively induced in this manner (30). Likewise, our cultured adult UE and VE retain their basic original morphology when grown with heterologous stroma, further indicating the similarity between cultured and normal epithelia.

The normal functioning of cultured VE and UE recombined with homologous stroma and grown *in vivo* is consistent with other reports that have shown that various cultured epithelia function normally when returned to their *in vivo* environment. Kollar and Kerley (31) demonstrated that cultured enamel organ epithelium reaggregated with either fresh or cultured dental papilla cells gives rise to normal teeth when transplanted *in vivo*. Cultured bovine corneal endothelial cells function normally when transplanted *in vivo* onto cat corneas denuded of their endothelium (32), and cultured mammary epithelial cells grafted into a cleared mammary fat pad *in vivo* develop into a normal, functional mammary ductal network (33).

In summary, our results indicate that cultured VE and UE reexpress normal morphology (i.e., the UE is simple columnar; the VE is stratified and cycles between a keratinized and mucified state) and estrogen responsiveness when reassociated with homologous stroma and grown *in vivo*. Thus, the loss of estrogen responsiveness in culture is not due to a transformation of the cells or the selection of a nonresponsive subpopulation but is more likely due to the altered conditions *in vitro*.

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