Implantation of the mouse blastocyst into a progesterone primed uterus occurs after an increase in circulating estrogen levels in the mouse. The cellular events first require changes in the uterine epithelial surface permitting attachment of the blastocyst. Changes induced by gonadal steroids are well documented in both the epithelium and stroma of the intact uterus, however, very little is known about how these changes are mediated. There is accumulating evidence in rodents that the proliferative response of uterine epithelium to estradiol in vivo probably requires interaction with the underlying stroma during development, before sexual maturity and at adulthood. A method of directly observing the cell types proliferating in response to estradiol in vitro was used in order to determine whether stromal interaction is required for epithelial response to estrogen. Primary cultures of uterine epithelium and stroma from either immature or adult mice in early estrus were maintained in a defined, serum-free medium, and were treated on day 2 of culture with $8 \times 10^{-9}$ M 17β-estradiol or control medium for 24 hours. Uterine epithelial and stromal cells were examined in monoculture and coculture, using cellular morphology and immunolocalization of cytokeratin to distinguish epithelial cells from stromal cells. Proliferation was assayed by antibody labeling of incorporated 5-bromo-2-deoxyuridine in individual cells. Estradiol-treatment increased the proliferation of the epithelium in cocultures of cells isolated from immature or adult mice (on the average of 2-fold and 4-fold, respectively). Proliferation
as high as 7-fold above controls occurred. Estradiol also stimulated proliferation in the stromal cells in coculture, albeit to a lesser extent. Treatment with 17β-estradiol suppressed proliferation of epithelial monocultures by 50% and stromal monocultures by 30% for cells isolated from immature mice. Estrogen-treatment of cells isolated from adult mice also decreased the proliferation of epithelial and stromal monoculture. The response of epithelium to estrogen in either cocultures or monocultures was greater than that of stromal cells. Density of the culture affected the baseline proliferation, which increased with increasing densities within the optimal range, and decreased at very high or very low culture densities. Cells were grouped from cocultures isolated from immature mice according to the type of cell and the type of cells each contacted directly. Epithelial cells that had physical contacts to both stromal and epithelial cells in the estrogen-treated coculture accounted for approximately 80% of the estradiol-stimulated proliferation in these cocultures. The specificity of the estradiol stimulation was shown by the addition of either 10⁻⁵ or 10⁻⁷ M tamoxifen to cocultures with estradiol, which prevented the estradiol-stimulated increase in proliferation. These results demonstrate that stromal cell interaction is necessary for the estrogen-induced proliferation of epithelium in vitro, and that this mediation requires cell contact with the epithelium or stromal-mediated changes in the microenvironment immediately around the epithelial cell.
Interactions Between Cultured Mouse Uterine Epithelial
and Stromal Cells During Estrogen-Induced Proliferation

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Chapter 1

Steroidally Induced Cellular Changes in the Endometrium During the Estrous Cycle in the Mouse – A Review of the Literature

1.1 Major Cell Types and Interactions within the Endometrium

1.1.1 Cell Types within the Endometrium

The uterus is composed of two compartments: the endometrium and myometrium. The endometrium consists of epithelium, stroma and the basement membrane which separates the two tissue types. The myometrium contains circular and longitudinal smooth muscle layers separated by and surrounded by connective tissue.

The simple, columnar epithelium which lines the luminal surface of the uterus has a number of tubular invaginations known as uterine glands. The number of glands present in the uterus varies among species from a relatively low
number of straight glands in rodents to a high number of tortuous glands present in primates [1]. Luminal and glandular epithelial cells have typical junctional complexes consisting of tight junctions, gap junctions and desmosomes on the lateral regions of their plasma membranes. These allow epithelial cells to maintain the integrity of the sheet and communicate with each other. Apical and basal surfaces of the cells are different in form and function. This polarity has been demonstrated by primarily basal uptake of methionine and secretion of prostaglandins, primarily apical secretion of proteins, and localization of a number of membrane components including apically located cell adhesion molecule-105 (CAM-105) [2, 3, 4].

Fibroblasts make up the cellular portion of the stroma and secrete the matrix in which they are embedded. Primary components of the stromal matrix are collagen type I, chondroitin sulfate proteoglycans, and fibronectin, while laminin and collagens type III, V and VI are present in lesser quantities [5, 6, 7]. Vascular, lymphatic and neural structures are also present in the stromal layer, as in other loose connective tissues.

The basement membrane separating the epithelium and stroma is a mesh of fibers produced by both cell types. Basement membrane components produced by epithelium are referred to as the basal lamina. Collagen IV, laminin, entactin/nidogen, and heparan sulfate proteoglycans make up the basal lamina primarily, but stromal cells may produce small amounts of these products as well [8]. Below the basal lamina layer of basement membrane is the lamina reticularis, which is produced by the stromal cells, and resembles the stromal extracellular
matrix. Collagen I, fibronectin and chondroitin sulfate proteoglycans constitute the lamina reticularis [9, 10].

1.1.2 Communication between Epithelial and Stromal Cells

Communication between cells could occur via direct contact between cells, through changes in attachments to basement membrane, or changes in molecules in the cell surface/basement membrane microenvironment. Extracellular matrix (ECM) molecules include basement membrane components as well as other matrix molecules such as those in the stromal matrix. Basement membrane components may be involved in facilitating communication between epithelial and stromal cells through intimate connections between basement membrane molecules, other ECM molecules, the basal epithelial surface, and the proximal surface of the stromal cells. In order to maintain these contacts, ECM components contain sequences which bind specifically to cell membrane proteins or regions on other matrix components. For instance, laminin binds collagen IV, the cell surface, and heparin [11]. Fibronectin binds to cellular receptors called integrins, collagen, heparan sulfate proteoglycans, and fibrin [12]. Nidogen binds collagen IV and laminin [13].

The presence of a basement membrane in culture as well as the type of ECM molecules in the basement membrane can influence morphology and physiology of the attached cell. Normal rat hepatocytes maintain mRNA synthesis and stability typical to their in vivo tissue state only when both a hormonally defined serum-free medium and a basement membrane are provided for the cultured cells [14].
Culture of insulinoma cell lines on Matrigel, an artificial basal lamina-like matrix isolated from Engelbreth Holm Swarm tumors of mice, improved their production of insulin and response to glucose [15]. Mammary epithelium grown on mammary basement membrane maintains a 160-fold higher production of casein and lactalbumin than cells cultured on plastic [16]. Pulmonary epithelial cells cultured on laminin maintained differentiated characteristics, whereas pulmonary epithelial cells cultured on fibronectin lost their differentiated state [17].

Rat uterine epithelial cells remain polarized (have defined apical and basal surfaces like *in vivo*) when cultured on Matrigel-coated filters. This polarization was demonstrated by greater protein secretion from the apical, as compared to the basal surface (9:1), and a larger uptake of radioactive methionine from the basal, compared to the apical surface (5:1) [4]. Polarized epithelial cells secrete from their apical surfaces molecules known to be upregulated by estrogen such as prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$), complement component C3, the secreted portion of the IgA receptor, and uterine secretory protein-1 (USP-1) [2, 3, 4]. CAM-105 is also present on the apical surface of the polarized cells [4]. Uterine epithelial cells in these experiments were always cultured in the presence of 17$\beta$-estradiol, so the possibility that these secreted proteins could be produced in this culture situation without estrogen-stimulation cannot be ruled out. However, the lack of production of PGF$_{2\alpha}$ by non-polarized epithelium cultured in the presence of estradiol does confirm that the polarized morphology resulting from culture on Matrigel and access to medium through the basal cell surface was necessary for at
least some physiological conditions of the cell [3].

The matrix can alter gene regulation by hormones \textit{in vitro} in a tissue specific manner to recreate a more \textit{in vivo}-like response [18]. Mammary epithelial cells isolated from pregnant or lactating mice and cultured on ECM produce large amounts of transferrin mRNA in response to lactogenic hormones as \textit{in vivo} [16]. Mammary epithelium does not show this response to hormones in the medium when cultured on plastic. Hormones can also alter the composition of the extracellular matrix through stimulating deposition of new matrix molecules or degradation of the old matrix molecules. Fibroblasts increase fibronectin biosynthesis and osteoblasts increase collagen I biosynthesis in response to glucocorticoids [19, 20]. Estriol, 17\(\beta\)-estradiol, or interleukin 1\(\beta\) stimulated collagenase production by cervical cells in culture [21]. Changes in composition of the matrix could in turn alter the interactions between epithelial and stromal cells.

It is unclear how hormonal response is modified by matrix components. Two possible mediators are extracellular matrix-induced cell shape change and changes in the population of growth factors bound to ECM molecules within the basement membrane [18]. Folkman and Moscona demonstrated that cell shape can alter proliferation [22]. They cultured different cell lines on coated plastic which created varying degrees of adherence by the cells. Several different cell shapes ranging between flattened and round resulted, and as the cells became more flattened they had a higher incidence of cell division as measured by incorporation of tritiated thymidine. Matrix induced shape change and growth factors may have synergistic
effects on cells. Corneal epithelium cultured on plastic has a flattened morphology and does not increase proliferation in response to epidermal growth factor (EGF), but is sensitive to fibroblast growth factor (FGF). When corneal epithelium is cultured on collagen which allows a columnar shape to be maintained, the cells do not proliferate in response to FGF, but proliferate when EGF is added [23].

Extracellular matrix molecules often contain binding sites for growth factors or sequences which themselves can cause growth factor-like effects by binding to growth factor receptors. Growth factors effect cell division by causing cells to become competent to divide (an early event in G₁), or by causing the progression of competent cells through G₁ from an arrested point [24]. Heparan sulfate proteoglycan chains contain sequences which bind to basic fibroblast growth factor (bFGF), albeit with a lower affinity than that of its dominant cell surface receptor [25]. One member of the EGF family, HB-EGF, binds heparin [26]. Glycosaminoglycans isolated from the ovary interact or complex with EGF as shown by the co-precipitation of angiogenic activity with the glycosaminoglycan fraction [27]. Laminin has been shown to contain EGF-like repeats, which can stimulate DNA synthesis only in cell lines that contain EGF receptors [28]. Nidogen and the fibronectin receptor have also been shown to contain EGF-like repeats [13, 29]. Matrigel has been shown to contain bFGF, transforming growth factor-beta (TGF-β) and tissue plasminogen activator (TPA) [25, 30, 31]. There is evidence to suggest that either EGF, TGF-α, which has similar structure and function to EGF and acts through the EGF receptor, or both are present in Matrigel since cultured kidney epithelium form
tubules on Matrigel or on protein-stripped Matrigel with either EGF or TGF-α added [31]. These observations have been used to suggest that the ECM could act as a reservoir for growth factors. Changes produced in the microenvironment around the cell including degradation of ECM molecules by the steroid-stimulated release of hydrolases from the cell could release growth factors bound to the ECM. These growth factors then would be free to bind the higher affinity cell surface receptors and facilitate cellular changes such as progression from G₀ to G₁ of the cell cycle.

Epithelial and stromal cells directly contact one another in at least some species besides sharing connections to ECM molecules. Transmission electron micrographs of human endometrium showed that basilar epithelial projections through the basal lamina increased in response to increasing plasma estrogen levels during the menstrual cycle. Cytoplasmic projections are sparse during the luteal phase, but increase in number and form increased numbers of gap junctions with other epithelial processes during the proliferative phase when estrogen is highest. These complex projections also connect with stroma cell projections growing up through the basal lamina during the mid-proliferative phase [32]. Gap junctions allow the passage of chemical or electrical signals between cells, and have been implicated in cell communication [33, 34, 35]. Thus there are many possible ways for the matrix and/or stroma to communicate with the epithelium; an interaction that may be necessary for the epithelial response to estrogen.
1.2 The Estrous Cycle in the Laboratory Mouse

The estrous cycle is the female reproductive cycle in non-primate mammals. It is characterized by the remodeling and slight thickening of the uterine endometrium so that it can support the implantation and growth of an embryo in the event of fertilization during estrus. Growth of the endometrium is due to hypertrophy (increased cell size due to higher fluid volume of the organ) and hyperplasia (increased cell division) of both epithelial and stromal cell layers in response to increased estrogen and progesterone.

The following description of the estrous cycle summarizes the most generally accepted understanding of the process [36, 37, 1, 38]. The estrous cycle is controlled by changes in secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary, which cause changes in the plasma levels of estrogen and progesterone secreted by the ovary (Figure 1). Pulsatile releases of luteinizing hormone releasing hormone (LHRH) from the arcuate nucleus of the hypothalamus cause the pulsatile release of LH and FSH from the anterior pituitary. Several neurotransmitters are involved in the release of LHRH, including noradrenergic, dopaminergic, and opioid neuronal networks. The mechanism of control of LHRH release, and how estrogen contributes by both positive and negative control is not fully understood. The release of FSH is also under positive control by activin and negative control by inhibin. Inhibin and activin are peptide hormones produced by the ovary, and differ only in one of the two
peptide subunits from each other. The production of inhibin is probably stimulated by gonadotropins. The ovary also produces \( \beta \)-endorphin, adrenocorticotropin hormone (ACTH), \( \alpha \)-melanocyte stimulating hormone (\( \alpha \)-MSH), vasopressin, and oxytocin. However, the physiological role of ovarian peptide hormones is still unclear. The release of LH and FSH occurs approximately every hour (70-100 minutes), and each subsequent pulse contains increasingly larger amounts of LH and FSH. The amount of FSH released is approximately half of LH released in any pulse. Luteinizing hormone receptors are present on the plasma membranes of thecal, granulosa, and stromal cells in the ovary. The binding of LH to its receptor activates the enzymes needed to convert cholesterol to progesterone in both cell types, and the enzymes necessary to convert progesterone to androstenedione. Androstenedione in a thecal cell is converted to testosterone, or can be transported to a granulosa cell where it is converted to estrone. Testosterone is also transported to the granulosa cell where it can be aromatized to 17\( \beta \)-estradiol. Aromatase activity is stimulated by the binding of FSH to its receptors which are present exclusively on granulosa cell membranes. The enzymes responsible for converting cholesterol to progesterone in the granulosa cell are also stimulated by FSH. Estrogen causes the increase of estrogen receptors in LH responsive cells of the ovary which produce estrogen, therefore, the signal is amplified between diestrus and proestrus and estrogen release increases at a higher rate.

The mouse completes an estrous cycle every four to five days as measured by the state of the the vaginal epithelium which can be assessed by flushing the vagina
with water or saline and examining the fluid microscopically [39]. The correlation between vaginal lavage and the histological state of the uterus and the vagina was described in 1922 even though the hormones responsible had not yet been discovered [40]. During diestrus the vaginal lavage is dominated by leukocytes, and contains only few rounded epithelial cells. The high number of leukocytes in the lavage occurs because the cervical epithelium, like the uterine endometrium, is thinnest at this stage allowing the leukocytes to infiltrate the epithelium more easily. Plasma progestagen levels are slightly elevated during metestrus and diestrus due to progesterone and 20α-dihydroprogesterone secretion from the interstitial cells of the ovary [36]. These cells have been referred to as the “permanent corpus luteum” of rodents [41]. As the cycle progresses, the vaginal lavage shows increasing numbers of rounded epithelial cells, and by the end of early estrus, the presence of cornified epithelial cells. The increase of epithelial cells in the lavage is indicative of the estrogen-promoted growth of the epithelial layers of both the vagina and uterus [42, 36]. Estrogen also causes the cornification of the vaginal epithelium after the stratified squamous epithelium reaches a critical thickness [43, 44, 45]. Estrus vaginal lavage contains only cornified epithelial cells. Leukocytes collect in the subepithelial zone during metestrus and invade the epithelial layer [40]. The reappearance of leukocytes in the lavage occurs in metestrus when degeneration of the epithelial layers of the vagina and uterus is taking place. Flakes of the uterine luminal epithelium and layers of cornified vaginal epithelium are lost during metestrus of each cycle, then replaced during the next cycle through stimulation.
by estrogen. However, the luminal epithelium continues to be present as a layer throughout the mouse estrous cycle [46].

![Graph showing changes in estrogen and progestagens](image1)

Figure 1. Relative changes in the concentrations of estrogen and progestagens during the rat estrous cycle and early pregnancy (reproduced from Svalander [47]).

1.3 Molecular Mechanisms of Steroid Action

Steroid hormones exert their effects on target cells by binding to specific receptors. Immunohistochemical studies have localized progesterone, estrogen, and androgen receptors to the cell nucleus even in the absence of hormone [48]. Several studies using anti-glucocorticoid receptor antibodies reported glucocorticoid receptors in the cell cytoplasm [49, 50, 51, 52]. Another immunohistochemical study showed that this cytoplasmic immunoreactivity is due to diffusion of the glucocorticoid receptor from the nucleus in the absence of hormone [53]. And recently, another immunocytochemical study has localized the unliganded glucocorticoid receptor
exclusively to the nucleus in hepatoma cells [54]. Therefore, the glucocorticoid receptors are probably also localized to the nucleus regardless of the presence of hormones. There are cases where steroids bind to a receptor located on the plasma membrane as well. Frog eggs have progesterone receptors on their plasma membranes that bind progesterone released from the ovary, and stimulate germinal vesicle breakdown and release of the primary oocyte from meiotic arrest [55]. Corticosterone binds to sites on the neuronal cell membrane in the newt brain and rapidly suppresses male reproductive behavior [56]. Steroid receptor proteins belong to a large superfamily of ligand-activated DNA binding transcription effectors including the vitamin D3 and vitamin A receptors, Drosophila juvenile hormone receptor, thyroid receptor, orphan receptors which are similar in structure to the family but do not have identified ligands, as well as the glucocorticoid receptors and sex steroid receptors [57, 58].

Steroids circulate in the plasma primarily bound to steroid binding proteins (SBP). Only 1-2% of testosterone, estrogen, and progesterone are not bound to SBP in human blood plasma [59]. Measurements of unbound estrogen in rat plasma have shown that the free fraction of estrogen is < 1% in immature rats, but rises to 4% at maturity [60]. Originally, the consensus was that steroid binding proteins acted as a simple buffer reserve for sex steroid, and the unbound steroids were the active portion of the hormonal pool [59]. However, the bound steroid may be the active fraction. Two types of evidence indicate that steroid binding proteins may affect transfer of hormone to the cell: (1)
Steroid binding proteins have been detected in cells other than the site of synthesis [61, 62, 63, 64]. (2) Membrane specific binding sites for steroid binding proteins have been isolated from cell membranes from hyperplastic prostate, decidual endometrium, pre-menopausal endometrium, endometrial adenocarcinoma, liver, and post-menopausal breast [65, 66, 67]. Two opposite models have been presented to explain these evidence: (1) The model derived from research on prostate states that the binding of steroid-SBP to a membrane binding site for SBP inhibits entry of the steroid into the cell [68]. (2) The model derived from research on decidual endometrium states that binding of the steroid-SBP to its binding site facilitates steroid transport into the cell [66].

The activated steroid-receptor complex binds to the DNA and activates a cascade of gene transcription by a well studied series of events. The consensus is that steroids enter the cell by diffusion through the plasma membrane and move by simple diffusion to the nucleus where they bind the appropriate receptor [58]. Some researchers believe that movement through the cytoplasm is facilitated by cytoplasmic components. High affinity binding sites for estrogen and testosterone were found on the microsomal membranes of rat uterine tissue and ventral prostate, respectively [69]. These sites were hypothesized to act as carriers to move steroids into the nucleus.

The unliganded steroid receptor protein exists complexed to several proteins including heat-shock proteins 70 and 90, and is unable to bind DNA in this state [58]. The binding of the steroid molecule to the receptor causes dissociation
of other proteins and mediates receptor activation through allosteric change in the receptor followed by a covalent modification [58]. The trans-activation activity of the estrogen receptor is located on the C-terminus of the receptor and becomes active only after ligand binding [70]. It is generally accepted that phosphorylation of the receptor by serine and threonine protein kinases (also tyrosine on the estrogen receptor) have some role in the final activation of the steroid/receptor complex [57, 71]. The receptor/steroid complex dimerizes then binds to enhancer sequences called steroid response elements which are located 5' to or within the genes that the steroid activates or represses in the cell. These enhancer regions are palindromic and contain two half-sites, each of which binds a DNA binding region on one of the dimerized receptor proteins through the interaction of the DNA with two zinc finger motifs on each receptor. Finger swap experiments have shown that the amino terminal zinc finger determines the target gene specificity [57]. Ligand-receptor-complex binding to the DNA activates a cascade of gene transcription thought to be brought about by the stabilization of transcription factors situated at the promoter of the gene with the bound steroid response element. The stabilization could be brought about by unspecific or ubiquitous interactions between transcription factors and the hormone receptor [72]. Some of these transcripts code for regulatory proteins themselves (early gene products) which activate other tissue specific structural genes (late genes) associated with the steroid action.

Through conventional methods steroids cannot effect changes in cells which do not contain receptors. Therefore, autoradiographic or immunohistochemical
analysis of tissues, or quantitative assays for the appropriate receptor are commonly performed to determine whether the cell is able to respond directly to steroids. Receptor isolation techniques allow a portion of the estrogen or progesterone receptors to leak out of the nucleus into the cytoplasmic fraction. Therefore, the total number of receptors is usually estimated by adding the number of receptors from both fractions together. Sometimes receptors isolated in the nuclear fraction are the only receptors measured because such receptors are a better measure of those that are biologically active and closely associated with the chromatin at the time of extraction.

When two or more steroid hormones are accessible to cells, there can be synergistic responses by cells which are different from the response to each individual hormone. For instance, when ovariectomized mice injected daily with three doses of progesterone before injection of a single dose of estradiol, the number of mitoses in the uterine stromal cells increase dramatically (1.4 ±0.2 to 143.0 ±17.3 stromal mitoses per uterine section) [73]. Without progesterone priming mitoses increase slightly above untreated controls (0.2 ±0.2 to 4.5 ±1.1 stromal mitoses per uterine section). Administration of a large dose of dexamethasone 20-25 minutes prior to estrogen injection inhibits estradiol induced uterine weight gain [74]. Dexamethasone administration at various times after estradiol injection also reduced long term growth in the uterus [74]. Through studies measuring the quantity of nuclear-bound, receptor-estrogen complexes and the baseline rates of proliferation, it has been hypothesized that the effect of dexamethasone on proliferation is indirect and
actually affects estrogen-independent proliferation [75, 74]. Hydrocortisone is less potent than dexamethasone, however, hydrocortisone may decrease the baseline level of cell division in cultures where it is a medium supplement. Hydrocortisone is a common additive to serum-free medium, and glucocorticoid-receptor binding can cause the upregulation of the glucocorticoid receptor in stimulated cells [76]. Also, the location of a high-affinity progesterone-binding site on the glucocorticoid receptor was discovered and characterized in AtT-20 cells [77]. So the amount of progesterone binding to the glucocorticoid receptor may also be increased by the regular addition of hydrocortisone to the media, and make measurements of progesterone action based solely on progesterone receptor extraction and analysis less accurate.

1.4 Estrogen and Progesterone Action in the Endometrium

Estrogen and progesterone cause endometrial cells to undergo hypertrophy, hyperplasia, initiate gene activation for a number of molecules, and increases vascularization of the endometrium following the release of estrogen-induced chemotactic factors which cause neutrophil infiltration of the stroma. Progesterone’s primary target is the stroma, however, it also acts to decrease some estrogenic effects in the epithelium [78]. Estrogen’s primary target is the epithelium where it causes upregulation of steroid receptors, stimulates transcription of many growth factors, their receptors, proto-oncogene products, cell surface molecules and secretory products
Estrogenic responses are grouped into early and late effects. Early responses include imbibition of water and increases in blood flow resulting in hypertrophy, glucose oxidation, lipid synthesis, RNA polymerase activity, incorporation of nucleotide precursors into RNA, incorporation of amino acids into proteins, and transcription and translation of oncogene products including some growth factors and their receptors. These events occur 1-6 hours after estrogen treatment. Early responses can be induced by weak estrogens such as estriol and estrone. These estrogens have a lower affinity for the estrogen receptor than 17β-estradiol, and are retained in the nucleus less than six hours. Late responses include hyperplasia, continued stimulation of polymerase activity, and increases in DNA and protein synthesis. Estrogen must be retained in the nucleus for 6-8 hours for the late responses to occur. Strong estrogens such as 17β-estradiol or the synthetic estrogen diethylstilbestrol stimulate late effects. A single dose of estriol or estrone cannot stimulate late effects ordinarily. However, if the dose of estriol or estrone is large enough or administered multiple times, then the hormone continues to occupy the receptor and late effects can be stimulated. Estrone can also be converted into 17β-estradiol, which can initiate late responses. Estrone is a more potent stimulator of proliferation in the mouse uterine epithelium than estriol [86], inducing approximately 60% of the proliferative response, and 80% of the RNA transcription that 17β-estradiol stimulates [87, 88]. Estriol is more efficient at inducing protein synthesis than estrone [89]. It stimulates hyperplasia in the mouse uterine epithe-
lium, but does not protect against apoptosis as $17\beta$-estradiol does [90]. Some of the relative potencies of estriol and estrone may be due to the differences in the vehicles (saline or oil) in which the hormones were dissolved and injected [91]. Diethylstilbestrol and $17\beta$-estradiol are the estrogens most commonly used recently, since they elicit all the estrogenic responses strongly in vivo.

The earliest noted transcriptional regulation by estrogen is an increase in proto-oncogene products which act as transcription activators themselves. These include c-fos, c-myc, c-jun, jun-B, jun-D and H-ras [92, 82, 93]. Maximal increases in mRNA of the fos and jun family occur three hours after $17\beta$-estradiol stimulation. These products are the primary constituents of the AP-1 transcription activator, which could be involved in promoting the $G_0$ to $G_1$ transition causing quiescent cells to enter mitosis, or in promoting a number of other gene functions. Jun-B can act as a negative regulator of c-jun, so that AP-1 may perform different functions if levels of its constituents are altered [94, 95]. C-fos, c-jun and c-myc can also be regulated by tissue plasminogen activator in the endometrium, showing that non-steroidal activation may compete with steroidally induced activation to provide cross-talk between estrogen and other factors in the endometrial environment [94].

Other early responses include stimulation of growth factors and their receptors. Estrogen has been shown to stimulate EGF, insulin-like growth factor (IGF), their receptors, TGF-$\beta$, and colony stimulating factor-1 (CSF-1) in the endometrium. Both EGF and IGF stimulation can cause hyperplasia. Epidermal
growth factor stimulated cell division in cultured endometrial cells was inhibited by concurrent treatment with the antiestrogen, hydroxytamoxifen [80]. Basic fibroblast growth factor has also been shown to be present in the basal lamina and matrix of the rodent uterus, but the amount does not change during the estrous cycle [96].

The different early responses may work as part of a cascade to induce late responses to estrogen. Estrogen stimulation of growth factors or their receptors, and correlations between proto-oncogene expression and growth factor expression have been shown in other cell and organ types. Estrogen induction of DNA synthesis has been shown to be mediated by TGF-β in rat granulosa cells in culture [97]. Transforming growth factor-β stimulates the production of the extracellular matrix components: collagen, fibronectin, integrin, tenascin, and proteoglycans [98]. It can also inhibit or stimulate cell division depending on cell type and cell location [99]. The varying effects which TGF-β can initiate, and the fact that it is stimulated by estrogen in the endometrium make it a possible mediator of at least some of the diverse responses of the endometrium to estrogen during estrous cycling and preparation for pregnancy. The growth factors that are induced by estrogen themselves induce “estrogenic” type responses. EGF stimulates tissue plasminogen activator mRNA in rat granulosa cells. EGF, IGF-1 and cyclic AMP upregulate progesterone receptors in cultured endometrial cells, all of which can be prevented by the addition of antiestrogens (ICI 164,384, tamoxifen, and 4-OH-tamoxifen). Elevated TGF-β1 and 3 are associated with ras and myc increases in
reconstituted mouse prostate [100].

Estrogen also induces changes in cell surface and secretory products. Changes in cell surface molecules may influence cell interactions with other cells and the basement membrane. Changes in association with basement membrane molecules can facilitate migration, cell cycling, or other processes such as differentiation. Estradiol also increases the basally expressed syndecan, which binds cells to collagens and fibronectin, and integrin, the receptors for collagen, fibronectin, and laminin respectively [101, 102, 103]. Estrogen stimulates the turnover of heparan sulfate proteoglycan (HSPG) chains on the luminal surface of the epithelium [104], and the production of uterine secretory protein-1 (USP-1) in rodents [85].

Effects of estrogen on the lipid metabolism of the rat uterus in vivo were noted as early as 1972 [105]. Estrogen stimulates the incorporation of tritiated inositol into inositol phospholipids and the breakdown of phosphatidylinositol into inositol polyphosphates in vivo in the mouse uterine epithelium [106, 107]. These effects occur 6-9 hours after injection of the hormone and have been blocked by antiestrogen or progesterone treatment, so it is unlikely that they are the result of non-traditional estrogen action pathways [108, 107]. Because this phospholipid turnover occurs before the onset of estrogen-induced DNA synthesis, membrane signal transduction may have some role in the stimulation of cell division by estrogen.
1.5 Estrogen and Cell Cycling in the Mouse Endometrium

Estrogen was first described as the factor which initiated estrus and as a "stimulator of mitoses in special tissues" [109]. Allen and Doisy described the extraction and partial purification of "theelin" from ovine liquor folliculi in 1923 [109]. Injection of this preparation into ovariectomized mice or rats stimulated estrus, as measured by vaginal lavage and histological examination of the uterus and vagina. Injection of "theelin" into a variety of strains of ovariectomized mice produced increased numbers of mitoses in the epithelia of the uterus, vagina, and breast [42]. Allen described the increase in mitoses as a "true hyperplasia" because the number of mitoses was unusually high for the tissue and a significant amount of new tissue was produced [42].

The number of mitoses in the luminal epithelium of ovariectomized mice treated with estradiol was higher than in ovariectomized controls, and increased as the dosage of estradiol increased [73]. A single injection of 0.1 μg of estradiol per mouse increased the mean number of mitoses per 5 μm section of uterine horn from zero to 50. Doses as high as 2.5 μg were injected, but could not increase the mean number of mitoses any higher than 65 per section. The number of mitoses in the epithelium also increased in a time dependent manner after estrogen injection. The number of mitoses began to increase at 12 hours, reached a maximum near 24 hours, and did not decrease to control levels until after 30 hours post-injection [42, 110, 86]. These investigations utilized colchicine to block all mitoses two hours
before the death and histological examination of the animal.

For over 50 years it has been known that the cells of the uterus maintain a low level of cell division. The number of mitoses in one 10 μ section of mouse uterine horn varies from 0.3% to 4% during the estrous cycle [111]. The DNA content of the uterus also remains relatively constant with the lowest content occurring at diestrus as measured by incorporation of radioactive phosphate [111]. Most studies of cell division in the uterus use some way of enhancing these numbers by labeling S-phase or blocking cytokinesis with colchicine or colcemid. It is also necessary to address the responses of the different cell types of the uterus separately. A large increase in cell division in the epithelium appears as a very small change in the entire uterus if it is the only tissue responding to the stimulus because the epithelium only constitutes 5-10% of the uterus by mass [112].

The establishment of estrogen as a stimulator of cell division initiated research on how estrogen affected the cell cycle. The DNA content of the uterus of the estrogen-treated ovariectomized mouse did not change appreciably, but the RNA content increased with estrogen treatment [113]. The nuclear volume of the uterine epithelial cells increased cyclically during the estrous cycle with a peak volume during metestrus, approximately 24 hours after the estrogen peak in the cycle indicating that changes in nuclear volume are within the time of estrogen stimulated events [114, 111]. These data led Salvatore to propose that estrogen was responsible for the replication of the genome, and that DNA replication may not always be immediately followed by mitosis [115]. Later experiments showed that
after DNA was synthesized, the cell would continue through mitosis and divide because the amount of DNA per cell did not change according to the photoelectric density of the Feulgen reaction of the uterine cell nuclei [116]. Therefore, each round of DNA synthesis must be followed by mitosis without the cell entering a quiescent period in G2. Bullough suggested that estrogen stimulated mitoses through the activation of the glucokinase pathway and subsequent flow of energy into the cell allowing the synthesis of DNA followed by mitosis [117]. This assumed that a large population of uterine epithelial cells were ready to enter S-phase when the cell energy increased. Swann maintained that this was an unlikely mechanism of estrogen action in tissues with low mitotic indices, such as the uterus, and that instead estrogen caused a switch in the cell metabolism to one that preferred synthesis of DNA and proteins necessary for mitosis [118].

Interest in how a hormone affects the different stages of the cell cycle fueled research about the cell cycle in unstimulated and stimulated epithelial and stromal cells. Injection of ovariectomized mice with estrone did not shorten mitosis, but did decrease the length of interphase in the uterine epithelial cells [110]. The average lengths of the cell cycles were estimated for luminal and glandular epithelial cells to be 270 hours and 156 hours, respectively [119]. Das stated, however, that the cell cycle was much too variable to obtain an accurate estimate, but that he could conclude that estrogen shortened the overall cycle time [119]. Other estimates of the length of the epithelial cell cycle have ranged from 82 to 757 hours, to illustrate the extent of the variation possible [110, 120, 87, 121, 122, 123].
Since Alfert had shown that G2 was not appreciably long, it was probable that G1 and/or S-phase was shortened by estrogen to cause the decrease in the cycle length [116]. Estimates of the mean length of S-phase and G1 varied considerably between researchers. This was attributed to differences in the type of estrogen used, strain of mouse used, and method used to determine to the lengths of the cell cycle stages [119]. Estimates of the mean length of S-phase in the unstimulated epithelial cell varied from 5 to 10.5 hours, and in the estrogen-stimulated cell from 2.2 to 7.7 hours [110, 120, 87, 121, 122, 119, 124]. Each report demonstrated that the mean time of S-phase was decreased by estrogen-treatment. This was most likely due to the elimination of longer S-phases in unstimulated cells, rather than an overall shortening of S-phase in each cell because labeled mitosis curves generated in the method of Quastler and Sherman showed that the length of S-phase in unstimulated cells varied and the length of S-phase in estrogen-treated cells did not [125, 119, 110]. Quastler and Sherman labeled mouse intestinal cells with tritiated thymidine and plotted the percent of labeled mitoses at varying times after labeling, then used equations for the kinetics of the cellular population to estimate cell cycle duration and stage length in the different cell types present in the examined tissue [125]. They state that if the descending and ascending limbs of the curve have different slopes, then the length of S-phase is variable. The reported mean length of G1 in unstimulated cells was not reported with confidence because it varied so widely, but estrogen appeared to shorten G1 [123, 124, 87, 121, 119, 110, 120, 126]. Smith and Martin later hypothesized that estrogen stimulated
proliferation by increasing the probability that any epithelial cell would become committed to one round of cell division [127], and this commitment would result in the shortening of G1.

Although most literature states that estrogen only appreciably stimulates cell division in the uterine epithelium, a closer examination shows that a small amount (1/10 to 1/4 of the stimulation in the epithelium) of cell division is stimulated in the stroma by injection of estrogen alone into ovariectomized mice. The number of stromal cells increased from 15 to 20 x 10^2 at 24 hours after estradiol injection [86]. The mean number of stromal mitoses increased from 0.2 ±0.2 to 4.5 ±1.1 per section 24 h after a single injection of estradiol [73]. Ovariectomized mice that were injected with [14C]lysine and estradiol showed that the total protein increased in the stroma half as much as the total protein in the epithelium. Also, increases in acidic nuclear proteins and histones occurred to a lesser degree in the stroma at the same time as increases occurred in the epithelium [128]. If the ovariectomized mouse was first primed with three daily injections of progesterone then a single injection caused a synchronized entry of stromal cells into S-phase then mitosis for one round of cell division [73].

Estrogen may act on the epithelium by first inducing changes in the underlying stroma. Estrogen does not initiate a proliferative response (measured by 3H-thymidine incorporation) in uterine epithelial cells cultured apart from stroma with or without a basement membrane. Increases in hypertrophy, protein synthesis, and secretion can be shown in uterine epithelium cultured on collagen, Matrigel
or nitrocellulose filters coated with Matrigel [4, 3]. However, the possibility that
the response when cultured on Matrigel may be due at least in part to the pres­
ence of growth factors sequestered in Matrigel has not been ruled out [25, 30, 31].
There was one attempt to ascertain whether estrogen administered to cocultured
epithelium and stroma caused increased proliferation in the epithelium by mea­
suring incorporation of tritiated thymidine. The total thymidine incorporation for
the culture increased in the presence of estrogen, though the amount attributable
to stroma could not be identified [129]. Estrogen receptors do not appear in
C57BL/J6 mouse uterine epithelial cells until the fifth or sixth day after birth,
but estrogen administered on the second day after birth, when estrogen receptors
are present only in the nuclei of stromal cells, does cause epithelial cell prolifera­
tion [130, 131]. There is also in vivo evidence indicating stromal-epithelial cell
contact may be necessary for steroidogenic effects in the epithelia of other or­
gans including prostate, seminiferous tubule, vagina and cervix [112]. All of this
evidence taken together strongly suggests that interaction between stroma and
epithelium may be necessary for the estrogen-induced proliferation of the uterine
epithelium in vivo.
Chapter 2

Estradiol-17β Stimulates Hyperplasia of both Uterine Epithelial and Stromal Cells in Coculture, but not in Separate Culture in Serum-free Defined Medium

2.1 Abstract

There is indirect evidence that the in vivo proliferative response of uterine epithelium to estradiol in rodents requires interaction with the underlying stroma during development, before sexual maturity and at adulthood. To examine this potential requirement directly, the proliferative response of epithelium to estrogen in the presence of stroma was carried out in vitro. Uterine epithelial and stromal cells were isolated separately from immature or adult mice, and were maintained as monocultures or cocultures in defined, serum-free medium with or without $8 \times 10^{-9}$ M 17β-estradiol. To measure proliferation, the incorporation of BrdU into the cell’s DNA during three hours of labeling was assayed in individual
cells by immunolabeling of incorporated 5-bromo-2-deoxyuridine. Immunolabeling of cytokeratin and cell morphology were used to distinguish epithelial from stromal cells. Treatment of cocultures with estradiol for 24 hours increased the proliferation of epithelial and stromal cells relative to controls (1.5- to 4-fold and up to 2-fold, respectively), whereas, in monocultures of epithelial or stromal cells estradiol decreased the number of BrdU-incorporating cells (50% and 20% of controls, respectively). The epithelial cells within a coculture were categorized according to the type of cells with which they maintained physical contacts. Approximately 80% of the epithelial cells that responded to estradiol were touching both stromal and other epithelial cells. These results demonstrate that stromal cells mediate the estrogenic proliferative response in vitro, and that this mediation requires cell contact or stroma-mediated changes in the microenvironment immediately around the epithelial cell.

2.2 Introduction

For over 50 years it has been known that estrogen stimulates increased cell division, or hyperplasia, in the mouse uterine epithelium in vivo [42]. However, it is still unclear whether estrogen acts directly on the epithelium through its own receptors or requires interactions with the underlying stromal cells. It has been well documented that 17β-estradiol administered to ovariectomized or immature rodents in vivo causes hyperplasia of the epithelium [42, 73, 86], hypertrophy [86],
and increased vascularization of the endometrium [132, 91]. Estrogen also induces transcriptional activation of genes encoding the progesterone receptor [79], estrogen receptor [133], a number of growth factors and their receptors [92, 134], enzymes [135, 136, 137, 138, 139, 140, 141, 142, 143, 144], cell surface and secretory molecules [85, 47, 104, 145, 146, 147, 3, 148], and proto-oncogene products thought to be involved in mediating other estrogenic responses [92, 93].

Recently evidence has accumulated that implicates several growth factors and oncogene products as intermediates in estrogen action in several tissues. In cultured endometrium, EGF stimulated increased levels of progesterone receptors in epithelial cells, and was inhibited by anti-estrogen treatment [80]. An antibody specific to EGF inhibited estrogen-induced growth and differentiation of the mouse uterus and vagina in vivo [149]. Administration of EGF prevented indomethacin-induced inhibition of estriol-induced embryo implantation in the mouse [150]. Treatment of cultured rat granulosa cells with a neutralizing antibody to TGF-β inhibited estrogen-induced DNA synthesis [97]. Estradiol increased the amount of TGF-β in rat pituitary tumors that were growth-inhibited by estradiol, but did not change TGF-β levels in tumors that were insensitive to estradiol [151]. Estrogen may also initiate some of its effects through non-genomic mediators. The inositol triphosphate signal transduction system was maximally activated in the mouse uterine epithelium six hours after injection of either diethylstilbesterol or 17β-estradiol [106, 107, 108]. This increase in phosphoinositide turnover was inhibited by the administration of anti-estrogens or progesterone [106, 108].
Hyperplasia is a late response to estrogen, and occurs within a distinct range of time after stimulation. Mitoses begin to increase 12 hours after estrogen injection with the peak stimulation of cell division occurring at 24 hours and decreasing by 30 to 36 hours after estrogen administration in vivo [73]. Several peptide growth factors and proto-oncogene products that are upregulated by estrogen can also induce cell division, including IGF-I [134], EGF [149], Fos, Myc, and the Jun family [95]. All of this evidence taken together suggests that the stimulation of cell division by estrogen is likely to result from a complex mechanism involving at least some of these intermediates.

Culture of primary isolated cells from the endometrium is an ideal method for examining the mechanism of estrogen action without the complex interactions that exist in vivo. However, estrogen does not stimulate uterine [152], mammary [153], or vaginal [154] epithelial cell division when cultured as epithelial monolayers on plastic. Estrogen also does not increase proliferation of vaginal or uterine epithelium cultured on collagen or Matrigel, an artificial basement membrane prepared from Engelbreth Holm Swarm sarcoma of mice [155, 156, 4]. Implication of stroma as an intermediate in the induction of proliferation in epithelium was shown when uterine or vaginal epithelium were recombined with stroma in a collagen gel and grown under the kidney capsule in mice; the epithelium responded to estrogen by increased cell division [157]. An attempt to duplicate this interaction using mouse uterine epithelial and stromal cells cocultured on tissue culture plastic in the absence of the complex humoral milieu of the intact mouse showed an overall
increase in thymidine incorporation in response to estradiol [129]. Unfortunately, no attempt was made to exclude the possibility that the increased incorporation included or was due exclusively to stromal cells. Thus, despite considerable indirect evidence suggesting that stroma mediates the hyperplastic response of epithelium to estradiol, the question still has not been rigorously tested.

We have investigated this question using primary isolated mouse uterine epithelial and stromal cells cocultured or cultured separately. The proliferative response of morphologically and immunocytochemically identified stromal and epithelial cells to estradiol was directly observed by the visualization of 5-bromo-2-deoxyuridine incorporated into nucleus during DNA synthesis. We confirm that epithelial cells require interaction with stromal cells for this response and that proliferation in the interacting stromal cells is also enhanced by physiological levels of 17β-estradiol. We also demonstrate by analysis of specific cell groupings that the response requires either physical contact or short range signals between epithelial and stromal cells.

2.3 Materials and Methods

2.3.1 Materials

DMEM/F12 mixture was purchased from Gibco (Grañ Island, NY). Tissue culture supplements, 17β-estradiol, trypsin, pancreatin V, collagenase IV, extravidin, and 2,3 diaminobenzidine were purchased from Sigma (St. Louis, MO). Polyclonal
Cytokeratin Immunostaining Kit was purchased from BioGenex (San Ramon, CA). Monoclonal antibody against 5-bromo-2-deoxyuridine was purchased from DAKO Corporation (Carpenteria, CA). Anti-mouse IgG conjugated to biotin was purchased from Vector (Burlingame, CA). The chromogen 4-chloro-1-naphthol was purchased from BioRad (Richmond, CA).

2.3.2 Animals

Immature and 6-week old female CF-1 mice were purchased from Charles River Laboratories (Wilmington, MA) or bred and raised in the facility at this university. Mature CF-1 females (6 weeks old) were staged for early estrus of the estrous cycle by vaginal lavage [39], and sacrificed by cervical dislocation immediately before each experiment. Immature females were sacrificed between 21 and 25 days after birth. Animals were maintained by the University Laboratory Animal Resources staff in accordance with NIH guidelines and fed ad libitum.

2.3.3 Cell Culture

Uteri were cleaned of fat and mesometrium then slit open longitudinally and cut into four pieces. Uteri were washed with Hanks’ Balanced Salt Solution (BSS) followed by calcium- and magnesium-free BSS (CMF). Epithelial cell flakes were isolated by trypsin and pancreatin digestion as before [158]. After removal of the epithelium, uteri were incubated in CMF for 20 minutes then triturated gently with a wide bore pipette before removing the stroma to decrease the amount of
epithelium contaminating the stromal fraction. Stromal cells were loosened from
the uteri by digestion with 2.5 mg/ml collagenase for one hour at room temperature
with gentle shaking on a rotary shaker. After the collagenase was removed, the
uteri were triturated gently in plating medium with a wide-bore pipette to remove
cells. The stromal cells were washed twice in plating medium then counted for
plating.

The epithelial cell suspension was counted in a hemacytometer, however,
the densities were used only as estimations for plating since these were not single
cell suspensions and the number of cells per flake was estimated. An estimated
plating density between 100 and 200 cells/mm² was used for cultures. Actual cell
densities were determined at the time of fixation of the cultures. Stromal cell
suspensions were counted by hemacytometer and plated at the same densities as
the epithelial cells. Cocultures were prepared by allowing epithelium to attach for
24 hours then adding an equal number of stromal cells to the culture, as previ­
ously described [129]. Stromal cells used for cocultures were plated into a 25 cm²
culture flask after the cell isolation and removed from the flask the following day
by trypsinization then recounted in the presence of Trypan blue before replating.
Cells were grown on glass coverslips in 35 mm culture dishes to facilitate easy re­
moval of culture for immunocytochemistry. Cultures used for growth curves were
plated directly into 35 mm culture dishes.

All cultures were maintained at 37°C in a humidified chamber with 5%
carbon dioxide and 95% air in a defined, serum-free maintenance medium con-
sisting of DMEM/F-12 mixture supplemented with insulin (5\(\mu g/ml\)), transferrin (10 \(\mu g/ml\)), hydrocortisone (10\(^{-6}\)M), essentially globin free BSA (2mg/ml), penicillin(100 IU/ml) and streptomycin (0.1mg/ml). Plating medium also contained fetuin (1mg/ml) and was replaced at 24 hours with maintenance medium. Cultures were maintained for four days with daily medium changes to eliminate the possibility of depletion of the medium additives. Steroids were first dissolved in ethanol then diluted to the appropriate concentration in medium, with the concentration of ethanol never exceeding 0.1% in the culture medium. Cultures were incubated with 0.01 M 5-bromo-2-deoxyuridine (BrdU) for 3 hours to label proliferating cells. The labeling medium was replaced with medium containing 10\(^{-4}\)M thymidine for one hour before fixation to remove unincorporated BrdU from the cell cytoplasm and decrease background staining during immunocytochemistry.

2.3.4 Immunocytochemical Staining

Cultures on coverslips were sequentially immunostained for BrdU and for the epithelial cell marker, cytokeratin. All incubations were performed at room temperature unless otherwise specified. Coverslips containing cultures were removed from culture dishes, rinsed in BSS, and fixed for 15 minutes in 100% methanol at 4\(^\circ\)C. After fixation coverslips were incubated for one hour at 37\(^\circ\)C in 2 N HCl to denature the DNA, then neutralized with two rinses of borate buffer followed by PBS (pH = 7.4). Coverslips were incubated in horse serum diluted in PBS to block nonspecific binding. A mouse monoclonal antibody to BrdU was applied and incubated for
one hour at 37°C. Afterward coverslips were incubated for 20 minutes each with biotinylated antibody against mouse immunoglobulin then Extravidin conjugated to peroxidase. Coverslips were washed twice with PBS between antibody incubations to remove un-bound antibody from the coverslips. The antibody against BrdU was visualized using the chromogen 2,4-diamino benzidine (DAB) which produced a brown precipitate in nuclei containing DNA that had incorporated BrdU. Following immunostaining for BrdU, the cultures were immunostained for cytokeratin according to the procedure outlined in the manufacturers’ directions. The anti-cytokeratin was visualized by the chromogen 4-chloro-1-Naphthol which forms a blue precipitate. Precipitated DAB inhibits local peroxidase activity [159, 160], so that the 4-chloro-1-naphthol precipitates specific to areas where anti-cytokeratin binds as long as the DAB background remains low. If the DAB background is high all of the peroxidase activity is inhibited and none of the 4-chloro-1-naphthol precipitates. Initially primary or secondary antibodies were replaced with diluted serum or PBS to determine background staining. Both PBS and serum produced the same results, therefore, PBS replaced for the primary antibodies was used as the control for each subsequent experiment.

2.3.5 Gathering and Analysis of Data

A glass reticle etched with a 0.6 mm² grid hatch-marked into 100 square fields was used to count cells. A starting point within the immunostained area was chosen at random, and neighboring fields were counted as in the method of counting cells
on blood smears [161]. The number of fields counted on each slide was used to determine the cell density of each slide. Counting continued until 2000 cells had been counted for each slide and categorized according to cell type, the presence of incorporated BrdU, and the cell types each cell was physically contacting.

One-tailed simple sign tests [162] were used to test the hypothesis that estrogen-treatment of cocultured cells increased the number of labeled cells per the total cells counted from a treatment group, and the hypothesis that estrogen-treatment of monocultured cells decreased the number of labeled cells per the total cells counted from a treatment group. Cell count data was also analyzed graphically.

2.4 Results

2.4.1. Growth and Purity of Cultures

Epithelial cells were dislodged from the luminal surface of the uterus in flakes that maintained their cellular junctions during the isolation and culture procedures. Typically, central cells in the flake were more rounded, and divided more frequently than the flattened cells on the periphery. Cells isolated from adult mice that were initially associated as flakes at plating often separated from one another during culture and formed loosely attached groups (see Figure 2). These cells maintained a polygonal shape and size similar to cells on the periphery of conventional epithelial flakes and maintained some cell contacts to their nearest neighbors.
through thin, stretched cytoplasmic processes. These cells divided more often than cells in typical flakes. It is likely that the increased proliferation resulted from the loss of contact inhibition as the cells separated from one another. Immunostaining for the localization of a polyclonal antibody to human cytokeratin showed that all of these cells contained cytokeratin, however, they were less immunoreactive to the anti-cytokeratin than cells in typical flakes which were similarly flattened.

The baseline growth rates of epithelial and stromal cells at various densities were estimated by continuous observation of marked areas in cultures maintained in a defined, serum-free medium on tissue culture plastic. The number of cells in a randomly chosen 0.175 mm² area on the culture dish was counted 12 hours after plating. This area was then marked and monitored during six days of culture at 12 hour intervals (see Figure 3). Epithelial flakes required up to 24 hours to orient their basal surface downward and attach to the culture substrate, therefore, 24-hour cultures were used to assess the number of cells successfully plated.

Epithelial cells isolated from the uteri of immature mice grew better and were more predictable than cells isolated from adult mice. A summary of the proliferation rate for cell densities at 24 hours after plating is included in Table 1. Optimal densities spanned a large range especially for epithelial cells isolated from immature mice.
Figure 2. Epithelium isolated from adult mice in early estrus after three days of culture. Cultures were labeled with BrdU three hours before fixation, then double immunolabeled for cytokeratin (at arrow) and BrdU (at arrowhead and throughout the flake). Phase contrast (a,c) and bright field (b,d) photographs of the same field are included to highlight both cell morphology and immunostaining. Note that the cells in the upper portion of the photo (a,b) are separated from one another and maintain a higher rate of proliferation than the cells in the typical epithelial flake in the lower portion of the photo. Also, the cytoplasmic stain for anti-cytokeratin is less intense in the cells that constitute the separated flake. Only the typical epithelial flake morphology was present in cultures of cells isolated from immature mice. An epithelial culture incubated with PBS in place of anti-BrdU and anti-cytokeratin is included (c,d) to demonstrate background staining. × 344.
Figure 3. Growth of epithelium and stroma in serum-free culture. Cultures were maintained in a defined, serum-free medium for six days. The number of cells was recorded every 12 hours for several areas in each culture. A summary of the growth rates of epithelium isolated from immature mice (A) or adult mice in early estrus (B), and stroma isolated from immature mice (C) or adult mice in early estrus (D) plated at varying densities is shown. Each line represents the averaged growth of several areas that had similar starting densities. Each point is the average number of cells/mm² from at least three areas. Bars represent +S.E. Points without bars represent less than three observations. Note that cells isolated from immature mice (graphs on the left) increase in number during six days of culture. Whereas, the cells isolated from adult mice (graphs on the right) maintain their culture density or double once during the six days.
Table 1. Growth rates of uterine cells cultured in a defined, serum-free medium. Numbers represent the density (cells/mm²) at 24 hours after plating.

<table>
<thead>
<tr>
<th>Rate of Proliferation</th>
<th>Cell type</th>
<th></th>
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<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Epithelium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Immature</td>
<td>Adult</td>
<td>Immature</td>
<td>Adult</td>
<td></td>
</tr>
<tr>
<td>no significant doubling</td>
<td>&lt; 2</td>
<td>&lt; 25</td>
<td>&lt; 14</td>
<td>&lt; 20</td>
<td></td>
</tr>
<tr>
<td>doubling time 1–3 days</td>
<td>3–100</td>
<td>27–150</td>
<td>60–100</td>
<td>60–100</td>
<td></td>
</tr>
<tr>
<td>rapid division then decline</td>
<td>&gt; 120</td>
<td>&gt; 80</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In preliminary experiments (not shown), the purity of stromal and epithelial monocultures was observed by comparing immunostaining for cytokeratin and vimentin. As expected, stromal cells did react selectively with antibodies against vimentin, but not with anti-cytokeratin. Epithelial cells reacted selectively with anti-cytokeratin, but not anti-vimentin. The purity of the epithelial and stromal monocultures is described in Figure 4. Occasionally epithelial flakes were observed in stromal cultures. If stromal cells contaminated epithelial cultures, they were usually associated with each other as groups or whorls. The stromal cells which formed whorls in culture were not fibroblastic in appearance, and were probably myeloid-derived cells from the uterine stroma. Epithelial cells isolated from adult mice were contaminated with stromal cells more frequently than epithelium from immature mice. Yet stromal contamination was never higher than 4% in any epithelial monoculture (see Figure 4). Although the epithelial contamination
of stromal monocultures occasionally reached as high as 20%, the percentage of epithelial cells which contacted stromal cells in these cultures was always less than 1%. Epithelial and stromal cells isolated from either immature or adult mice were observed during 6 days to determine baseline division at various densities (see Figure 3). In all experiments only specifically identified cells were used in calculations.

Figure 4. The purity of uterine cell monocultures. The bars represent all of the monocultures of epithelial and stromal cells. They are divided into fractions representing the percent of the population that contained each level of contamination of the other cell type. Numbers inside of the bars indicate the percentage of contaminating cell type present in each fraction.

A coculture response, determined by at least a doubling of proliferating cells in E2-treated cultures, was observed only when the minor cell type constituted at
least 25% of the total cell population in the coculture. Three quarters of stromal cultures isolated from adult mice ranged between 190 and 400 cells/mm² at fixation, and 80% of the stromal cultures isolated from immature mice ranged between 60 and 600 cells/mm² at fixation. Whether they were isolated from immature or adult mice, 67% of the epithelial cultures ranged between 90 and 430 cells/mm². Including both cell types, 80% of the cocultures were between 100 and 475 cells/mm² at the time of fixation.

2.4.2 Effects of Steroid Treatment on Proliferation

Epithelial proliferation increased significantly in cocultures treated with 8 x 10⁻⁹ M 17β-estradiol for 24 hours. Proliferation of the stromal cells within the cocultures also increased in response to treatment with 17β-estradiol, but to a lesser degree (see Figures 5, 6 and 7). The fraction of cocultured epithelial cells from immature mice incorporating BrdU increased by 1.5-fold, while those from adult mice in early estrus showed a 4-fold increase. Experiments involving cells isolated from adult mice showed more variability, most likely resulting from the different populations of epithelial cell flakes which made up most cultures as well as the more variable plating densities and growth rates observed in cultures of cells isolated from adult mice. The number of cocultured immature stromal cells that incorporated BrdU increased 2-fold above the control with estrogen treatment. Stromal cells isolated from both immature or adult mice increased proliferation in response to estrogen-treatment in coculture with the variability again higher in cells isolated
from adult mice. Estrogen stimulated proliferation of stroma cells only when they were cocultured with epithelium. Thus, it appears that the stromal-epithelial interaction that is required for estrogen-induced epithelial proliferation is reciprocal.

Although proliferation was stimulated in cocultured stromal cells less than in epithelial cells, stromal cells accounted for the majority of the cells that incorporated BrdU in each coculture. The number of BrdU-labeled stromal cells ranged between 60 and 230 per thousand cells, for experiment densities other than those with less than 40 cells/mm² or greater than 900 cells/mm². Epithelial cells ranged between only 5 and 60 labeled cells per thousand cells consistently.

Cells within the cocultures differed by the types of cell-cell contacts they maintained at the time of treatment and fixation of the culture. When the population of cells counted for each coculture was divided into groups of cells that maintained the same types of cell-cell contacts, the estrogen-stimulated increase of proliferation in immature epithelial cells occurred in approximately 80% of epithelial cells which physically contacted both epithelial and stromal cells (see Figures 8 and 9). Similar results were obtained when the stromal cell population was grouped according to their cell-cell contacts. However, the response of stromal cells that physically contacted both epithelium and stroma to estradiol was more variable. This group of stromal cells appeared to be divided into one group that increased proliferation with estrogen-treatment and one group that remained near control levels of proliferation (see Figures 8 and 9). This type of variability may indicate that other factors within this culture system could have affected the response of
these stromal cells to estradiol.

Treatment of either epithelial or stromal monocultures with $8 \times 10^{-9}$ M 17$\beta$-estradiol for 24 hours suppressed proliferation (see Figures 10 and 11). The number of labeled nuclei in monocultures of either immature epithelium or stroma treated with $8 \times 10^{-9}$ M 17$\beta$-estradiol were 50% and 20% lower than non-treated control cultures, respectively. Estrogen inhibited monocultured epithelium and stroma isolated from adult mice by 20% and 50%, respectively. A similar suppression of proliferation by estrogen was recently reported for mouse uterine epithelium cultured on Matrigel or collagen substrates [156].

The estradiol-stimulated increase in the incorporation of BrdU in cocultures was inhibited by treating the cocultures concurrently with either $1.8 \times 10^{-5}$ M or $1.8 \times 10^{-7}$ M tamoxifen (see Figure 12), a weak antiestrogen shown to inhibit estrogen-stimulated upregulation of progesterone receptors in cultured mouse endometrial cells [163]. Treatment of cocultures with tamoxifen alone did not reduce or increase the proliferation of either cell type, so subsequent controls for these experiments were treated with tamoxifen alone.
Figure 5. Changes in proliferation in response to estrogen-treatment of monocultures and cocultures of uterine cells isolated from immature mice. The amount that proliferation increased or decreased from the control was determined by dividing the number of cells that incorporated BrdU per 1000 cells in the estrogen-treated culture by the number of cells that incorporated BrdU per 1000 cells in its parallel control culture (•). The amount of control proliferation is represented by 1 on the y-axis. The bars represent the median increase or decrease of proliferation from the control cultures. Cells from the same isolation were plated for paired control and estrogen-treated cultures in each experiment. There were at least 10 cell isolations per group. Increases in proliferation of epithelial cells \( (P = 0.002) \) and stromal cells \( (P = 0.0899) \) within cocultures treated with estradiol were analysed by one-tailed simple sign tests. The same method was used to analyze the suppression of proliferation in estradiol-treated epithelial monocultures \( (P = 0.0547) \) and stromal monocultures \( (P = 0.017) \).
Figure 6. Estrogen-induced changes in proliferation of monocultured and cocultured uterine cells isolated from adult mice in early estrus. Experiments were conducted in the same manner as explained for cells isolated from immature mice in the legend to Figure 5. The bars represent the median of the fold-change in proliferation as compared to the parallel control cultures. The fold-change in proliferation for each experiment is shown (●). Paired estrogen-treated and control cultures were from the same cell isolation, and there were at least 10 cell isolations per group. The level of control proliferation is represented as 1 on the y-axis.
Figure 7. Estrogen-induced proliferation of cocultured endometrial cells isolated from immature mice. Cultures were maintained in control serum-free medium (a,b) or treated with $8 \times 10^{-9}$ M 17β-estradiol (c,d), then labeled with BrdU three hours before fixation. They were double immunolabeled for anti-BrdU (arrow) and anti-cytokeratin (at arrowhead and throughout flake) (a,b). Phase contrast (left) and bright field (right) pictures of the same field are included to highlight both cell morphology and immunostaining. $\times$ 344.
Figure 8. Estrogen-induced proliferation of cocultured uterine cells isolated from immature mice and grouped according to their cell-cell interactions within the culture. The number of proliferating cells per thousand in each estradiol-treated culture was divided by the number of proliferating cells per thousand in each parallel control culture (•). The bars represent the median for each group. In order to determine whether direct contact effected estrogen stimulation of proliferation, all cells counted in the cocultures were grouped accordingly: epithelial cells with no contacts (E), epithelial cells touching other epithelial cells (EE), epithelial cells touching only stromal cells (ES), epithelial cells contacting both cell types (E-ES), stromal cells with no contacts (S), stromal cells touching other stromal cells (SS), stromal cells touching only epithelial cells (SE), and stromal cells contacting both cell types (S-ES). Note that the largest estrogen-induced increase in proliferation is in the group of epithelial cells directly contacting both other epithelial cells and stromal cells.
Figure 9. Estrogen-induced proliferation of uterine cells isolated from adult mice in coculture grouped according to their cell-cell interactions within the culture. The number of proliferating cells per thousand in each estradiol-treated culture was divided by the number of proliferating cells per thousand in each parallel control culture (●). The control level of proliferation is represented as 1 on the y-axis. The bars represent the median for each group. In order to determine whether direct contact effected estrogen stimulation of proliferation, all cells counted in the cocultures were grouped accordingly: epithelial cells with no contacts (E), epithelial cells touching other epithelial cells (EE), epithelial cells touching only stromal cells (ES), epithelial cells contacting both cell types (E-ES), stromal cells with no contacts (S), stromal cells touching other stromal cells (SS), stromal cells touching only epithelial cells (SE), and stromal cells contacting both cell types (S-ES). Note that the variation is higher for cells isolated from adult mice than cells isolated from immature mice (Figure 8), however, similar trends are apparent. The increased proportion of estrogen-stimulated proliferation in epithelial cells not contacting stromal cells probably resulted from the presence of separated flakes in these cultures.
Figure 10. The estrogen-induced suppression of proliferation in monocultured uterine epithelial cells isolated from immature mice. Epithelial monocultures were maintained in control serum-free medium (a,b) or treated with $8 \times 10^{-9}$ M 17$\beta$-estradiol (c,d), then labeled three hours with BrdU before fixation. Cultures were double-immunolabeled for anti-BrdU (arrow) and anti-cytokeratin (at arrowhead and throughout flake) (a,b). Epithelium treated with 17$\beta$-estradiol shows decreased proliferation from the control culture in these similarly sized epithelial flakes. Phase contrast (left) and bright field (right) pictures of the same field are included to highlight both cell morphology and immunostaining. $\times$ 422.
Figure 11. The estrogen-induced suppression in proliferation of monocultured uterine stromal cells isolated from immature mice. Stromal monocultures were maintained in control serum-free medium (e,f) or treated with $8 \times 10^{-9}$ M 17β-estradiol (g,h), then labeled three hours with BrdU before fixation. Cultures were double-immunolabeled for anti-BrdU (arrow) and anti-cytokeratin (no immunoreactivity). Treatment of stromal monocultures with 17β-estradiol reduced the proliferation of the stromal cells, although this is not readily apparent from examining the cultures visually. Phase contrast (left) and bright field (right) pictures of the same field are included to highlight cell morphology and immunostaining. × 422.
Figure 12. Tamoxifen inhibited estrogen-induced proliferation in cocultured stromal and epithelial cells. The number of proliferating cells per thousand in the estradiol-treated or estradiol- and tamoxifen-treated culture was divided by the number of proliferating cells per thousand in the parallel control culture treated with tamoxifen alone. The control level of proliferation is represented as 1 on the y-axis. The bars representing uterine cells isolated from immature mice include only one experiment. The bars representing uterine cells isolated from adult mice show the mean of two experiments.
2.5 Discussion

Through direct examination of cell morphology and BrdU incorporation during culture in a defined, serum-free medium, we have shown that estrogen increases the proliferation of epithelial cells only when they are cocultured with stromal cells. While *in vivo* studies in ovariectomized and immature mice have shown that proliferation increases dramatically in response to estrogen [73], there is still debate as to whether estrogen exerts this effect directly through binding to estrogen receptors within the uterine epithelium or via some kind of stromal mediation. Neonatal mice injected with 17β-estradiol prior to the presence of estrogen receptors in the uterine epithelium showed increased epithelial proliferation and secretory activity in response to estrogen [130]. Estrogen receptors are not present in the mouse uterine epithelium until 3-5 days after birth depending on the strain of mouse [131, 130], but stromal cells contain estrogen receptors during uterine development, sexual immaturity, and adulthood in rodents [164]. The uterine epithelial cells should not have been able to respond to estrogen stimulation in these experiments if estrogen acts on the epithelium through its own estrogen receptors. Epithelial and stromal tissues were isolated from ovariectomized adult mice, recombined in collagen, then transplanted under the kidney capsule of adult female mice. The epithelium in these implants proliferated in response to estrogen rises during the estrous cycle [157]. These experiments were all performed *in vivo* where the cells still had access to plasma-borne hormones and growth factors, and the possibility
that endocrine-acting factors in the plasma were affecting the epithelium could not be excluded.

Estrogen has never been shown to increase the proliferation of mouse uterine epithelium cultured alone on plastic or on various matrices [4, 156, 152]. However, uterine epithelium cultured on substrates, including Matrigel (an artificial basal lamina produced by Engelbreth Holmes Swarm Sarcoma cells), collagen, or Matrigel-coated filters does respond to estrogen by hypertrophy and increased production of various proteins that are known to be stimulated by estrogen in vivo [4]. Hypertrophy [91] and increased production of specific estrogen-inducible proteins [165] are known to occur more quickly after estrogen stimulation in vivo than the onset of increased DNA replication and cell division [73, 86]. These experiments provide evidence that estrogen exerts some effects on the epithelial cell directly through binding to the epithelial estrogen receptors, but that the stimulation of proliferation requires a more complex series of events, probably involving the stroma.

We have demonstrated that in addition to the stimulation of epithelial proliferation by estradiol in epithelial-stromal cocultures stromal cells are also stimulated, albeit to a lesser extent. In vivo experiments measuring changes in tritiated thymidine incorporation, mitoses, nuclear volume, tritiated cytidine incorporation into RNA, and nucleolar volume have also shown slight stimulation of the stroma 24 hours after estrogen injection into ovariectomized mice without progesterone priming [111, 86, 110, 73]. However, the epithelium makes up only 5-10% of the
uterus by mass, whereas the stroma makes up 30-35% of the uterus [112], therefore, measurements of estrogen-induced incorporation of tritiated thymidine into the whole uterus, whole endometrium, or cocultured stroma and epithelium [129] is not representative of the response of epithelium alone. The accurate measurement of the estrogen-induced proliferative response in uterine cells requires that proliferating cell types be identified specifically.

We found that the proliferation of stromal and epithelial cells cultured separately was suppressed by treatment with estrogen. This was also recently shown for mouse uterine epithelium cultured on plastic, collagen, or Matrigel substrates by measuring BrdU incorporation [156]. There are a number of possible explanations for this reduction. Fukamachi and McLachlan hypothesized that estrogen protected the cultured epithelial cells against apoptosis (programmed cell death), and this resulted in a slower division rate and fewer cells detaching from the substrate [156]. However, they did not measure detached cells or cell death directly in these cultures. It is also possible that estrogen shifts the cell’s physiology away from one preparing for cell division, and towards one that emphasizes the production of estrogen-induced proteins and secretory products unrelated to cell division. Increased protein production is stimulated by estrogen in monolayer cultures of epithelium.

We have shown that epithelial cells maintaining physical contacts to both epithelial and stromal cells in coculture constitute the majority of the cells stimulated to proliferate in the presence of estradiol in vitro. Using transmission electron
microscopy, epithelial projections through the basement membrane that connect to stromal cells and other epithelial cells via gap junctions have been shown to increase following the increasing concentration of circulating estrogen during the menstrual cycle in human endometrium [32]. The number of gap junctions on these processes also increase with increasing plasma estrogen levels, indicating that increased communication between stromal and epithelial cells and between epithelial cells may result during estrogen stimulation. Taken together this evidence supports the hypothesis that direct contact is required for stromal mediation of estrogen action on epithelial proliferation in the uterus. Alternatively, stromal cells may be secreting short-lived epithelial proliferation factors into the area immediately surrounding the epithelial cell in response to estrogen action in the stroma. A possible mechanism for such factors may be gene derepression. If genes required for cell division are being constitutively repressed without stromal cell intervention in response to estrogen, the stromal cell, could release factors that remove this repression. One experiment examining the effect of stromal-conditioned medium on epithelial cells did not result in a difference in proliferation between estrogen treated and control cultures [129]. However, the possibility that a short-lived factor is released from the stroma and requires close contact between stromal and epithelial cells still could not be ruled out. The short-lived factor could influence epithelial cells directly, as a growth factor, or alter the extracellular matrix, as a matrix-degrading enzyme, to cause the movement of growth factors bound to ECM molecules to the higher affinity epithelial cell surface receptors. The factor may also act directly on the
epithelial cell to alter its interaction with the matrix through regulating the cell surface matrix receptors which could cause the epithelial cell to change shape and alter cell proliferation [166, 18, 22].

Previous whole animal or organ culture studies have implicated the stroma as a mediator of estrogen-induced proliferation of epithelium [167, for a review]. However, the only attempt to directly examine the role of isolated stromal cells in estradiol-induced epithelial cell proliferation was ambiguous because no attempt was made to identify responding cell types [129]. The present work demonstrates for the first time by direct examination of responsive cell types in vitro, that uterine epithelial cells require close association with stromal cells for a proliferative response to estradiol. Furthermore, the interaction between epithelial and stromal cells appears to be reciprocal in that stromal proliferation in response to estradiol also requires association with epithelial cells. The results provide strong evidence for either direct stromal-epithelial contact or the release of short-lived factors from the stroma that act only within the very short distances between these two intimately associated cell populations.
BIBLIOGRAPHY


APPENDICES
Appendix A

Summary of Data
Table 2. Estrogen-induced proliferation of cocultured epithelium and stroma isolated from immature mice. The left side of each column summarizes the number of BrdU-labeled cells per 1000 cells for each experiment. The density of each culture at fixation (in cells/mm²) is included on the right side of each column. Each row includes data from one cell isolation.

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Table 3. The suppression of proliferation in response to estrogen-treatment of monocultured epithelium and stroma isolated from immature mice. The left side of each column summarizes the number of BrdU-labeled cells per 1000 cells for each experiment. The density of each culture at fixation (in cells/mm²) is included on the right side of each column. Each row includes data from one cell isolation.

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Table 4. The suppression of proliferation in response to estradiol-treatment of monocultured epithelium and stroma isolated from adult mice. The left side of each column summarizes the number of BrdU-labeled cells per 1000 cells for each experiment. The density of each culture at fixation (in cells/mm²) is included on the right side of each column. Each row includes data from one cell isolation.

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Table 5. Estrogen-induced proliferation of cocultured epithelium and stroma isolated from adult mice in early estrus. The left side of each column summarizes the number of BrdU-labeled cells per 1000 cells for each experiment. The density of each culture at fixation (in cells/mm²) is included on the right side of each column. Each row includes data from one cell isolation.

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Appendix B

Supporting Experiments

B.1 Measurement of Estradiol Stimulation by Incorporation of $^3$H-Thymidine

Preliminary experiments were modeled after Inaba et al. [129], and used the incorporation of tritiated thymidine into the nuclei of cells synthesizing DNA during a one hour labeling period. The measurement of the proliferation of epithelial and stromal monocultures or cocultures in this manner was inconclusive. Variability of the densities of cultures within and between experiments contributed strongly to the variability in these experiments. Using this method it was not possible to quantify which cell type incorporated the label in these cultures. Some of the variability in data from monocultured cells may have been due to low purity of monocultures. Inconsistent stromal/epithelial cell ratios in cocultures probably contributed to differing amounts of stimulation and increased variability in data from cocultures. These experiments were based on the amount of protein in each homogenate, and
estrogen-stimulation of increased protein production could have masked increases in proliferation in cocultures. Tables 6, 7 and 8 show a summary of the results of these experiments recorded in DPM/µg protein. Suppression of proliferation by estrogen-treatment of monocultures is present for most monocultures. However, estrogen-treatment of cocultures show no evident trends.

Table 6. Changes in proliferation resulting from estrogen-treatment of mono- and cocultured epithelium and stroma isolated from adult mice in estrus. Numbers reported are DPM of incorporated tritiated thymidine per µg protein in each culture extract. Two types of medium bases were used in these experiments: Medium 1 base was deficient DMEM/F-12 (sigma) with components readded except for phenol red. Medium 2 was complete DMEM/F-12 (Gibco) containing phenol red.

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<td>105</td>
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<td>18</td>
<td>38</td>
<td>22</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 7. Changes in proliferation resulting from estrogen-treatment of mono- and cocultured epithelium and stroma isolated from immature mice in estrus. Estrogen-response of mono- and cocultured epithelium and stroma isolated from immature mice. Numbers reported are DPM of incorporated tritiated thymidine per µg protein in each culture extract. The description of Media types is included legend of Table 6.

<table>
<thead>
<tr>
<th>Medium Type</th>
<th>Control Epithelium</th>
<th>Estrogen-Treated Epithelium</th>
<th>Control Stroma</th>
<th>Estrogen-Treated Stroma</th>
<th>Control Coculture</th>
<th>Estrogen-Treated Coculture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium 1</td>
<td>18</td>
<td>22</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>65</td>
<td>4</td>
<td>4</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Medium 2</td>
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<td>8</td>
<td>20</td>
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<td>43</td>
<td>35</td>
<td>12</td>
<td>13</td>
<td>31</td>
<td>40</td>
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</tbody>
</table>

Table 8. Changes in proliferation in response to estrogen-treatment of mono- and cocultured epithelium and stroma isolated from four week old mice. Numbers represent DPM of incorporated tritiated thymidine per µg protein in each culture extract. The description of Media types is included legend of Table 6.

<table>
<thead>
<tr>
<th>Medium Type</th>
<th>Control Epithelium</th>
<th>Estrogen-Treated Epithelium</th>
<th>Control Stroma</th>
<th>Estrogen-Treated Stroma</th>
<th>Control Coculture</th>
<th>Estrogen-Treated Coculture</th>
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</thead>
<tbody>
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<td>Medium 1</td>
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<td>14</td>
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<td>47</td>
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<td>6</td>
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<td>7</td>
<td>6</td>
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<tr>
<td>Medium 2</td>
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<td>26</td>
<td>3</td>
<td>3</td>
<td>46</td>
<td>70</td>
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</tbody>
</table>
B.2 Immunocytochemistry and Culture Morphology

Preliminary immunostaining showed that all epithelioid cells which made up flakes or groups contained cytokeratin in their cytoplasm. Cytokeratin localization also showed that some epithelial cells did exist as single cells in culture. These cells were sometimes polygonal in shape and sometimes more elongated as migrating cells.

Epithelial cultures from adult mice in early estrus contained typical epithelial flakes as well as flakes in which the epithelial cells separated from one another. Cells in separated flakes were polygonally shaped and maintained thin, stretched connections to their nearest neighbors. These cells were untouched by neighboring cells on at least two sides, and divided at unusually high rates when compared to epithelium in typical flakes. The most probable explanation of this increased proliferation was the loss of contact inhibition in the separated flakes. The separated epithelial cells immunostained for cytokeratin, however, the staining was not as strong as in cells in typical epithelial flakes. This could have been due to their high division rate since each cell would not have as much time between successive divisions to accumulate cytokeratin. It is also possible that these cells are more actively migrating, and producing cytoskeletal molecules other than cytokeratin because of this. Single cells which appeared migratory contained less cytokeratin as measured by relative immunoreactivity.

Stromal cells exhibited two distinct morphologies in culture. The typical
stromal cell appeared stellate and immunostained for vimentin, an intermediate filament found specifically in fibroblasts. A small population of stromal cells appeared in whorls of cells with very small, round nuclei. These cells also immunostained for vimentin, and divided much less often than typical fibroblastic stromal cells since it was rare to find this smaller type of stromal cell labeled with BrdU. Stromal whorls were present more often in cocultures with very low densities of stroma (less than 20\%). Cells in the uterine stroma are derived from two lineages, paramesonephric ductal mesenchyme and myeloid [1, page 90]. It is probable that the stellate, fibroblastic appearing cells are derived from the mesenchyme of the paramesonephric duct, and that the smaller cells which appeared in whorls were derived from the myeloid lineage.

Macrophages were common in cultures isolated from immature animals, but were rarely present in cultures isolated from adult mice. Macrophages were present in or near epithelial flakes. They sometimes stained positively for cytokeratin, but not for vimentin (however, very few were present in vimentin immunostained slides so this may have been an unfair sample size). These cells contained between 2 and 18 nuclei per cell, almost always being an even number. Either all nuclei incorporated 5-bromo-2-deoxyuridine or none within a single cell, and size was within an appropriate range for macrophages.
Figure 13. Mouse uterine epithelium double-immunostained using DAB for anti-BrdU (brown) and 4-chloro-1-napthol for anti-cytokeratin (blue). Phase contrast (a) and bright field (b) pictures of the same field are included to highlight both cell morphology and immunostaining. Note that a mitotic figure (arrow) which incorporated BrdU during the three hour labeling period is present. × 1260.
Figure 14. Mouse uterine stroma double-immunostained using DAB for anti-BrdU (brown) and 4-chloro-1-napthol for anti-cytokeratin (blue). Phase contrast (a) and bright field (b) pictures of the same field are included to highlight cell morphology and immunostaining. $\times 1260$. 