Establishment of a Primary Culture Model of Mouse Uterine and Vaginal Stroma for Studying *In Vitro* Estrogen Effects

KEIKO INADA,* SHINJI HAYASHI,* TAISEN IGUCHI,†,‡ AND TOMOMI SATO*,[1]

*Graduate School of Integrated Science, Yokohama City University, Yokohama 236-0027, Japan; †The Graduate University for Advanced Studies and Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, Okazaki 444-8787, Japan; and ‡CREST, Japan Science and Technology Corporation, Kawaguchi 332-0012, Japan

Effects of 17β-estradiol (E2) on uterine and vaginal epithelial cell proliferation could be mediated by stromal cell–derived paracrine factors. To study the epithelial-stromal interactions in mice, an *in vitro* model of uterine and vaginal stromal cells of immature mice is essential. Therefore, we established a primary culture model of stromal cells both from uterus and vagina and examined the effect of E2 on proliferation of cultured stromal cells. We found that E2 stimulated proliferation of stromal cells from both organs *in vitro*, showing an increase in the number of cells and the percentage of 5-bromo-2′-deoxyuridine (BrdU)–labeled cells. Interestingly, vaginal stromal cells responded to lower E2 than uterine stromal cells in proliferation (10^{-12} *M* vs. 10^{-8} *M*) and BrdU labeling ($10^{-14} - 10^{-10}$ *M* vs. $10^{-10} - 10^{-6}$ *M*). To examine the effect of E2 *in vivo*, cells were grafted into the subrenal capsule of the host mice and grown for 2 weeks. The BrdU labeling in cultured stromal cells was increased by E2 *in vivo*. To examine the effect of cultured stromal cells on epithelial cell proliferation, uterine and vaginal epithelium of adult mice were separated, recombined with the cultured stromal cells, and grafted under the renal capsule of hosts for 3 weeks. Epithelial cells recombined with cultured stromal cells showed simple columnar morphology in uterine grafts and stratified and keratinized morphology in vaginal grafts under the influence of the hormonal environment of the hosts. The BrdU labeling in epithelial cells was increased by E2, suggesting that cultured stromal cells can stimulate epithelial cell proliferation. In conclusion, we established a primary culture model of uterine and vaginal stromal cells, which can be mitogenically stimulated by E2 *in vitro* and *in vivo* after being grafted under the renal capsule. This culture system will be useful for investigating the underlying molecular mechanisms of uterine and vaginal epithelial-stromal interactions. Exp Biol Med 231:303–310, 2006

Key words: uterus; vagina; stromal cells; estrogen

Introduction

Female reproductive organs show structural and functional changes under the control of sex hormones 17β-estradiol (E2) and progesterone (P). E2 can stimulate proliferation of uterine and vaginal epithelial cells through the estrogen receptor (ER), which is normally expressed in both epithelial and stromal cells (1). E2 was considered to act directly through epithelial ER on epithelial cell mitogenesis.

Although ERα was not detectable in uterine epithelium, E2 stimulated cell proliferation of uterine epithelium in neonatal mice (2). In tissue recombinant experiments, ERα-negative epithelium, derived from the ERα knockout mouse uterus recombined with ERα-positive stroma, showed proliferation following estrogen stimulation, whereas wild-type epithelium recombined with ERα-negative uterine stroma did not proliferate (3–5). These reports suggested that epithelial cell proliferation could be mediated indirectly by stromal ERα. In culture conditions, E2 stimulated DNA synthesis in human endometrial epithelial cells co-cultured with stromal cells in a transfilter system (6). Thus, estrogen signals can be translated in the stroma into a growth-regulating message for the epithelium. This theory is supported by several observations in different tissues: the stroma of mammary glands is able to support both growth and differentiation of epithelial cells (7), and androgenic effects on prostatic epithelial cells are mediated *via* stromal cells (8).

Several hormonally regulated growth factors, such as

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[1] To whom correspondence should be addressed at Graduate School of Integrated Science, Yokohama City University, 22–2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan. E-mail: tomomi@yokohama-cu.ac.jp

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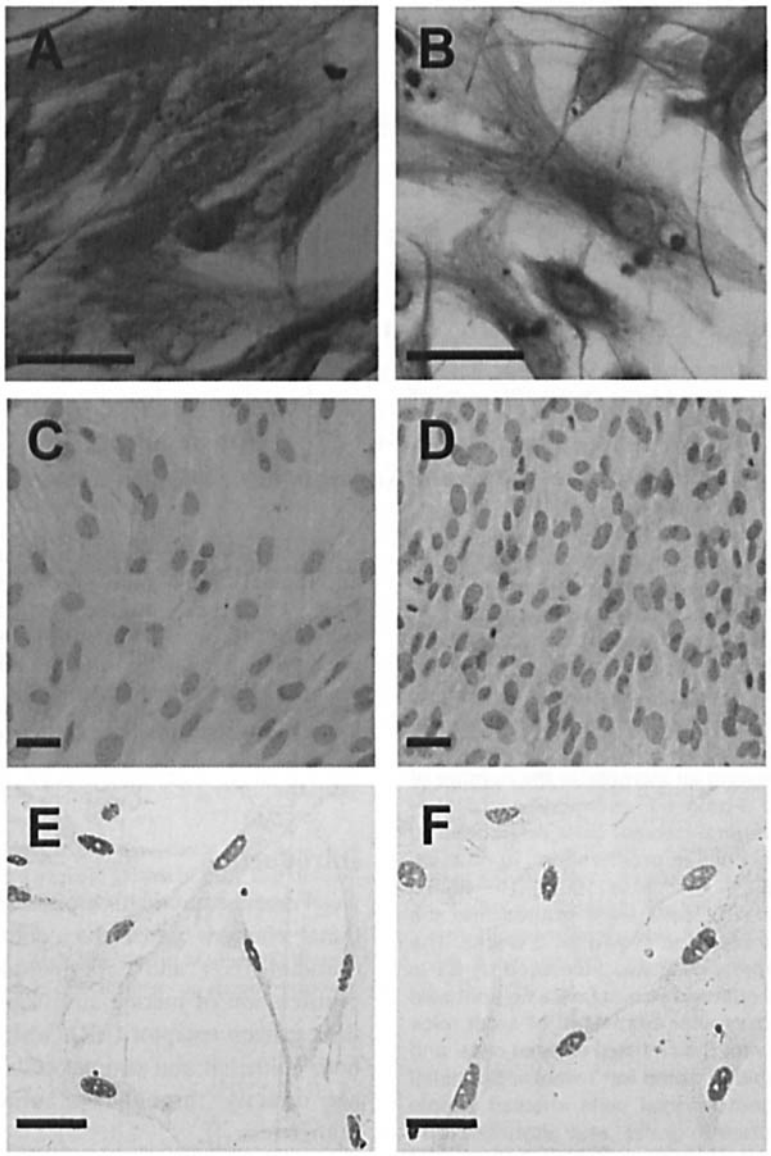

Figure 1. Immunocytochemical detection of (A, B) vimentin and (C, D) cytokeratin 8 in cultured (A, C) uterine and (B, D) vaginal stromal cells. Immunocytochemical detection of ERα in (E) cultured uterine and (F) vaginal stromal cells treated by 10^{-8} *M* E2 for uterus and 10^{-12} *M* E2 for vagina, respectively. Bar = 50 μm.

epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), transforming growth factor-α (TGF-α), and fibroblast growth factor (FGF), have been identified in mouse uterus and vagina. The importance of these growth factors in epithelial-stromal interaction has been proposed (9–12). However, recent reports suggested that EGF-receptor (EGFR) knockout-mouse uterus and vagina could respond to E2 with a marked proliferation of epithelium, suggesting EGFR signaling was not essential for estrogen-inducible epithelial growth in the uterus and vagina (13). Thus, the mechanism of epithelial-stromal interaction is still unclear, and other paracrine factors might be present in the stroma.

In this study, to clarify the epithelial-stromal interactions in mouse uterus and vagina, we established a primary culture model of stromal cells and compared the sensitivity of uterine and vaginal stromal cells with E2 stimulation in cell proliferation and 5-bromo-2′-deoxyuridine (BrdU) incorporation *in vitro*. Also, E2 effect was examined on the BrdU incorporation of grafted stromal cells with or without epithelial cells under the renal capsule.

Materials and Methods

Animals. Two- and eight-week-old female mice of the C57BL/6J Jcl strain (CLEA Japan Inc., Tokyo, Japan) were used for the isolation of stromal cells and epithelial cells, respectively. These animals were maintained in a temperature-controlled (23°–25°C) and artificially illuminated (12 hrs of light from 0800–2000 hrs) room and were fed CF-2 (CLEA Japan Inc.) and tap water *ad libitum*. All

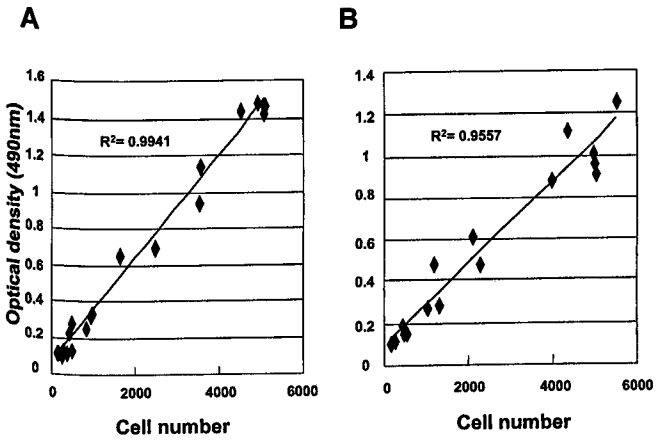

Figure 2. Correlation of MTS assay with the cell number. After the MTS assay, cells were fixed with methanol, stained with Giemsa's solution, and dried. The cell number was counted, and the relationship between optical density (OD_{490}) and the cell number was estimated. (A) Uterine stromal cells. (B) Vaginal stromal cells.

animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all experiments were approved by the Institutional Animal Care Committee of the Yokohama City University.

Uterine and Vaginal Stromal Cell Isolation. Five to six uteri and vaginae were removed from 2-week-old mice to avoid endogenous estrogen, slit longitudinally, and placed into trypsin (0.5%; Gibco BRL, Gaithersburg, MD) dissolved in Hank's balanced salt solution (HBSS; Sigma Chemical Co., St. Louis, MO) at 4°C for 1 hr, then at room temperature for 1 hr. After trypsinization, uteri and vaginae were vortexed for 10 secs. The supernatant containing epithelial cells was discarded, and uteri and vaginae were washed twice with 5 ml HBSS to remove epithelial cells. Ten mililiters trypsin (0.05%) plus 1 ml DNase (0.04%; Sigma Chemical) were added. Uterine and vaginal fragments were briefly suspended and placed at 37°C for 20 mins. Every 10 mins in incubation, uteri and vaginae were vortexed for 10 secs. The supernatant containing stromal cells was transferred to a new tube containing 1 ml of fetal bovine serum (FBS; Gibco BRL). Tissues were washed once with 5 ml of HBSS, and the wash was added to the tube. Then cells were collected by centrifugation. Cell viability was assessed by trypan blue exclusion test, and it was usually more than 80%.

Uterine and Vaginal Stromal Cell Culture. Isolated stromal cells were seeded in 10-cm tissue culture plates, 96-well tissue culture plates, and 4-well culture slides (FALCON, Becton Dickinson Labware, Franklin Lakes, NJ), and incubated at 37°C in a humidified, 5% CO_2 atmosphere in air. Stromal cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) containing 10% FBS and 1% penicillin-streptomycin (Gibco BRL). After preculture for a week, cells were cultured in DMEM/Nutrient Mixture F-12 (DMEM/F-12; Gibco BRL), without phenol red containing 5% dextran-coated charcoal-treated FBS (DC-FBS) and 1% penicillin-streptomycin. Independent experiments were carried out at least three times for each study.

Cultured Stromal Cells and Tissue Graft. To prepare grafts of tissues and cells, cultured stromal cells were collected from 10-cm tissue culture plates using trypsin (0.25%) and centrifuged for 5 mins at 300 *g*. Then, these cell pellets were placed on the agar gel and incubated alone, or fresh epithelial tissues isolated from 8-week-old mouse uterus and vagina using trypsin (1%) were put on the stromal cell pellets and incubated at 37°C overnight. Two to three drops of HBSS containing 20% FBS were added into the recombinants. The grafts were transplanted under the renal capsules of female host mice (8-weeks-old). After 3 weeks, the grafts were collected and processed for histological examination (hematoxylin-eosin stain or BrdU immunohistochemistry).

Immunocytochemistry. For vimentin and cytokeratin detection, cultured stromal cells were fixed in 70% ethanol for 30 mins, dipped eight times into cold water, and incubated in 5% normal goat serum (Vector Laboratories, Burlingame, CA) for 30 mins. The cells were incubated with antivimentin mouse monoclonal antibody (1:20 dilution, DAKO Corp, Carpinteria, CA), anticytokeratin 8 mouse monoclonal antibody (1:50 dilution, PROGEN Biotechnik GmbH, Heidelberg, Germany), or mouse IgG as a negative control at 4°C overnight, respectively.

For ERα detection, E2 (10^{-8} *M* for uterus or 10^{-12} *M* for vagina) was added into the culture medium, and cells

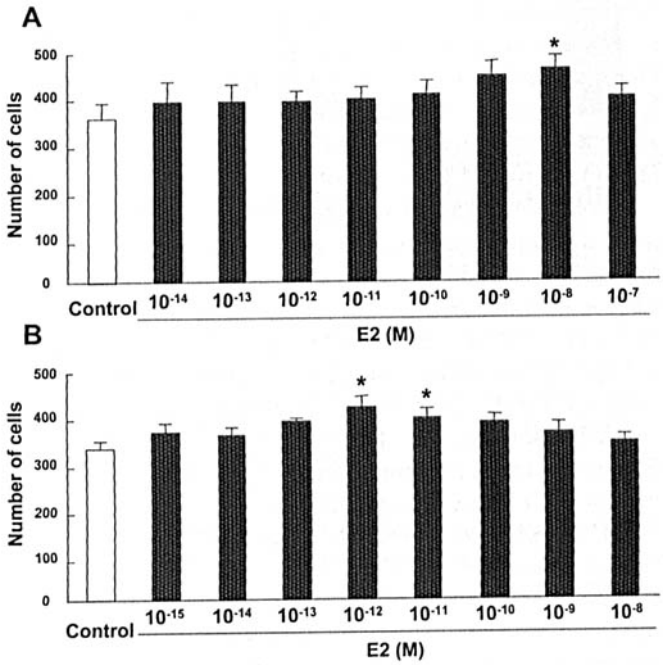

Figure 3. Effects of E2 on cell proliferation in (A) cultured uterine stromal cells and (B) vaginal stromal cells were analyzed by MTS assay. Data are expressed as the mean ± SEM of three different experiments. * $P < 0.05$ compared with the control.

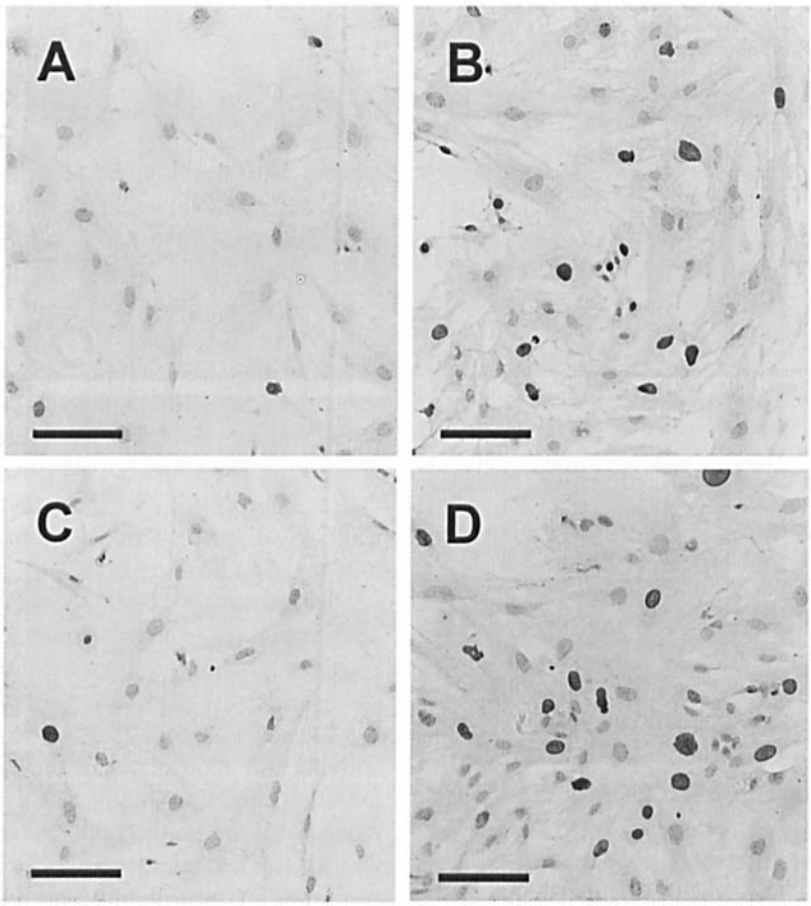

Figure 4. Immunocytochemical detection of BrdU labeled cells in (A, B) cultured uterine and (C, D) vaginal stromal cells. Cells were treated with (A, C) the vehicle alone or (B, D) with E2 (10^{-8} *M* for the uterus and 10^{-12} *M* for the vagina), respectively. Bar = 100 μm.

were incubated for 2 days. Cultured stromal cells were fixed in 4% paraformaldehyde at 4°C for 15 mins, and nonspecific binding was blocked in 5% normal horse serum (Vector Laboratories) for 30 mins. Then, cells were incubated with anti-ERα mouse monoclonal antibody (1:500 dilution, DAKO Corp) at 4°C overnight.

Following incubation with the primary antibody, cells were incubated with biotinylated anti-mouse IgG or anti-rabbit IgG for 30 mins. Amplification of the antigen-antibody complex was achieved by using Vectastain Elite ABC kit (Vector Laboratories). Peroxidase visualization was performed using 1 mg/ml diaminobenzidine (DAB; Sigma) and 0.1% hydrogen peroxide for 5 mins.

MTS Assay. To determine the mitogenic effects of E2 on the cultured stromal cells, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay (CellTiter 96 AQ_{ueous} nonradioactive cell proliferation assay kit; Promega Corp, Madison, WI) was performed. In this method, colored formazan products are created within the viable cells, and absorbance at 490 nm is measured with a microplate reader. The relationship between cell number and the amount of MTS formazan products was examined.

Isolated stromal cells were first placed on a 96-well tissue culture plate at a density of 10^4 cells per well. After the 24-hr preincubation period, the culture medium containing the E2 was replaced. Twenty four hours later, the amount of formazan after 4 hrs incubation with assay reagents was determined by measuring absorbance at 490 nm, and the number of cells in a well was estimated.

BrdU Immunostaining. For BrdU immunocytochemistry, cultured stromal cells were incubated in the medium containing E2 (10^{-5} *M*–10^{-10} *M* for uterus or 10^{-8} *M*–10^{-16} *M* for vagina) or a vehicle (ethanol) for 21 hrs. BrdU (0.01 *M*) was added to the culture medium, and 3 hrs later, cells were harvested and fixed in 70% ethanol for 30 mins.

For BrdU immunohistochemistry, hosts were ovariectomized 2 weeks after the grafting. Seven days later, hosts were given a single ip injection of E2 (5 μg/kg body weight) or of sesame oil alone. After 14 hrs, hosts were injected ip with BrdU (10 mg/100 g body wt) and killed 2 hrs later by cervical dislocation. Grafts and uterine and vaginal tissue of hosts were removed, fixed in 10% formalin neutral buffer solution, and embedded in paraffin.

Cells and sections were quenched in 0.3% hydrogen peroxide in methanol for 30 mins to remove endogenous peroxidase activity and treated with 2 *N* HCl for 20 mins

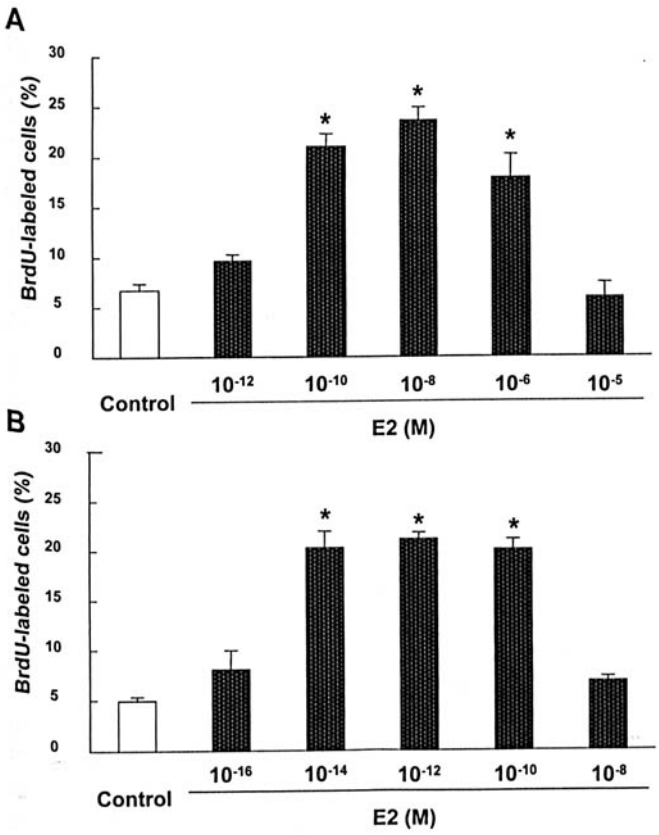

Figure 5. The percentage of BrdU-labeled cells in cultured (A) uterine and (B) vaginal stromal cells. Data are expressed as the mean percentage of labeled cells of three independent experiments ± SEM. * $P < 0.05$ compared with the control.

and 0.1% trypsin at 37°C for 30 mins to retrieve the antigen. To block nonspecific binding, cells and sections were incubated in 10% FBS albumin in phosphate-buffered saline (PBS), then incubated with anti-BrdU-POD, Fab fragments (1:15 dilution, Roche Diagnostics GmbH, Mannheim, Germany) at 4°C overnight. Peroxidase visualization was performed using 0.4 mg/ml DAB, dissolved in 0.05 *M* Tris/HCl buffer containing 0.68 mg/ml imidazole, and 0.1% hydrogen peroxide for 15 mins. The number of BrdU-labeled cells per 300 cells were counted in uterine and vaginal epithelium and stromal cells.

Statistical Analysis. The statistical significance of the difference between the control and the respective experimental groups was evaluated by Student's *t* test or Mann-Whitney *U* test.

Results

Immunocytochemical Analysis of Cultured Stromal Cells. Uterine and vaginal stromal cells were easily separated by trypsin and gentle vortexing. Immunocytochemical analysis showed that cultured uterine and vaginal stromal cells expressed vimentin (Fig. 1A and B), but not cytokeratin 8 (Fig. 1C and D), whereas cultured epithelial cells expressed cytokeratin 8 (data not shown).

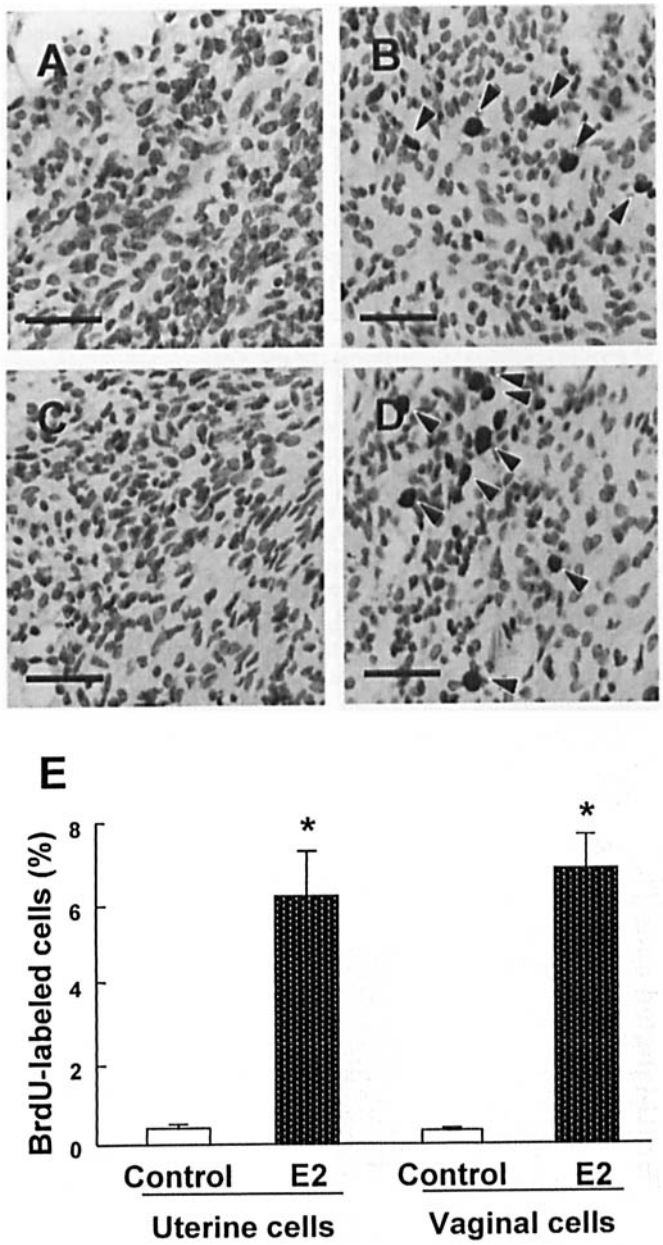

Figure 6. Effects of E2 on BrdU-labeling in cultured (A, B) uterine and (C, D) vaginal stromal cells in grafts. Hosts were injected with (A, C) oil or (B, D) 5 μg E2/kg body weight, respectively. Arrowheads shown in (B) and (D) indicate BrdU-labeled cells. Bar = 100 μm. (E) The percentage of BrdU-labeled cultured uterine and vaginal stromal cells in grafts. Data are based on three mice for each treatment group and expressed as the mean percentage of labeled cells ± SEM. * $P < 0.05$ compared with the control.

Expression of ERα was examined immunocytochemically. Immunoreaction of ERα was detected mainly in the nucleus of the uterine and vaginal cultured cells (Fig. 1E and F), whereas no staining was observed without the ERα antibody (data not shown).

Effects of E2 on the Proliferation of Stromal Cells *In Vitro*. The number of cultured stromal cells was measured by MTS assay. The amount of formazan products

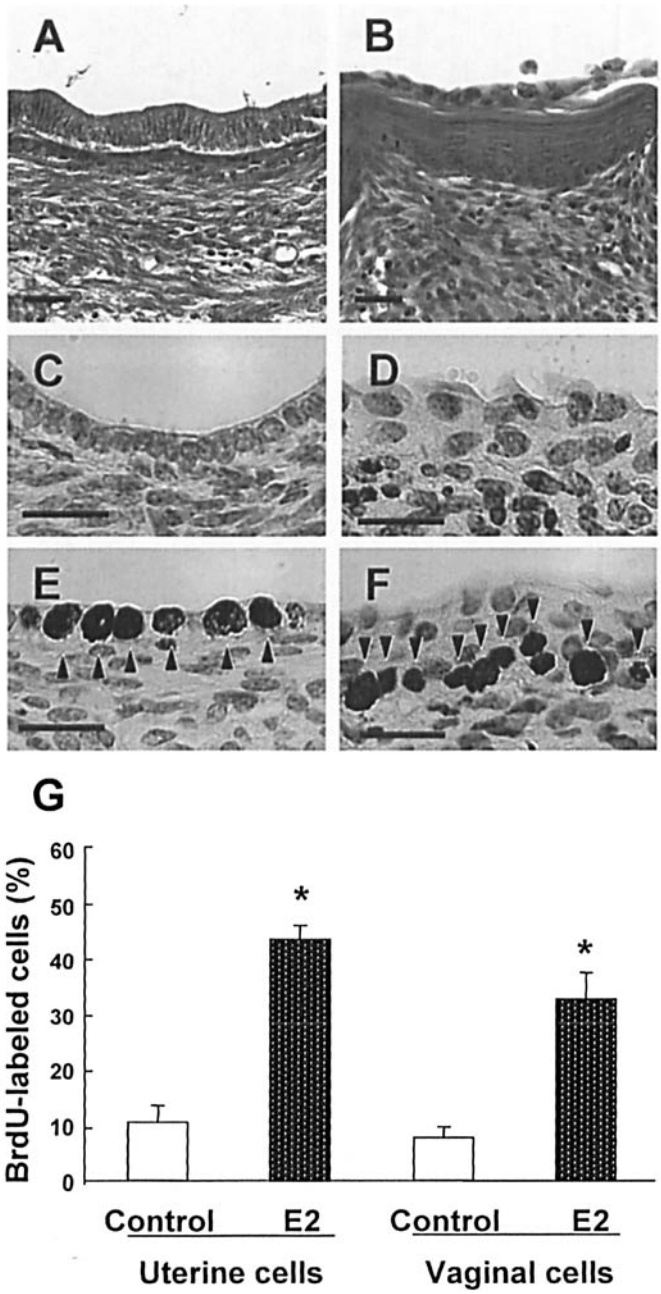

Figure 7. Histology of grafted stromal cells recombined with epithelial tissue from adult mouse. Uterine stromal cells recombined with (A) uterine epithelial tissue and (B) vaginal stromal cells recombined with vaginal epithelial tissue. Bar = 50 μm. Immunohistochemical detection of BrdU-labeled cells in (C, E) uterine and (D, F) vaginal grafts. Hosts were injected with (C, D) oil or (E, F) 5 μg E2/kg body weight, respectively. Arrowheads shown in (E) and (F) indicate BrdU-labeled cells. Bar = 20 μm. (G) The percentage of BrdU-labeled uterine and vaginal epithelial cells recombined with cultured stromal cells. Data are based on three mice for each treatment group and expressed as the mean percentage of labeled cells ± SEM. * $P < 0.05$ compared with the control.

was well correlated with the number of cells in culture (Fig. 2). After 24 hrs of culture with E2, the number of cultured stromal cells was increased by 10^{-8} *M* E2 in uterine cells and 10^{-12} and 10^{-11} *M* E2 in vaginal cells compared with the control (Fig. 3).

To examine the effect of E2 on DNA synthesis of the cultured stromal cells, BrdU-labeled cells were detected immunocytochemically. The percentage of BrdU-labeled cells was significantly increased by 10^{-10} *M*, 10^{-8} *M*, and 10^{-6} *M* E2 in uterine cells and 10^{-14} *M*, 10^{-12} *M*, and 10^{-10} M E2 in vaginal cells 24 hrs after E2 stimulation, respectively (Figs. 4 and 5).

Growth of Cultured Stromal Cells *In Vivo*. Grafts of cultured uterine and vaginal stromal cells showed stromal morphology. The percentage of BrdU-labeled cells was significantly increased by a single injection of 5 μg/kg body weight E2 compared with oil controls (Fig. 6). In the BrdU-labeling index of uterine and vaginal stromal cells, no differences were found between grafts and hosts.

Histology of Grafted Stromal Cells Recombined with Epithelial Tissue. Uterine epithelium recombined with cultured uterine stromal cells showed a simple columnar morphology without glands typical of normal uterine luminal epithelium. Vaginal epithelium recombined with cultured vaginal stromal cells showed stratification and keratinization depending on the estrous cycle of the hosts (Fig. 7A and B). The percentage of BrdU-labeled epithelial cells was significantly increased by 5 μg E2/kg body weight (Fig. 7C–G). In the BrdU-labeling index of uterine and vaginal epithelial cells, no differences were found between grafts and hosts.

Discussion

The proliferation of uterine and vaginal epithelial cells is regulated by E2 through stromal cells *via* paracrine factors (9–12). Several growth factors were candidates as paracrine factors in the epithelial-stromal interaction of reproductive organs. However, these growth factors are not essential for estrogen-induced epithelial cell growth on the basis of a number of experiments using knockout mice (13–15). In the present study, we established a primary culture model of uterine and vaginal stromal cells and examined the effects of E2 on cultured stromal cells.

Using trypsin, we isolated uterine and vaginal stromal cells from 2-week-old mice. Isolated stromal cells were positive for vimentin but negative for cytokeratin, indicating that these cells were derived from stromal cells but not from epithelial cells. In addition, cultured stromal cells expressed ERα and showed cell proliferation in response to E2 *in vivo* and *in vitro*. Because immature uterine stroma, but not adult stroma, exhibited E2-induced cell proliferation (16), cultured stromal cells from immature mice maintained their characteristics. E2 inhibited proliferation of stromal monocultures (17) and did not enhance cell growth of vaginal epithelial cells (18) in a serum-free medium. In the present study, significant cell proliferation in response to E2 was observed in the medium containing 5% DC-FBS, suggesting the presence of associating factors in the serum for E2-mediated cell proliferation. We have also reported that E2,

P, or a combination of E2 + P stimulated proliferation of trophoblastic cells in a medium containing 10% FBS (19).

Interestingly, vaginal stromal cells responded to lower E2 than uterine stromal cells in both proliferation (10^{-12} *M* vs. 10^{-8} *M*) and BrdU labeling ($10^{-14} - 10^{-10}$ *M* vs. $10^{-10} - 10^{-6}$ *M*), indicating that uterine and vaginal stromal cells have different responses to E2. In the developing rat Müllerian duct, region-specific cell proliferation by DES was found, suggesting the presence of different functional mechanisms for cell growth and differentiation in female reproductive tracts (20). Tanahashi *et al.* (21) have established several epithelial and fibroblastic cell lines from $p53^{-/-}$ mouse vagina, and these cell lines showed distinct morphology and characteristics. Thus, organ- and cell-type specificity could be present throughout the development, which may result in a different response to hormones. Application of this culture system will be useful for investigating differences between uterine and vaginal stromal cells. In addition, the concentrations of E2 that stimulated BrdU labeling did not increase the cell number after 24 hrs, indicating that timing of the cell proliferation analysis was not appropriate. However, low-dose IGF-I increased the replicating cells in the uterine epithelial cells after 5-hrs incubation, but the same dose did not affect cell number on 5 days in culture (11). It has been suggested that an increase in DNA synthesis did not correlate with an increase in cell number in epithelial cell cultures. Further studies are needed to analyze cell numbers at 48 hrs after E2 stimulation.

Epithelial tissue of adult mouse recombined with cultured stromal cells showed typical morphology of the normal uterine and vaginal epithelium and a significant increase of BrdU-labeled cells *in vivo* after E2 injection. Increases in BrdU-labeled cells in epithelia of grafts were also stimulated by E2, indicating that cultured stromal cells can respond to E2 and result in cell proliferation of both epithelial and stromal cells. Cooke *et al.* (22) have demonstrated that grafted recombinants of cultured uterine and vaginal stromal cells in a collagen gel mix and fresh epithelia were histologically normal and stimulated cell proliferation by E2. In this study, we cultured stromal cells without a collagen matrix and found that their characteristics still remained in both *in vivo* and *in vitro* experiments. The percentage of BrdU-labeled cells in grafts was similar to that in host uterus and vagina, suggesting that cultured stromal cells maintained their responsiveness to E2.

In conclusion, we have established a primary culture system for studying hormonal effects *in vitro* and found that the dosage of E2 inducing maximal cell proliferation and BrdU incorporation showed a large difference between uterine stromal cells and vaginal stromal cells. This primary stromal culture system is useful for studying the molecular mechanism of estrogenic effects on these tissues. Furthermore, the primary stromal cell system is suitable for screening the expression of mRNAs or proteins in response to E2 as well as environmental estrogenic chemicals. Grafted stromal cells with epithelial cells under the renal capsule showed proliferation in response to E2. Using this stromal culture system, underlying molecular mechanisms of epithelial-stromal interactions in the uterine and vaginal tissues can be studied in the near future.

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