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# 자궁내막 세포의 분리 및 배양 그리고 불멸화된 세포주의 확립

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## Endometrial Cell Culture: Isolation, Characterization, and Immortalization.

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**목 적:** 본 실험의 목적은 자궁내막세포를 분리 및 배양법 확립과 함께 불멸화 시키는 것이다.

**방 법:** 자궁내막에서 상피세포(epithelial cells)와 기질세포(stromal cells)의 분리는 Satyawaroop 등(1979)의 방법에 기초를 두었다. 자궁내막에서 상피세포와 기질세포의 순수 분리도를 확인하고, 불멸화된 기질세포에서 SV40 large T antigen을 확인하기 위하여 면역형광 염색(immunocytochemistry)과 Western blot 기법을 이용하였다. 정상 기질세포의 경우 subconfluence (60%) 상태에서 transfection을 진행하였다. 순수 분리된 plasmid DNA와 Qiagen 사의 superfect를 이용하여 transfection을 실시하였다.

**결 과:** 본 연구에서 우리는 두 가지 형태의 자궁내막 세포의 분리 및 배양에 성공하였다. 상피세포는 다면체의 형태를 띠며, 선(grandular) 조직의 조각으로부터 나선형으로 자란다. 기질 세포는 길쭉한 형태를 띠며, 상피세포에 비해 오래 살고, 빠르게 증식하여 나란한 형태로 배열된 세포 다발(cell bundle)을 형성한다. 이렇게 분리된 세포들은 95%의 균질성을 보였으며, 면역형광염색과 western blot을 통해 확인 하였다. 한편 SV40(Simian Virus 40) large T 항원을 암호화 하고 있는 염기 서열을 포함한 플라스미드 벡터로 안정적인 트랜스펙션을 시킴으로써 불멸화 된 자궁내막의 기질 세포주를 확립하였다. 불멸화 된 세포는 그 세포가 유래한 정상의 세포와 동일한 표현형을 가지고 있었다.

**결 론:** 본 연구에서, 우리는 자궁내막에서 상피세포(epithelial cells)과 기질세포(stromal cells)를 분리하여 배양법을 확립하였다. 동시에 SV40 large T antigen을 이용하여 불멸화된 세포주를 확립하였다. 이렇게 확립된 세포주는 자궁의 생리작용 연구 및 자궁내막증(Endometriosis)과 자궁암(Endometrial cancer) 등과 같은 여러 자궁관련 질병 연구에 많은 도움이 될 것으로 사료된다.

**Key Words:** Endometrial stromal cells, Endometrial epithelial cells, SV40(Simian Virus 40) large T antigen, Immortalization

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In vitro cell culture have been developed to study the physiology, biology and pharmacology of several organs such as breast, kidney, liver, prostate, bone and thyroid, either normal or malignant. Endometrial tissue represents also an interesting model to understand the role of intrinsic and extrinsic factors, ie, hormones and growth factors, involved in it`s normal and pathologic development and it`s cyclic growth[1]. Endometrium is one of the most rapidly renewing tissues of the body, and the proliferation of endometrial cell is believed to be dependent mainly on estrogen. The endometrium is composed of epithelial and stromal cells, containing fibroblast, myofibroblast and infiltrating hematopoietic cells. Especially, stroma can promote epithelial development or differentiation in the female reproductive tract [2~4].

While, human diploid cells have a limited life span, ending in replicative senescence, in contrast to cell lines derived from tumors, which show an indefinite life span and are immortal[5~6], suggesting that replicative senescence is a tumor suppression mechanism. In this study, we successfully immortalized normal human endometrial stromal cells using SV40 large T antigen. Therefore, this cell line may give rise the useful tool for understanding and exploring endometrial biology and disease including cancer and endometriosis.

## MATRIALS AND METHODS

### 1. Tissue specimens

Human endometrial tissue was obtained from normal cycling patients who had undergone hysterectomy. Tissue samples (2-3g) were obtained from the proliferative (days 5-14) and secretory phase (days 15-26) from women who received no hormonal therapy within the prior 30 days. Endometrial tissue was transported to the laboratory in isolation media consisting of M199/F12 culture media (Gibco Life Technologies, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Gibco Life Technologies, Gaithersburg, MD, USA) plus antibiotic and antimycotic agents to yield final concentrations of 200 units penicillin, 0.2 mg/ml streptomycin and 0.5 µ ml amphotericin-B (antibiotic/antimycotic solution, Sigma, St Louis, MO, USA).

### 2. Isolation of endometrial epithelial and stromal cells

Endometrial tissue was rinsed in HBSS to remove blood and debris. Separation of glandular and stromal components was based on a modification of the work of Satyaswaroop and colleagues[7] and Julia and colleagues[8]. After gentle centrifugation (600 g) the supernatant was removed, and the tissue was placed on a 100-mm plastic tissue culture dish (Corning-Costar, Cambridge, MA, USA). The entire procedure was performed under a sterile laminar flow hood. The tissue was minced with sterile scalpels into 1-2mm fragments and digested with collagenase (2 mg/ml; Sigma, St Louis, MO, USA) in M199/F12 (as above) for 2.5 h at 37°C on a shaking incubator. The tissue digest was vigorously pipetted and added to a stacked sterile wire sieve assembly with number 100 wire cloth sieve (140 µ size; Newark Wire Co., Newark, NJ, USA), followed by a number 400 wire cloth sieve (37 µ ). After the endometrial digest was added to the top of the sieve assembly, the epithelial glands were retained in the number 100 and 400 sieves while the stromal cells passed through to the receptacle below. The glands were back-flushed out of the sieves onto a 100 mm sterile dish using the same medium. Any stromal cells remaining with the glands were further separated by selective adherence to plastic tissue culture dishes for 1h. Stromal cells were collected from the lower receptacle, pelleted by centrifugation and resuspended in isolation medium. Red blood cells were removed by careful washing after stromal cells attached to 100 mm sterile dish.

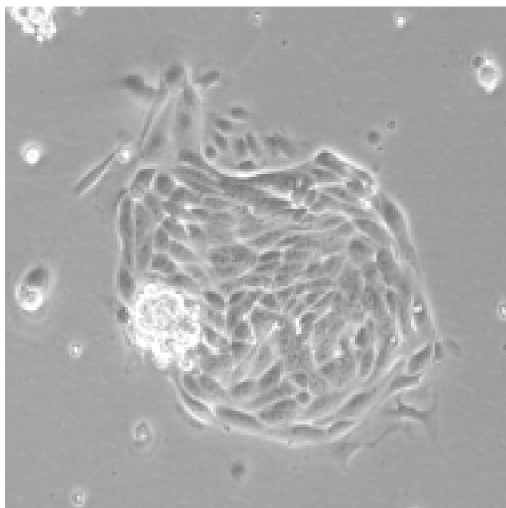
### 3. Cell culture medium

Stromal cells were maintained in medium consisting of a 1:1 mixture of M199:Ham's F12 media

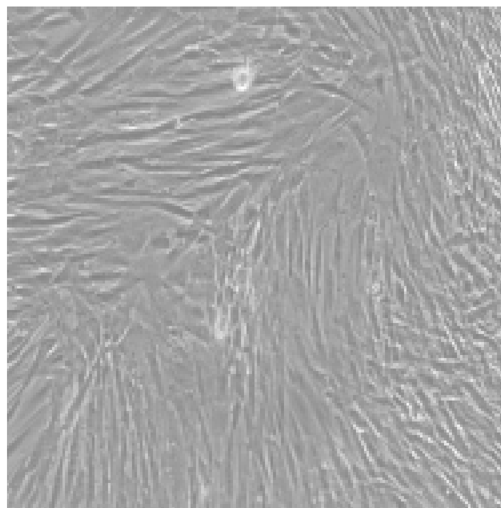
(Gibco Life Technologies) supplemented with 10% FBS(Gibco Life Technologies), ITS+[containing insulin (0.62 μ ml), transferrin (0.62 μg/μℓ), and selenium (0.62 ng/ml), bovine serum albumin (125 μg/μℓ) and linoleic acid (52.6 μ ml) (Sigma, St Louis, MO, USA)] plus 100 units penicillin, 0.1 mg/ml streptomycin and 0.25 μ ml amphotericin B (antibiotic/antimycotic solution, Sigma) according to the method of Julia T et al[9]. Culture medium was routinely changed every 2-3 days. The epithelial gland cells required little or no serum and were cultured in media consisting of M199 and F12 (1:1) with added bovine pituitary extract (BPE; 2ml/l) (Collaborative Biomedical Products, Bedford, MA, USA), ITS+ (concentrations as above), and antibiotic and antimycotic agents as described above.

### 4. Immunocytochemistry

To confirm the purification of the stromal cells and the expression of transfected SV40 large T antigen, immunocytochemical analysis was performed using cytokeratin 8/18 (Santa Cruz Biotechnology, CA, USA) as a marker of epithelial cells, vimentin (Chemicon, Temecula, CA, USA.) as a marker of stromal cells, and monoclonal antibody against SV40 large T antigen (Santa Cruz Biotechnology, CA, USA). For immunocytochemistry, cultures were rinsed with PBS and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. They were then treated with ice cold 100% methanol for 10 min, then 100% acetone for 5 min, then 0.4% Triton X-100 in PBS for 10 min, with triple PBS rinses each between treatments. Samples were treated with 2% horse serum and 2% goat serum in PBS containing 4% BSA (PBS/BSA) for 100 min at 37°C to block non-specific binding of primary antibodies. Antibodies were diluted in PBS/BSA plus 2% horse and 2% goat sera at 1:100 for cytokeratin, 1:100 for vimentin, and 1:100 for antibody against SV40 large T antigen. The primary antibodies were incubated on cells for 1h at 37°C. Samples were rinsed three times with PBS. Fluorescent secondary antibodies were added concurrently: FITC anti-mouse IgG diluted 1:100 (Zymed Laboratories, Inc., San Francisco, CA, USA) in PBS/BSA



**A**



B

**Figure 1.** Photomicrographs of endometrial epithelial and stromal cells. The different cultures were observed with a phase-contrast inverted microscope. Stromal cells typically apprear spindle shaped and reach confluence within a short period of times (**A**). Epithelial cells derived from the glandular epithelium initially presented a whorled morphology, but within 2 weeks cell integrity deteriorated (**B**).

plus 2% horse and 2% goat sera for 45 min at 37°C. Slides were rinsed with PBS and coverslips added. Fluorescence was visualized using a fluorescent microscope.

### 5. Western blot analysis

Western blot analysis was performed as described previously[10,11]. Cells were grown in a 100 mm tissue culture dish (Nunc, Rochester, NY, USA) to the same confluency and were then treated with test compounds. Proteins were extracted with 20% SDS solution containing 1 mM phenylmethylsulfonyl fluoride (a protease inhibitor), 10 mM iodoacetoamide, 1 mM leupeptin, 1 mM antipain, 0.1 mM sodium orthovanadate and 5 mM sodium fluoride. Protein content was determined using the DC assay kit (Bio-Rad, Hercules, CA), and separated on 12.5% SDS-PAGE according to the method of leupeptin. They were then transferred to nitrocellulose membranes at 100 V, 350 mA for 1 h. All antibodies were used according to the manufacturers instructions and protein bands were detected using an ECL detection kit (Amersham, Piscataway, NJ).

### 6. Immortalization of endometrial stromal cells

The plasmid pMK 16, containing SV40 replication origin defective gene (SV40 ori-), was used in this study. Normal cell were transfected 12 day in culture when subconfluent. This purified plasmid DNA was transferred into the cells using DNA superfect(Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. Brifly, 1.5$\mu g$ plasmid and 40$\mu g$ DNA superfect in 3ml serum free medium were added to culture which had been washed twice with serum free medium. After 24h incubation at 37°C, fresh medium was added to the culture. A successful transfection was assessed after 5 or 6 days by a sharp increase of cell doubling time and SV40 large T antigen immunostaining. Growth curves of normal or transfected endometrial stromal cells have been evaluated: $2\times10^5$ cells were plated in 75cm$^2$ flask and culture as reported above. Medium was changed every 2 day. The proliferation potential of transformed clones was determined by their total CPDL (cumulative population-doubling levels) using the formula cpdl = ln ($Nf/Ni$)/ln 2, where $Ni$ and $Nf$ are initial and final cell numbers, respectively, and ln is the natural log. The initial cell number was 2 x $10^5$ for each propagation.

## RESULTS

### 1. Isolation of normal endometrial cells

Epithelial cells were polyhedral and grew as islands in a whorl-like pattern around glandular fragment, and stromal cells were spindle-shaped, more long-lived and grew rapidly to form parallel bundles of cells as shown in Figure 1. Stromal cells typically apprear spindle shaped and reached confluence within a short period of times. Epithelial cells derived from the glandular epithelium initially presented a whorled morphology, but within 2 weeks cell integrity was deteriorated.

### 2. Characterization of endometrial cells

The intermediate filaments vimentin and cytokeratin were detected immunocytochemically to proof the epithelial or stromal origin of the cultivated cells. Cytokeratin 8/18(the marker of epithelial cells) was positive for the epithelial cells, whereas vimentin (the marker of stromal cells) was positive for stromal cells as shown in Figure 2.

### 3. Immortalization of endometrial stromal cells

1-2 weeks after transfection (with SV40 large T antigen), rapidly growing colony were observed. After conflunecy, they lost contact inhibition and grow as overlayer. Transfected endometrial stromal cells were tested for the expression of several specific antigens previously observed in normal endometrial cells. The expression of sv40 large T antigen was tested by immunocytochemistry and showed a strong nuclear straining in 100% cells of immortalized endometrial

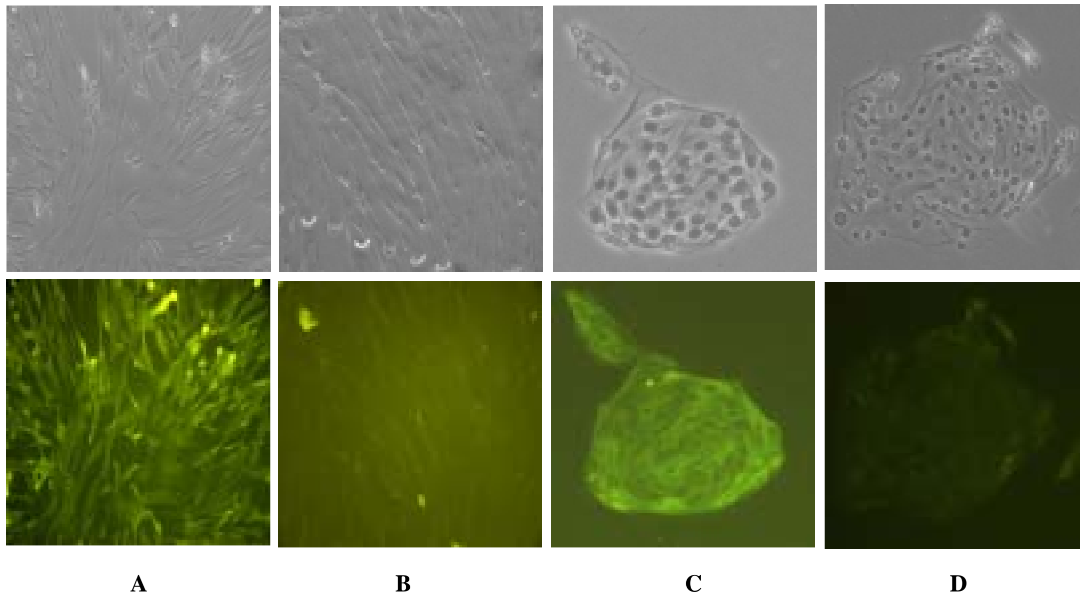
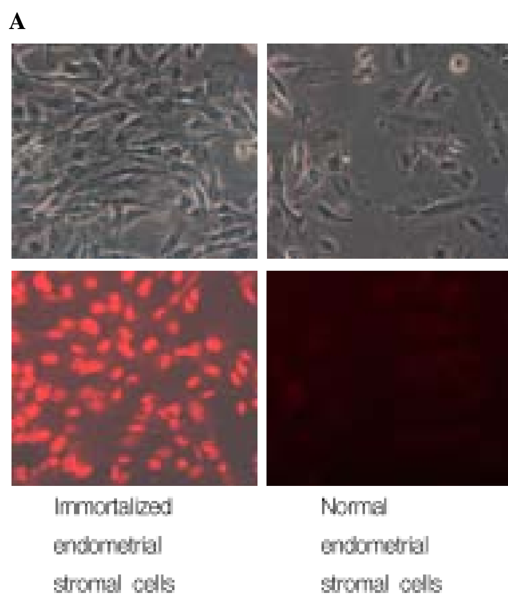



**Figure 2.** Immunofluorescent staining of endometrial epithelial and stromal cells. Stromal cells immunostained for vimentin (**A**), but stromal cells were not stained for cytokeratin as shown (**B**). However, Epithelial cells immunostained for cytokerain (**C**), but epithelial cells were not stained for vimentin (**D**).



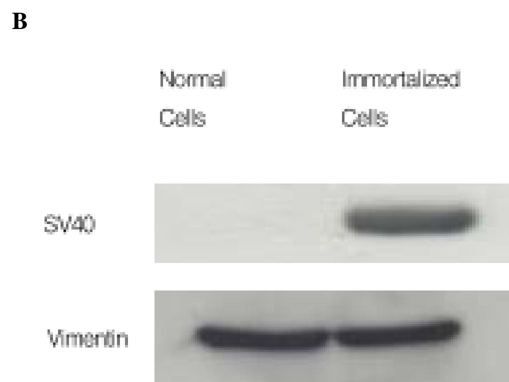




**Figure 3.** The SV40 large T antigen expression of normal endometrial stromal cells and immortalized endometrial stromal cells. Both in immunostaining (**A**) and western blot analysis (**B**), SV40 large T antigens strongly expressed in immortalized endometrial stromal cells, but, in normal endometrial stromal cells, SV40 large T antigens were not expressed.

stromal cells as shown in Figure 3. We carried out with cumulative population-doubling levels (CPDLs) of immortalized endometrial stromal cells to study of growth characteristics and production. This result suggests that, immortalized endometrial stromal cells had an extended life span (CPDL60) as shown in Figure 4. The transfected endometrial stromal cells were showed by 100% positive vimentin expression

and negative for cytokeratin8/18 expression as shown in Figure 5.

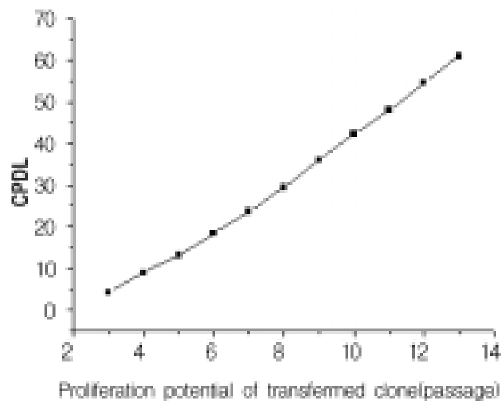



**Figure 4.** Cumulative growth curve of transformed cells at immortalization. We carried out with cumulative population-doubling levels (CPDLs) of immortalized endometrial stromal cells to study of growth characteristics and production. This result suggests that, immortalized endometrial stromal cells had an extended life span.

## DISCUSSION

The aim of this work was the isolation of highly purified human endometrial epithelial and stromal cells in order to set up a model system to study endometrial physiology, in particular endocrine and paracrine response and interaction. Stromal cells typically apprear spindle shaped and reached confluence within a short period of times. Epithelial cells derived from the glandular epithelium initially presented a whorled morphology, but within 2 weeks cell integrity deteriorated. We also tried to develop a more stable model for these studies by immortalization of endometrial stromal cells.

The pure endometrial stromal cells were immortalized by SV40 large T antigen using a similar protocol with Kang *et al*[12,13]. These endometrial cells have integrated the SV40 genome and large T antigens were shown by immunostaining analysis. Phenotypic characteristics of transfected cells were similar to

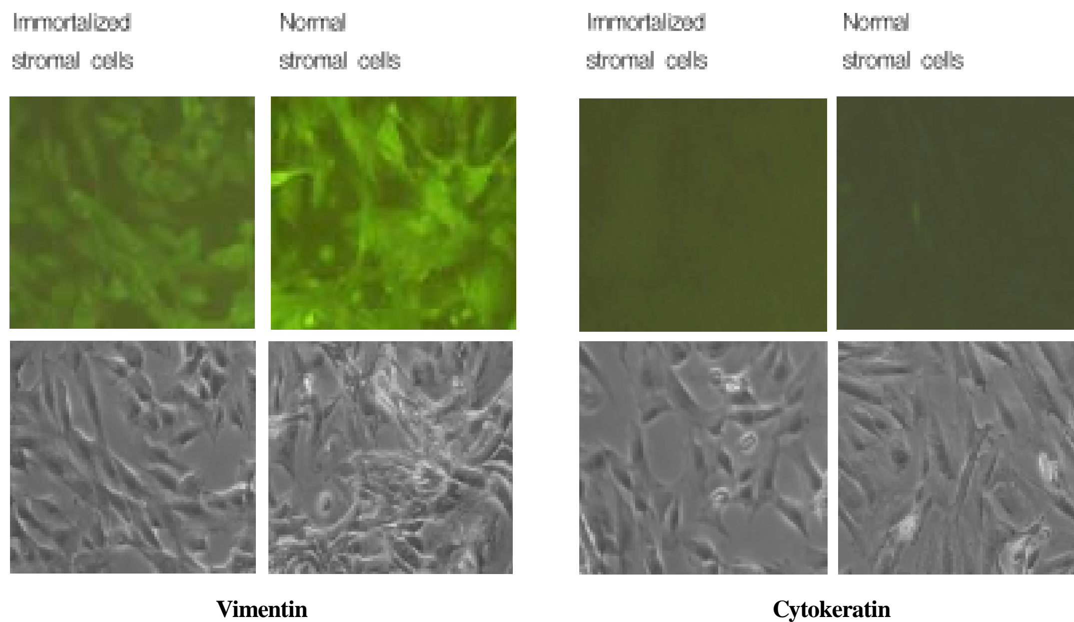



**Figure 5.** Immunochemical trait of immortalized endometrial stromal cells and normal endometrial stromal cells. Phenotypic characteristics of transfected cells were similar to those of their normal corresponding endometrial cells. Transfected endometrial stromal cells were showed by 100% positive vimentin expression and negative cytokeratin8/18 expression.

those of their normal corresponding endometrial cells. However, the frequency of immortalization is quite low. For example, Shay and Wright (1989) determind a frequency of $10^{-7}$ of immortal cells in a SV 40 transformed human fibroblast (SV/HF) culture. The SV40 large T antigens were known to interact with anti-oncogene products such p53 and Rb, and may be involved in the dysregulation of proliferation and differentiation-associated genes[15,16]. Therefore, these immortalized cells could be useful for further study of paracrine interaction, and the initial stages of transformation and endometrial cancer.

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