In Vitro Model of Stromal and Epithelial Immortalized Endometriotic Cells

Mariarosaria Boccellino,[1] Lucio Quagliuolo,[1] Annarita Verde,[1] Raffaele La Porta,[1] Stefania Crispi,[2] Maria Teresa Piccolo,[2] Antonio Vitiello,[1] Alfonso Baldi,[1,3*] and Pietro Giulio Signorile[3*]

[1]Department of Biochemistry and Biophysics, Second University of Naples, Naples, Italy
[2]Gene Expression & Human Molecular Genetics Laboratory, Institute of Genetics and Biophysics, CNR, Naples, Italy
[3]Fondazione Italiana Endometriosi, Rome, Italy

ABSTRACT

Endometriosis is a relatively common chronic gynecologic disorder that usually presents with chronic pelvic pain or infertility. It results from implantation of endometrial tissue outside the uterine cavity. Despite its frequency and its impact on quality of life, the understanding of pathogenesis of endometriosis remains incomplete and its treatment remains controversial. In this work, we established a suitable in vitro model system of immortalized human endometriotic cell line taking advantage of the human telomerase reverse transcriptase. The results demonstrate that these cells retain the natural characteristics of endometrial cells in term of phenotype and of functional expression of estrogen and progesterone receptors, without chromosomal abnormalities. In conclusion, these cells are potentially useful as an experimental model to investigate endometriosis biology. J. Cell. Biochem. 113: 1292–1301, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: ENDOMETRIOSIS; HTERT RETROVIRUS; ESTROGEN RECEPTORS; STEROID HORMONES

Endometriosis is defined as the proliferation of tissue similar to endometrium but residing outside of the uterine cavity. It is one of the most common gynecological diseases, often requiring surgical treatment (Bulun, 2009). The disease affects up to 10% of all reproductive-aged women and the prevalence rises to 20–50% in infertile women (Rapkin et al., 2000; Taylor et al., 2002; Baldi et al., 2008).

Endometriotic lesions are primarily located on the pelvic peritoneum and ovaries but can also be found in the pericardium, pleura, lung parenchyma, and even in the brain (Giudice et al., 1998; Laschke and Menger, 2007; Signorile et al., 2009b). Implants can result in substantial morbidity, including pelvic adhesions and pain, allergies, fatigue, bowel problems, and infertility, often requiring extensive medical and surgical treatments (Behera et al., 2006; Redwine, 2006). Hence, this disease is costly and both physically and psychologically debilitating. The etiology of the disease is still unknown, being retrograde menstruation, coelomic metaplasia or both the most recognized hypotheses (Starzinski-Powitz et al., 2001; Gazvani and Templeton, 2002a; Slater et al., 2005). A different theory of special interest is based on a defect in embryogenesis (Signorile and Baldi, 2010). To support the latter theory, our research group has recently shown the presence of ectopic endometrium in a significant number of human female fetuses (Signorile et al., 2009a, 2010a; Signorile et al., 2011). Although the exact molecular mechanisms underlying this phenomenon remains to be clarified, data from an animal model of endometriosis created in our laboratory suggests alterations in the fine tuning of female genital structures organogenesis due to abnormal activation of the estrogen morphogenetic pathway during the fetal life (Signorile et al., 2010b).

However, apart from the origin, there is a general agreement that endometriosis is associated with a local inflammatory response, and that vascularization at the site of invasion plays a decisive role in the pathogenesis of the disease (Haskill et al., 1988; Starzinski-Powitz et al., 2001; Gazvani and Templeton, 2002ab; Slater et al., 2005). Although it is well accepted that endometriotic lesions are composed of stromal and epithelial glandular cells, little is known about the development and characteristics of the cell types contributing to the pathogenesis of endometriosis. Nevertheless, studies on the

molecular, cellular, and pathophysiological parameters in endometriosis are limited by scarcity of in vitro model systems such as endometriotic cell lines. In particular, the lack of a stable cellular model renders the study in this area difficult, because of the extremely short life span of primary cultured endometrial cells in vitro.

One major mechanism that accounts for the limited life span of primary cultured cells is telomere-based replicative senescence. Telomeres are the specialized nucleo-protein structures, which are located at the ends of eukaryotic chromosomes. Without complete replication of telomeric DNA, the telomeres slowly shorten until cell division is arrested. Telomerase is a ribonucleoprotein complex that extends and maintains the telomeres. Activation of this enzyme is, therefore, required for cells to overcome replicative senescence and obtain the ability to divide without limit (Counter et al., 1992).

The aim of the present study was to establish a new in vitro model system of immortalized human endometriotic cell line taking advantage of the human telomerase reverse transcriptase (hTERT). Our results demonstrate that these cells do not have transformed phenotypes and retain the natural characteristics of endometrial cells without chromosomal abnormalities, and thus are potentially useful as an experimental model to investigate endometriosis biology.

MATERIALS AND METHODS

ISOLATION AND CULTURE OF HUMAN ENDOMETRIAL STROMAL AND EPITHELIAL CELLS

Deep endometriotic tissue samples were obtained, by laparoscopy, from patients with histologically confirmed endometriosis during the proliferative phase of the menstrual cycle. Isolation and culture of human endometrial stromal cells and endometrial epithelial cells were made as previously described (Osuga et al., 1995; Koga et al., 2001). Fresh endometrial biopsy specimens collected in sterile medium were rinsed to remove blood cells. The tissues were minced into small pieces and incubated in phenol-red free DMEM/F-12 containing type I collagenase (0.25%) and deoxynuclease I (15 U/ml) for 120 min at 37°C. The resultant dispersed endometrial cells were separated by filtration through a 40-μm nylon cell strainer. Endometrial epithelial cells were retained by the strainer, whereas dispersed stromal cells passed through the strainer into filtrate. Epithelial cells in the filtrate were collected by centrifugation and resuspended in DMEM/F-12 containing 10% heat-inactivated FBS, 100 U/ml of penicillin, 0.1 mg/ml of streptomycin, and 0.25 μg/ml amphotericin B. Endometrial stromal cells were seeded in a 100-mm culture plate and kept at 37°C in a humidified 5% CO_2-95% air atmosphere. Endometrial epithelial cells were collected by back-washing the strainer with complete DMEM/F-12 medium, seeding in a 100-mm plate, and incubation at 37°C for 60 min to allow contaminated stromal cells to attach to plate wall. The non-attached epithelial cells were recovered and cultured in the complete medium in a 100-mm culture plate. The purity of stromal and epithelial cells was confirmed immunocytochemically. Finally, the cells were grown to confluence and, then, were seeded onto six-well dishes for subsequent immortalization.

IMMUNOCYTOCHEMISTRY

Endometriotic stromal and epithelial cells were cultured on glass slides for 24 h, fixed with 70% ethanol rehydrated through a graded alcohol series and washed in phosphate-buffered saline (PBS). PBS was used for all subsequent washes and for antiserum dilution. Sections were quenched sequentially in 3% hydrogen peroxide in aqueous solution and blocked with PBS-6% non-fat dry milk (BioRad, Hercules, CA) for 1 h at room temperature. Slides were then incubated at 4°C overnight at 1:100 dilution with the following antibodies: mouse monoclonal antibody against cytokeratin 7 (clone OV-TL 12/30) and CD10 (clone M7308) (Dako Laboratories, Carpinteria, CA). After three washes in PBS to remove the excess of antiserum, the slides were incubated with diluted anti-mouse biotinylated antibodies (Vector Laboratories, Burlingame, CA) at 1:200 dilution in PBS-3% non-fat dry milk (BioRad) for 1 h. All the slides were then processed by the ABC method (Vector Laboratories) for 30 min at room temperature. Diaminobenzidine (Vector Laboratories) was used as the final chromogen and hematoxylin was used as the nuclear counterstaining. Negative controls for each tissue section were prepared by leaving out the primary antiserum. Positive controls constituted of tumor tissues expressing either cytokeratin or vimentin, were run at the same time. All samples were processed under the same conditions.

RETROVIRAL VECTORS AND INFECTION

Endometrial stromal and epithelial cells were sub-cultured onto six-well dishes and allowed to recover for 4 days. Cells were, then, infected with the amphotrophic hTERT retroviral vectors supernatant in the presence of 8 μg/ml polybrene (first infection). Six to eight hours later, the viral supernatant from the first infection was removed and the cells were re-infected with fresh retrovirus supernatant with polybrene (second infection). Eighteen hours after incubation, the viral supernatant was removed and the appropriate complete growth medium was added to the cells and incubated at 37°C with 5% CO_2. Seventy-two hours after incubation, cells were cultured with Puromycin to select stable cell-line generation. Ten to fifteen days after selection, the clones were selected and isolated for expansion and screened for positive ones.

KARYOTYPING

Cells were harvested when 50–70% confluent with actively dividing cells present. Colcemid was added to a final concentration of 0.04 mg/ml at least 2 h before harvesting. Cells were trypsinized and harvested to produce G-banded metaphase preparations. Karyotype analysis and documentation were performed by a computerized acquisition and analysis system (Applied Spectral Imaging). A minimum of 20 cells were analyzed from each immortalized and parent primary cell culture for chromosomal clonal structural or numerical abnormalities.

REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION (RT–PCR)

Total RNAs were extracted by using Trizol reagent (Invitrogen), strictly according to the manufacturer's instructions. The purity and quantity of total RNA were determined by Experion analysis (BioRad). For each sample, reverse-transcription reaction was performed using High Capacity cDNA Reverse Transcription Kits

(Applied Biosystems) following manufacturer's instructions and starting from 1 μg total RNA. First-strand cDNA was synthesized with the use of 1 μg total RNA. The total product cDNA (20 μl) was diluted with 60 μl H$_2$O, then 4 μl were used for the PCR reaction using the AmpliTaq Gold DNA Polymerase (Applied Biosystems). The RT-PCR negative control and a PCR negative control without cDNA were also used. Specific primers were used for each receptor analyzed: Er-alpha Forward 5′-GCAGACAGG-GAGCTGGTTCA-3′; Reverse 5′-GCCTTTGTTACTCATGTGCC-3′; ER-beta Forward, 5′-GGCAACTACTTCAAGGTTTCGAG-3′; Reverse, 5′-ACTGAGACTGTGGGTTCTGGGAG-3′; PGR Forward 5′-TTAC-CATGTGGCAGATCCCACAG-3′; Reverse, 5′-ACCATCCCTGCCAA-TATCTTGGG-3′. All the primers were compared to the human genome to exclude any significant homology with BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi BLAST program). Amplicons were visualized on a 1% agarose.

MTT ASSAY

MTT assay is based on the conversion of the yellow tetrazolium salt to purple formazan crystals by metabolically active cells and provides a quantitative estimate of viable cells. Briefly, 1.0×10^4 cells were incubated in triplicate in a 96-well plate in the presence or absence of the indicated test samples in a final volume of 0.2 ml for various lengths of time at 37°C. Thereafter, 20 μl MTT solution (5 mg/ml in PBS) was then added to each well. After a 3-h incubation at 37°C, 100% (v/v) of a solubilization solution (10% 1 N HCl in 2-propanol) was added to each well. Finally, the plates were shaken and the optical density at 570 nm was measured using a multi-well plate reader (Microplate Reader; BioRad). Percent cell viability was calculated as cell viability of the experimental samples/cell viability of the control samples ×100. At least three independent experiments were performed.

RESULTS

ISOLATION AND CHARACTERIZATION OF HUMAN ENDOMETRIOSIC CELLS

We have successfully established epithelial and stromal cell monolayer cultures from endometriotic biopsies. The samples were minced and digested in a collagenase solution, then the resultant dispersed endometrial cells were separated by filtration in epithelial cells that retained by the strainer, and in stromal cells that passed through the strainer.

Morphologically, the epithelial cells exhibited a typical honey-comb-like morphology, while the stromal cells exhibited the typical fibroblastoid spindle-shape morphology (Fig. 1A,B). Next, we
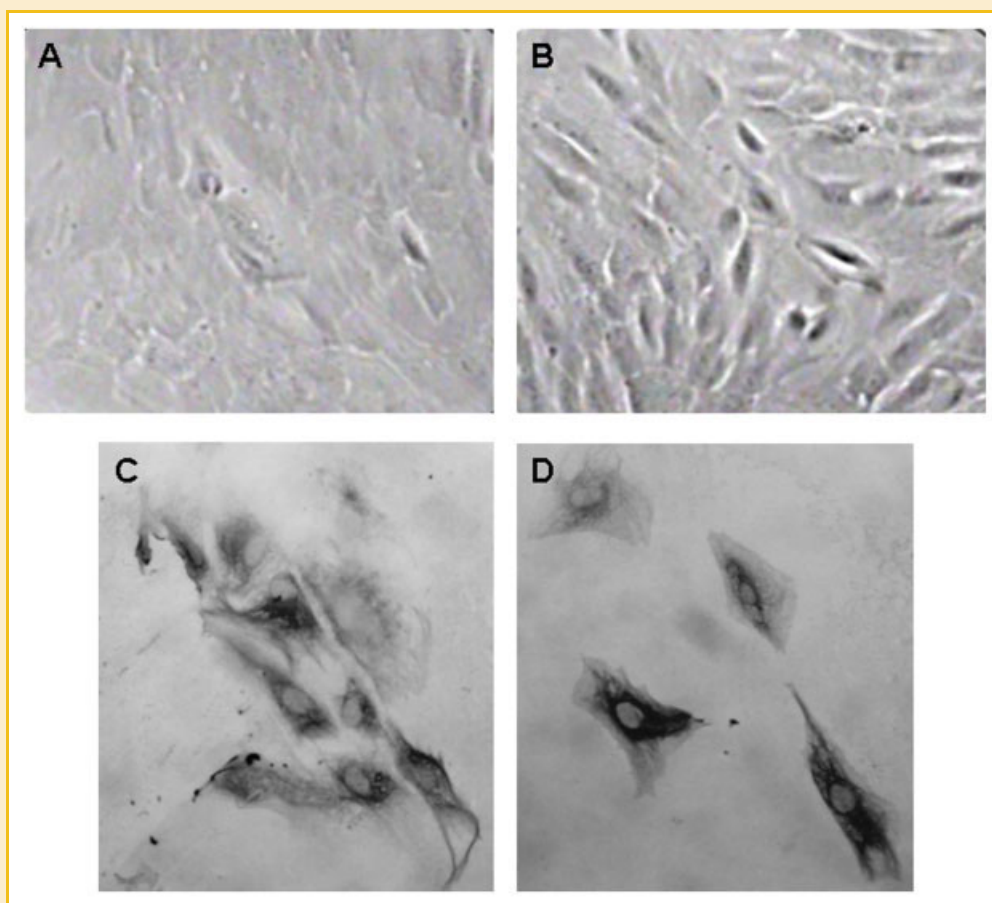

Fig. 1. The upper panel shows micrographs representative of cell shape. A: Epithelial cells; (B) stromal cells. Original magnification ×400. The bottom panel shows immunohistochemical analysis. C: Epithelial cells were positive for cytokeratin 7; (D) stromal cells were positive for CD10. Original magnification ×400.

verified the epithelial and stromal origin by immunocytochemistry. Cytokeratin 7 is a well-established markers of the gynecological tract (Wang et al., 2001), while CD10 is expressed in the stromal cells of endometriosis (Groisman and Meir, 2003). As shown in Figure 1C,D, epithelial cells expressed cytokeratin 7 while stromal cells were positive for CD10.

IMMORTALIZATION OF HUMAN ENDOMETRIAL CELLS: RETROVIRAL TRANSFECTION OF hTERT

To attempt immortalization, we plated the cells in a six-well plate 24 h before infection with the density around 30–40% confluence and, then, we exposed to retrovirus supernatant hTERT in the presence of 8 μg/ml polybrene. Polybrene is a polycation that neutralizes charge interactions to increase binding between the pseudoviral capsid and the cellular membrane. The microscopic observation revealed a normal cell growth, in fact, 24 h after infection, both the epithelial cells and the stromal had a confluence of about 80% (Fig. 2). This suggests that the infection has not caused any damage cellular activity. For both cell lines, the comparison between primary controls and their immortalized model did not show specific abnormalities in cell morphology, as shown in Figure 2.

Then, we proceeded to the selection with puromycin. The selection period lasted 15 days, having the cells daily observed by optical microscopy. The non-transformed parent cells died within 72 h after puromycin addition. Under phase-contrast microscopy, at the sixth day of selection, the transformed endometriotic cells went through a crisis and most of them died (Fig. 3). Another observation, performed at the end of the selection period, showed a resumption of growth of immortalized cells for both lines (Fig. 4). In particular, the immortalized cell lines (epithelial and stromal) showed a recovery of cell growth, mainly localized around a few clones (Fig. 4). This indicates the successful immortalization of proliferating clones that are resistant to puromycin. These cells were amplified for several generations resulting in the two cell lines of interest that have been subjected to further characterization.

RT–PCR DETECTION OF hTERT EXPRESSION

We have experimentally confirmed the successful immortalization by RT-PCR analysis for the expression of telomerase gene. hTERT mRNAs were detected in cultured epithelial and stromal cells from both transformed endometriotic cells and non-transformed parental cells. Indeed, as shown in Figure 5, elevated hTERT
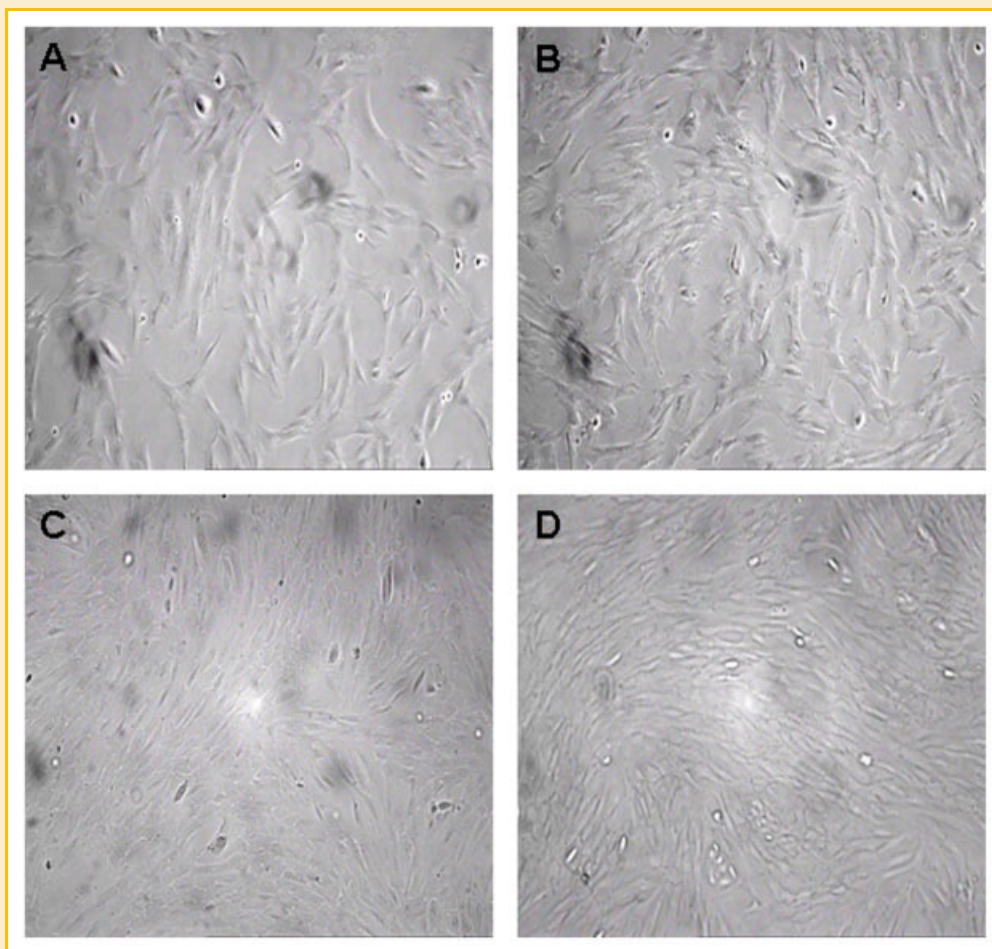

Fig. 2. Micrographs representative of cell growth 24 h after infection. A,B: Epithelial cells primary and immortalized, respectively. C,D: Stromal cells primary and immortalized, respectively. Original magnification ×100.
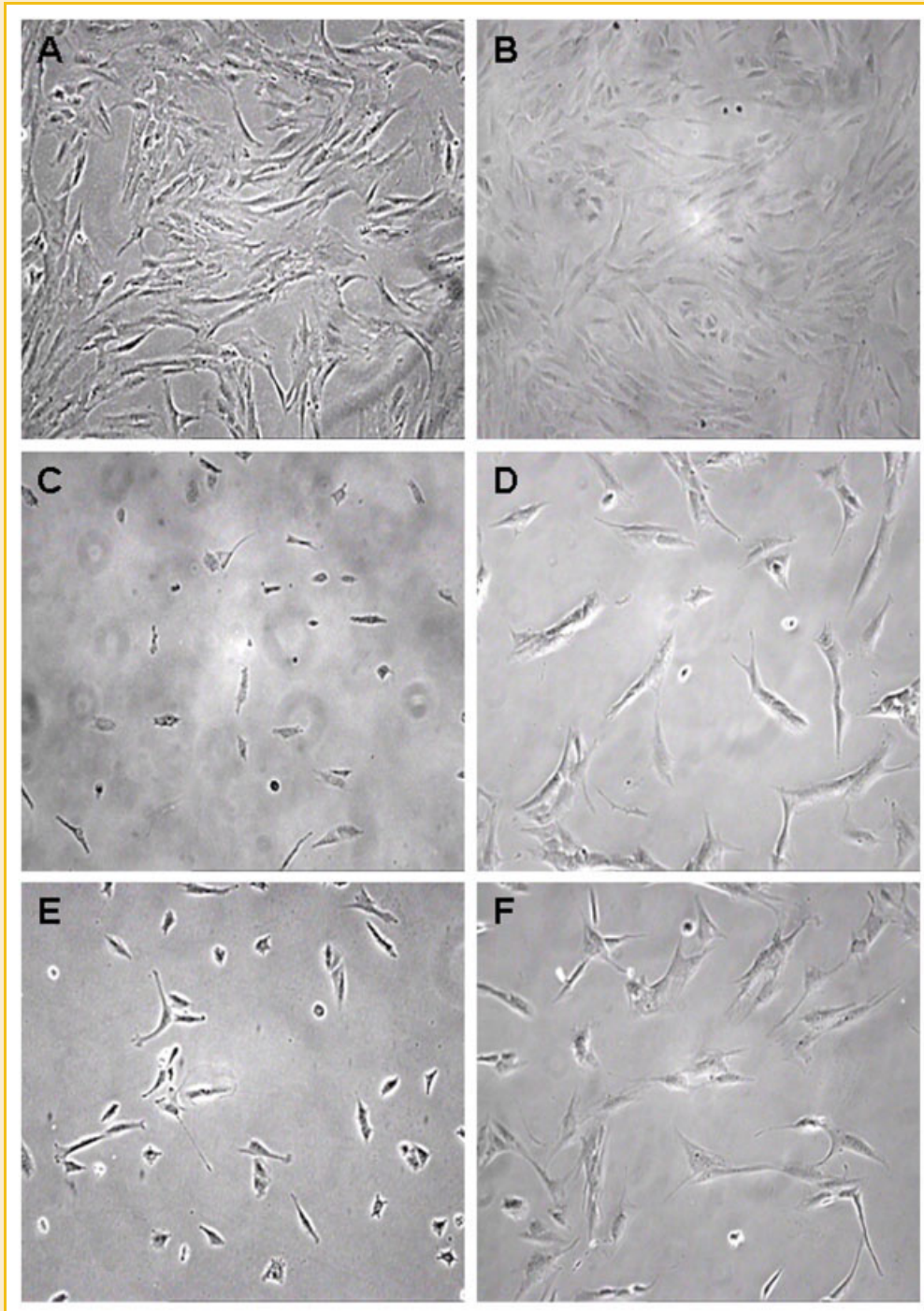
Fig. 3. Micrographs representative of cell growth at the sixth day of selection of epithelial cells (A, C, and E) and stromal cells (B, D, and F). A and B are primary controls; (C and D) primary controls in selection; (E and F) immortalized model in selection. Original magnification ×100.

transcript levels were detected in the immortalized lines but not in their controls.

CHARACTERIZATION OF HUMAN IMMORTALIZED ENDOMETRIAL CELLS

Morphology. In order to verify that immortalization did not result in any kind of cellular alterations, we undertook a cytogenetic and morphological characterization. Under phase contrast microscopy, the epithelial cells retained their typical morphology in the honeycomb, while in the stromal cells their characteristic elongated morphology as can be seen in Figure 6A,B.

Immunocytochemistry. Immunocytochemical markers for the characterization of the cytoskeleton showed a profile consistent with that obtained for the primary cell lines. As shown in
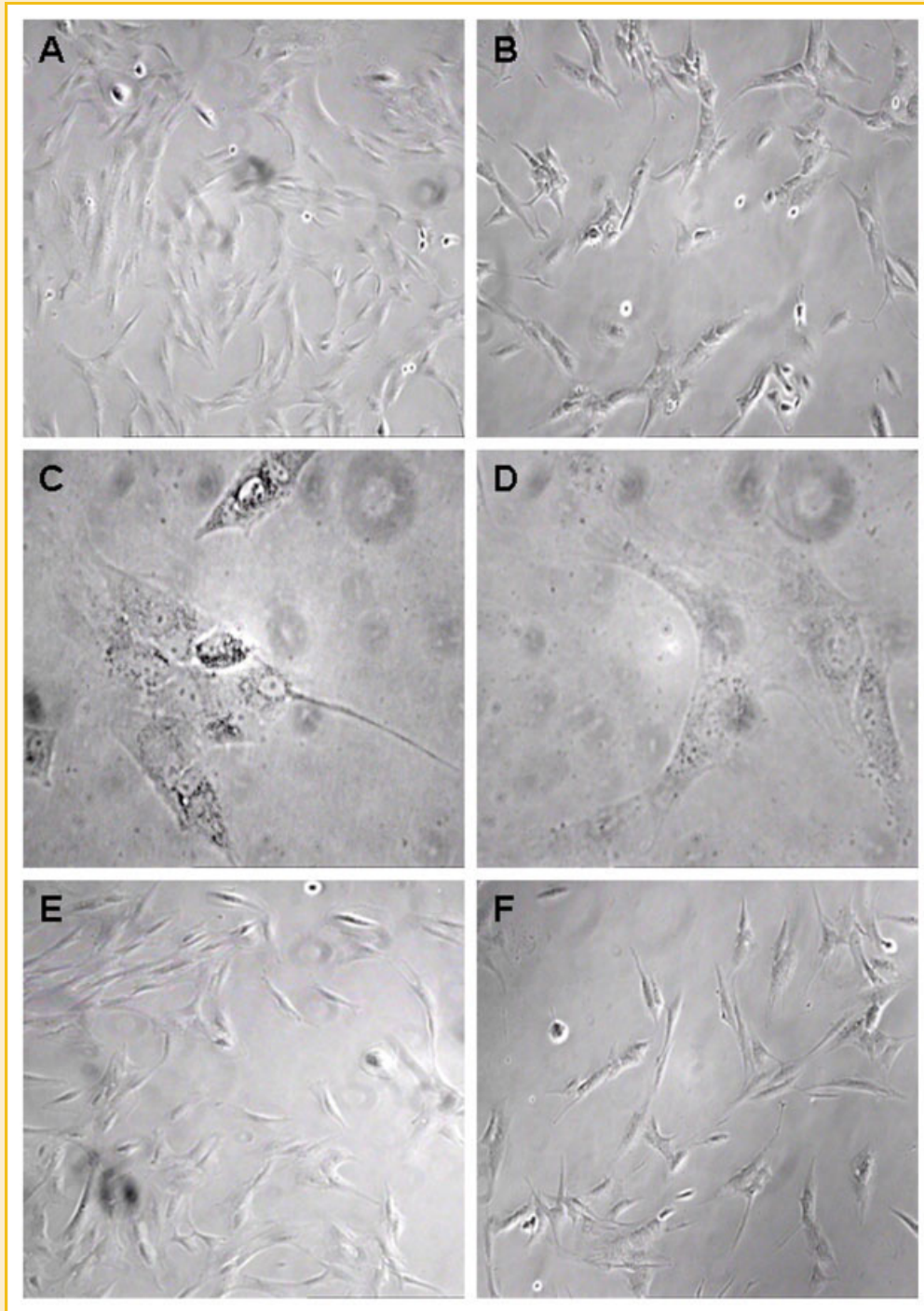
Fig. 4. Micrographs representative of cell growth at the end of the selection period of epithelial cells (A, C, and E) and stromal cells (B, D, and F). A and B are primary controls; (C and D) primary controls in selection; (E and F) immortalized model in selection. Original magnification ×100 (A, B, E, and F) and ×400 (C and D).

Figure 6C,D, the immortalized epithelial cells are positive for cytokeratin 7 and negative for CD10, while the immortalized stromal cells are positive for CD10 and negative for cytokeratin 7.

Karyotype. To investigate the effect of immortalization on cell karyotypes, the cytogenetic analysis was performed using standard G-banding analysis. This method allowed a homogeneous staining of all chromosomes, showing their size and morphology. A minimum of 20 metaphases per cell line immortalized and their primary controls were examined. The results obtained from either parental or immortalized cell lines revealed no clonal structural or numerical chromosome abnormalities (data not shown).

RT-PCR detection of estrogen and progesteron receptors. We next examined whether the line immortalized (epithelial and stromal) maintained the typical endometrial receptors. In particular,
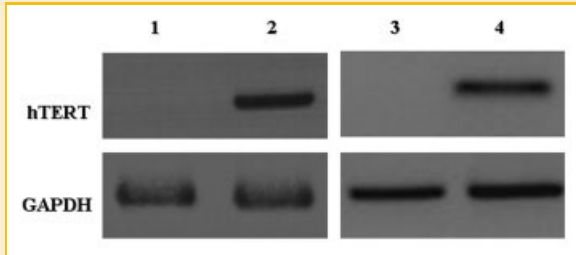
Fig. 5. RT–PCR detection of hTERT expression. Lines 1 and 3, primary cells epithelial and stromal, respectively; lines 2 and 4 immortalized cells epithelial and stromal, respectively. PCR products derived from GAPDH mRNA were used as controls.

α, β estrogen, and progesterone receptors were detected in cultured cells by RT-PCR. The results shown in Figure 7 indicate that cells presented both estrogen receptors, although the β receptors are expressed more than α. Moreover, in both lines the transcript for progesterone was detected. All GAPDH amplification revealed the 240-bp fragment corresponding to the length of the cDNA, but no 354-bp products representing genomic DNA contamination were detected. PCR controls, which did not include cDNA in the reaction, revealed no amplification products.

Hormone effects on primary and immortalized cells. Then, we examined the responsiveness of cells to two-steroid hormones. Primary and immortalized cells, both epithelial and stromal, were incubated in media with 5% charcoal-treated fetal calf serum for 48 h and, then, treated with 17 β-estradiol (E2) or 17 α-ethynyl estradiol at 10 nM for different time periods. Cell proliferation was examined by MTT assay. We first examined epithelial lines. As shown in Figure 8, the immortalized cells were more responsive to both hormones compared with their primary control. Indeed, treatment of immortalized lines for 48 h with E2 or 17 α-ethynyl estradiol resulted in twofold increases in cell proliferation, which was significantly higher than that observed with the primary lines. In the stromal lines, the response was lower in immortalized cells and was slower in the primary. In fact, the treatment for 48 h with both hormones produced a proliferation increase of 1.5-fold than control. These results indicate that the cellular model obtained retains the functional characteristics.

DISCUSSION

In the present study, we established a stable in vitro culture system of endometriotic cells that will benefit the study of endometriosis biology and carcinogenesis. In fact, primary endometrial epithelial
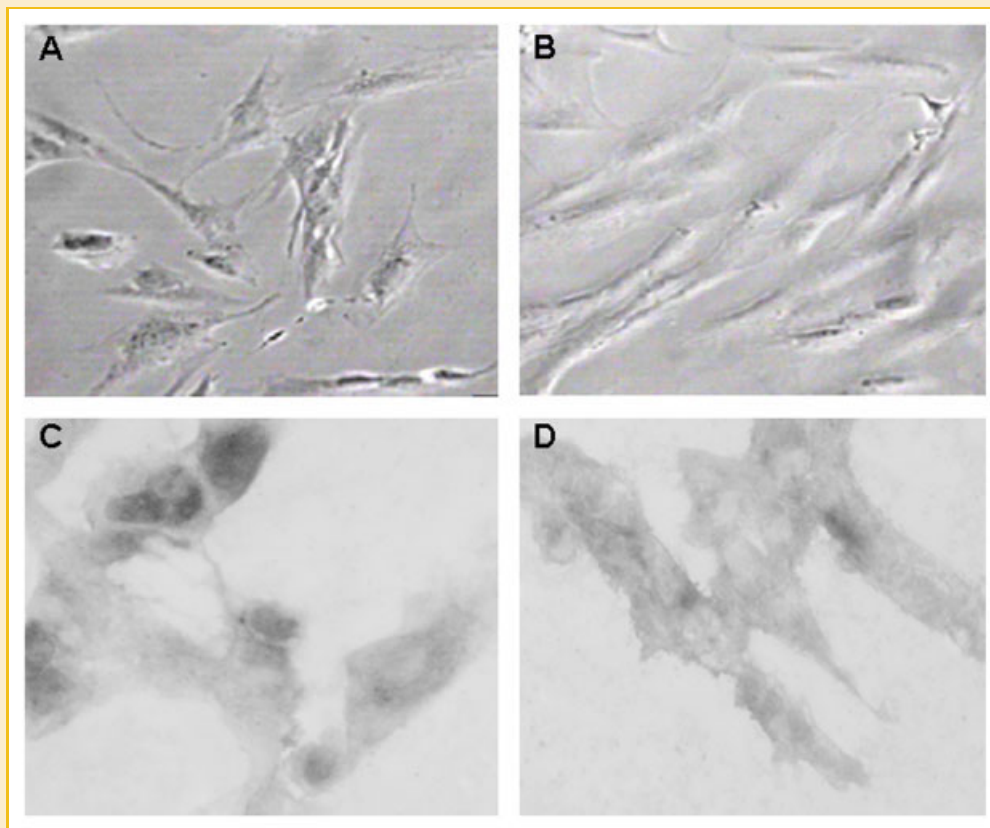

Fig. 6. The upper panel shows micrographs representative of cell shape of immortalized model. A: Immortalized epithelial cells; (B) immortalized stromal cells. Original magnification ×100. The bottom panel shows immunohistochemical analysis of immortalized model. C: Immortalized epithelial cells were positive for cytokeratin 7; (D) immortalized stromal cells were positive for CD10. Original magnification ×400.
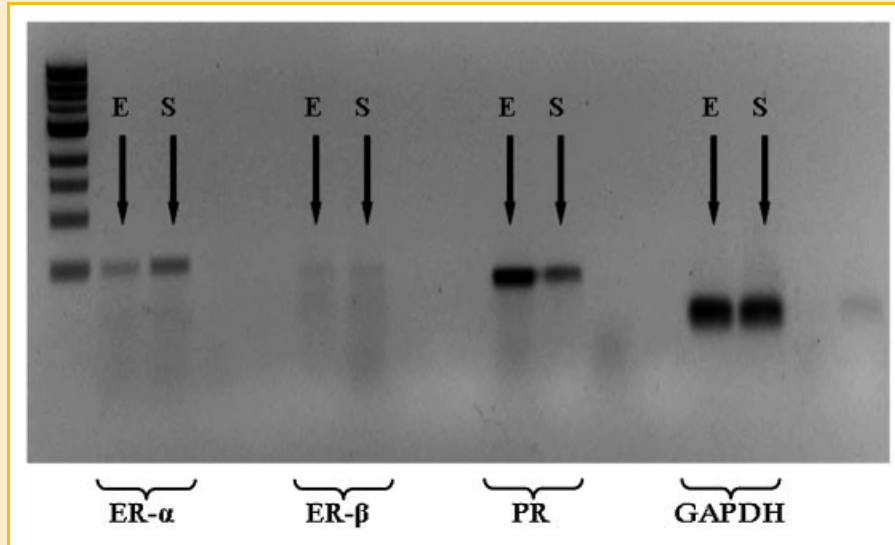
Fig. 7. Gene expression of steroid receptors in immortalized epithelial (E) and stromal (S) cells. Total mRNA isolated from cells was reverse-transcribed and amplified by PCR using primers for α, β estrogen (ER-α, ER-β), and progesterone (PR) receptors. PCR products derived from GAPDH mRNA were used as controls.

cells fall into senescence within 2 weeks when cultured on plastic dishes, and more complete understanding of endometriosis pathogenesis has been delayed because of a lack of a suitable in vitro culture model. We obtained primary cultures from women endometriotic biopsies by digesting with collagenase. Based on morphology and immunocytochemical staining, the primary cultures contained two different cell types: epithelial cells with a honeycomb-like morphology, expressing cytokeratin 7 and cells of fibroblastoid spindle-shape morphology, stromal cells, containing CD10. Then, we established immortalized human endometriotic cell line that retained the normal functions and characteristics of the primary cells.

Several methods exist for immortalizing mammalian cells in culture. The most recently discovered approach to cell immortalization is through the expression of telomerase reverse transcriptase protein (TERT), particularly for cells that are most affected by telomere length (e.g., human). This protein is inactive in most somatic cells, but when hTERT is exogenously expressed, the cells are able to maintain sufficient telomere lengths to avoid replicative senescence. Analysis of several telomerase-immortalized cell lines has verified that cells maintain a stable genotype and retain critical phenotypic markers. Through the introduction of an hTERT cDNA, we produced endometrial stromal and epithelial cells with greatly extended life spans. Insertion of hTERT resulted in reconstitution of the telomerase activity, elongation of telomere length, and extend life span. Whereas control cells stopped dividing after about 15–20 doublings, the hTERT-expressing endometrial cells have exceeded 80 population doublings and are continuing to divide at normal rates. Recent studies have shown that expression of hTERT proteins produced immortalized human fibroblasts and keratinocytes without transformation to a malignant phenotype (Dickson et al., 2000; Farwell et al., 2000). Consistent with these observations, the immortalized cell lines (epithelial and stromal) demonstrated

growth characteristics of normal cells but, unlike the parent cells, did not cease to grow after extended culture time. Occurred immortalization has been verified both empirically and experimentally. In fact, the immortalized cells of both lines have shown resistance to the selection with the antibiotic, other than their primary controls. On the other hand, the experimental confirmation that the cells obtained were actually in continuous culture, was obtained by evaluating the expression of hTERT gene by RT-PCR analysis, that showed the presence of the gene only in the transfected lines and not in controls.

The most important concern about using a cell culture model for molecular and cellular studies of endometriosis is whether these cell lines maintain their in vivo characteristics. First, immortalized cells express appropriate cell-specific cytoskeletal elements: cytokeratin 7 for epithelial cells and CD10 for stromal cells, furthermore, these cells did not differ by microscopic morphology. Next, we have evaluated the presence of molecules also found in endometriotic lesions in vivo that included markers such as estrogen receptor-α and -β, and progesterone receptor. Our results indicate that continuous lines contain estrogen receptor-α and low amounts of estrogen receptor-β while progesterone receptor is more expressed in the stromal lines. Finally, our data also demonstrate a functional estrogen response in these cells; in particular treatment of immortalized epithelial cells with 17 β-estradiol (E2) or 17 α-ethynyl estradiol, induced cell proliferation greater than in the primary controls. Regarding stromal lines, treatment with the same hormones produced a less marked response and more slowly. In the final analysis, morphological, cytogenetic and functional analyses showed a perfect overlap between the primary and continuous lines.

In conclusion, in this work we have created a valid and appropriate model of experimental study that can be used to evaluate the complex molecular and genetic mechanisms underly-
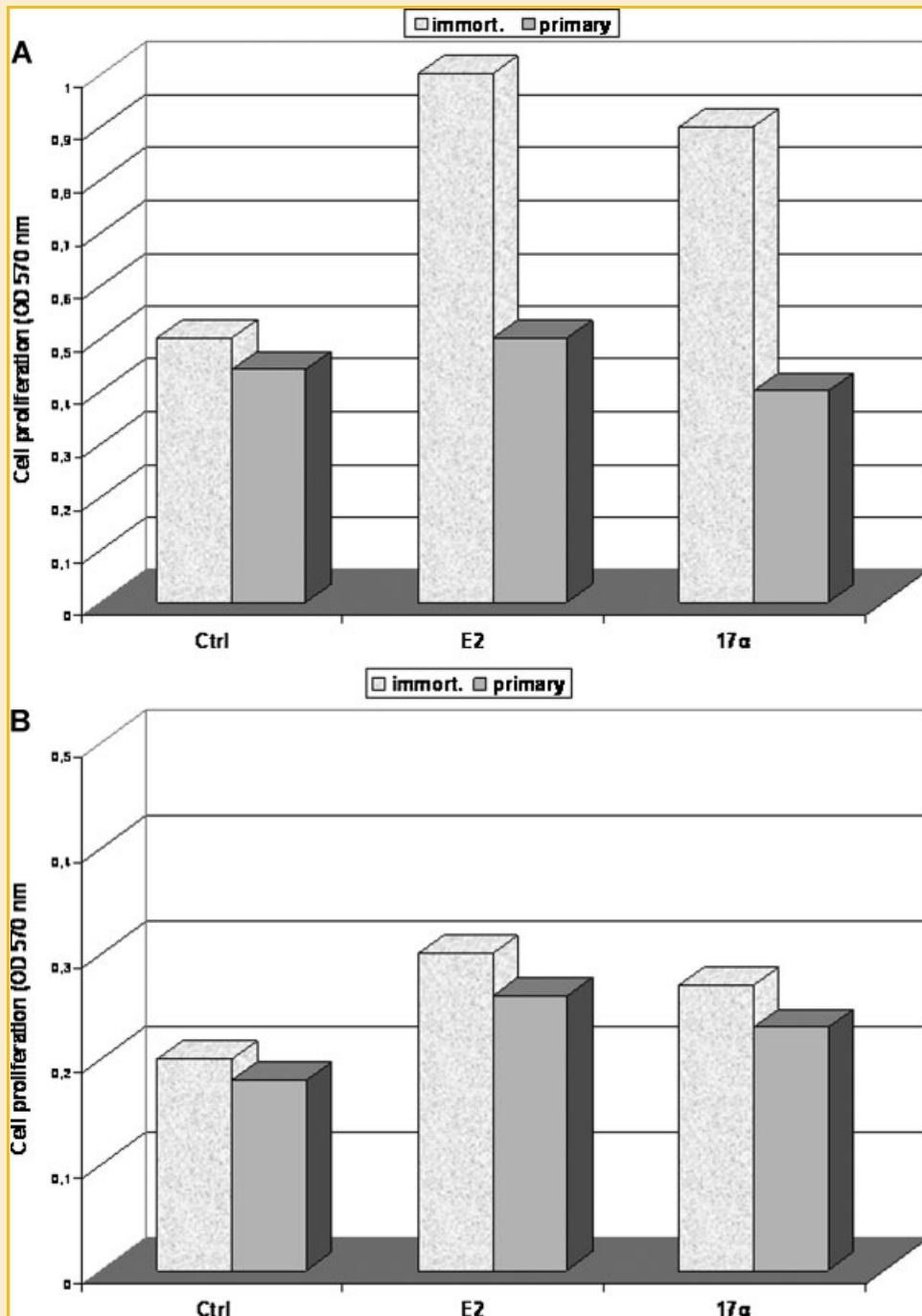
Fig. 8. Responsiveness of epithelial (A) and stromal (B) cells to two-steroid hormones. Primary and immortalized cells of both lines were seeded, cultured in media with charcoal-treated serum for 48 h, and stimulated with vehicle alone (Ctrl) or with 17 β-estradiol (E2) or 17 α-ethynyl estradiol (17-α) for 48 h at 10 nM. MTT proliferation assay were then performed. Results are the average of four wells in each sample.

ing endometriosis and, not least, the evaluation of effects of molecules that can act on therapeutic targets.

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