Regulation of functional steroid receptors and ligand-induced responses in telomerase-immortalized human endometrial epithelial cells

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Abstract

Information on the regulation of steroid hormone receptors and their distinct functions within the human endometrial epithelium is largely unavailable. We have immortalized human primary endometrial epithelial cells (EECs) isolated from a normal proliferative phase endometrium by stably transfecting the catalytic subunit (hTERT) of the human telomerase complex and cultured these hTERT-EECs now for over 350 population doublings. Active hTERT was detected in hTERT-EECs employing the telomerase repeat amplification assay protocol. hTERT-EECs revealed a polarized, non-invasive epithelial phenotype with apical microvilli and production of a basal lamina when grown on a three-dimensional collagen–fibroblast lattice. Employing atomic force microscopy, living hTERT-EECs were shown to produce extracellular matrix (ECM) components and ECM secretion was modified by estrogen and progesterone (P4). hTERT-EECs expressed inducible and functional endogenous estrogen receptor-alpha (ER-alpha) as demonstrated by estrogen response element reporter assays and induction of P4 receptor (PR). P4 treatment down-regulated PR expression, induced MUC-1 gene activity and resulted in increased ER-beta transcriptional activity. Gene activities of cytokines and their receptors interleukin (IL)-6, leukemia inhibitory factor (LIF), IL-11 and IL-6 receptor (IL6-R), LIF receptor and gp130 relevant to implantation revealed a 17 beta-estradiol (E2)-mediated up-regulation of IL-6 and an E2- and P4-mediated up-regulation of IL6-R in hTERT-EECs. Thus, hTERT-EECs may be regarded as a novel in vitro model to investigate the role of human EECs in steroid hormone-dependent normal physiology and pathologies, including implantation failure, endometriosis and endometrial cancer.

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Introduction


Endometrial carcinoma cell lines, including ECC-1, HEC-1A, RL-95, Ishikawa and EN, have long been employed as experimental models but their usefulness is
limited since their transformed phenotype has partially resulted in a loss of physiological growth regulation and polarization or altered steroid hormone receptor regulation (Thie et al. 1995, Kooiman et al. 1999, Di Nezza et al. 2003, Farnell & Ing 2003, Isaka et al. 2003). In particular, primary endometrial epithelial cells (EECs) display a rapid decrease in proliferative capacity within a few culture passages (Mulholland et al. 1986, Classen-Linke et al. 1997, Arnold et al. 2001). In addition, the process of dedifferentiation includes down-regulation of steroid hormone receptors (Schatz et al. 1990, White et al. 1990). However, due to the lack of experimental models retaining steroid hormone responsiveness there is conflicting evidence on the effects of 17 beta-estradiol (E2) on EECs (Marshburn et al. 1992, Zhang et al. 1995, Dardes et al. 2002).

Recently, hTERT overexpression has been employed as a novel strategy to immortalize human somatic cells, including human uterine leiomyoma and normal myometrial cells, human skin fibroblasts and retinal pigment cells (Bodnar et al. 1998, Counter et al. 1998, Carney et al. 2002). The catalytic subunit hTERT of the ribonucleoprotein telomerase complex is the rate-limiting factor for telomerase activity in normal human somatic cells facilitating the elongation of chromosomal telomeres (Counter et al. 1998). It is highly pertinent that immortalization of human somatic cells by virtue of overexpression of hTERT does not interfere with normal cellular physiology (Jiang et al. 1999, Carney et al. 2002).

In the normal human endometrium, telomerase activity has been exclusively detected during the proliferative phase of the cycle and localized to glandular epithelial cells at the base of the endometrial crypts within the stratum basale (Kyo et al. 1997, Tanaka et al. 1998, Yokoyama et al. 1998). These basal glandular epithelial cells provide a recurrent source for the cellular restitution of the endometrial epithelial lining during the proliferative phase of the cycle. In isolated primary EECs, E2 was unable to sustain telomerase activity, which has been reported to cease within 8 days of culture resulting in the senescence of primary EECs (Varma et al. 1982, Tanaka et al. 1998).

In the present study we present a novel hTERT-immortalized human endometrial epithelial cell line (hTERT-EECs) which displays a stable epithelial phenotype. Hormonally responsive to the actions of ovarian steroid hormones, estrogen receptor (ER)-alpha induced the expression of a functional P4 receptor (PR), which, in turn, affected expression of ER-beta in these immortalized cells. The hTERT-EEC cell line may provide a unique in vitro cellular model to study the molecular endocrine involvement of human EECs in the normal human endometrium and in impaired endometrial function, such as endometriosis and implantation failure.

Materials and methods
Isolation and immortalization of human EECs
Primary EECs were isolated from a healthy human endometrium staged day 7 of the proliferative phase of the cycle based on cycle days and inspection of the endometrium by an experienced gynecopathologist (J B). This study was approved by the University Ethical Committee and the patient had given written, informed consent. The nulliparous patient, aged 37, had undergone surgery because of uterine myomatosis. A modification of the isolation protocol by Satyaswaroop et al. (1979) was used. Briefly, several endometrial tissue specimens from the region of the uterine corpus were cut into 1–3 mm² pieces, washed in PBS, digested for 45 min at 37 °C in PBS with 4 mg/ml BSA (Sigma) containing 2·5 mg/ml collagenase (CLSII, Worthington type); Biochrom, Berlin, Germany) and 25 µg/ml DNase (Sigma) and passed through a 250 µm sieve to remove mucous material and undigested tissue. Stromal cells were separated from epithelial cells by sequential sieving through 70 µm and 40 µm nylon sieves with stromal cells passing into the filtrate. The remaining EECs on top of the filter were backwashed with PBS and incubated for a further 30 min at 37 °C in PBS containing 4 mg/ml collagenase, 1 mg/ml hyaluronidase (Sigma), 0·17 mg/ml DNAse and 1 mg/ml proteinase K (Sigma) to further separate into single epithelial cells from the isolated glands. After centrifugation, cell pellets were washed once at 4 °C in culture medium consisting of Ham’s F-12 minimal essential medium (MEM) (Biochrom) substituted with 2 mM l-glutamine (Life Technologies, Karlsruhe, Germany), 10% fetal calf serum (FCS) (Biochrom), 160 ng/ml bovine insulin (Life Technologies) and 1 nM E2 (Sigma), including the antibiotics streptomycin (100 µg/ml), penicillin (100 µg/ml) and amphotericin B (0·5 µg/ml) (all Sigma). EECs were resuspended in the same medium at 37 °C and seeded into six-well dishes coated with collagen IV (Greiner, Solingen, Germany). From 2 days of culture onwards, EECs were cultured in medium devoid of antibiotics.

Prior to transfection, the EECs were passaged into fresh six-well culture dishes. On the second or third day following isolation of primary cells transfection was performed under serum-free conditions for 6 h at 60–80% cellular confluence employing the Lipofectamine PLUS transfection kit (Life Technologies) and 1, 5 and 10 µg of the eukaryotic expression plasmid pCIneo hTERT plasmid (generously provided by Prof. R Weinberg, Whitehead Institute, MA, USA). The transfection medium was replaced by normal culture medium overnight, and the day after transfection cells were passaged in fresh normal culture medium. Selection of stable transfecants started 48 h later on these highly proliferating cells with culture medium.
containing 600 µg/ml geneticin (Life Technologies). Starting from cell passage 18, stable hTERT transfectants of EECs (hTERT-EECs) were further characterized. Stable hTERT-EECs transfectants were cultured in normal E2-free medium. Non-transfected primary EECs or EECs transfected with the empty pcIneo plasmid died at passages four or five, approximately 18–26 days after isolation. Of the ten hTERT-EECs clones isolated we report here the characterization of clone, hTERT-EEC B37.

Telomerase repeat amplification protocol (TRAP)

Telomerase activity in primary EECs and in hTERT-EECs was determined with the TRAPeze telomerase detection kit (Intergen Company, Oxford, UK) according to kit instructions. Briefly, primary human EECs were used 5 days following isolation and hTERT-EECs were used at passage 45 corresponding to 250 population doublings. Cells (10⁴) were lysed for 30 min at 4°C in CHAPS lysis buffer provided with the kit, snap-frozen on dry ice and aliquots were stored at −80°C until used.

E2 and P4 stimulation

For stimulation studies with E2 at 1 and 10 nM for 24–48 h, hTERT-EECs were grown in phenol red-free medium (Promocell, Heidelberg, Germany) supplemented with 10% charcoal-stripped FCS (steroid hormone depleted FCS; Biozol, Eching, Germany) for at least 3 days. hTERT-EECs were primed with 1 nM E2 prior to the incubation for 48 h with 50–500 ng/ml P4 or with 10⁻⁵ M of the stable derivative medroxyprogesterone acetate (MPA) (both Sigma).

Proliferation assays

Ki-67 cell proliferation assay

In order to have a complementary measure of active cell proliferation beyond the standard methods of thymidine or bromodeoxyuridine (BrdU) incorporation, we developed an alternative to the ELISAs reported by Frahm et al. (1998, 1999). Ki-67 was selected as a marker because of evidence that its cellular expression has a direct relationship with function/type of cellular events or disease progression (Barzanti et al. 2000).

Europium (Eu) labeling This assay is based on DELFIA technology (time-resolved fluorescence). An aliquot of 200 µg/ml of Ki-67 (sc-15402) rabbit polyclonal antibody (Autogen Bioclear UK Ltd, Calne, Wilts, UK) was desalted using a Microspin G-25 centrifugal column (Amersham Biosciences) in order to remove azide, which interferes with Eu labeling. The antibody (100 µl) was then combined with 10 µl labeling buffer (500 mM Na₂CO₃, pH 9·2). Sephadex G-25 (Amersham) was soaked in elution buffer (50 mM Tris–HCl containing 9 g NaCl/l and 0·5 g NaN₃/l, pH 7·8) prior to being packed into a 30 × 1 cm plastic column and allowed to settle overnight. The Ki-67 was then labeled using an Eu labeling kit according to the manufacturer’s instructions (Perkin-Elmer UK Ltd, Beaconsfield, Bucks, UK). Briefly, 125 µl labeling buffer containing the Eu labeling reagent were added to 125 µl Ki-67 antibody in labeling buffer and incubated overnight at room temperature (RT). The G-25 column was equilibrated with 90 ml elution buffer, the Eu+Ki-67 antibody mixture was loaded and 60 fractions of 1 ml were collected. The fractions were diluted 1:10 000 in DELFIA enhancer solution (containing the following per liter: 1 ml Triton X-100, 1·4 g phthalic acid, 6 ml glacial acetic acid, 1 ml tri-n-octyolphosphine oxide dissolved at 19 mg/ml ethanol and 0·5 ml 4,4,4-trifluoro-(2-naphthyl)-1,3-butanediol dissolved at 8 mg/ml ethanol, pH 3·2) and counted in a 96-well microtiter plate using a 1234 DELFIA fluorometer (Perkin-Elmer). Two peaks of Eu were detected, the first containing labeled Ki-67 antibody, the second containing free Eu. The 1 ml fractions comprising the first peak were combined and stabilized (heavy metal-free BSA; Perkin-Elmer) was added (0·1% of final volume). The labeled anti-Ki-67 stock solution was then stored at 8°C.

DELFIA Ki-67 assay On the day of the assay the culture dishes to be assayed were decanted and tapped dry over filter paper. Two hundred microliters of Triton X-100 in 70% ethanol were added to each well and the dishes incubated for 30 min at RT to permeabilize cell membranes. Dishes were then decanted and 100 µl Eu-labeled anti-Ki-67 antibody (60 µl stock Eu-labeled Ki-67 in 9 ml culture media as detailed below) were added to each well. After 30 min shaking incubation at RT the plates were washed three times with a plate washer containing DELFIA wash buffer (1 ml Tween-20/1 distilled water). Two hundred microliters of DELFIA enhancer were then added to each well and the dishes counted as above after 5 min shaking incubation.

MTT cell viability assay

On the day of the assay the culture dishes were decanted and 10 µl of 5 mg MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide)/ml added to each well. The dishes were then incubated for a further 4 h at 37°C in a water-saturated 95% CO₂ incubator to allow development of formazan salt. The MTT was then removed and 100 µl DMSO (Sigma) were added to each well and left for 20 min until color developed.
Absorbance was read at 690 nm using an Anthos HT111 plate reader (Labtech, Salzburg, Austria).

BrdU incorporation proliferation assay A colorimetric BrdU cell proliferation ELISA (Roche Diagnostics) was used according to manufacturer’s instructions. Briefly, on the day of assay 20 µl BrdU labeling solution were added to each well, except for negative controls which received no BrdU, and incubated for 2 h at 37 °C in a water-saturated 95% CO₂ incubator. The culture dishes were inverted and tapped dry onto filter paper and 200 µl FixDenat added to each well and left for 20 min at RT. The dishes were then drained and blocked with 200 µl/well of ELISA blocking reagent (Roche) for 30 min at RT. After decanting, 100 µl anti-BrdU solution were added to each well and the dishes incubated for 30 min at RT. The dishes were drained again, washed and incubated with 100 µl/well of substrate solution for 10 min at RT. Finally 25 µl 1 M H₂SO₄ were added to each well and incubated for 1 min on a shaker at 300 r.p.m. The absorbance was measured at 450 nm within 5 min (Anthos HT111 plate reader; Labtech).

E2 induction of cell proliferation In order to investigate the E2 induction of proliferation in hTERT-EECs, two experiments were carried out and repeated at least twice. In the first series of experiments, rows of wells were plated out with between 0 and 20 000 cells in 96-well culture dishes and incubated for 72 h in normal culture medium supplemented with 10% FCS. The medium was then replaced with either steroid-free culture medium or normal culture medium plus 1 nM E2 (Sigma) and incubated for a further 48 h. Cell viability and proliferation were then determined using the MTT, BrdU and Ki-67 assays. In the second series of experiments, rows of wells were plated out with between 0 and 20 000 cells in 96-well culture dishes and incubated for 72 h in normal culture medium supplemented with 10% FCS or culture medium supplemented with 10% charcoal-treated FCS to remove steroid hormones. Thereafter, medium was replaced with steroid-free culture medium or normal culture medium plus 1 nM E2 and incubated for a further 48 h. Cell viability and proliferation were then determined using the MTT, BrdU and Ki-67 assays.

Three-dimensional (3D) culture of hTERT-EECs on a fibroblast/collagen lattice

A dermal equivalent fibroblast/collagen matrix was employed as a 3D culture system for hTERT-EECs (Hoeller et al. 2001). Briefly, 8 vol of acidic collagen (3 mg/ml collagen I and III in 12 mM HCl; Biochrom) and 1 vol of 10-fold Dulbecco’s MEM (Dulbecco’s MEM (DMEM) with 4:5 g/l d-glucose; Biochrom) were neutralized with 1 M sodium hydroxide. One vol of human foreskin fibroblasts (1 × 10⁵ cells/ml) in FCS was added and 4 ml of the mixture were poured immediately into polycarbonate membrane tissue culture inserts (2.5 cm diameter, 0.4 µm pore size; Nunc, Roskilde, Denmark). The inserts were placed into six-well culture plates (Falcon-Becton Dickinson, Franklin Lakes, NJ, USA) and filled with 2 ml culture medium. After complete polymerization, dermal equivalents were covered by culture medium which was composed of DMEM/Ham’s F-12 (1/1) high glucose, low calcium with r-glutamine (PAA, Linz, Austria) with 10% FCS, 1-8 × 10⁻⁴ M adenine (hydrochloride), 10⁻¹⁰ M cholera toxin, 2 × 10⁻⁹ M 3,3’,5-triiodo-l-thyronine (sodium salt) (all Sigma), 10 ng/ml human recombinant epidermal growth factor (EGF), 5 µg/ml human recombinant insulin (both Roche), 4 µg/ml hydrocortisone (Serva, Heidelberg, Germany) and 5 µg/ml transferrin (human HOLO, iron-saturated; Promocell). Two days after casting the dermal equivalents, hTERT-EECs at passage 40 grown to subconfluence were detached from the culture flask and seeded at 1 × 10⁶ cells per well. Seven days later the inserts were lifted onto polypropylene stoppers, the medium inside the insert was changed to high calcium (1.2 mM) and cultures were then cultivated at the air–liquid interface for another 7 days. For transmission electron microscopy, 3D gels were immersed in 2-2% phosphate-buffered glutaraldehyde solution for 2 h, postfixed for another 2 h in phosphate-buffered OsO₄, dehydrated in graded series of ethanol and embedded in Araldite. Ultrathin sections (0·1 µm) were examined with a Phillips EM 300 transmission electron microscope.

Atomic force microscopy (AFM)

Imaging of living hTERT-EEC surface structures and extracellular matrix (ECM) components was analyzed by AFM contact mode (Bischoff et al. 2003). hTERT-EECs at 1 × 10⁵ cells were seeded onto 1 cm² cover slips and cultured to 60-80% confluency. For AFM analysis, cells were thoroughly rinsed three times with PBS without Ca²⁺/Mg²⁺. Measurements were performed in constant force contact mode by cantilever probes with very low spring constants (about 0.06 N/m). The force was adjusted to the minimum possible, to approach the probe softly to the surface and avoid probe–sample interactions. Since drying-up processes strongly change the cell surfaces, the observations of the humid cells were performed for a maximum time period of 90 min. The influence of estrogen and P4 on the secretion of ECM produced by hTERT-EECs was investigated by culturing the cells in estrogen-depleted culture medium for 3 days prior to exposure to 10 nM E2 for 48 h. To determine the role of P4 on ECM production,
E2-primed hTERT-EECs were exposed to 1 μM MPA for 24 h.

**Immunohistochemistry**

For immunocytochemistry, hTERT-EECs cells at 80% confluence were washed once in PBS, fixed in Bouin’s solution and embedded in paraﬁn. For cytokeratin staining, antigen retrieval was performed by incubation of the sections with proteinase K (30 μg/ml for 30 min at 37 °C) and endogenous alkaline phosphatase was inactivated with 20% acidic acid in distilled water for 30 s prior to saturation of non-speciﬁc protein binding sites with 10% normal goat serum for 1 h at RT. The mouse monoclonal antibodies to cytokeratin (clone MNF 116) and vimentin (clone V9) (both Dako, Hamburg, Germany) were diluted in PBS plus 0·1% Tween-20 (PBS-T) containing 10% goat normal serum at 1:250 or 1:500 respectively, and incubated at 4 °C overnight. Sections were washed in PBS-T and incubated with an alkaline phosphatase-conjugated goat anti-mouse Ig secondary antibody (Dianova, Hamburg, Germany) at 1:250 for 1 h at RT. Specific binding was visualized with the alkaline phosphatase substrate (Dianova) was incubated for 1 h at RT at 1:20 000 in PBS-T. After washing, speciﬁc binding was visualized with an 3% solution of H2O2 in methanol. After washing in PBS, sections were incubated with a 1:200 dilution of biotinylated goat anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 30 min at RT. Detection of bound antibody was accomplished using the avidin-biotin complex method (Elite.Kit; Vector). Following several washing steps, a peroxidase-conjugated goat anti-mouse Ig secondary antibody (Dianova) was incubated for 1 h at RT at 1:20 000 in PBS-T. After washing, speciﬁc binding was visualized with an ECL detection reagent on ECL Hyperﬁlm (both Amersham).

**Western blot analysis**

For the immunodetection of ER-alpha, hTERT-EECs were grown in estrogen-free culture conditions for 5 days reaching 80% confluence in 25 cm² ﬂasks and lysis was performed in a cell lysis buffer containing 2% SDS and 10% saccharose in 63 mM Tris for 30 min at 4 °C. The lysate was boiled for 5 min at 90 °C and centrifuged to pellet the cell debris. The amount of protein was determined using a protein assay kit (BioRad) and a spectrophotometer at 595 nm. The lysate was stored at −80 °C until used. Protein extracts (30 µg/lane) were run on a 12% SDS polyacrylamide gel and proteins were blotted onto a nitrocellulose membrane (Amersham). After saturation of non-speciﬁc protein binding sites with 5% milk in PBS-T for 2 h at RT, membranes were incubated in blocking solution at 4 °C overnight with a mouse monoclonal antibody to human ER-alpha (1:100) (Clone D-12; Santa Cruz). Following several washing steps, a peroxidase-conjugated goat anti-mouse Ig secondary antibody (Dianova) was incubated for 1 h at RT at 1:20 000 in PBS-T. After washing, speciﬁc binding was visualized with an ECL detection reagent on ECL Hyperﬁlm (both Amersham).

**RNA isolation, RT- and quantitative RT-PCR (Q-RT-PCR)**

Total RNA was isolated with Trizol reagent (Life Technologies). The amount of mRNA isolated was determined by spectrophotometry at 260 and 280 nm (Sambrook et al. 1989). Primers and PCR conditions used for RT-PCR are listed in Table 1. The RT-PCR reactions were carried out in 50 μl solution containing 1 μl cDNA, 5 μl 10× Advantage cDNA polymerase mix buffer, 100 μM dNTP, 10 pmol of each primer (Table 1) and 2·5 U Taq DNA-polymerase (Life Technologies). The PCR cycles consisted of an initial denaturation for 3 min at 95 °C, followed by 40 cycles of denaturation at 95 °C and annealing at 60 °C, both for 1 min each, and
Table 1 Oligonucleotide primers employed in normal and quantitative RT-PCR analysis

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<th>Primer</th>
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</table>

Table 1 Oligonucleotide primers employed in normal and quantitative RT-PCR analysis

an elongation step for 2 min at 72 °C and a final extension cycle for 10 min at 72 °C.

For quantitation, 1 μl of the reverse transcriptase reaction mixture was added to 25 μl reaction mixture consisting of 1 × Advantage2 reaction buffer, 1·5 U Taq polymerase (Clontech, Heidelberg, Germany), 0·2 × SYBR Green (Biozym, Hess. Oldendorf, Germany), 200 μM each dNTP, and 0·5 μM of each primer listed (Table 1). A negative control without template was included. Assays were done in triplicates in a Rotor-Gene 2000 (LTF, Wasserburg, Germany). Initial denaturation at 95 °C for 30 s was followed by 40 cycles with denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 20 s. To verify the single PCR products melting curves were generated and amplicons were cloned and sequenced bidirectionally. The fluorescence intensity of the double-strand specific SYBR Green, reflecting the amount of formed PCR product, was read after each elongation step at 82 °C. Relative quantitation of gene expression was performed with the software Rotor-Gene version 4·6 (LTF, Wasserburg, Germany) in comparative quantitation mode. This mode allowed the comparison between differently treated samples relative to a control sample. The second derivative of the raw data was taken to calculate the take off point. Based on the take off point and the reaction efficiency, the relative concentration of each sample was calculated in comparison with the control sample. Standard deviations were determined by t-test.

Estrogen response element (ERE) reporter assay

Proliferative hTERT-EECs cultured under estrogen-free conditions for 5 days were transiently transfected with an ERE luciferase reporter plasmid (generously provided by Dr Silke Kietz, Karolinska Institute, Huddinge, Sweden) employing the Lipofectamine Plus transfection kit (Life Technologies). Culture medium was changed 6 h after transfection, and after 24 h of transfection hTERT-EECs were incubated for another 24 h with 10 nM E2 or 1 μM diethylstilbestrol (DES) diluted in estrogen-free medium. Cells were washed once with PBS, lysed for 15 min at RT with cell culture lysis reagent (Promega, Heidelberg, Germany) and supernatants were stored at −80 °C until used. Luciferase activity was determined with the firefly luciferase substrate (Promega) in a Series 2 luminometer (Berthold Detection Systems, Pforzheim, Germany). Estrogen-free cultured hTERT-EECs transfected with the luciferase reporter plasmid served as the negative control.

Flow cytometry analysis

Cells were detached from six-well plates using Accutase (PAA). Following two washes in 4 °C PBS, standard surface membrane immunofluorescence techniques were used. Cells were stained with either CD10 or CD13 monoclonal antibodies (both Becton Dickinson, Heidelberg, Germany) or an IgG1 isotype control (Becton Dickinson) at 4 °C for 40 min. After two washings with 4 °C PBS containing 0·1% sodium azide, cells were labeled with the phycoerythrin-conjugated goat anti-mouse IgG secondary antibody (Dianova) at 4 °C for 30 min, washed three times and fixed using 1% paraformaldehyde in PBS. Fluorescence was analyzed in a Becton Dickinson Calibur fluorescence activated cell sorter (FACS) using Cellquest software. Ten thousand cells per sample were counted. Mean fluorescence intensity (MFI) was calculated as sample MFI minus control antibody MFI.

Statistical analysis

The cell proliferation analyses were performed using the Statview 5 program (Abacus Concepts, Inc., Berkley, CA, USA). All results are presented as means ± S.E.M. Because the proliferation data were not normally distributed, the effects of treatments on proliferation and viability were determined using the non-parametric Mann–Whitney test. The relationship between Ki-67 and BrdU proliferation assays was analysed by simple linear correlation with significance established using
Fischer's z statistic. Results from the quantitative RT-PCR analyses were based on three independent cell culture experiments and PCR analysis for each of the cDNA samples was repeated at least twice. Results are presented as means ± S.E.M. P values of \( P < 0.05 \) were considered as statistically significant.

Results

Human primary EECs in the first and second passage following isolation were immortalized by lipid-mediated transfection with the catalytic subunit of the human telomerase complex (hTERT). Of the several epithelial cell clones derived after single cell cloning, we have been continuously culturing clone hTERT-EEC B37 for 69 passages or an estimated 370 population doublings in selection medium containing 600 µg/ml geneticin. By contrast, untransfected primary EECs underwent senescence after five or six passages. The TRAP assay revealed active telomerase in hTERT-EECs and weaker hTERT activity in primary EECs derived from the first passage after isolation (Fig. 1).

Starting at passage 20 onwards, hTERT-EECs were further characterized. The hTERT-EECs expressed the nuclear proliferation marker Ki-67 (Fig. 2A) and displayed typical epithelial cell morphology. Immunocytochemistry was positive for the epithelial cell marker cytokeratin (Fig. 2B) and cells were devoid of immunostaining for the stromal cell markers vimentin (Fig. 2C), CD10 and CD13 (Fig. 3), demonstrating the epithelial nature of hTERT-EECs. As shown by confocal laser scanning microscopy, confluent hTERT-EECs expressed membrane-anchored immunoreactive epithelial adhesion marker E-cadherin at lateral cell contacts (Fig. 2D) and revealed contact inhibition when grown to confluence on collagen-coated cell culture dishes. Employing specific primers (Table 1), RT-PCR analysis of untreated hTERT-EECs revealed transcripts for the integrin subunits alpha 3, alpha 6, beta 1, beta 3 and beta 4 (data not shown).

When cultured to confluence on collagen IV-coated cell culture dishes, hTERT-EECs displayed contact inhibition, remained viable for 2 weeks with daily changes of culture medium and after renewed passaging continued to grow normally. The hTERT-EECs were non-invasive in a fibroblast/collagen lattice employed as a 3D culture system. Growing as a continuous epithelial lining, hTERT-EECs displayed a polarized phenotype, producing a basal lamina towards the collagen matrix and displaying apical microvillous surface structures as shown by transmission electron microscopy (Fig. 2G).

AFM revealed extensive deposition of ECM components deposited by neighboring hTERT-EEC cells (Fig. 4A). ECM production and composition were altered in the presence of E2 and P4. hTERT-EEC cells cultured in normal medium or in estrogen-free medium supplemented with 1 nM E2 produced large amounts of tubular-shaped ECM structures with diameters of 60–120 nm (Fig. 4B and D). The same cells cultured in estrogen-free medium and then co-stimulated with E2 (1 nM) plus MPA (10⁻⁶ M) produced an amorphous ECM layer which was sticky to the AFM cantilever. Tubular-shaped ECM structures observed under the influence of P4 had taken on a mucus-like appearance (Fig. 4C).

hTERT-EECs expressed transcripts for ER-alpha, ER-beta and PR and displayed nuclear localization of immunoreactive ER-alpha and PR proteins (Fig. 2E and F). Both QT-RT-PCR and Western analysis revealed up-regulation of ER-alpha transcript (Fig. 5) and ER-alpha protein (Fig. 6), following culture of hTERT-EECs in estrogen-free medium for 3 days. Consecutive exposure to E2 (10⁻⁸ - 10⁻⁹ M) for 24 h caused a significant down-regulation of ER-alpha at the transcript (Fig. 5) and protein level (Fig. 6). Expression levels of ER-beta transcripts remained unaltered under these conditions (Fig. 5). Functionality of the induced endogenous ER-alpha was demonstrated by transient

Figure 1 Telomerase activity was detected in human primary EECs of the first passage on day 3 of culture (lane 1) and hTERT-EEC B37 at passage 40 (lane 2). Heat-inactivated hTERT-EEC B37 (lane 3) and CHAPS lysis buffer only (lane 4) served as negative controls. Positive control template for active telomerase served as positive control (lane 5). Telomerase activity was determined by TRAP. As expected, early passage primary EECs still demonstrated some telomerase activity (lane 1). Despite their long-term culture (> 2 years), stable hTERT-EECs transfectants revealed active hTERT (lane 2).
Figure 2 Localization of key cell markers in hTERT-EECs. Immunocytochemical staining of paraformaldehyde (PFA)-fixed, paraffin-embedded hTERT-EEC B37 cells with antibodies against the nuclear proliferation marker Ki-67 (A), the epithelial cell marker cytokeratin (B), the stromal cell marker vimentin (C), ER-alpha (E) and PR (F). Immunofluorescent labeling for the epithelial adhesion molecule E-cadherin (D) was performed on 4% PFA-fixed hTERT-EEC B37 cells grown to confluency on collagen-coated glass slides. Magnifications: (B) ×200; (D) ×600; all others ×400. (G) Polarized hTERT-EEC B37 display a basal lamina (BL) and apical microvillous structures (arrows), as shown by transmission electron microscopy of hTERT-EEC B37 cells grown on a 3D collagen/fibroblast lattice for 2 weeks. Magnification: ×90 000.
transfection assays with an ERE-luciferase reporter plasmid. Exposure to either 1 µM DES or 10 nM E2 initiated a strong induction of luciferase reporter activity (Fig. 7). Exposure to E2 strongly up-regulated transcripational activity of the PR gene, a classic ER-alpha target gene in the endometrium (Figs 5 and 8A). The E2-induced PR appeared to be functional as demonstrated by the significant down-regulation of PR-transcripts following exposure of the hTERT-EECs to P4 or the stable metabolite MPA (Fig. 8A).

The influence of E2 on viability and proliferation of hTERT-EECs was assessed with the MTT cell viability assay and both BrdU and Ki-67 proliferation assays. On average over the series of experiments the correlation between Ki-67 and BrdU proliferation assays was high ($r = 0.88–0.98$, $P < 0.001$) except that BrdU incorporation became asymptotic more quickly than Ki-67 expression at higher cell numbers. When the cells were not given at least 72 h of steroid-free incubation (Fig. 9a–c), there was no significant change in cell viability or proliferation following E2 treatment, except for a significant increase in BrdU incorporation at 20,000 cells/well (up to 16% increase, $P < 0.05$). However, when the cells were given at least 72 h in the absence of steroids (Fig. 9d–f) both BrdU incorporation and Ki-67 expression were significantly increased by subsequent exposure to E2 (up to 53 and 18% respectively, $P < 0.001$). These results are easily explained by the up-regulation of ER-alpha expression under estrogen-deprived culture conditions (Figs 5 and 6). Cell viability was not influenced by estrogen irrespective of the medium used (Fig. 9b). P4 treatment for 48 h with 500 nM P4 or 1 µM MPA on estrogen-primed PR-positive hTERT-EECs did not stimulate proliferation in either assay (data not shown).

In an attempt to determine the suitability of our hTERT-EECs as an in vitro model to study endometrial epithelial cell physiology, we analyzed the expression of the interleukin (IL)-6, IL-6 receptor (IL6-R), leukemia inhibitory factor (LIF), LIF receptor (LIF-R) and gp130 isoforms which are known to be important mediators of endometrial function during implantation (for specific primers see Table 1; primers for gp130 according to Sherwin et al. 2002). Both IL-6 and IL6-R were expressed by hTERT-EECs. Up-regulation of IL-6 transcripts was detected by Q-RT-PCR in hTERT-EECs upon exposure to estrogen (Fig. 8B) with P4 or MPA having no further effect. Estrogens only moderately affected IL-6R expression in hTERT-EECs. However, exposure of the cells to P4 or MPA caused an up-regulation of IL6-R (Fig. 8B). Employing specific
PCR primers reported by Sherwin et al., RT-PCR for the four known gp130 isoforms revealed the exclusive presence of the full-size gp130 transcript in hTERT-EECs. Transcriptional activity for LIF and LIF-R was weak and, like gp130, remained unaffected by ovarian steroid hormones (data not shown). The highly glycosylated membrane-anchored mucin MUC-1 has been implicated in endometrial receptivity. MUC-1 gene activity was up-regulated in hTERT-EECs upon treatment with E2 and this transcriptional activation was further enhanced in the presence of P4 (Fig. 8C; Table 1). Induction of ER-beta gene activity in hTERT-EECs...
was dependent on the presence of a functional PR and higher concentrations of P4 or MPA (Fig. 8D).

**Discussion**

Here we present a novel, hormonally responsive telomerase-immortalized human endometrial epithelial cell line (hTERT-EEC) which has conserved the ability of a fine-tuned regulation of steroid hormone receptors. These hTERT-EECs derived from primary endometrial glandular epithelial cells of the proliferative phase have been cultured for over 350 population doublings (65 passages) and displayed a stable epithelial phenotype. Kyo et al. (2003) have recently described the immortalization of human EECs from a late proliferative phase of the cycle by viral transfection with HPV E6 and E7 additional to hTERT to overcome Rb/p16 mediated telomerase-independent early senescence described in certain epithelial cells. In contrast to the method used by Kyo et al. (2003), our primary EECs derived from an early stage of the menstrual cycle (day 7) and cells had been liberated from the isolated endometrial glands by an additional enzymatic treatment. This may have resulted in a higher percentage of individual EECs with stem cell-like characteristics to be transfected. As demonstrated for primary mammary epithelial cells and conjunctival keratinocytes, a sub-population of isolated epithelial cells lacks p16 expression, escapes senescence stage M0 and can be immortalized by introducing hTERT only (Foster et al. 1998, Kiyono et al. 1998, Rheinwald et al. 2002).

hTERT-EECs revealed cellular contact inhibition when cultured at confluency as a monolayer on collagen-coated culture dishes for more than 2 weeks and, when re-seeded, remained viable, metabolically active and proliferative cells. By contrast, established human endometrial carcinoma cell lines frequently employed in studies on EEC physiology have lost normal epithelial anchorage-dependent growth control (Isaka et al. 2003). Cellular polarization is a critical parameter affecting numerous cell functions (Yeaman et al. 1999) and has been shown in primary EECs to be important for embryo attachment and implantation (Meseguer et al. 2001) and to enhance protein secretion (Negami & Tominaga 1989). hTERT-EECs cultured in a 3D collagen/fibroblast matrix displayed a polarized, non-invasive phenotype as illustrated by the production of a basal lamina and the formation of microvilli at the apical cell membrane. Thus, when cultured under

Figure 5 Steroids modulate steroid receptor gene expression in hTERT-EECs. Quantitative RT-PCR was performed on hTERT-EEC B37 cells precultured under estrogen-free conditions for 72 h. Thereafter, cells were treated without E2 or 10 nM E2. In addition, the hTERT-EECs were primed with 1 nM E2 for 24 h to induce PR production prior to treatment with 100 ng/ml P4. Hormonal treatment periods for E2 and P4 were 24 h (1d; d=day) and 48 h (2d). E2 resulted in a specific and lasting down-regulation of ER-alpha, but not ER-beta, which was unaffected by E2. E2 treatment caused an up-regulation of PR at both days of treatment. One hundred nanograms/ml of P4 did not influence ER-beta transcripts. 1d columns show a representative result from three independent experiments which was set at 1 (100%) and served as references to determine the relative means±S.E.M. (P<0.05) of the QPCR results derived from the stimulation assays.
Figure 6 Representative Western blot (n=3) demonstrating the specific up-regulation of ER-alpha protein (67 kDa) in hTERT-EEC B37 when cultured in estrogen-free medium (C). Cells cultured in normal medium plus 10% FCS (A) or in medium supplemented with 10 nM E2 (B) were devoid of immunoreactive ER-alpha indicating that E2 and traces of estrogens present in FCS are sufficient in suppressing ER-alpha expression in hTERT-EECs.

appropriate culture conditions, the hTERT-EECs have conserved a phenotype resembling native human EECs in vivo. Primary human EECs were reported to retain their polarization for a limited time period only (Classen-Linke et al. 1997, Negami & Tominaga 1989) and, when grown on ECM material from an Engelbreth-Holm-Swarm tumor (MatrigelR), showed increased protein secretion (Negami & Tominaga 1989). This Matrigel, however, is supplemented with several growth factors including transforming growth factor-β, EGF, insulin-like growth factor (IGF)-I, basic fibroblast growth factor and platelet-derived growth factor, and these growth factors and/or the tumor matrix itself may perturb physiological EEC responses. Similarly, the addition of growth factor supplements derived from crude protein extracts of bovine brain to the culture medium of human primary EECs would be regarded as a drawback (Zhang et al. 1995). By contrast, hTERT-EECs have been continuously cultured independently of the presence of numerous growth factors.

AFM showed that live hTERT-EECs deposited long tubular-shaped ECM structures, ranging in diameter from 60 to 120 nm. Similar to a previous report on reticulin fiber production in human menstrual cells cultured on collagen gels (Kamelle et al. 2002), the production of ECM by hTERT-EECs was influenced by the ovarian steroid hormones. Estrogen treatment of the ER-alpha-positive hTERT-EECs resulted in an increased secretion of tubular-shaped ECM structures. In the presence of P4, production of these ECM structures was greatly reduced and replaced by a mucus-like secretion, which proved sticky as determined by AFM contact scanning mode. These findings in hTERT-EECs may reflect a physiological secretory response of normal endometrial glandular epithelium to P4.

Responsiveness to estrogen and P4 is an important characteristic of the EEC. The human endometrial carcinoma cell lines Ishikawa, RL-95, ECC-1, KLE, HEC-1A and EN revealed altered or impaired hormonal responsiveness (Thie et al. 1995, Jazaeri et al. 2001, Dardes et al. 2002, Di Nezza et al. 2003, Farnell & Ing 2003, Isaka et al. 2003). In hTERT-EECs, the level of expression of ER-alpha, but not ER-beta, was regulated by estrogen, demonstrating different regulatory processes to affect the transcriptional activation of the two human ER isoforms in hTERT-EECs. Cultured under estrogen-free conditions, hTERT-EECs responded with a marked induction of ER-alpha gene activity that was reflected in increased production of ER-alpha protein. By contrast, estrogens in the culture medium proved to be strong repressors of ER-alpha production by hTERT-EECs. Down-regulation of ER-alpha following estrogen treatment has recently been shown in endometrial glands of ovariectomized macaques (Wang et al. 2002), in endometrial epithelial and stromal cells of immature ewes (Meikle et al. 2000) and in the human endometrial carcinoma cell line ECC-1 (Dardes et al. 2002). hTERT-EECs produced a functional ER-alpha that was clearly responsive to estrogen or the synthetic estrogenic compound DES, as demonstrated by a strong induction of luciferase using a ERE-luciferase reporter plasmid. In agreement with observations in primary human EECs (Zhang et al. 1995, Classen-Linke et al. 1997) and Ishikawa cells (Lessey et al. 1996), E2 induced PR expression in hTERT-EECs, which is a classic endometrial ER target gene (Milgrom et al. 1973, Classen-Linke et al. 1997, Brandenberger et al. 1999, Saegusa & Okayasu 2000, Borthwick et al. 2003). The dose-dependent decrease of PR transcriptional gene activity in the presence of P4 indicated a functional PR signaling pathway in hTERT-EECs as had been described for primary EECs (Classen-Linke et al. 2000, Spencer & Bazer 2002). We employed MUC-1 gene expression to provide further evidence for P4-induced physiological responses by this cellular endometrial model system. Expression and secretion of the highly glycosylated membrane anchored protein MUC-1 from glandular and luminal EECs is increased during the secretory phase of the cycle and MUC-1 is believed to act as an anti-adhesive factor of the receptive endometrium (Aplin et al. 1996, Meseguer et al. 2001). MUC-1 is down-regulated locally by the blastocyst at the site of embryonic attachment (Meseguer et al. 2001).
P4 caused increased MUC-1 gene activity in estrogen-primed PR-positive hTERT-EECs, suggesting these cells to be a suitable model for studies on P4 signaling in human EECs. Altered P4 signaling resulting in reduced epithelial secretory functions has been linked to clinical problems such as recurrent miscarriage (Aplin et al. 1996). In hTERT-EECs, P4, but not E2, affected the balance between ER-alpha and ER-beta expression by up-regulating ER-beta mRNA. Within the human glandular epithelium increased ER-beta gene activity has been described during the late secretory phase of the cycle (Critchley et al. 2002). An altered relationship of ER-alpha to ER-beta expression has been detected in endometriotic stromal cells (Brandenberger et al. 1999) and was linked to endometrial carcinoma (Fujimoto et al. 2000, Saegusa & Okayasu 2000, Jazaeri et al. 2001). ER-beta appears to be important for the regulation and control of estrogen-mediated effects within the human endometrium. Thus, hTERT-EECs may qualify as a suitable cellular model to investigate factors disturbing this fine-tuned balance and regulation of the different steroid hormone receptors potentially leading to endometrial disease.

There are conflicting results on the expression of ER-alpha within human EECs (Marshburn et al. 1992, Zhang et al. 1995, Dardes et al. 2002). The hTERT-EECs showed a down-regulation at the gene and protein level of ER-alpha by its ligand E2. This would explain the lack of proliferative response of the hTERT-EECs during long-term incubation with E2. By contrast, estrogen-free culture conditions caused the hTERT-EECs to induce expression of a functional endogenous ER-alpha allowing for proliferation to resume upon exposure to E2. These results clearly demonstrated that the ER-alpha is an essential component of the estrogen-mediated growth-promoting effect in hTERT-EECs and may help to explain contradictory reports on the effect of E2 on EEC proliferation. In human primary EECs cultured under estrogen-free culture conditions prior to E2 treatment, E2 had a similar growth-promoting effect (Zhang et al. 1995). By contrast, Marshburn et al. (1992) did not observe any proliferative response to E2 in primary EECs grown on ECM. However, in this study exposure of the cells to E2 had not been preceded by estrogen-free culture conditions, thus suggesting that these EECs did not express sufficient amounts of ER-alpha for E2 to be effective. An estrogen-induced, but IGF-I-mediated, paracrine effect on the proliferation of isolated human EECs was discovered in a co-culture system with endometrial stromal cells (Pierro et al. 2001). This indirect estrogen-induced proliferative effect on EECs may, in part, be explained by our observation that, regardless of the culture conditions used, isolated primary human endometrial stromal cells constitutively express ER-alpha.

Members of the IL-6 family of cytokines are known key regulators of implantation in the endometrium (Sherwin et al. 2002). IL-6 expression in human EECs is regulated by hypoxia, IL-1 and steroid hormones and its expression is highest during the mid-secretory phase suggesting a role in embryo implantation (Sherwin et al. 2002, von Wolff et al. 2002). Increased IL-6 secretion by EECs has been reported in women suffering from...
endometriosis (Piva et al. 2001), recurrent abortion and unexplained infertility (Tseng et al. 1996, von Wolff et al. 2000). The human glandular endometrial epithelium expresses IL6-R, LIF-R and gp130, a heterodimerization partner and signal transducer protein for both cytokine receptors (Cork et al. 2002, Sherwin et al. 2002). Therefore, we investigated whether the hTERT-EECs may serve as a suitable cellular model for studies on the physiological role of human EECs during implantation. hTERT-EECs had been cultured estrogen-free for 3 days and were then primed with 10 nM E2 for 24 h to induce PR expression. Incubation without E2 for 4 days (1); E2-deprived cells were treated with 10 nM E2 for 24 h only (2), and subsequently 1 nM E2+various concentrations of P4: 50 ng/ml P4 (3), 100 ng/ml P4 (4), 500 ng/ml (5) or 1 µM MPA (6). (A) A differential regulation of PR gene expression by steroid hormones was demonstrated by the suppressive effect of P4 and MPA on PR gene activity in hTERT-EEC B37. A similar partial suppression of PR gene expression was observed for all concentrations of P4 employed, suggesting a saturable effect of P4 at 50 ng/ml or 1 µM MPA. (B) E2 has stimulatory effects on the IL-6 system of hTERT-EECs. A 4-fold up-regulation of IL-6 gene activity in hTERT-EECs resulted from E2 treatment. By contrast, a slight but significant transcriptional up-regulation of IL6-R was observed at higher concentrations of P4 and MPA, whereas E2 alone or in combination with 50 ng/ml P4 was unable to affect IL6-R expression. This would indicate that in hTERT-EECs both ovarian steroids were able to differentially affect the IL6 ligand–receptor system. (C) Treatment of estrogen-deprived, ER-alpha-induced hTERT-EECs with 10 nM E2 caused an up-regulation in MUC-1 transcriptional gene activity which was further enhanced in the presence of P4 and MPA. (D) P4 induced ER-beta gene activity at 500 ng/ml (4) or 1 µM MPA (5). The lower P4 concentrations used for the incubations shown in Fig. 5 were not sufficient to induce ER-beta expression (see Fig. 5). Data is represented as means±S.E.M. of three independent experiments. *P≤0·05 significance compared to E2 free cultured cells.

Figure 8 Quantitative RT-PCR analysis from three independent incubations demonstrated the responsiveness of hTERT-EECs towards the steroid hormones E2 and P4. Prior to incubation with P4 and the synthetic P4 analogue MPA, hTERT-EECs had been cultured estrogen-free for 3 days and were then primed with 10 nM E2 for 24 h to induce PR expression. Incubation without E2 for 4 days (1); E2-deprived cells were treated with 10 nM E2 for 24 h only (2), and subsequently 1 nM E2+various concentrations of P4: 50 ng/ml P4 (3), 100 ng/ml P4 (4), 500 ng/ml (5) or 1 µM MPA (6).
Figure 9. E2 effects on hTERT-EECs viability and proliferation. Cell viability (MTT, a, d) and cellular proliferation as determined by BrdU ELISA (b, e) and Ki-67 assay (c, f) are shown. Representative graphs in (a–c) show the effects of the addition of 10 nM E2 (●) or medium only containing 10% FCS (○) on cells following 72 h of incubation in steroid-supplemented medium. Graphs (d–f) show the effects of 10 nM E2 on cells following 72 h incubation in either steroid-free medium (●) or steroid-supplemented medium (○). Pre-treatment of hTERT-EECs for 72 h with steroid-free medium was required to stimulate cell proliferation with 10 nM E2 treatment for 48 h (e, f). This proliferative effect was abolished when hTERT-EECs had been incubated in the presence of estrogens derived either from FCS which had not been estrogen-freed or from E2 added to the culture medium. Results of single experiments are shown, with values expressed as means±S.E.M. with significance denoted by stars (*P < 0.05) and (**P < 0.001) as determined by the Mann–Whitney test.
controlled and potentially functional cytokine-receptor system within the immortalized human endometrial epithelial cell model.

In conclusion, hTERT-EECs may be a suitable novel human endometrial cellular model linking studies on the molecular and endocrine role of EECs with relevant clinical pathologies, such as endometriosis, implantation and its failures and endometrial carcinogenesis.

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