Human endometrial epithelial telomerase is important for epithelial proliferation and glandular formation with potential implications in endometriosis

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STUDY QUESTION: How does regulation of telomerase activity (TA) in human endometrial epithelial cells (EEC) by ovarian hormones impact on telomere lengths (TL) and cell proliferation?

SUMMARY ANSWER: Healthy endometrial epithelial cell proliferation is characterized by high TA and endometrial TL changes according to the ovarian hormone cycle, with shortest TL observed in the progesterone dominant mid-secretory phase, when TA is lowest, implicating progesterone in the negative regulation of TA and TL.

WHAT IS KNOWN ALREADY: Critical shortening of telomeres may result in permanent cell cycle arrest while the enzyme telomerase maintains telomere length (TL) and replicative capacity of cells. Telomerase expression and activity change in the human endometrium with the ovarian hormone cycle, however the effect of this on endometrial TL and cell growth is not known.

STUDY DESIGN, SIZE, DURATION: A prospective observational study, which included endometrial and blood samples collected from 196 women.

PARTICIPANTS/MATERIALS, SETTING, METHODS: We studied endometrial samples from five different groups of women. Endometrial and matched blood TL and circulating steroid hormones were studied in samples collected from 85 women (Group 1). Fresh epithelial and stromal cell isolation and culture in vitro for TL and TA was done on endometrial biopsies collected from a further 74 healthy women not on hormonal therapy (Group 2) and from 5 women on medroxyprogesterone acetate (MPA) for contraception (Group 3). The epithelial TL and telomerase protein expression was examined in active, peritoneal, ectopic endometriotic and matched uterine (eutopic) endometrial samples collected from 10 women with endometriosis (Group 4); the in vivo effect of mifepristone on telomerase protein expression by immunohistochemistry (IHC) was examined in endometrium from 22 healthy women in mid-secretory phase before (n = 8), and after administering 200 mg mifepristone (n = 14) (Group 5). TA was measured by telomere repeat amplification protocol (TRAP) assay; TL by qPCR, and Q-FISH; cell proliferation was assessed by immunoblotting of histone H3 and 3D-culture to assess the ability of EECs to form spheroids; telomerase reverse transcriptase protein levels and Ki-67 (proliferative index) were assessed with IHC.

MAIN RESULTS AND THE ROLE OF CHANCE: Endometrial TLs correlated negatively with serum progesterone levels (r = -0.54) and were significantly longer than corresponding blood TLs (4893 ± 929 bp versus 3955 ± 557 bp, P = 0.002) suggesting a tissue-specific regulation. High TA and short TLs were observed in proliferating EECs in vivo and in vitro. During the progesterone dominant mid-secretory phase endometrial TL were significantly shorter compared with the proliferative phase (P = 0.0002). Progestagen treatment

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Introduction

The human endometrium is a highly dynamic tissue undergoing repetitive monthly cycles of growth, differentiation, shedding, and regeneration throughout a woman’s reproductive life. This cycle of endometrial cell proliferation and growth is regulated by ovarian steroid hormones (Hapangama et al., 2015). Telomerase remains inactive in most somatic cells apart from those with self-renewing abilities such as the endometrium (Williams et al., 2001; Hapangama et al., 2008). Telomerase is a ribonucleoprotein complex consisting of two core components, the RNA-part containing the template region for telomere synthesis, hTERC and the catalytic telomerase reverse transcriptase (hTERT). The main function of telomerase is the maintenance of the telomeres that cap the ends of chromosomes thereby regulating the proliferative lifespan of a cell; when telomeres shorten to a critical length the cell undergoes senescence or apoptosis. In addition to maintaining telomere length, telomerase may also have a role in proliferation and modulation of the expression of growth-promoting genes (Belair et al., 1997; Greider, 1998; Smith et al., 2003). In healthy endometrium telomerase activity (TA) correlates with proliferation; it is typically associated with the glandular epithelial cells and is regulated in a menstrual-phase dependent manner, suggesting a regulatory role for sex steroids (Tanaka et al., 1998; Williams et al., 2001). hTERT is a critical determinant of TA (Kyo et al., 1999) and is a target of estrogen and progesterone (Kyo et al., 1999; Wang et al., 2000). This has implications for endometrial proliferative diseases, such as endometriosis, defined as the presence of endometrial cells outside the uterine cavity and endometrial cancer; both pathologies associated with estrogen action and high TA. Progesterone antagonizes estrogen-induced proliferation in the endometrium and lack of progesterone activity on estrogen primed endometrium increases the risk of endometrial cancer whilst endometriosis is commonly associated with progesterone resistance (Bulun, 2010). Telomere length (TL) in peripheral blood mononuclear cells has been proposed as a potential biomarker of ageing and disease risk. Shortened TL has been linked to endometrial cancer (Smith and Yeh, 1992; Akbay et al., 2008) while elongated TL has been observed in the endometrium of women with endometriosis (Hapangama et al., 2008, 2009). Although progesterone is therapeutically given to inhibit the growth of estrogen-dependent cancers and in the management of endometriosis, little is known about the impact of progesterone on normal endometrial TA and TL. We report herein that like TA, TL changes with cyclical (hormone dependent) endometrial epithelial cell (EEC) proliferation in vivo and in ectopic lesions in endometriosis. Furthermore, inhibition of TA compromises endometrial epithelial cell proliferation and affects the ability of such cells to form glandular-like structures in a 3D culture.

Materials and Methods

Patient groups

Human endometrial samples for the measurement of TL were obtained from 85 fertile women who have each had at least one live birth, not on any hormonal therapy and a matched venous blood samples were obtained simultaneously from 70 of these women (Group 1). Peripheral blood mononuclear cell (PBMC) TL was available for 53 women and circulating estradiol (E2) and progesterone were also measured on 70 samples by radioimmunoassay, as previously described (Hapangama et al., 2008).

Endometrial biopsies were collected from a further 74 healthy women, who have had at least one full term pregnancy, without a history of recurrent miscarriage or infertility, not on hormonal therapy (Group 2) and from 5 women on medroxyprogesterone acetate (MPA) for contraception (Group 3) for fresh cell isolation and culture as previously described (Hapangama et al., 2008). Endometrial biopsies were also collected from healthy fertile women in mid-secretory phase before (n = 8), and after 200 mg mifepristone (n = 14) as previously published (Hapangama et al., 2002) (Group 5) and examined with immunohistochemistry (IHC).
Endometrial biopsies were dated using histological criteria (Noyes et al., 1975), the date of last menstrual period and hormone profile. All women were of reproductive age (18–46 years) with further demographic data presented in Table I. Ethical approval for the study was obtained from Liverpool Adult Research Ethics committee (LUREC09/H1005/55 and 11/H1005/4) and Lothian Research Ethics Committee (Institutional Review Board) (Hapangama et al., 2008).

**Immunohistochemistry and immunofluorescence**

Three-micrometre paraffin sections underwent antigen retrieval at pH6 in citrate buffer (Hapangama et al., 2012). Primary antibodies used: Ki67 (clone MM1, Leica Biosystems Ltd, Newcastle Upon Tyne UK) and telomerase (ab27573, Abcam, Cambridge UK). Detection was with ImmPRESS anti-mouse/rabbit polymer and visualization with ImmPACT DAB (Vector Laboratories, Peterborough, UK). Non-immune IgG was used as negative control. Ki67 was evaluated as percentage of immunostaining positive cells and an arbitrary four-point semi-quantitative scoring scale (0, negative/no staining; 1, weak; 2, strong; 3, very strong) was used to analyse telomerase staining as previously described (Hapangama et al., 2008). For immunofluorescence (IF), EECs in monolayer were fixed in 10% (w/v) NBF (neutral-buffered formalin, Sigma-Aldrich, Dorset, UK) and immunostained for pan-cytokeratin (C9231, Sigma-Aldrich, Dorset, UK) and F-actin (FITC-Phalloidin, PS282, Sigma-Aldrich, Dorset, UK). Images were collected and processed as described previously (Valentijn et al., 2013).

**TA and TL assays**

TA was measured using the TeloTTAGGG TRAP assay (Telomere Repeat Amplification Protocol assay; Roche Diagnostics, Ltd, Burgess Hill, UK) using 1 μg of lysate and mean TL was measured with qPCR in the blood, tissue and cells as previously described (Hapangama et al., 2008, 2009; Valentijn et al., 2013). Telomerase activity was measured as absorbance at 450 nm using 1 μM for 72 h to inhibit TA in monolayer cultures of epithelial cells. Dosing regime was based on previous publications (Goldblatt et al., 2009; Marian et al., 2010; Mender et al., 2013) and a trial on epithelial cells derived from a patient (in-house) with endometrial adenocarcinoma maintained in monolayer culture.

**Telomere Q-FISH**

Quantitative fluorescence in situ hybridization (Q-FISH) was performed on 3 μm-thick sections using a Cy-3 labelled PNA (peptide nucleic acid) telomere oligonucleotides (Panagene, Daejeon, Korea) (Hewitt et al., 2012). Nuclei were stained with DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride, Vector Laboratories, Peterborough, UK). Digital images were captured using a Nikon IF Microscope Lens-Nikon Plan Fluor 100x/1.30 oil OFN25 DIC H/N2 MRH01902 using the programme NCIS and analysed using Image J. The mean fluorescence intensity of telomere spots in each of the nuclei was quantified relating TL to pixel brightness. Freehand drawing around the nuclei on the DAPI image was transposed to the PNA red filtered image and measurement of the mean relative TL within each nuclei was gained. All epithelial cell nuclei (confirmed by the expression of cytokeratin in sequential sections) in the high magnification and at least 10 fields of vision per section were analysed. A control sample of mouse gut was included with each run to ensure uniformity and reproducibility.

**Explant culture**

Endometrial biopsies were washed several times in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Phenol-red free, Life Technologies, Paisley, UK) to remove blood. The tissue was cut into 1–2 mm³ pieces and placed into 24-well plates with medium [DMEM/F12, 5% charcoal-stripped FBS (fetal bovine serum, Sigma-Aldrich, Dorset, UK), Primocin (Source Bioscience, Nottingham, UK)], containing 10⁻⁶ M β-estradiol (E2) or 10⁻⁶ M medroxyprogesterone acetate (MPA). All hormones were from Sigma-Aldrich, Dorset, UK and prepared as 1 mg/ml stocks in ethanol. Treatment was for 24 h, after which harvested tissue was stored at −80°C for TRAP assay. Control treatments received ethanol only.

**Isolation of human endometrial epithelial cells**

Epithelial and stromal fractions were separated from freshly harvested endometrial tissue as previously described (Valentijn et al., 2013). Freshly isolated epithelial cells were positively selected using EpCAM (epithelial cell adhesion molecule, CD326) microbeads (#130-061-101; Miltenyi Biotec Ltd, Surrey, UK) according to the manufacturer’s instructions and were pooled and stored at −80°C. EpCAM-depleted stromal cells were plated in DMEM/F12 medium (Sigma-Aldrich, Dorset, UK), 10% FBS (Sigma-Aldrich, California, USA). Both were used at 1 μM for 72 h to inhibit TA in monolayer cultures of epithelial cells. Dosing regime was based on previous publications (Goldblatt et al., 2009; Marian et al., 2010; Mender et al., 2013) and a trial on epithelial cells derived from a patient (in-house) with endometrial adenocarcinoma maintained in monolayer culture.

**Table I  Patient demographics, median value and the range are shown.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (years)</th>
<th>Height (m)</th>
<th>Weight (kg)</th>
<th>BMI (kg/m²)</th>
<th>Parity I + (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36 (18–46)</td>
<td>1.58 (1.5–1.78)</td>
<td>73 (50–119)</td>
<td>28 (18–45)</td>
<td>100</td>
</tr>
<tr>
<td>Group 2</td>
<td>38 (23–45)</td>
<td>1.60 (1.48–1.75)</td>
<td>74 (49–112)</td>
<td>30.7 (19.9–42)</td>
<td>100</td>
</tr>
<tr>
<td>Group 3</td>
<td>26 (22–30)</td>
<td>1.62 (1.5–1.72)</td>
<td>57 (49–72)</td>
<td>21.7 (19.6–29.7)</td>
<td>80</td>
</tr>
<tr>
<td>Group 4</td>
<td>38 (24–47)</td>
<td>1.64 (1.53–1.7)</td>
<td>60 (51–76)</td>
<td>24.5 (21.5–27.9)</td>
<td>70</td>
</tr>
<tr>
<td>Group 5</td>
<td>34 (26–45)</td>
<td>1.64 (1.55–1.77)</td>
<td>70 (51–90)</td>
<td>27.6 (21–40)</td>
<td>100</td>
</tr>
</tbody>
</table>
Dorset, UK), Primocin (Source Bioscience, Nottingham, UK) for 24 h to minimize the amount of contaminating blood cells. Stromal cell were collected by trypsinisation and stored at –80 °C. Representative aliquots of the epithelial and stromal fractions (sorted by EpCAM microbeads) were analysed by sodium dodecyl sulfate-Poly acrylamide gel electrophoresis (SDS-PAGE) and immunoblotting for expression of cytokeratin and vimentin. Cells maintained in culture were assessed morphologically and by flow cytometry for the expression of CD9, a glandular epithelial marker and CD13, a stromal marker (Valentijn et al., 2013).

Primary culture (2D and 3D) of human endometrial epithelial and stromal cells

Primary human epithelial and stromal cells were maintained in monolayer culture in DMEM/F12, 10% FBS, and Primocin. For epithelial cells, medium was supplemented with 50 ng/ml EGF (epidermal growth factor, Sigma-Aldrich, Dorset, UK). For 3D culture, short-term (16–36 h post-plating) monolayer epithelial cultures were embedded in Matrigel (BD Biosciences, Oxford, UK) and maintained in DMEM/F12 medium supplemented with insulin-transferrin-selenium (ITS (insulin-transferrin-seleniumG); Life Technologies, Paisley, UK) and 50 ng/ml EGF with medium changes every 1–2 days (Valentijn et al., 2013). For inhibition of TA, spheroids were allowed to form for 3–5 days. Imetelstat and a control mismatch oligonucleotide, both at 5 μM were added on Day 5 of culture and the spheroids monitored daily for a further 5 days. Spheroids that had formed on Day 5 of culture with a diameter of at least 50 μm were assessed by microscopy and scored. On Day 5 of treatment, the spheroids were assessed morphologically by microscopy and the number of intact spheroids with obvious lumen was counted. The number of intact spheroids remaining on Day 5 of treatment was expressed as a percentage of spheroids that had formed on Day 5 of the initial culture.

Monolayer endometrial cultures comprising stromal and epithelial cells were grown in DMEM/F12 with 2% charcoal-stripped FBS (Sigma-Aldrich, Dorset, UK) for 24 h prior to treatment with E2, P4 (progesterone; Merck, Nottingham, UK). After a further 24 h, cells were treated with estrogen (E2; 10⁻⁸ M) alone or in combination with progesterone (P4; 10⁻⁶ M) for 5 days. The medium containing the hormones was changed every 1–2 days. Cells were subsequently analysed for TA by TRAP.

MTT and senescence assays

Cell viability was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, Dorset, UK) assay. Briefly primary epithelial cells were seeded into 96-well plates (10 000 cells/well) and allowed to attach overnight prior to treatment with Imetelstat or the mismatch control for 72 h. MTT solution (5 mg/ml) was added to each well (10 μl/100 μl medium) for 4 h. The medium was removed and then 100 μl acidified isopropanol was added to dissolve the formazan precipitate before absorbance was measured at 570 nm using a microplate reader (Multiskan Ascent, Thermo Scientific, UK). Cell viability was expressed as a percentage of the untreated control.

Senescent human endometrial cells in culture were identified using a pH-dependent β-galactosidase assay (Dimri et al., 1995).

SDS-PAGE and immunoblotting

Cells were extracted in RIPA (radioimmunoprecipitation assay) buffer supplemented with protease (P8340, Sigma-Aldrich, Dorset, UK) and phosphatase (PhosSTOP, Roche Diagnostics Ltd, Burgess Hill, UK) inhibitors. Lysates were analysed by SDS-PAGE under reducing conditions on precast 4–15% gradient gels (Mini-PROTEAN TGX, Bio-Rad, Herfordshire, UK) and transferred to polyvinylidene difluoride membrane (Bio-Rad, Herfordshire, UK). Primary antibodies used included: cytokeratin 18 (M7010, Dako, Ely, UK), vimentin (ab45939, Abcam, Cambridge, UK), GAPDH (Glyceraldehyde 3-phosphate dehydrogenase, G9545, Sigma-Aldrich, Dorset, UK) and phosphohistone H3 ([Ger10], ab5176, Abcam, Cambridge, UK). HRP (horse radish peroxidase)-linked secondary antibodies were from ThermoScientific, Loughborough, UK. Signal detection was performed using SuperSignal™ West Dura chemiluminescent substrate (Thermo Scientific, Loughborough, UK) and CL−Xposure film (Thermo Scientific, Loughborough, UK).

Statistical analysis

Statistical analysis was performed using GraphPad prism. Student’s t-test and non-parametric equivalent tests were used as appropriate to determine significance between groups, paired samples and treatments. Pearson’s correlation coefficient was used to test the association between TL with demographic and endocrine data. Criterion for significance was P < 0.05. All data are presented as mean ± standard error of the mean (SEM).

Results

Endometrial mean tissue TLs are longer than donor-matched peripheral blood mononuclear cells TLs and correlate with serum progesterone levels suggesting a tissue-specific regulation

To test the tissue-specific regulation of the endometrial TL, the mean TL (TL) was measured in endometrium collected from 85 women (Group 1, proliferative phase, n = 18; mid-secretory phase, n = 38, and late-secretory phase n = 17) and in matched PBMC collected simultaneously in 53 of these women. The PBMC TL was significantly shorter than in the matched endometrial tissue TL (3955 ± 557 bp (base pairs) versus 4893 ± 929 bp, n = 53 pairs; paired t-test *P < 0.0001, Fig. 1A) from the same woman when all phases of the menstrual cycle were analysed, and there was no correlation between the PBMC and endometrial tissue TL (r = −0.05, Fig. 1B) of individuals suggesting a tissue-specific regulation of TL in endometrial cells. There was no correlation between endometrial TL and the demographic features such as age (Fig. 1C) and BMI (Fig. 1D).

To evaluate the effect of ovarian hormones on endometrial TL, the data were analysed according to the menstrual cycle phase. The endometrial TL changed according to the cycle and were shortest in the progesterone dominant mid-secretory phase (Kruskal–Wallis test, 4 groups, P = 0.0003, Fig. 1E) where progesterone levels were either absent or low. While there was no correlation with serum estrogen (data not shown), endometrial TL negatively correlated with circulating progesterone levels (n = 70 pairs, r = −0.54, Fig. 1F).

High TA and short TLs are features of proliferating EECs in vivo and in vitro

To confirm that high TA is associated with EEC proliferation, freshly isolated endometrial epithelial and stromal cells (n = 5) in the proliferative phase (the epithelial proliferation in these samples was confirmed by Ki67 expression by IHC; Supplementary data Fig. S1A) were examined. Routinely cultured epithelial cells were > 80% positive for CD9; stromal cells were > 95% positive for CD13 (Supplementary data, Fig. S1B and C). The EpCAM+ sorted EECs showed significantly higher TA (1.57 ± 0.16 versus 0.93 ± 0.36, n = 5 pairs, paired t-test, P = 0.01; Fig. 2A, AU for arbitrary units of absorbance at 450 nm) with a shorter TL
Endometrial telomere length (TL) varies according to the menstrual cycle and does not correlate with peripheral blood mononuclear cells (PBMC) TL, age or BMI. The TL of endometrial tissue and PBMC TL in normal fertile healthy women were measured by qPCR. (A) In paired samples endometrial TL was significantly longer than PBMC TL ($n = 53$, ***$P = 0.0001$). Endometrial TL did not correlate with PBMC (B; $n = 53$ pairs), age (C; $n = 69$ pairs) or BMI (D; $n = 67$ pairs). (E) In endometrial tissue TLs were shortest in the mid-secretory phase (Kruskal–Wallis test, ***$P = 0.0005$) of the menstrual cycle when progesterone levels are maximal. Proliferative phase ($n = 13$), early-secretory phase ($n = 12$), mid-secretory phase ($n = 33$), late-secretory phase ($n = 17$). Mean ± SEM are presented. (F) Negative correlation of endometrial TL with serum progesterone levels in secretory phase samples ($n = 70$ pairs, $r = -0.54$, *$P < 0.0001$). Serum progesterone was measured by radioimmunoassay (RIA).
(5167 ± 1332 bp versus 8598 ± 1422 bp, n = 5 pairs, paired t-test, \(P = 0.0002\), Fig. 2B) compared with the corresponding EpCAM-depleted stromal population from the same sample. TA and TL in the proliferating EEC were negatively correlated (\(n = 5\) pairs, \(r = -0.994, P = 0.0005\); Fig. 2C), suggesting that the proliferating epithelial cells with short TL were associated with high TA. EpCAM+ EEC TLs in the secretory phase showed no correlation with TA (\(n = 5\) pairs, \(r = 0.24\), Fig. 2F) and had lower TA levels [these epithelial cells were not proliferating shown by absent or low Ki67 (Supplementary data, Fig. S1)]. Stromal cell TL remained significantly longer than epithelial cell TL in both proliferative and secretory phases (Fig. 2B and E).

**Effect of Imetelstat on epithelial and stromal cells**

Since TA is associated with cell proliferation we tested the effect of telomerase inhibition on proliferation of epithelial cells in culture. In cultures of epithelial and stromal cells, TA was higher in epithelial cells where it increased initially and persisted for longer (Fig. 3A and B). Long-term culture of EEC was associated with a senescent phenotype (Fig. 3C; enlarged cell morphology and β-galactosidase staining). Imetelstat produced a dose-dependent inhibition of telomerase (IC\(_{50}\) 1.5 μM) in a primary endometrial epithelial cancer cell line with high telomerase activity (Fig. 3D). The IC\(_{50}\) for telomerase inhibition ranges from 0.1 to 1 μM in most tumour cell lines tested (Joseph et al., 2010). 1.0 μM Imetelstat inhibited TA on average by >60% in monolayer cultures of normal healthy primary EECs treated for 72 h (\(n = 4\) pairs, Paired t test, \(*P = 0.02\), Fig. 3E) and this was associated with a decrease in the expression of the mitotic marker, phosphohistone H3 (Ser10) (\(n = 5\) pairs, t-test, \(**P = 0.009\), Fig. 3G). Note that while Imetelstat inhibited TA in the primary endometrial epithelial cancer cell line (Fig. 3C), it did not affect the phosphorylation status of phospho-histone H3 (Fig. 3G). Imetelstat only affected epithelial cell viability at 100 μM as assessed by MTT assay (\(**P = 0.002\), Fig. 3F). Inhibition of TA in stromal cells by 1.0 μM
Imetelstat affects telomerase activity (TA) and proliferation, but not viability of endometrial epithelial cells. (A) Epithelial cells and (B) stromal cells were maintained in monolayer culture for the indicated times prior to harvesting for telomere repeat amplification protocol (TRAP) assay. For each time point, n ≥ 4; Patient group 2. (C) Epithelial cells maintained in long-term culture had a phenotype consistent with senescence. Note the enlarged cells and positive blue stain for β-galactosidase in the micrographs (representative of n = 5). (D) Epithelial cells were isolated from an adenocarcinoma of the human endometrium and maintained in culture as a cell line. The cells were treated with the concentrations of Imetelstat indicated for 72 h prior to TRAP. TRAP activity is expressed as a percentage relative to the activity of the mismatch control (mean ± SEM for n = 3 separate experiments). (E) Epithelial cells were maintained in culture for up to 3 days and then treated with 1 μM Imetelstat or mismatch control oligonucleotide for a further 72 h prior to TRAP assay. TRAP activity is expressed as a percentage of the mismatch control (n = 4). T-test, *P = 0.02. (F) Human endometrial epithelial cells (HuEEC) were directly seeded into 96-well dishes, allowed to attach and treated the next day with Imetelstat or the mismatch control at the concentrations indicated for 72 h. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Note significant loss in cell viability at 100 μM (Mann–Whitney test, P = 0.002). (G) Cultures of normal epithelial cells and an adenocarcinoma of the endometrium treated with Imetelstat or mismatch control as before, and immunoblotted for phospho-H3 [phosphohistone H3 (Ser10)]. Histone H3 is only phosphorylated on Ser 10 during mitosis. Shown is a representative blot (top) of normal epithelial cells (n = 5) and the adenocarcinoma (representative of two separate experiments) and densitometric analysis (bottom). T-test, **P = 0.009. (H) Stromal cells were grown for 24 h and then treated with 1 μM Imetelstat or mismatch control oligonucleotide for 72 h prior to TRAP assay. Telomerase activity is expressed as a percentage of the mismatch control. T-test, ***P = 0.0004.
Imetelstat in monolayer culture for 72 h was on average >90% (n = 4 pairs, paired t-test, \( ***P = 0.0004; \) Fig. 3H) when measurable TA was present.

To test if telomerase inhibition affects formation of glands, primary EECs forming gland-like spheroids in 3D after 3–5 days in culture (Fig. 4A) were treated with 5 \( \mu \)M Imetelstat or the mismatch control for 5 days. Imetelstat significantly affected the architecture of the glands reducing the number of intact spheroids (Fig. 4B) compared with control (n = 3 pairs, paired t-test; \( ^*P = 0.03 \)). Imetelstat treated spheroids failed to proliferate and increase in size beyond Day 5 of treatment. Given the effect of Imetelstat on the architecture of the spheroids we speculated that Imetelstat might affect the cytoskeleton. We treated EECs in monolayer with 5 \( \mu \)M Imetelstat (same concentration as that used for spheroids) or the mismatch control for 5 days and immunostained for F-actin (Phalloidin) and cytokeratin. After 5 days of treatment with Imetelstat, the cells adopted a rounded morphology and actin filaments were concentrated along the cell membrane (Fig. 4C). The distribution of cytokeratin was similarly affected (Fig. 4C).

**In vivo and in vitro treatment with progesterone inhibits endometrial epithelial TA**

The effects of endogenous and exogenous progesterone in vivo on EEC TA were determined on freshly isolated EECs from women in the proliferative phase (n = 8), secretory phase (n = 8) and MPA-treated (n = 5). TA was significantly higher in the proliferative phase samples compared with the secretory phase and MPA-treated samples (Kruskal–Wallis test, \( ***P = 0.0005; \) Fig. 5A). In explant cultures of endometria collected during the proliferative phase, MPA treatment (24 h) in vitro produced a significant reduction in TA (n = 4 pairs, paired t-test, \( ^*P = 0.01; \) Fig. 5B). Rather than look at separate cultures of epithelial and stromal cells, we reasoned that a combined culture of the two cell types physically interacting would provide more useful information on the effect of progesterone on estrogen induced TA than separate cultures. Endometrial cultures comprised of both epithelial and stromal cells showed >50% reduction in TA (Fig. 5C) after 5 days of treatment with progesterone.

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**Figure 4** Imetelstat affects the morphology of endometrial epithelial cells (EECs) in monolayer and 3D culture. (A) Micrographs showing the formation of an endometrial epithelial spheroid embedded in Matrigel on the days indicated. Scale bar, approx. 100 \( \mu \)m. (B) On Day 5 of culture the number of spheroids was counted and 5 \( \mu \)M imetelstat or mismatch control added and cultured for a further 5–10 days. The number of intact spheroids remaining after 5 days of treatment was scored and expressed as a percentage of those formed on Day 5 before treatment started. \( N = 3 \) for each treatment group (T-test, \( ^*P = 0.03 \)). Micrographs show positive nuclei stained with 4',6-Diamidino-2-Phenylindole, dihydrochloride (DAPI) of spheroids in 3D culture on Day 5 of treatment. Scale bar 100 \( \mu \)m. (C) Effects of 5 \( \mu \)M Imetelstat or mismatch control on F-actin and cytokeratin in EECs in monolayer treated for 5 days. EECs were seeded in chamber slides (Nunc, Sigma-Aldrich, Dorset, UK) and allowed to attach overnight prior to treatment and analysis of the cytoskeleton by immunofluorescence. Representative of three separate experiments.
Progesterone is associated with changes in endometrial telomerase. (A) Telomerase activity (TA; arbitrary units of absorbance at 450 nm [AU]) in freshly isolated endometrial epithelial cells (EECs) is repressed by endogenous progesterone in secretory phase normal cycling endometrium (Mann–Whitney test, **P = 0.005, n = 8/phase) and in endometrium treated exogenously with synthetic progestagen (medroxyprogesterone acetate [MPA], Group 3, n = 5, Mann–Whitney test *P = 0.001). (B) In explant cultures of human endometrium, MPA (10^{-6} M) but not estradiol (E2) (10^{-8} M) had a significant impact on TA after 24 h. n = 4, paired t-test, *P = 0.01 (vehicle versus MPA). (C) In monolayer cultures comprised of both epithelial (E) and stromal (S) cells (micrograph), E2 and P4 significantly reduced TA compared with E2 alone (n = 3, paired t-test, *P = 0.02). (D) Progesterone antagonism with mifepristone increases telomerase immunostaining intensity in endometrial glands in vivo. For each time point, n ≥ 3, Group 5, **P = 0.003. Micrographs of telomerase immunostaining in mid-secretory phase (0 h after) and 36 h after 200 mg mifepristone administered in mid-secretory phase. Also shown are a positive control for telomerase expression (endometrial cancer) and a negative control (post-menopausal endometrium). The telomerase immunoreactivity in the stromal cells did not change significantly after mifepristone treatment.
and E2 compared with E2 alone (n = 3 pairs, paired t-test, *P = 0.02). Administration of 200 mg of the progesterone antagonist mifepristone in the mid-secretory phase in healthy women increased immunoreactivity for the telomerase protein hTERT in endometrial glands after 24 h, with significant changes in semi-quantitative scoring at 24 and 36 h (n = 22, Kruskal–Wallis test, **P = 0.003; Fig. 5C). Telomerase immunoreactivity in stromal cells did not change significantly after mifepristone treatment (Fig 5D).

**Figure 6** Peritoneal ectopic lesions have longer telomeres and higher telomerase expression. (A) Telomerase length (TL) in matched secretory phase uterine (uterine) and ectopic endometrium was assessed by fluorescence in situ hybridization (FISH) using peptide nucleic acid (PNA) telomere probe. The relative fluorescence intensity was stronger in the glandular structures of the ectopic lesion compared with uterine (eutopic) endometrium (paired t-test, P = 0.01, n = 5, Group 4). Data from at least 3000 epithelial nuclei per field/section and around 6000–10 000 per matched sample were analysed. (B) Secretory phase endometrium and peritoneal ectopic lesions were stained by immunohistochemistry for telomerase. Representative micrographs for eutopic (left) and ectopic (right), n = 10. Note the strong staining in the gland-like structures in the ectopic lesion. Scale bar 100 μM.

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**Longer relative mean TL in ectopic glandular-like epithelium**

Progesterone resistance is a hallmark of ectopic endometriotic lesions and we examined glandular epithelial cells for differences in TL with qFISH. Ectopic endometriotic lesions (Group 4) were associated with significantly higher epithelial telomeric signal than in matched corresponding uterine (eutopic) endometrium from the same women (n = 5 pairs; paired t-test, P = 0.01) suggesting longer relative mean TL in ectopic endometriotic glands (Fig. 6A). Agreeing with this observation, the ectopic peritoneal endometriotic lesions showed increased immunoreactivity for telomerase protein hTERT compared with the matched functional uterine (eutopic) endometrium (n = 10 pairs, Fig. 6B).

**Discussion**

The dynamic changes in TA according to the ovarian cycle have been well documented in the human endometrium (Kyo et al., 1997; Tanaka et al., 1998; Williams et al., 2001) but little is known about its downstream effects particularly on endometrial TL. In humans, mean PBMC TL decreases with age (Frenck et al., 1998) and in addition, there seems to be a correlation between TL in different tissues of the same individual (Friedrich et al., 2000; von Zglinicki et al., 2000; Takubo et al., 2002) suggesting that PBMC TL could serve as a surrogate for relative TL in other tissues. Although there is a positive correlation between reproductive life span and PBMC TL (Aydos et al., 2005; Lin et al., 2011), we demonstrate herein that endometrial TL does not correlate with matched PBMC TL in a large, well characterized group of fertile women not on any hormonal therapy and without any endometrial pathology. This suggests a tissue specific, possibly hormonal regulation of the endometrial TL. Since a fully functional endometrium can be regenerated from the thin post-
menopausal endometrium with the provision of exogenous ovarian steroid hormones (Paulson et al., 2002), it is the only reproductive organ not reported to show irreversible age-related changes. This endometrial age-defiance might include the apparent tissue specific TL preservation, presumably due to the ability of the hormonal induction of TA.

The human endometrium is a complex, multi-cellular, hormone-responsive tissue. The observation that isolated epithelial and stromal cells from dissociated endometrium showed differential TLs and TA levels is a novel finding adding a further level of complexity to endometrial telomere biology.

We now demonstrate, we believe, for the first time that endometrial TL changes according to the ovarian hormone cycle using a reproducible and validated qPCR method (Martin-Ruiz et al., 2014), with the shortest TL observed in the progesterone dominant mid-secretory phase, when TA is known to be at its lowest level (Tanaka et al., 1998; Kyo et al., 1999; Williams et al., 2001). Fractionated endometrium showed that EEC TL is shorter than that of the corresponding stroma throughout the cycle, whilst TA was higher in the EEC than in the corresponding stroma during the proliferative phase. In normal human somatic cells, telomere attrition triggers replicative senescence (Bodnar et al., 1998). Shorter TL would impose a limit on the proliferative life span in EECs and thus higher TA in these cells would allow them to maintain TL at a certain length thereby preventing mitotic arrest. Studies have shown that high TA, telomerase mRNA and hTERT protein are features of the endometrial proliferative disease, endometriosis (Kim et al., 2007; Hapangama et al., 2008; Mafra et al., 2014) and we now further demonstrate that ectopic lesions with a postulated hallmark of progesterone resistance phenotype (Bulun, 2010) have relatively longer TLs than the eutopic functional cells. Therefore, direct inhibition of TA with Imetelstat in EEC may bypass the pathological progesterone resistance and may prevent EEC proliferation and propagation of endometriotic lesions.

Endometrial TA as assessed by TRAP was reported to be localized to the glandular epithelial cells (Tanaka et al., 1998; Yokoyama et al., 1998); however, we now demonstrate measurable TA in the stromal compartment of the endometrium using the more sensitive TRAP-ELISA (Fajkus, 2006). TA is a marker of cell proliferation (Belair et al., 1997) and persisted in our short-term cultures of EECs while the cells were proliferating but was reduced upon senescence, which is consistent with previous work (Tanaka et al., 1998; Yokoyama et al., 1998). Moreover, inhibition of TA in short-term proliferating EEC cultures with 1 μM Imetelstat for 72 h was associated with a reduction in the expression of the mitosis marker, phosphorylated histone H3. This was in contrast to the adenocarcinoma where TA was affected by Imetelstat, but the phosphorylation status of histone H3 was not, suggesting a disconnection between TA and proliferation in endometrial cancer. Although our work focused mainly on the endometrial epithelial compartment, the direct inhibition of TA in stromal cells in culture appears to be potentially relevant and warrants further investigation. The stromal cells of the endometrium are persistently proliferative throughout the menstrual cycle, while the epithelial cells are highly proliferative specifically during the proliferative phase (Jurgenesen et al., 1996).

It is now well established that a 3D cell culture system more closely mimics natural tissues and organs than cells grown in 2D (Yamada and Cukierman, 2007). For this reason EECs were grown in 3D culture in order to recapitulate the architecture of the glands in vivo, to study the effects of telomerase inhibition. In 3D culture, under defined serum-free conditions with proliferation being driven by EGF, epithelial cells formed spheroid structures. There was a more profound effect of TA inhibition in 3D culture compared with 2D culture. Inhibition of TA in 3D was associated with an inability of spheroids to proliferate and expand. Additionally, the architecture of the spheroids was adversely affected such that after 5 days of treatment there was a loss of spherical form, suggesting an effect on the cytoskeleton which was confirmed by treating 2D cultures of EECs with Imetelstat. Imetelstat altered the cell morphology and growth in breast (Goldblatt et al., 2009) and lung (Mender et al., 2013) cancer cell lines. The profound effects of telomerase inhibition in 3D culture suggest an important role for telomerase in normal glandular epithelial function of the endometrium.

Observational studies suggest that TA in the endometrium is activated by estrogen and repressed by progesterone. TA is at its highest during the proliferative phase and progressively decreases during the secretory phase, becoming undetectable during the mid-secretory when progesterone levels peak (Kyo et al., 1997; Saito et al., 1997; Tanaka et al., 1998; Williams et al., 2001). In further support of these observations, we have demonstrated that women treated with MPA (as Depo-Provera) for 6 months, had a marked reduction in endometrial epithelial TA. Depo-Provera is known to induce pronounced reduction in endometrial epithelial proliferation (Lu et al., 2013). Also, in monolayer endometrial cultures consisting of both stromal and epithelial cells that allow these cell types to physically interact, we demonstrated that estrogen and progesterone significantly reduced TA compared with estrogen alone. Although there was a modest effect of MPA on TA in our explant cultures, the culture time was short and in addition to epithelial and stromal cells have endometrial cells and lymphocytes, both of which are reported to possess TA (Norrbck et al., 1996; Hsiao et al., 1997). The anti-proliferative effect of progesterone in the endometrium is exerted via the stroma, and both progesterone receptor (PR) and glucocorticoid receptor (GlucR) are postulated to be involved in progesterone and MPA action in the endometrium (Henderson et al., 2003).

The mechanisms by which hTERT expression is regulated by progesterone appear to be complex because the hTERT promoter lacks a canonical progesterone-responsive element (Wang et al., 2000). In a breast cancer cell lines, MPA inhibited hTERT mRNA transcription even in the presence of estrogen (Wang et al., 2000; Lebeau et al., 2002) and arrested cells in the late G1-phase (Lange et al., 1999) with the induction of p21 (Wang et al., 2000). It is known that cell cycle arrest is associated with down-regulation of telomerase (Belair et al., 1997). Interestingly p21 is induced in the secretory phase of the menstrual cycle when progesterone levels are high (Toki et al., 1998). There is evidence for cell cycle-dependent regulation of telomere synthesis in human cells (Tomlinson et al., 2006). The telomerase complex consisting of, hTR and hTERT, is targeted to telomeres specifically in the late S phase of the cell cycle when telomeres are lengthened (Hug and Lingner, 2006). We suggest that the different telomere lengths in proliferative and mid-secretory phase endometrium might reflect the negative effect of progesterone on cell cycle during the mid-secretory phase with cells accumulating in the late G1 phase. The mid-secretory phase is characterized as being metabolically active with differentiation of the stromal cells (Yang et al., 2011). Therefore, at this juncture the negative effects of progesterone on estrogen induced hTERT in the endometrial epithelium might also be indirect. In contrast, treatment with mifepristone (a PR antagonist) during the progesterone dominant mid-secretory phase when TA is low/undetectable (Williams et al., 2001), was associated with a significant increase in endometrial glandular
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telomerase immunoreactivity after 24–36 h. This coincides with reduced serum progesterone levels (Hapangama et al., 2002) and increased endometrial cell proliferation (Cameron et al., 1997). All women receiving mifepristone at mid-luteal phase developed vaginal bleeding and endometrial shedding by 48 h, therefore these 48 h samples may contain endometrial cells that were undergoing apoptosis and necrosis (associated with shedding). We speculate that this is the reason for the lack of a persisting increase in telomerase expression by 48 h. As mifepristone is capable of inhibiting glucocorticoid and progesterone receptors, its effect on telomerase expression is unclear. However, in healthy women progesterone had been previously shown to reverse the mid-cycle effects of mifepristone (Batista et al., 1992).

Consistent with the antagonistic effects of progesterone on estrogen-induced proliferation in the endometrium (Hapangama et al., 2015), our study further implicates progesterone in the negative regulation of both TA and TL. The mechanisms by which progesterone regulates telomerase activity in the endometrium requires further investigation which might reveal novel pathways that can be targeted in treating endometrial proliferative diseases. Of particular interest was the effect of telomerase inhibition in vitro on epithelial cell proliferation in 2D and 3D cultures, suggesting that telomerase might be an attractive target in developing new therapies for proliferative disorders of the endometrium.

Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors’ roles
D.K.H. obtained the Ethical approval, and conceived the study design. D.K.H., A.J.V., G.S. formulated experiments, analysed and interpreted data, produced figures and formulated the first draft. The samples were collected by D.K.H., H.O.D.C. and N.T. Experiments were carried out and data collected by A.J.V., D.K.H., N.T., and G.S. and D.K.H. and G.S. were involved in obtaining funding. H.O.D.C. was involved in data interpretation, and manuscript preparation and revision. All authors approved the submitted final version of the manuscript.

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Conflict of interest
None declared.

References


