Human Reproduction Vol.23, No.7 pp. 1511–1519, 2008 doi:10.1093/humrep/den172
Advance Access publication on May 2, 2008

Endometriosis is associated with aberrant endometrial expression of telomerase and increased telomere length

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BACKGROUND: In order to test our hypothesis that endometriosis is associated with abnormal expression of telomerase and telomere lengthening in endometrium, we assessed endometrial expression of the human telomerase enzyme and telomere length (TL). METHODS: This prospective pilot study, included 29 women with symptomatic, surgically diagnosed endometriosis (Group 1) and 27 healthy, fertile, symptom-free women without endometriosis (Group 2, confirmed by laparoscopy). Seventeen women in Group 1 and 15 women in Group 2 had endometrial biopsies taken on Day 21 $\pm$ 2 of the cycle. A further 12 women in each group were biopsied on Day 26 $\pm$ 2. Telomerase and estrogen receptor beta (ERβ) expression was evaluated by immunohistochemistry. Mean TL was determined by quantitative PCR. RESULTS: The endometria of fertile healthy women showed either weak or no telomerase immunoreactivity throughout the luteal phase. Immunostaining for telomerase was significantly increased during the implantation window and the premenstrual endometria of women with endometriosis ($P < 0.0001$). This was associated with a loss of stromal and vascular ERβ immunostaining ($P < 0.05$). The mean TL were significantly longer in endometria of women with endometriosis during the implantation window ($P = 0.005$), indicating the biological relevance of our novel finding of telomerase in benign endometrium. There was positive correlation of the circulating estradiol with peripheral blood TL in women. CONCLUSIONS: We speculate that aberrant endometrial expression of telomerase mediates alterations in cell fate that enhance proliferation, contributing to the pathogenesis of endometriosis.

Keywords: telomerase; endometriosis; telomere length; telomerase repeat amplification protocol; secretory phase endometrium

Introduction

Telomeres, the non-coding tandemly repeated DNA sequences (5-TTAGGG-3) that protect all eukaryotic linear chromosomal ends, are vital for maintaining chromosomal integrity and cell stability (Greider, 1996). In somatic cells, the telomere is shortened with each cell division. The critical shortening of telomeres with cell division may function as a mitotic clock, by which normal cells count their division and eventually signal their senescence or apoptosis (Harley and Villeponteau, 1995). Telomere shortening can be prevented by a specialized reverse transcriptase telomerase. Most somatic cells have undetectable levels of this enzyme (Harley and Villeponteau, 1995; Greider, 1996). Consequently, with every cycle of cellular growth and division, there is a steady decline in telomere length (TL) (Vaziri *et al*., 1993). In contrast, cells and tissues that express telomerase show preservation of the TL (Vaziri *et al*., 1993; Harley and Villeponteau, 1995; Greider, 1996; Wright *et al*., 1996) which allows these cells to overcome replicative senescence and to divide indefinitely.

Telomerase expression specifically correlates with cell proliferation (Yan *et al*., 2004) and most endometrial cancers express high levels of telomerase (Oshita *et al*., 2000). In the healthy endometrium of premenopausal women, telomerase activity has been studied by the detection of mRNA or by telomerase repeat amplification protocols (TRAP) (Tanaka *et al*., 1998; Williams *et al*., 2001). These studies suggest that telomerase expression in the functional layer of the endometrium varies according to the cyclical secretion of ovarian hormones. Many authors have reported high telomerase activity during the proliferative phase (Kyo *et al*., 1997; Tanaka *et al*., 1998; Williams *et al*., 2001). The largest study, which investigated functional endometrium during the luteal phase, reported either absent or weak activity during the mid-luteal phase [window of implantation (WOI)] and the late luteal phase of normal fertile women (Williams *et al*., 2001). However, the endometrium is composed of several cell types, including stromal, glandular, epithelial and leukocyte, and the location of telomerase expression within these differing compartments

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is not known. There are no reports of the functional relevance of cyclical telomerase expression, i.e. whether cyclical telomerase expression is associated with endometrial TL in the benign endometrium.

The importance of understanding endometrial cell kinetics is highlighted by the clinical condition of endometriosis. Endometriosis is defined as the presence of endometrial like glands and stroma at ectopic sites beyond the uterine cavity. Endometriosis-associated symptoms have a huge cost both in economic and social terms. Out of various theories put forward to explain the pathogenesis of endometriosis, retrograde menstruation (viable endometrial tissue refluxed at the time of menstrual shedding implants on pelvic organs) has gained the widest acceptance (Sampson, 1927). Various cellular adhesion molecules and metalloproteinases have been implicated in the process (Sillem *et al.*, 1998; Starzinski-Powitz *et al.*, 1998). There is evidence that retrograde menstruation occurs in >90% of women (Halme *et al.*, 1984) yet even with the most generous estimates the prevalence of endometriosis in the general population is around 10% (Cramer and Missmer, 2002). Thus, it is unclear why some women develop endometriosis whereas others do not.

The eutopic endometrium of women with endometriosis seems to differ from that in healthy women, e.g. their endometrial cells are resistant to apoptosis (Dmowski *et al.*, 1998). Furthermore, global gene expression studies have shown an up-regulation of endometrial gene families that are involved in mitosis and an increase in the endometrial expression of selected anti-apoptotic genes with a simultaneous decrease in pro-apoptotic genes (Braun *et al.*, 2007; Burney *et al.*, 2007). Transfection of endometrial cells with vectors expressing telomerase is sufficient for immortalization of these cells, and this technique is being widely used in cell-culture experiments (Kyo *et al.*, 2003). These observations led us to develop a hypothesis for the pathogenesis of endometriosis under which sloughed endometrium that has been expelled into the pelvic cavity via retrograde menstruation has enhanced replicative capacity and an ability to persist in an adverse environment, which promotes ectopic implantation. We hypothesize that endometriosis is associated with abnormal expression of telomerase and telomere lengthening or stabilization in the luteal phase endometrium. The only study on this topic published to date suggested a possibility of increased endometrial telomerase mRNA expression throughout the menstrual cycle in women with endometriosis (Kim *et al.*, 2007). There have not yet been any studies localizing the telomerase activity at a cellular level and evaluating the associated changes that telomerase expression has on endometrial TL.

As the first step towards testing our hypothesis, we carried out a prospective, pilot study. Our aims were:

(i) to examine the sites of telomerase expression within the endometrium using immunohistochemistry;
(ii) to assess whether there are any differences in endometrial telomerase expression in healthy women and women with endometriosis;
(iii) to determine whether endometrial TL differs between healthy women and women with endometriosis;
(iv) to determine whether the estrogen receptor subtype beta (ERβ) is differentially expressed in healthy women and women with endometriosis during the luteal phase.

Materials and Methods

Fifty-six premenopausal women (18–46 years) with regular menstrual cycle (25–31 day) not taking any hormonal treatments were recruited to this prospective, single-centre, observational, non-therapeutic, pilot study. Group 1 consisted of 29 women with surgically diagnosed endometriosis. Group 2 consisted of 27 fertile women who were recruited prior to laparoscopy while undergoing female sterilization and were found to have no detectable endometriosis and had no history of symptoms or prior diagnosis of the condition. A pipelle endometrial sampler (Prodimed, France) was used to obtain a sample of endometrium. Seventeen women in Group 1 and 15 women in Group 2 had their endometrial biopsies taken during the WOI (cycle days 21 ± 2), and a further 12 women in each group had biopsies taken in the late luteal phase (cycle days 26 ± 2). Blood samples were taken at the time of the endometrial biopsy for the assessment of peripheral blood TL and circulating estradiol (E_2) and progesterone by radioimmunoassay. Informed written consent was obtained from all participants, and the study was approved by Liverpool Adult Local Research Ethics Committee.

Endometrial samples were split into two parts; one was fixed for 24 h in 4% buffered formalin, rinsed and embedded in paraffin. The other part was immediately snap frozen with liquid nitrogen and was kept at −70°C for DNA/RNA studies. All endometrial samples were designated to cycle stage based on morphological criteria (dated according to recent modifications of Noyes criteria by two experienced pathologists; Murray *et al.*, 2004), last menstrual period date and circulating hormone profile.

Telomerase immunohistochemistry

Five-micrometer sections of endometrial specimens were deparaffinized and re-hydrated. A high-temperature antigen retrieval was performed by pressure cooking the slides in 0.01 M citrate buffer at pH 6 for 4 min. Endogenous peroxidase was quenched in 3% H_2O_2. After washing with Tris-buffered saline (TBS), the non-specific antibody binding was blocked with 10% non-immune goat serum (Vectastain, Vector Laboratories, Inc., Peterborough, UK) for telomerase or in a non-immune rabbit serum (DAKO, UK) in TBS containing 5% bovine serum albumin (Sigma) for ERβ. The sections were then incubated with rabbit polyclonal anti-human telomerase primary antibody (ab27573; Abcam, Cambridge, UK) or mouse monoclonal anti-human ERβ primary antibody (MCA 1974S; Serotec Ltd., Oxford, UK) at 1:100 dilution overnight at 4°C in a humidifying chamber. After washing, the sections were incubated with labelled streptavidin biotin polymer (EnVision, DAKO), and incubating with diaminobenzidine developed brown positive staining. The sections were lightly counterstained with Mayers' haematoxylin and mounted.

For telomerase, as a negative control, the primary antibody was replaced by rabbit immunoglobulin G (Dako, USA). Endometrial adeno-carcinoma, tonsillar tissue, breast carcinoma and proliferative phase functional endometrial sections were used as external positive controls. Known telomerase negative myometrial smooth muscle was used as external negative tissue control (Yan *et al.*, 2004). For ERβ, normal serum negative controls and proliferative phase functional endometrial positive controls were used.

All slides were scored blind by two independent observers prior to breaking the code. An arbitrary four-point semi-quantitative scoring scale (0, negative/no staining; 1, weak; 2, strong; 3, very strong) as

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previously described (Hapangama *et al*., 2002) was used to analyse telomerase expression on the basis of a global assessment of all slides. Once the slides had been independently scored, the values were tabulated and an overall score for each section was determined after consultation between the two observers before the code was broken. This scoring system is a standard method used previously (Wang *et al*., 1998; Critchley *et al*., 2001; Hapangama *et al*., 2002; Refaat *et al*., 2008) and was validated by demonstrating a high correlation between objectively measured immunoreactivity (computerized image analysis system) and subjective semi-quantitative scores by a trained observer in a subset of tissue sections (Wang *et al*., 1998).

TL measurement by real-time PCR

Genomic DNA was extracted from endometrium and peripheral blood mononuclear cells using QIAamp DNA mini kit (Qiagen). DNA concentration was adjusted to 5 ng/μl in H_2O. TL was measured on an iCycler real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) as previously described (Martin-Ruiz *et al*., 2004) with the following modifications. Telomere PCRs included 100 nM primer Tel A (5′CGGTTTGTTTGGGTTTGG GTTTGGGTTTGGGTTTGG GTT-3′), 300 nm primer Tel B (5′GGCTTG CCTTACCCTTACC CTTACCCTTACCCTTACCCT-3′), 15 ng genomic DNA, 0.1 M SYBR green (Sigma-Aldrich Co.) and 1 M Platinum Quantitative PCR Supermix-UDG (Invitrogen Ltd, Paisley, UK) in a 30 μl reaction. The reactions were set-up in quadruplicate in 96-well plates. Each plate includes four DNA quantity standards (serial dilutions of a reference DNA sample giving final DNA quantities of between 15 and 1.87 ng per reaction), one negative control and three internal controls represented by three samples of genomic DNA with known TL (3, 5.5 and 9.5 kb). The PCR cycle conditions include an initial denaturation step at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 56°C for 60 s. Mean TL is expressed in base pairs (bp).

Trap assay for telomerase activity

To validate our immunohistochemical assay of telomerase, we carried out a PCR-enzyme-linked immunosorbent assay-based TRAP assay using the Telo TAGGG telomerase PCR kit (Roche, GmbH, Mannheim, Germany) to measure telomerase activity. Biopsy samples were homogenized in TRAP-assay buffer for 30 min, centrifuged and protein level was determined using Bradford method (Bio-Rad Laboratories). One microgram protein lysate was used for all samples, including hTERT over expressing fibroblasts and OVCAR-3 ovarian carcinoma cells positive controls. PCR was carried out for 30 cycles and the conditions were according to the manufacturers' manual (Roche, GmbH, Mannheim, Germany). Optical density (OD) was measured at 450 nm in a multi-Ascent multi-well plate reader. Normal MRC-5 fibroblasts and water were used as negative controls. MRC-5 fibroblasts resulted in an OD of 0.006, whereas hTERT-over expressing fibroblasts and OVCAR-3 resulted in reference values of 1.44 and 1.99.

Statistical analysis

The Statistical Package for the Social Sciences version 14.0 for Windows statistical programme was used for all calculations. Semi-quantitative scores from different groups were compared by a non-parametric test (Kruskall-Wallis). Two-way analysis of variance was used to examine the effects of endometriosis and the timing of the biopsy on mean TL. Pearson's correlation coefficient was used to test the association between mean TL with age, BMI, weight and height.

Results

Demographics

Women who had biopsies in the mid-luteal phase were similar in age in both groups [mean 37 (SD ± 5) versus 38 (±5); $P = 0.30$]; however, in the late luteal phase, younger women were biopsied in the endometriosis group [31 (SD ± 8) versus 33 (±7); $P = 0.01$]. BMI, height, weights or cycle lengths were not significantly different in each group, whereas age did differ between groups ($P = 0.006$, Table I). Forty-eight percentage of women in Group 1 had mild to moderate (American Fertility Society Stage I/II) endometriosis whereas the remainder had severe disease.

Telomerase immunohistochemistry

Control tissue

Paraffin sections of endometrial adenocarcinoma, tonsilar tissue, breast carcinoma and proliferative phase functional endometrial tissue were used as positive controls and specific brown nuclear telomerase staining was seen in all (Fig. 1a–c). No staining was observed in either the immunological negative control (Fig. 1d) or the external negative control tissue (myometrium) (Fig. 1e).

Control group

In healthy fertile women, telomerase staining was either weak or absent in all tissue compartments studied during the mid- and late luteal phase endometrium (Fig. 2a and b).

Endometriosis group

The secretory phase endometrial tissue from women suffering with endometriosis showed increased immunoreactivity versus healthy women both in the mid-luteal and late luteal phase. This increase in telomerase expression was highly significant in all tissue compartments studied (gland, stroma, luminal epithelium and in endothelial cells, $P < 0.0001$), except perivascular cells ($P = 0.17$) in the mid-luteal phase. Telomerase expression was also significantly increased in glands and endothelial cells ($P < 0.0001$), stroma ($P < 0.001$) and luminal

Table I. Demographic features of women with endometriosis (Group 1) and fertile healthy women (Group 2).

	Group 1 Median (range) $n = 29$	Group 2 Median (range) $n = 27$	*P*-value
Age (years)	35 (18–46)	39 (25–44)	0.006
BMI	27 (17–38)	29 (21–36)	0.22
Weight (kg)	73 (52–97)	80 (59–95)	0.51
Height (cm)	170 (147–179)	164 (155–177)	0.21
Cycle length (days)	28 (25–31)	28 (25–30)	0.51

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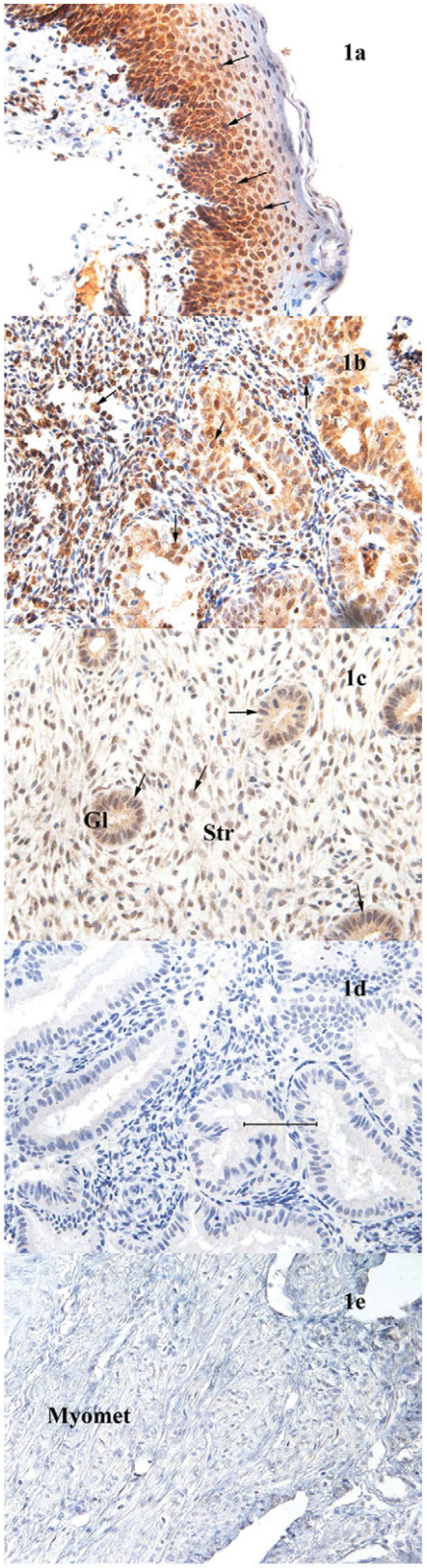

Figure 1: Immunoexpression of telomerase in control tissues. External positive control tissues; tonsilar Cortex (**a**), endometrial carcinoma (**b**), and proliferative phase endometrium (**c**). Immunological negative control with DAKO rabbit negative immunoglobulin G (**d**) and external negative tissue control (myometrium) (**e**) showing no staining are also included. Scale bar **d** = 200 μm; positive immunoexpression, brown (arrow). Endometrial glands (**Gl**), stroma (**Str**) and myometrial smooth muscle cells (**Myomet**) are labelled.

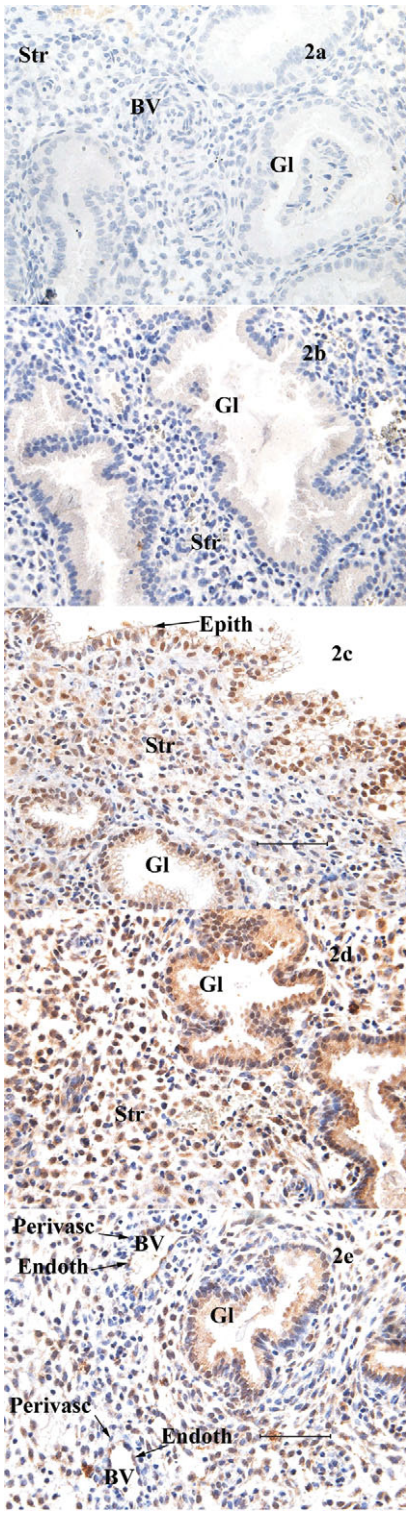

Figure 2: Immunoexpression of telomerase in the secretory phase endometria; **Gl, Str,** surface epithelium (**Epith**), blood vessels (**BV**), perivascular tissue (**Perivasc**) and vascular endothelium (**Endoth**) highlighted. Scale bar **d** = 200 μm; positive immunoexpression, brown (arrows). Healthy fertile women in control groups had absent or weak endometrial telomerase staining. Note the absence of telomerase immunoexpression in healthy fertile women during the WOI (**a**) and the late luteal phase (LLP) (**b**). Significantly increased telomerase staining was seen in the endometria of women with endometriosis in WOI (**c**) and in the LLP (**d**). In women with endometriosis, there was absent telomerase staining in the perivascular region; note the absent endometrial telomerase staining in the perivascular region during the WOI in a woman with endometriosis (**e**).

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epithelial cells ($P = 0.005$) in the late luteal phase endometrium. The peri-menstrual stromal leucocytes also showed positive telomerase staining. Telomerase immunoreactivity was weak and not significantly different to the healthy women in perivascular cells ($P = 0.33$) (Fig. 2c–e, Table II). There was no association between the stage of endometriosis and telomerase expression ($r = 0.37$, $P = 0.06$).

Trap assay

We tested three control and three endometriosis samples. All control samples from Group 2 did have either very low or negative telomerase (range 0.001–0.13) and endometriosis samples were either strongly positive or had low values of telomerase activity (range 0.092–1.74) (Table III).

Endometrial TL

Mean TL was assessed in 15 endometrial samples from Groups 1 and 2 in the mid-luteal phase. A further 10 endometrial samples from Group 1 and 12 from Group 2 were studied in the late luteal phase. The mean TL appeared to be longer in the mid-luteal phase endometrial tissue of the endometriosis group (Fig. 3), and it was confirmed by their significant [$F(1,44)=5\,305\,779$, $P < 0.005$] interaction in the analysis of variance. The endometrial mean TL did not correlate with age ($r = 0.19$), weight ($r = 0.19$), height ($r = 0.11$) or BMI ($r = 0.16$).

Blood TL

The mean TL in peripheral blood was assessed in 25 samples in each group and there was no significant interaction in the analysis of the variance [$F(1,33) = 284\,642$, $P < 0.36$] among groups. The mean TL in peripheral blood was positively correlated with the circulating E_2 level ($r = 0.35$, $P = 0.01$). There was no correlation with the mean peripheral blood TL and age ($r = -0.08$), weight ($r = -0.27$), height ($r = 0.13$) or BMI ($r = 0.34$). There was no significant correlation between the tissue TL and peripheral blood TL ($r = -0.002$).

ERβ immunohistochemistry

In healthy women, the positive ERβ staining seen in all cellular compartments of the proliferative endometrium decreased in the luteal phase epithelial fraction, but persisted in the stromal cells. In these women, perivascular and endothelial cells in the secretory phase showed persistent ERβ staining. Women with endometriosis showed either weak or negative ERβ immune reactivity in all the endometrial tissue compartments during the luteal phase. This difference was significant in stroma ($P = 0.02$), perivascular ($P < 0.05$) and endothelial ($P < 0.0001$) compartments (Fig. 4).

Progesterone and E_2 level

The women with endometriosis did not differ from normal women in circulating progesterone (mean progesterone for WOI 8.61 ng/ml versus 7.38 ng/ml, $P > 0.72$; Mean progesterone for late luteal phase 2.54 ng/ml versus 4.20 ng/ml, $P > 0.12$) or E_2 (Mean E_2 for WOI 423 pmol/l versus 393 pmol/l, $P > 0.90$; Mean E_2 for late luteal phase 217 pmol/l versus 341 pmol/l, $P > 0.23$) levels in the luteal phase. Furthermore, the endometrial TL or telomerase expression did not correlate with either the serum E_2 ($P = 0.26$) or progesterone level ($P = 0.46$). However, the blood TL positively correlated with the circulating E_2 levels ($r = 0.35$, $P = 0.01$).

Table II. Mean semi-quantitative scores for telomerase immunohistochemistry staining.

Groups		Telomerase glands	Telomerase stroma	Telomerase epithelium	Telomerase perivascular	Telomerase endothelial
Fertile Control WOI (Group 2)	Mean	0.00	0.30	0.07	0.07	0.00
	N	17	17	16	16	16
	SD	0.00	0.45	0.26	0.26	0.00
Endometriosis WOI (Group 1)	Mean	1.93	2.13	1.82	0.25	1.82
	N	15	15	14	14	14
	SD	0.86	0.64	0.77	0.43	0.67
Fertile Control LLP	Mean	0.37	0.67	0.37	0.25	0.00
	N	12	12	12	12	12
	SD	0.48	0.49	0.48	0.45	0.00
Endometriosis LLP	Mean	2.38	2.29	2.09	0.73	1.82
	N	12	12	11	11	11
	SD	0.64	0.75	0.54	0.78	0.87

WOI, window of implantation; LLP, late luteal phase.

Table III. Correlation between the mean values for TRAP assay, immunohistochemistry (IHC) semi-quantitative scores and mean tissue and peripheral blood TL in six samples where TRAP assay was carried out.

Groups		TRAP	IHC glands	IHC stroma	IHC epithelium	IHC perivscular	IHC endothelial	Tissue TL (bp)	Blood TL (bp)
Endometriosi (Group 1)	Mean	0.96	1.66	2.33	1.66	0.33	2.00	4860.66	3466.00
	N	3	3	3	3	3	3	3	3
	SD	0.83	0.58	0.57	0.58	0.58	0.00	535.15	653.24
Fertile healthy (Group 2)	Mean	0.05	0.00	0.33	0.00	0.00	0.00	3818.67	3562.00
	N	3	3	3	3	3	3	3	1
	SD	0.06	0.00	0.58	0.00	0.00	0.00	524.02	0.0

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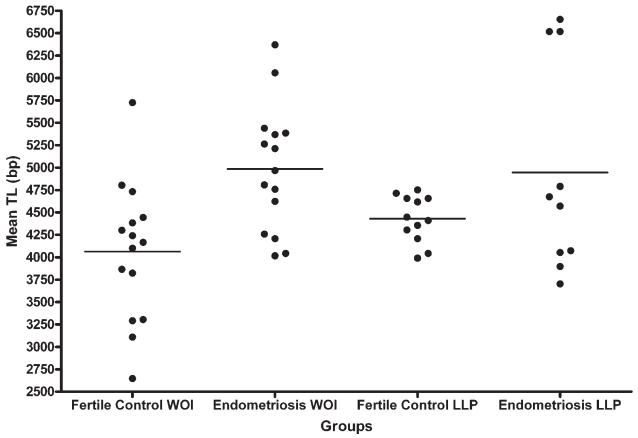

Figure 3: Scatter graph showing the mean TL of endometrial tissue of women in different groups studied. The differences in groups reached statistical significance only in the WOI. Analysis of variance revealed evidence of a statistically significant interaction between timing of the endometrial biopsy in the luteal phase and the disease status (whether the woman has endometriosis or not; $F(1,44) = 5\ 305\ 779$, $P < 0.006$). Horizontal bar=median.

Discussion

This study is the first to examine the expression of telomerase at a cellular level in eutopic endometrium from women with and without endometriosis. The biological relevance of the telomerase expression we report is demonstrated by a finding of increased TL among women with high levels of telomerase, i.e. those with endometriosis. Furthermore, we confirm for the first time the highly accepted yet so far not proven hypothesis that circulating E_2 level positively correlates with blood TL.

The involvement of telomerase in determination of cell fate has usually been assessed by means of RNA or DNA-based techniques which neglects the importance of cellular level telomerase expression in a particular tissue. Almost all recent studies (Wu *et al*., 2006) that explored immunodetection of telomerase used a mouse monoclonal antibody [NCL- human telomerase reverse-transcriptase (hTERT)] as the immunoprobe. This antibody was claimed to have high specificity and reproducibility (Yan *et al*., 2004). However, early in 2006, Wu *et al*. demonstrated that this NCL-hTERT antibody in fact recognized nucleolin rather than telomerase (Wang *et al*., 2000). We have shown that immunohistochemistry can be used to detect a signal for telomerase at a cellular level in paraffin embedded endometrial sections using a polyclonal rabbit anti-telomerase antibody which identify the amino acids 174–341 region of human telomerase (Abcam, Cambridge, UK). The signal was limited to the nuclei of the endometrial cells. We used a variety of human tissues that are known to express high levels of telomerase and also confirmed the observed telomerase immunoreactivity with the telomerase activity detected by more established methods such as TRAP (Table III). To our knowledge, this is the first report to immunolocalize telomerase in the non-malignant premenopausal endometrium. Our results agree with recent data which illustrated an increase in endometrial telomerase mRNA and enzyme activity in women with endometriosis (Kim *et al*., 2007).

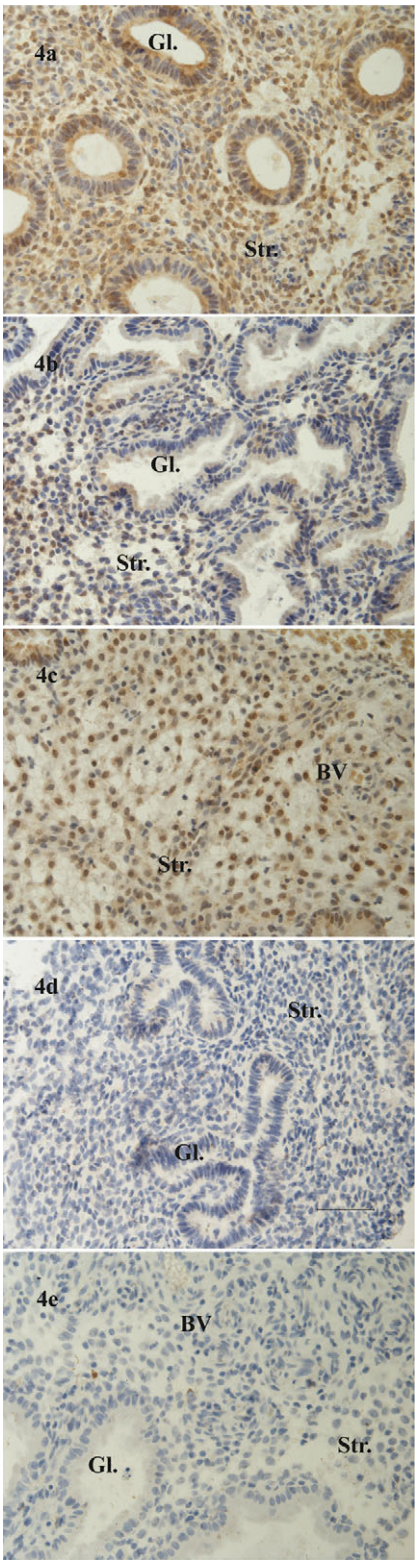

Figure 4: Immunoexpression of estrogen receptor beta (ERβ) in human endometrium.
Immunoexpression of ERβ positive control, proliferative phase endometrium (**a**); **Gl**., **Str**. and **BV** highlighted. Scale bar **d** = 200 μm; positive immunoexpression, brown. Healthy fertile women in control groups had positive endometrial ERβ staining. Note the presence of positive ERβ immunostaining in the stromal cells of healthy fertile women during the WOI (**b**) and the LLP (**c**). Absent or weak ERβ staining was seen in the stromal cells (including the vessels) of women with endometriosis in WOI (**d**) and in the LLP (**e**).

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In accordance with previous PCR-based studies, we have shown either weak or absent telomerase expression in the mid-luteal and late luteal endometrium of healthy premenopausal women (Kyo *et al*., 1997; Tanaka *et al*., 1998; Williams *et al*., 2001). Significantly higher expression of endometrial telomerase was immunolocated in most tissue compartments in the luteal phase endometria of endometriosis sufferers.

While progesterone is the dominant hormone in the luteal phase, the level of circulating E_2 also peaks around the WOI. Estrogen modulate telomerase via a direct classical estrogen response element in the telomerase promoter (Kyo *et al*., 1999; Williams *et al*., 2001; Kondoh *et al*., 2007) and progesterone exerts diverse effects on telomerase mRNA expression without involving the classical progesterone response element (Wang *et al*., 2000). The circulating estrogen and progesterone values did not differ significantly between the endometriosis and healthy women, and this agrees with the available evidence (Radwanska and Dmowski, 1981; Cahill and Hull, 2000). Therefore, we examined the expression of ERβ, which is the predominant ER subtype (Critchley *et al*., 2001; Lecce *et al*., 2001) in the luteal phase endometrium of normal fertile women. In agreement with previous authors, luteal phase endometrium of healthy women showed weak or no glandular ERβ staining, whereas positive staining was detected in all other cellular compartments (Wang *et al*., 2000; Critchley *et al*., 2001; Lecce *et al*., 2001). Furthermore, our results confirm the previous observations that endometriosis is associated with a decrease in endometrial ERβ expression (Jackson *et al*., 2007). Loss of ERβ is associated with cell proliferation and up-regulation of telomerase (Stettner *et al*., 2007) hence our results suggest that in the endometrial stromal and vascular compartments telomerase expression may be regulated via ERβ.

In women with endometriosis, the mid-luteal endometrial cells had significantly higher mean TL compared with the healthy fertile group. The variation in TL associated with age is not relevant to our results because the women included in both groups who had biopsies in mid-luteal phase were of similar age. This observation of the positive correlation of high endometrial telomerase expression with longer mean TL during the WOI suggests that variation in protein expression is biologically relevant since it is consistent with the hypothesis that telomerase activity is associated with extension of telomeric DNA. In normal women, suppression of telomerase activity during the WOI may instigate a shift of endometrial cellular activity from proliferation to maximize differentiation and apoptosis, which may promote implantation and invasion by an early embryo. Among women with endometriosis, telomerase expression may make the endometrium during 'WOI' to be 'hostile' rather than receptive to an invading embryo. Studies on various cell proliferative indices have shown the endometrial cells of women with endometriosis to have greater proliferative ability (Ulukus *et al*., 2006). Furthermore, there is epidemiological evidence to support the link between endometriosis and recurrent conception failure after assisted reproduction (Koninckx, 1998).

In the late luteal phase, the endometrium of the endometriosis sufferers showed significantly greater telomerase expression than the healthy group. Despite this, the TL in the late luteal phase was not significantly different between the two groups. Although this could be due to the smaller sample size (12 versus 15+ in mid-luteal phase) in each group in the late luteal phase, we note that the significance of telomerase expression can be dissociated from an apparent effect on the TL. For example, telomerase expression without an effect on TL renders cells more resistant to apoptosis and growth arrest mediated by various factors (Forsythe *et al*., 2002) and telomerase expression regulates the expression of a key cell cycle regulator, cyclin D1 (Jagadeesh and Banerjee, 2006). Indeed, some authors have suggested that once a cell expresses telomerase the exact TL is of secondary importance in cell cycle dynamics (Karlseder *et al*., 2002) and that in some cells, alteration in telomere state rather than the actual length correlates with replicative senescence (Dong *et al*., 2005). Together these observations suggest that although we did not see an associated significant difference in TL throughout the cycle, the telomerase-expressing endometrial cells in the endometriosis group are likely to have a persistently greater proliferative ability than the non-telomerase expressing endometrial cells of the healthy group. We did not examine tissue obtained at the time of menses. If the pattern of aberrant telomerase expression is persistent throughout the cycle (Kim *et al*., 2007), then our results indicate that altered cell kinetics in endometriosis may allow sloughed endometrial cells to be 'aggressive', survive and give rise to endometriotic deposits in the peritoneal cavity.

Many authors have hypothesized the observed gender difference in blood TL to be due to high circulating E_2 levels (Serrano and Andres, 2004; Stindl, 2004). Our results for the first time confirm this, showing a positive correlation between circulating E_2 levels and the blood TL. Surprisingly, the mean endometrial TL did not correlate positively with the circulating E_2. This may be explained by the fact that only one type of cell (leucocytes) contributes to the mean TL in blood, whereas various cell types contribute to the mean endometrial cell TL. Furthermore, we have shown a differential expression of ERβ within the endometrium, suggesting a diverse response to E_2 by the different compartments. Previous studies have suggested a negative correlation between age and BMI with peripheral blood mean TL (Butler *et al*., 1998; Aviv, 2004; Valdes *et al*., 2005). We have not seen a correlation between age or BMI with the mean TL of peripheral blood or endometrial tissues. This could be due to the small sample size or the relatively limited age range seen in our patients. Of further interest is the fact that peripheral blood TL did not correlate with the mean TL of the endometrial tissue. Since endometrium shows a dynamic pattern of telomerase expression according to the monthly cyclical changes in ovarian hormones, it is likely that the endometrial TL will also change accordingly throughout the cycle. The peripheral blood TL on the other hand seems to be fairly stable over such a short period in time with changes happening at a relatively slow pace over years rather than months.

In summary, we have immunolocalized telomerase enzyme in the benign premenopausal endometrium providing detailed information on the cellular origin of endometrial telomerase activity. We have shown that telomerase expression is

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significantly increased in the endometrium of women suffering with endometriosis. This finding was associated with a simultaneous decrease in stromal and vascular ERβ expression in women with endometriosis. We have also shown that peripheral blood TL in women correlated with the circulating E_2 level. We speculate that aberrant endometrial expression of telomerase mediates alterations in cell fate that enhance proliferation, contributing to the pathogenesis of endometriosis. We therefore report the first immunohistochemical study suggesting a role for endometrial telomerase expression in the pathogenesis and disease process of endometriosis.

Acknowledgement

We would like to thank Miss Stephanie Brooks for her assistance with the steroid hormone assays and Dr Hima Vamulapalli for her involvement with the ERβ staining.

Funding

This work was supported by a RDF grant from the University of Liverpool and RCOG millennium grant to D.K.H.

References

Aviv A. Telomeres and human aging: facts and fibs. *Sci Aging Knowledge Environ* 2004;**51**:43.

Braun DP, Ding J, Shaheen F, Willey JC, Rana N, Dmowski WP. Quantitative expression of apoptosis-regulating genes in endometrium from women with and without endometriosis. *Fertil Steril* 2007;**87**:263–268.

Burney RO, Talbi S, Hamilton AE, Vo KC, Nyegaard M, Nezhat CR, Lessey BA, Giudice LC. Gene expression analysis of endometrium reveals progesterone resistance and candidate susceptibility genes in women with endometriosis. *Endocrinology* 2007;**148**:3814–3826.

Butler MG, Tilburt J, DeVries A, Muralidhar B, Aue G, Hedges L, Atkinson J, Schwartz H. Comparison of chromosome telomere integrity in multiple tissues from subjects at different ages. *Cancer Genet Cytogenet* 1998;**105**:138–144.

Cahill DJ, Hull MG. Pituitary-ovarian dysfunction and endometriosis. *Hum Reprod Update* 2000;**6**:56–66.

Cramer DW, Missmer SA. The epidemiology of endometriosis. *Ann N Y Acad Sci* 2002;**955**:11–22.

Critchley HO, Brenner RM, Henderson TA, Williams K, Nayak NR, Slayden OD, Millar MR, Saunders PT. Estrogen receptor beta, but not estrogen receptor alpha, is present in the vascular endothelium of the human and nonhuman primate endometrium. *J Clin Endocrinol Metab* 2001;**86**: 1370–1378.

Dmowski WP, Gebel H, Braun DP. Decreased apoptosis and sensitivity to macrophage mediated cytolysis of endometrial cells in endometriosis. *Hum Reprod Update* 1998;**4**:696–701.

Dong CK, Masutomi K, Hahn WC. Telomerase: regulation, function and transformation. *Crit Rev Oncol Hematol* 2005;**54**:85–93.

Forsythe HL, Elmore LW, Jensen KO, Landon MR, Holt SE. Retroviral-mediated expression of telomerase in normal human cells provides a selective growth advantage. *Int J Oncol* 2002;**20**:1137–1143.

Greider CW. Telomere length regulation. *Annu Rev Biochem* 1996;**65**: 337–365.

Halme J, Hammond MG, Hulka JF, Raj SG, Talbert LM. Retrograde menstruation in healthy women and in patients with endometriosis. *Obstet Gynecol* 1984;**64**:151–154.

Hapangama DK, Critchley HO, Henderson TA, Baird DT. Mifepristone-induced vaginal bleeding is associated with increased immunostaining for cyclooxygenase-2 and decrease in prostaglandin dehydrogenase in luteal phase endometrium. *J Clin Endocrinol Metab* 2002;**87**:5229–5234.

Harley CB, Villeponteau B. Telomeres and telomerase in aging and cancer. *Curr Opin Genet Dev* 1995;**5**:249–255.

Jackson KS, Brudney A, Hastings JM, Mavrogianis PA, Kim JJ, Fazleabas AT. The altered distribution of the steroid hormone receptors and the chaperone immunophilin FKBP52 in a baboon model of endometriosis is associated with progesterone resistance during the window of uterine receptivity. *Reprod Sci* 2007;**14**:137–150.

Jagadeesh S, Banerjee PP. Telomerase reverse transcriptase regulates the expression of a key cell cycle regulator, cyclin D1. *Biochem Biophys Res Commun* 2006;**347**:774–780.

Karlseder J, Smogorzewska A, de Lange T. Senescence induced by altered telomere state, not telomere loss. *Science* 2002;**295**:2446–2449.

Kim CM, Oh YJ, Cho SH, Chung DJ, Hwang JY, Park KH, Cho DJ, Choi YM, Lee BS. Increased telomerase activity and human telomerase reverse transcriptase mRNA expression in the endometrium of patients with endometriosis. *Hum Reprod* 2007;**22**:843–849.

Kondoh K, Tsuji N, Asanuma K, Kobayashi D, Watanabe N. Inhibition of estrogen receptor beta-mediated human telomerase reverse transcriptase gene transcription via the suppression of mitogen-activated protein kinase signaling plays an important role in 15-deoxy-Delta(12,14)-prostaglandin J(2)-induced apoptosis in cancer cells. *Exp Cell Res* 2007;**313**:3486–3496.

Koninckx PR. Biases in the endometriosis literature. Illustrated by 20 years of endometriosis research in Leuven. *Eur J Obstet Gynecol Reprod Biol* 1998;**81**:259–271.

Kyo S, Takakura M, Kohama T, Inoue M. Telomerase activity in human endometrium. *Cancer Res* 1997;**57**:610–614.

Kyo S, Takakura M, Kanaya T, Zhuo W, Fujimoto K, Nishio Y, Orimo A, Inoue M. Estrogen activates telomerase. *Cancer Res* 1999;**59**:5917–5921.

Kyo S, Nakamura M, Kiyono T, Maida Y, Kanaya T, Tanaka M, Yatabe N, Inoue M. Successful immortalization of endometrial glandular cells with normal structural and functional characteristics. *Am J Pathol* 2003;**163**:2259–2269.

Lecce G, Meduri G, Ancelin M, Bergeron C, Perrot-Applanat M. Presence of estrogen receptor beta in the human endometrium through the cycle: expression in glandular, stromal, and vascular cells. *J Clin Endocrinol Metab* 2001;**86**:1379–1386.

Martin-Ruiz C, Saretzki G, Petrie J, Ladhoff J, Jeyapalan J, Wei W, Sedivy JM, von Zglinicki T. Stochastic variation in telomere shortening rate causes heterogeneity of human fibroblast replicative lifespan. *J Biol Chem* 2004;**279**:17826–17833.

Murray MJ, Meyer WR, Zaino RJ, Lessey BA, Novotny DB, Ireland K, Zeng D, Fritz MA. A critical analysis of the accuracy, reproducibility, and clinical utility of histologic endometrial dating in fertile women. *Fertil Steril* 2004;**81**:1333–1343.

Oshita T, Nagai N, Ohama K. Expression of telomerase reverse transcriptase mRNA and its quantitative analysis in human endometrial cancer. *Int J Oncol* 2000;**17**:1225–1230.

Radwanska E, Dmowski WP. Luteal function in infertile women with endometriosis. *Infertility* 1981;**4**:269–277.

Refaat B, Amer S, Ola B, Chapman N, Ledger W. The expression of activin- A- and - B-subunits, follistatin, and activin type II receptors in fallopian tubes bearing an ectopic pregnancy. *J Clin Endocrinol Metab* 2008;**93**: 293–299.

Sampson JA. Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. *Am J Obstet Gynecol* 1927;**14**:422–425.

Serrano AL, Andrés V. Telomeres and cardiovascular disease: does size matter? *Circ Res* 2004;**94**:575–584.

Sillem M, Prifti S, Neher M, Runnebaum B. Extracellular matrix remodelling in the endometrium and its possible relevance to the pathogenesis of endometriosis. *Hum Reprod Update* 1998;**4**:730–735.

Starzinski-Powitz A, Gaetje R, Zeitvogel A, Kotzian S, Handrow-Metzmacher H, Herrmann G, Fanning E, Baumann R. Tracing cellular and molecular mechanisms involved in endometriosis. *Hum Reprod Update* 1998;**4**: 724–729.

Stettner M, Kaulfuss S, Burfeind P, Schweyer S, Strauss A, Ringert RH, Thelen P. The relevance of estrogen receptor-beta expression to the antiproliferative effects observed with histone deacetylase inhibitors and phytoestrogens in prostate cancer treatment. *Mol Cancer Ther* 2007;**6**: 2626–2633.

Stindl R. Tying it all together: telomeres, sexual size dimorphism and the gender gap in life expectancy. *Med Hypotheses* 2004;**62**:151–154.

Tanaka M, Kyo S, Takakura M, Kanaya T, Sagawa T, Yamashita K, Okada Y, Hiyama E, Inoue M. Expression of telomerase activity in human endometrium is localized to epithelial glandular cells and regulated in a

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menstrual phase-dependent manner correlated with cell proliferation. *Am J Pathol* 1998;**153**:1985–1991.

Ulukus M, Cakmak H, Arici A. The role of endometrium in endometriosis. *J Soc Gynecol Investig* 2006;**13**:467–476.

Valdes AM, Andrew T, Gardner JP, Kimura M, Oelsner E, Cherkas LF, Aviv A, Spector TD. Obesity, cigarette smoking, and telomere length in women. *Lancet* 2005;**366**:662–664.

Vaziri H, Schachter F, Uchida I, Wei L, Zhu X, Effros R, Cohen D, Harley CB. Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. *Am J Hum Genet* 1993;**52**:661–667.

Wang H, Critchley HO, Kelly RW, Shen D, Baird DT. Progesterone receptor subtype B is differentially regulated in human endometrial stroma. *Mol Hum Reprod* 1998;**4**:407–412.

Wang Z, Kyo S, Takakura M, Tanaka M, Yatabe N, Maida Y, Fujiwara M, Hayakawa J, Ohmichi M, Koike K *et al.* Progesterone regulates human telomerase reverse transcriptase gene expression via activation of mitogen-activated protein kinase signaling pathway. *Cancer Res* 2000; **60**:5376–5381.

Williams CD, Boggess JF, LaMarque LR, Meyer WR, Murray MJ, Fritz MA, Lessey BA. A prospective, randomized study of endometrial telomerase during the menstrual cycle. *J Clin Endocrinol Metab* 2001;**86**: 3912–3917.

Wright WE, Piatyszek MA, Rainey WE, Byrd W, Shay JW. Telomerase activity in human germline and embryonic tissues and cells. *Dev Genet* 1996;**18**:173–179.

Wu YL, Dudognon C, Nguyen E, Hillion J, Pendino F, Tarkanyi I, Aradi J, Lanotte M, Tong JH, Chen GQ *et al.* Immunodetection of human telomerase reverse-transcriptase (hTERT) re-appraised: nucleolin and telomerase cross paths. *J Cell Sci* 2006;**119**:2797–2806.

Yan P, Benhattar J, Seelentag W, Stehle JC, Bosman FT. Immunohistochemical localization of hTERT protein in human tissues. *Histochem Cell Biol* 2004;**121**:391–397.

Submitted on July 16, 2007; resubmitted on February 10, 2008; accepted on April 8, 2008

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