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Assessment of the proliferative status of epithelial cell types in the endometrium of young and menopausal transition women

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BACKGROUND: We determined protein and mRNA expressions of markers of normal human endometrial proliferation and hypothesized that dysregulation of the endometrial response to estradiol (E_2) and progesterone would be observed in the older menopausal transition (MT) women compared with mid-reproductive age (MRA) controls. METHODS: Endometrial biopsies were prospectively obtained from MRA and MT non-randomized healthy volunteers during proliferative ($\pm$ exogenous E_2) and secretory (MRA only) menstrual cycle phases. mRNA and/or nuclear protein expressions of proliferative markers (MKI67, PCNA and MCM2), cell-cycle regulators (cyclins A1, E1 and D1 and cyclin dependant kinase Inhibitor B; CCNA1, CCNE1, CCND1 and CDKN1B) and sex-steroid receptors [estrogen receptor (ER) and progesterone receptor (PR)] were assessed in endometrial lumen, gland and stroma. RESULTS: MRA women had significantly higher proliferative than secretory expression of MKI67, PCNA, MCM2, CCNA1, CCNE1, ESR1 and PGR in lumen and gland (minimal stromal changes), whereas CDKN1B protein expression was higher during the secretory phase. E_2-treatment of MT women led to relatively less MKI67 glandular protein expression compared with MRA women; no other age-related differences were observed. CONCLUSION: Although the MT does not appear to alter the proliferative cell phenotype of endometrial epithelium and stroma, the data suggest that prior to the MT, age is associated with a decrease in some proliferative markers and steroid receptor expression status within different endometrial cell types.

Keywords: cell cycle; endometrium; laser-capture; menopausal transition; proliferation

Introduction

Epithelial cell proliferation in the uterus is tightly regulated by the ovarian steroids, estrogen and progesterone. On the one hand, 17β-estradiol (E_2) drives uterine epithelial cell proliferation (Slayden and Brenner, 2004); on the other, progesterone acts to inhibit E_2-induced epithelial cell proliferation and promote its differentiation (Brenner and Slayden, 2005). In women, uterine stromal cell (SC) proliferation appears to depend upon E_2 alone (Jabbour *et al.*, 2006), probably reflecting the need to renew the human endometrial lining during each menstrual cycle. E_2 and progesterone exert these effects through their cognate receptor transcription factors, estrogen receptor 1 (alpha; ESR1) and progesterone receptor (PGR).

The endometrial proliferative status is regulated by oscillations of cell-cycle regulatory proteins such as the cyclin dependant kinases (CDKs) that act together with their cyclin (CCN) partners. Passage through the G1 to S phase checkpoint in the cell cycle depends upon the sequential activity of cyclin D (CCND), cyclin E (CCNE) and cyclin A (CCNA) together with their respective CDKs, which phosphorylate their nuclear substrates, members of the retinoblastoma family of proteins. The activity of these cyclin–CDK complexes can also be negatively regulated by CDK inhibitors (CDKNI), belonging to members of the INK4 (CDKN2A to CDKN2D) and CIP/KIP (CDKN1A to CDKN1C) families.

Endometrial hyperplasia (Anastasiadis *et al.*, 2000b), polyps (Anastasiadis *et al.*, 2000a) and adenocarcinoma (Parazzini *et al.*, 1991) are all disorders of proliferation that increase dramatically with reproductive ageing, suggesting that proliferation and/or endometrial response to E_2 are altered with ageing. Moreover, although the poorer reproductive outcomes of ageing women are primarily attributed to ovarian rather than endometrial dysfunction (Sauer, 1998), selective screening of women who undergo *in vitro* fertilization together with the administration of supraphysiological doses of exogenous E_2 and progesterone to facilitate implantation (Meldrum, 1993) may bias against the detection of an endometrial component for age-related implantation failure in the general population.

The purpose of this study was to improve our understanding of the mechanisms that regulate normal epithelial cell proliferation on a molecular level and to relate these findings to reproductive ageing. We have recently reported (Niklaus and Pollard, 2006) that in mice the isolation of luminal (LE) and glandular (GE) epithelial cells by laser capture microdissection (LCM) identified unique molecular signatures of these cell types under the influence of ovarian hormones. Thus, in addition to well-characterized antibody markers of epithelial proliferation (Tong and Pollard, 1999, 2002), we used the same technology to test the hypothesis that due to increased potential for proliferative abnormalities (Weiss *et al.*, 2006) and reduced fertility (Rowe 2006), reproductive ageing would be associated with dysregulation of proliferation in endometrial cell types.

Materials and Methods

Participants

The protocol (July 2002–July 2005) was approved by the Committee on Clinical Investigations/Institutional Review Board in accordance with the Declaration of Helsinki for Medical Research involving Human Subjects and was conducted at the General Clinical Research Center at the Albert Einstein College of Medicine. Written, informed consent was obtained from all participants of this study.

All participants were healthy volunteers with no history of infertility, recruited from the community. Endometrial samples were derived from 29 women in the age of 18–38 (mid-reproductive age, MRA) and 15 women in the age of 45–54 (menopausal transition, MT). Participants had not used hormonal contraception within 3 months prior to study inclusion and were at least 90% normal weight for height (Company, 1983). Screening done solely for study purposes included a negative Pap smear, negative urine pregnancy test and normal saline hysterosonogram. The MRA women all had regular 25–35-day menstrual cycles. MT participants also reported regular menses during their mid-reproductive years, but had changes in cycle regularity or at least 1 skipped menstrual period within the past 3 months, consistent with the Stages of Reproductive Aging Workshop definition of Stage-2 entry into the MT (Soules *et al.*, 2001).

Endometrial biopsy protocols

All endometrial biopsies were performed using a Pipelle catheter (Unimar Inc., Wilton, Conn), with the aim of sampling the fundal area and removing as intact a cylinder of tissue as possible. By placing the catheter $\geq$5 cm depth within the uterus and rotating it

Table 1. Patient demographics represented for each of the mid-reproductive age (MRA) and menopausal transition (MT) groups examined

	MRA-Prol	MRA-Prol-E_2	MRA-Sec	MT-Prol	MT-Prol-E_2	MRA Prol versus MRA-Sec	MRA Prol versus All Prol groups
N	22	3	13	7	8	*P*-values	
Age (years)	26.9 ± 0.9	30.3 ± 3.7	28.1 ± 1.4	47.1 ± 0.6*	48.8 ± 1.3*	0.587	<0.001*
Height (cm)	162.0 ± 1.0	162.1 ± 5.3	162.4 ± 1.4	162.0 ± 1.7	162.3 ± 2.7	0.929	0.998
Weight (kg)	71.0 ± 4.0	79.1 ± 8.2	74.9 ± 4.7	79.3 ± 5.0	79.1 ± 6.6	0.623	0.552
BMI (kg m^{-2})	27.0 ± 1.5	30.0 ± 2.5	28.5 ± 1.8	30.2 ± 2.4	30.1 ± 2.5	0.609	0.542
Number of smokers	1	0	1	4	0	1.000	0.015*
E_2 (nmol l^{-1})	0.52 ± 0.08	0.77 ± 0.44	0.48 ± 0.08	0.26 ± 0.08	1.22 ± 0.38*	0.701	0.011*
Progesterone (nmol l^{-1})	2.30 ± 0.39	0.78 ± 0.43	45.88 ± 6.97*	2.84 ± 0.82	2.18 ± 0.43	<0.001*	0.407

Data presented as mean ± SE. Significant differences compared to MRA proliferative (MRA-Prol), *$P < 0.05$. The estradiol (E_2)-treated MT group (MT-Prol-E_2) excludes the patient with the highest E_2 level (37 nmol l^{-1}) to calculate mean E_2 level ($n = 7$); she was included in all other demographic analyses ($n = 8$).

Table 2. Sequences of forward and reverse primers used for the different proliferative and sex-steroid receptor endometrial markers in the quantitative real-time PCR (QPCR) assay

Gene	Primer sequence	
	Forward	Reverse
MKI67	GTGGAAGTTCTGCCTACGGA	ATGTGCCCAATTTCTCAGGC
CCNA1	TGGATCAGAAAATGCCTTCC	CCCCTGCTCTAGTTCATCCA
CCNE1	ATCCTCCAAAGTTGCACCAG	AGGGGACTTAAACGCCACTT
MCM2	TGAACAAGTTCAGCCACGAC	CAAACCAGAATCCCAAGGAA
ESR1	CCTATCTCAGGGAGGGAAGG	TGCTCTCCAAGTCCCACTCT
PGR	CATACTCATGGCCATCAACG	TTCCAGCAAAGACCCCATAC

gently, up to 8 cm of tissue length was obtained from a single pass (Santoro *et al.*, 2000). Segments $\sim 5 \times 2$ mm were either fixed or placed in a cryomold filled with OCT and frozen on a bed of dry ice for immunohistochemical and mRNA studies, respectively. Subjects received 800 mg ibuprofen pre-procedure and lidocaine topical spray to the cervix (which was not instrumented).

Proliferative phase biopsies were performed on cycle day (CD) 12 ± 1 to target the time of maximal endometrial response to E_2 (Lessey *et al.*, 1988). Serum E_2 and progesterone were obtained on the day of the biopsy; the sample was excluded if progesterone ≥ 11.1 nmol l^{-1} (3.5 ng ml^{-1}) (Israel *et al.*, 1972). Because older reproductive women show deviations in follicular phase E_2 when compared with MRA women (Santoro *et al.* 2003), we treated a subset of MT women with exogenous transdermal E_2 (MT-Prol-E_2; Climara; Berlex, 100 and 50 μg per day) to control their cycle. E_2 levels for these treated MT women were targeted to achieve physiological circulating $E_2 \geq 0.44-0.55$ nmol l^{-1} (120–150 pg ml^{-1}) by CD 12 (Santoro *et al.*, 1998). A small subset of MRA women were also E_2-treated (MRA-Prol-E_2) as controls ($n = 3$). Thus, proliferative cycles were characterized either by no medication (MRA-Prol $n = 22$, MT-Prol $n = 7$) or E_2-treatment (MRA-Prol-E_2 $n = 3$, MT-Prol-E_2 $n = 8$) (Table 1). E_2-treated participants were non-smokers with no contraindications to exogenous E_2. Patches were added or subtracted according to serum E_2 levels measured every other day.

Secretory phase biopsies were also performed on 13 MRA women (MRA-Sec), who used urinary LH predictor kits (Ovukit Monoclonal Antibodies, Los Angeles, CA, USA) from day 11 onwards until detection of the LH surge (LHS). Subsequently, ovulation was confirmed by serum progesterone level ≥ 11.1 nmol l^{-1} (Israel *et al.*, 1972) drawn on the day of secretory biopsy. Endometrium was collected from one woman in the early luteal phase 4 days post-LHS, eight women in the mid-luteal phase 5–9 days post-LHS and four women in the late luteal phase 10–11 days post-LHS (Filicori *et al.*, 1984). Since cell-cycle markers are the focus of this study, the secretory phase was not investigated in the MT women.

After a rest period of ≥ 30 days, some of the participants underwent additional biopsies. Six MRA women had biopsies in both proliferative and secretory phases and three MRA women underwent untreated and E_2-treated proliferative cycles. Participants in the MT-Prol and MT-Prol-E_2 groups did not overlap. Due to the large amount of material needed for protein and mRNA analysis particularly for the proliferative phase, three untreated MT women, one E_2-treated MT woman and one MRA woman underwent additional proliferative biopsies; results from their samples were averaged.

Immunohistochemistry

Three measures of DNA synthesis were used as surrogate markers for cell proliferation: the antigen identified by monoclonal antibody Ki-67 (MKI67), proliferating cell nuclear antigen (PCNA) and minichromosome maintenance deficient 2, mitotin (*S. cerevisiae*) (MCM2). Monoclonal PCNA (sc-56) and ESR1 (sc-8005) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), MKI67 (MB67) from Neomarkers (Fremont, CA, USA) and MCM2 (ab-6153) from Abcam (Cambridge, MA, USA). Polyclonal CCNA1 (sc-751), CDKN1B (sc-528) and PGR (sc-538) were purchased from Santa Cruz. Biopsies from at least 17 MRA proliferative (MRA-Prol) and 7 secretory (MRA-Sec) women were used for immunohistochemistry (IHC) ($n = 6$ for CDKN1B). For the MT women, samples from at least four and up to seven women were examined for protein expression by the various antibodies listed.

As described elsewhere in detail (Tong and Pollard, 1999), to achieve optimal staining, in some cases, antibody microwave treatment of deparaffinized sections with 0.1 M citrate buffer (pH 6.0) was performed for the following antibodies; ESR1, PCNA, CCNA1 and MCM2 only. The primary antibody was either applied for 60 min at room temperature (MKI67 and CCNA1) or overnight at +4°C. Hence, the incubation temperatures/times were kept consistent per antibody to produce optimal staining, after which sections were washed in phosphate-buffered saline and then exposed to biotin-conjugated secondary antibodies (BD Pharmagen or Vector). Incubations in Vectastain ABC peroxidase (Vector) and nuclear staining with metal enhanced diaminobenzidine (Pierce, Rockford, IL, USA) with or without (MKI67 and CCNA1) 2.5% nickel ammonium sulphate (Sigma-Aldrich, St Louis, MO, USA) followed. Sections were counterstained with hematoxylin. Negative controls included omission of the primary antibody.

Positive IHC staining for each antibody (except CCND1 and CDKN1B) was quantified by random, blinded counting of at least 200 cellular nuclei from LE, GE and SC endometrial compartments. Randomized regions of the endometrium were chosen by joystick motion of two fields of view in an X/Y direction. As described by others (Brenner *et al.*, 2003), the protein expression was calculated as the proportion (%) of positively stained nuclei over the total number of nucleated cells present. Mean differences in counts of MKI67 and CCNA1 made by two blinded, independent observers for all cell types were within 2.5%, with the exception of proliferative phase MKI67 showing a 6% mean inter-observer difference. Thereafter, one observer determined the percent staining for the remaining antibodies.

Serum hormone assays

E_2 and progesterone were measured using DELFIA fluoroimmunoassays (Perkin-Elmer Life and Analytical Sciences; Wallac; Turku, Finland) as previously described (Santoro *et al.*, 2003). The limit of sensitivity for the E_2 assay was 0.05 nmol l^{-1}, and the intra- and inter-assay coefficient of variations (CVs) were 6.1 and 5.8, respectively. For progesterone, the limit of sensitivity was 0.8 nmol l^{-1} and the corresponding CVs were 4.9 and 7.0, respectively.

LCM and quantitative real-time PCR

As previously described (Niklaus *et al.*, 2002; Niklaus and Pollard, 2006), at least 20 unfixed, eosin-stained uterine cryosections were dried and subjected to LCM using the Arcturus Pix cell II instrument (Arcturus Engineering Inc., Mountain View, CA). LE and GE (not SC) were captured for mRNA analysis. Total RNA was extracted and assessed for adequate yield and quality, and cDNA was synthesized for quantitative real-time PCR (QPCR) assays as previously described (Niklaus and Pollard, 2006). Up to 6 primer pairs could be evaluated from this material. Briefly, 10 μl PCR reaction mixture consisted of 1× SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA), cDNA template, forward and reverse primers (ranging from 0.5 to 1 μM) and 2 μl cDNA.

Oligonucleotide primers were synthesized by Invitrogen Life Technologies Inc. from human mRNA sequences for MKI67, CCNE1, CCNA1, MCM2, ESR1 and PGR. The forward and reverse sequences of each of the genes tested are listed in Table 2. In preliminary experiments, serial dilutions of cDNA were analysed to confirm a linear relationship between cDNA content and quantity of product across the amplification range. As previously described, data was normalized to expression levels of the housekeeping gene 18s rRNA (Universal 18S Primer Pair, Ambion) (Niklaus and Pollard, 2006).

Relative gene expression of the MRA-Prol (internal calibrator) versus MRA-Sec as well as the MRA-Prol versus untreated MT-Prol/MT-Prol-E_2 was assessed using the $2^{-\Delta\Delta\ CT}$ method (Livak and Schmittgen 2001) as previously outlined (Niklaus and Pollard 2006). Samples from five MRA-Prol, five MRA-Sec, five MT-Prol and six MT-Prol-E_2 women were examined for LE and GE mRNA expression of MKI67, CCNE1, CCNA1, MCM2 (in this case 10 samples each of MRA-Prol and MRA-Sec), ESR1 and PGR. mRNA analyses for ESR1 and PGR were performed for proliferative groups only.

Statistics

After assessing normality, data were reported as the median and interquartile range (IQR) for nuclear protein counts and as the mean ± SE for all other parameters. Participant demographic data was analysed using Analyze-It software (Analyze-it, Leeds, England). The Fisher's Exact test was used for categorical data. The independent samples *t*-test and 1-way analysis of variance (ANOVA) were used for continuous data points, and the Dunnett-correction was applied for *post hoc* testing. Data from repeated samples for an individual patient within a study group were averaged to give a single data point. All other analyses were performed using SAS-software (SAS institute, Cary, NC, USA). The Wilcoxon-rank-sum and Mann–Whitney tests were applied for nuclear protein counts. Linear regression was employed to assess relationships of protein with serum sex-steroid levels and age. Where multiple comparisons are made for each marker, the lowest *P*-value is given. Significance was assessed at a two-tailed $P < 0.05$. For mRNA data, 95% confidence intervals (CIs) were calculated to assess significance of the $2^{-\Delta\Delta\ CT}$.

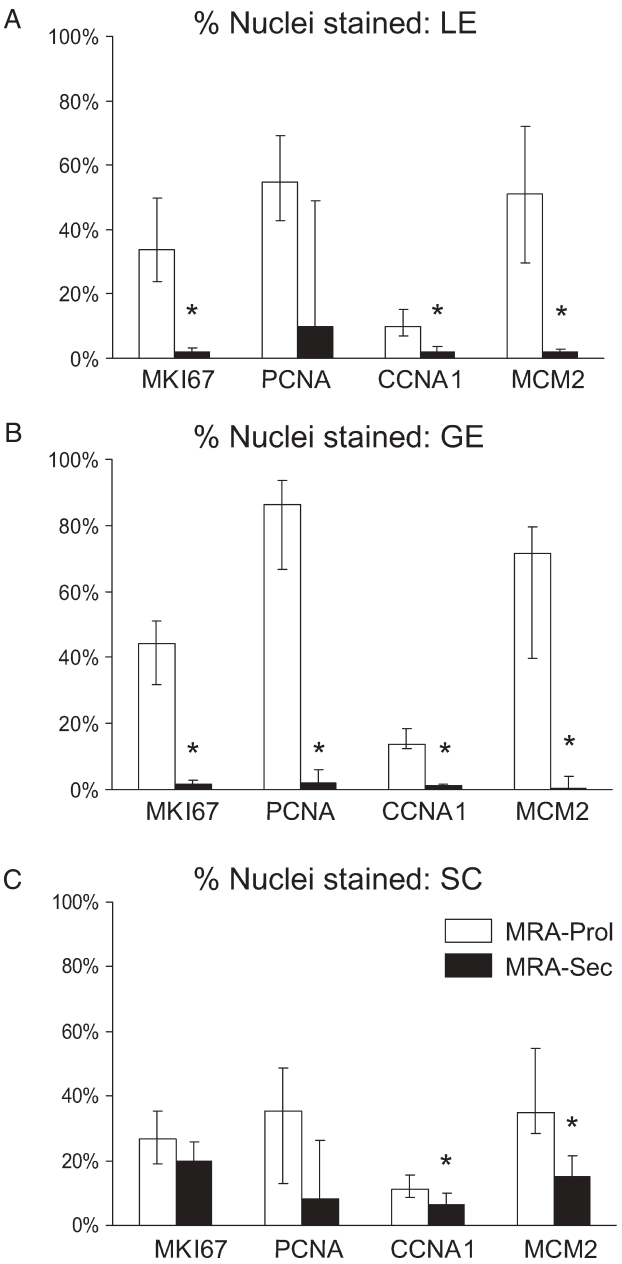

Figure 1. Measurements of cyclic nuclear protein expression for markers of DNA synthesis in mid-reproductive age (MRA) endometrium. Nuclear protein expression (% positive cells) for MKI67, PCNA, MCM2 and CCNA1 antibodies in lumen (LE; **A**), gland (GE; **B**) and stroma (SC; **C**) compartments of proliferative (MRA-Prol) (open bars) and secretory phase (MRA-Sec) (black solid bars) MRA endometrium. Data represented as median and interquartile range (IQR) (error bars). Significant differences between MRA-Prol versus MRA-Sec, $*P < 0.05$. The sample size examined for each antibody in each group was: MKI67, MRA-Prol ($n = 17$) and MRA-Sec ($n = 12$); PCNA, MRA-Prol ($n = 17$) and MRA-Sec ($n = 7$); CCNA1, MRA-Prol ($n = 17$) and MRA-Sec ($n = 12$); MCM2, MRA-Prol ($n = 18$) and MRA-Sec ($n = 9$).

Results

Participants and menstrual cycle characteristics

MRA and MT women ranged in age from 18–35 and 45–54 years respectively (Table 1). MRA women sampled in the proliferative (MRA-Prol) versus secretory (MRA-Sec) phase did not differ with respect to age ($P = 0.59$; independent samples *t*-test), weight ($P = 0.62$), height ($P = 0.93$), body mass index (BMI) ($P = 0.61$), or E_2 level ($P = 0.70$). As per design, progesterone levels were higher in the MRA-Sec (12.1–91.4 nmol l^{-1}) compared with the three proliferative groups ($P < 0.001$; 1-way ANOVA), which had statistically similar mean progesterone levels of 0.78–2.84 nmol l^{-1} (Table 1). One MT-Prol-E_2 woman had an E_2 level of 37 nmol l^{-1}; after excluding her from the mean E_2 calculation,

MT-Prol-E_2 still had significantly higher E_2 levels than the other proliferative groups ($P = 0.011$; 1-way ANOVA) (Table 1). This patient was analysed for all other parameters including progesterone level. Irrespective of E_2-treatment, the proliferative groups did not differ in weight ($P = 0.55$; independent samples *t*-test), height ($P = 0.99$), or BMI ($P = 0.54$), but more untreated MT-Prol women smoked ($P = 0.02$; Fisher's Exact test) (Table 1).

Protein and mRNA analysis of proliferative status

Mid-reproductive age

(i) Epithelial expression—Lumen (LE) and gland (GE):

In the GE, MKI67 ($P < 0.001$; Wilcoxon-rank-sum test), PCNA ($P < 0.001$) and MCM2 ($P < 0.001$) had more positively stained nuclei in proliferative than in secretory endometrium (Figs 1 and 2). MKI67 ($P < 0.001$) and MCM2 ($P < 0.001$) protein expressions were significantly greater in proliferative than secretory phase LE; wide variation in secretory phase PCNA precluded reliable comparisons (Fig. 1). Compared with untreated MRA-Prol, E_2-treatment of MRA women did not significantly alter MKI67 protein expression in LE and GE ($P > 0.4$; Mann–Whitney test, data not shown). Serum E_2 and progesterone levels did not show a linear relationship with any protein marker in the untreated MRA tissue ($P > 0.095$; linear regression, data not shown). Despite testing with two independent commercial antibodies, specific and reproducible staining for CCND1 was not obtained. CCNA1 nuclear expression was significantly greater ($P < 0.001$; Wilcoxon-rank-sum test) (Fig 1 and 2) in the proliferative compared with secretory LE and GE. Although not quantified, both cytoplasmic and nuclear CDKN1B expressions in the LE and GE showed dramatically increased intensity (with similar colour development times); nuclear staining increased from ~40% in the proliferative to ~95% in the secretory phase (Fig. 3).

LE and GE MKI67, MCM2, CCNA1 and CCNE1 (LE only) mRNA expressions were significantly greater during the proliferative than secretory phase of MRA women (excluded 1 CIs) (Fig. 4). PCNA and CCNA1 proliferative phase nuclear protein expressions were significantly higher in GE than LE ($P < 0.03$; Wilcoxon-rank-sum test) (Table 3). Conversely, secretory phase PCNA expression was significantly greater in the LE than in GE ($P = 0.027$) (Table 3). MKI67 and MCM2 did not exhibit epithelial differences in nuclear staining at either menstrual cycle phase ($P > 0.09$; Wilcoxon-rank-sum test) (Table 3).

(ii) Stroma (SC) expression

CCNA1 and MCM2 protein expressions increased in proliferative compared with secretory SC ($P<0.03$; Wilcoxon-rank-sum test) (Fig. 1C), but SC MKI67, PCNA (Fig. 1C) and CDKN1B (data not shown) showed no nuclear staining changes ($P > 0.05$). E_2-treatment of MRA women also did not alter MKI67

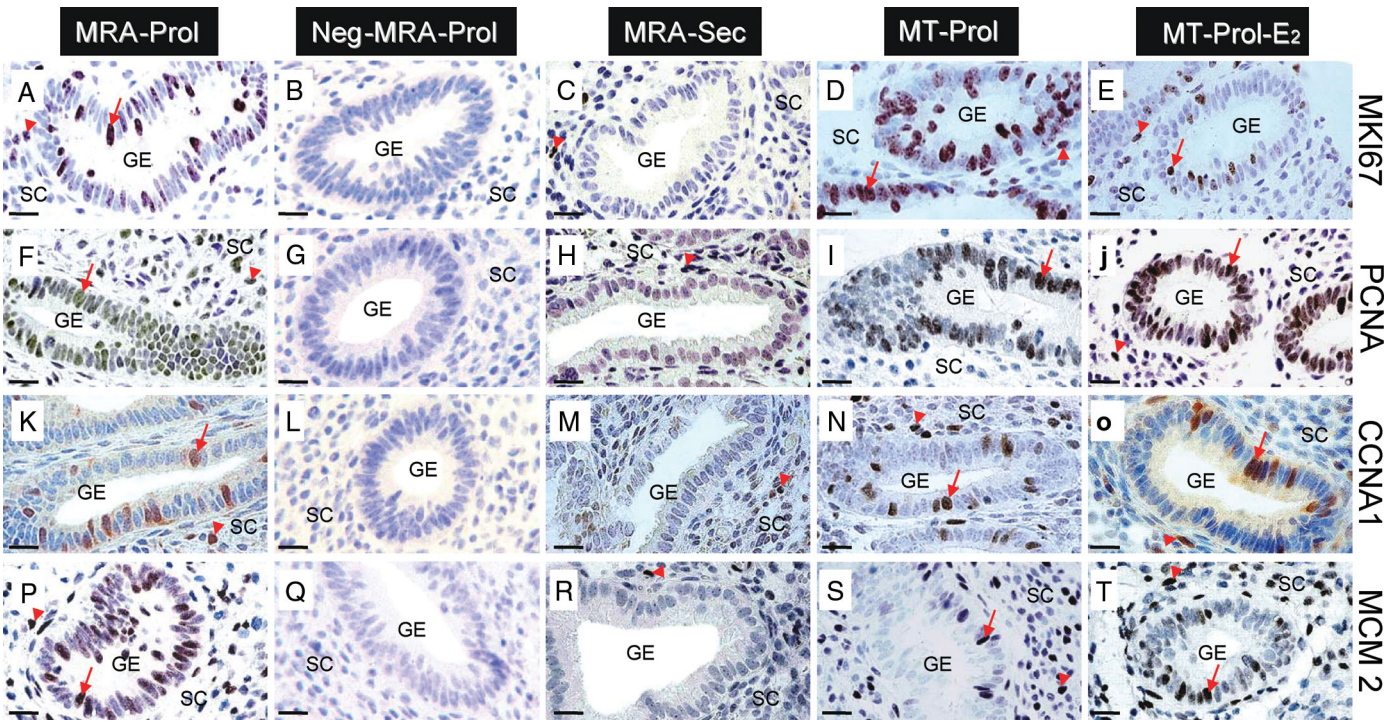

Figure 2. Immunohistochemistry (IHC) staining of cell-cycle markers in the endometrial glands (GE) of MRA and menopausal transition (MT) women. Endometrial GE protein expression of MKI67 (**A**–**E**), PCNA (**F**–**J**), CCNA1 (**K**–**O**) and MCM2 (**P**–**T**) was significantly greater during the proliferative (MRA-Prol) >than secretory (MRA-?A3B2 h 0,14Sec) phase of MRA women. There was a similar expression of all cell-cycle markers between MRA-Prol and MT-Prol groups. However, MKI67 and CCNA1 protein expressions were significantly greater in estradiol (E_2)-treated MT women (MT-Prol-E_2) than MRA-Prol. Negative controls are shown for each antibody with MRA-Prol endometrium (Neg-MRA-Prol). Nuclear staining in GE (arrows) and stroma (arrowhead) shown in red. Scale bar representative of each group is 20 μm. The sample size examined for each antibody is given (MRA-Prol as in Fig. 1) in parenthesis. For MKI67; MT-Prol ($n = 5$) and MT-Prol-E_2 ($n = 7$). PCNA; MT-Prol $n = 5$) and MT-Prol-E_2 ($n = 7$). CCNA1; MT-Prol ($n = 5$) and MT-Prol-E_2 ($n = 7$). MCM2; MT-Prol ($n = 5$) and MT-Prol-E_2 ($n = 5$).

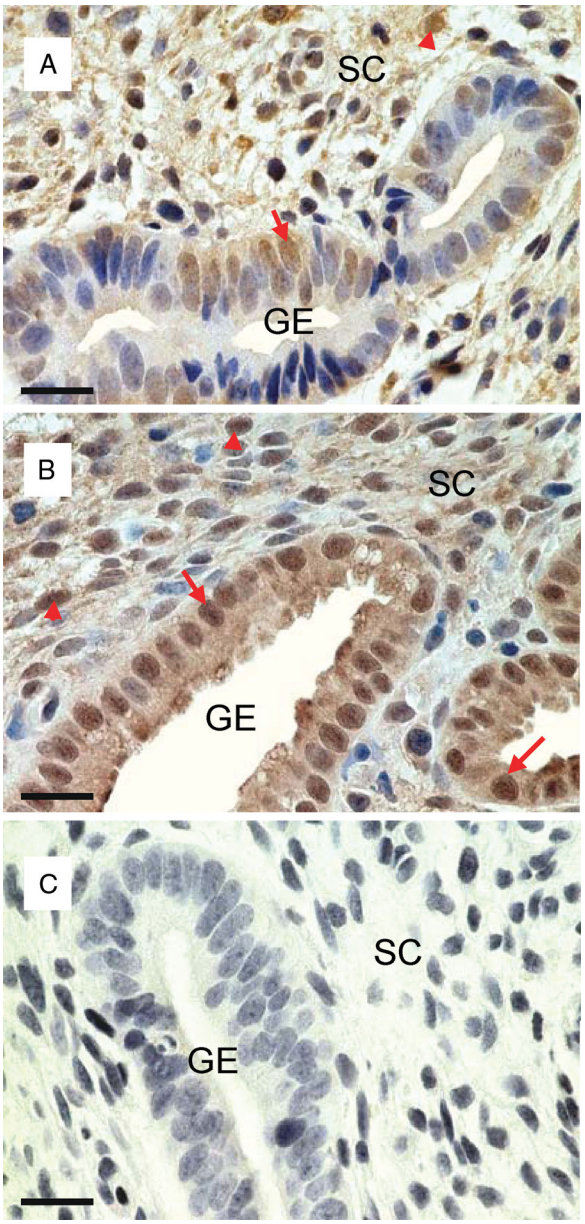

Figure 3. Cyclic protein expression of CDKN1B in MRA endometrium. IHC staining of paraffin sections showed less CDKN1B nuclear (red arrow) and cytoplasmic expressions in gland (GE) during proliferative (MRA-Prol) (**A**) than secretory (MRA-Sec) (**B**) phase. Nuclear stroma (SC) (arrowhead) expression appeared relatively constant across the menstrual cycle. Scale bar represents 20 μm. Negative control sections showing primary antibody omission are also shown (**C**).

protein expression in the SC when compared with no treatment ($P = 1$; Mann–Whitney test, data not shown).

Comparisons of MRA and MT women

(i) Proliferative (MRA-Prol) versus MT-Prol women:

The percentage of MKI67 and PCNA positively stained nuclei in both lumen (LE) and gland (GE) were similar between MRA-Prol and MT-Prol groups ($P > 0.2$; Mann–Whitney test) (Figs 2 and 5). Mean serum E_2 levels measured in these groups were similar at the time of the biopsy ($P = 0.07$; independent samples *t*-test) (Table 2). There were no differences in mRNA (CIs crossed 1; data not shown) or protein expression of CCNE1 (mRNA only), CCNA1 ($P > 0.7$; Mann–Whitney test) (Figs 2 and 5) or MCM2 ($P > 0.1$) between MRA-Prol and MT-Prol women in LE, GE or stroma (SC) compartments.

(ii) MRA-Prol versus E_2-treated MT (MT-Prol- E_2) women:

The elevated E_2 serum levels (Table 2) in MT-Prol-E_2 were associated with significantly decreased protein MKI67 expression in all three cell types ($P < 0.05$; Mann–Whitney test) and decreased GE CCNA1 expression compared with untreated MRA-Prol women ($P < 0.02$) (Figs 2 and 5). However, the LE, GE (except CCNA1) and SC protein expressions of PCNA, MCM2 and CCNA1 were similar in the MT-Prol-E_2 and untreated MRA-Prol women ($P > 0.09$; Mann–Whitney test) (Figs 2 and 5), as were mRNA expression of all markers of DNA synthesis (CIs crossed 1; data not shown). Because the age range of the MRA women spanned 20 years, we constructed a total of 12 (6 MRA-Prol and 6 secretory MRA-Sec) scatter plots of the 4 proliferative markers (MKI67, PCNA, CCNA1 and MCM2) in the 3 different cell types versus age. No significant relationships were observed among the MRA-Sec samples ($P > 0.05$ for all six scatter plots; data not shown). Among the MRA-Prol samples, nuclear protein expression of GE MKI67 and CCNA1 ($P < 0.05$; linear regression) (Fig 6A and B) and SC MCM2 ($P < 0.05$) (Fig. 6C) significantly decreased with age. We then assessed whether these correlations masked differences between MRA-Prol and the MT proliferative groups. After sub-stratifying the MRA samples by age (18–27 years and 28–38 years) for comparison to both MT groups, we observed no change in statistical outcomes.

Protein and mRNA analysis of sex-steroid receptors

MRA-Prol endometrium showed significantly greater ESR1 and PGR nuclear protein expressions than that of secretory (MRA-Sec) in lumen (LE), gland (GE) and stroma (SC; not PGR) compartments ($P < 0.002$; Wilcoxon-rank-sum test) (Figs 7 and 8). ESR1 and PGR protein expressions were significantly higher in GE than LE of MRA-Prol ($P < 0.03$; Wilcoxon-rank-sum test) but not MRA-Sec tissue ($P > 0.08$) (Table 3; Fig. 7).

The mRNA (CIs crossed 1; data not shown) and protein expressions (Figs 7 and 8) of sex steroid receptors did not differ between MRA-Prol and untreated MT (MT-Prol) groups in any cell compartment ($P > 0.09$; Mann–Whitney test). E_2-treated (MT-Prol-E_2) women had significantly elevated GE PGR and SC ESR1 expressions compared with untreated MT-Prol endometrium ($P < 0.04$; Mann–Whitney test; Figs 7 and 8).

Within the MRA group, luminal PGR expression significantly decreased ($P < 0.05$) with age (Fig. 5D), but similar to the proliferative markers, the statistical outcomes were unchanged after sub-stratifying MRA by age.

Discussion

In this study, we have demonstrated that human endometrium can be evaluated for molecular markers of proliferation using

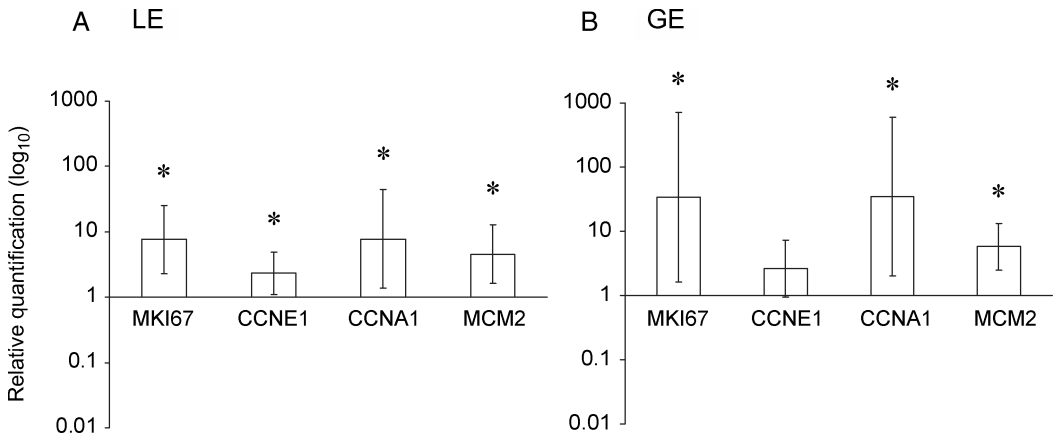

Figure 4. Quantitative mRNA expression of DNA synthesis markers in endometrial epithelia across the menstrual cycle of MRA women. Relative quantification was performed according to the comparative method ($2^{-\Delta\Delta CT}$) with proliferative (MRA-Prol) Δ CT as the internal calibrator. 95% Confidence intervals that do not cross 1 ($\log_{10}$) are considered significant as indicated by asterisk. Laser-capture microdissected samples of lumen (LE; **A**) and gland (GE; **B**) showed significantly greater mRNA expression of MKI67, CCNE1 (LE only), CCNA1 and MCM2 during the proliferative (>1) rather than secretory phase of MRA women. Number of women included for analysis of each marker for both MRA-Prol and MRA-Sec was 5 (except $n = 10$ for MCM2).

ex vivo tissue obtained from normal volunteers, generating data consistent both internally and with observations made in rodent models. Moreover, we demonstrated that lumen (LE) and gland (GE) can be successfully isolated from human endometrial biopsy material using LCM, thereby enabling insights specific to each tissue component that have not been previously explored. Previous studies that have utilized LCM for endometrial related research have only used this technology to collectively examine both epithelial cell types (Wu *et al.*, 2003, 2006) or only that of GE (Matsuzaki *et al.*, 2004, 2005, 2006). We evaluated a cohort of MRA women, presumably at their peak fertility and younger than those previously published (Gerdes *et al.*, 1984; Lessey *et al.*, 1988; Press *et al.*, 1988; Hayama *et al.*, 2002) to establish parameters of normal proliferative endometrium and to compare our findings to those in older reproductive aged women.

Over-expression of MKI67 (Cao *et al.*, 2002), CCND1 (Ozuysal *et al.*, 2005; Kayaselcuk *et al.*, 2006), CCNA1 (Shiozawa *et al.*, 1997; Kayaselcuk *et al.*, 2006) and CCNE1 (Oshita *et al.*, 2002) and under-expression of CDKN1B (Bamberger *et al.*, 1999), all markers featured in this study, have been linked to endometrial hyperplasia and/or carcinoma consistent with their roles in driving cell proliferation. Our finding that older MT women (MT group) had endometrium with very similar properties to the younger controls (MRA group) strongly implies that known mechanisms accounting for cell proliferation and secretory function are not impacted by age.

Within the 20-year age span of our MRA group, decreased nuclear protein expression of GE CCNA1, GE MKI67 and stromal MCM2 were observed, suggesting a decreased endometrial response to sex steroids that antedates the MT. The examination of multiple, non-independent markers within the MRA group could have led to the detection of some spurious associations. However, many outcomes with age were internally consistent, favouring reduced proliferation across the age span 18–38, despite relatively constant hormone production over this 20-year interval.

As gene ablation studies in mice have shown that the predominant mediator of mitogenic effects in the uterus is ESR1 (alpha) rather than ESR2 (beta) (Hewitt *et al.*, 2005) and human ESR2 affects primarily uterine pathology (Pedeutour *et al.*, 1998) and uterine vasculature (Critchley *et al.*, 2001), we focused on ESR1 for our study of normal endometrium. Concentrations of ESR1 are believed to parallel E_2 action, whereas PGR expression requires previous exposure to E_2 (Hewitt *et al.*, 2005). Consistent with these data, both ESR1 and PGR were higher in the LE and GE in proliferative

Table 3. Comparison of lumen (LE) versus gland (GE) nuclear protein counts during the proliferative (MRA-Prol) and secretory (MRA-Sec) phases in MRA women. Data presented as median and interquartile range. Significant differences between GE and LE, $*P < 0.05$.

Antibody	MRA-Prol				*P*-value (GE versus LE)	MRA-Sec				*P*-value (GE versus LE)
	N	LE	*N*	GE		*N*	LE	*N*	GE	
MKI67	18	34.28	18	44.20	0.091	12	2.2	12	1.3	0.319
PCNA	17	55.27	16	86.27	0.002*	7	10.68	7	2.5	0.027*
CCNA1	18	10.9	18	14.7	0.019*	12	2.3	12	1.1	0.671
MCM2	20	51.44	19	72.41	0.120	9	2.3	9	0.4	1.000
ESR1	19	48.30	19	89.13	<0.001*	9	1.4	9	0.2	0.644
PGR	19	66.41	19	79.26	0.030*	10	16.31	10	2.14	0.087

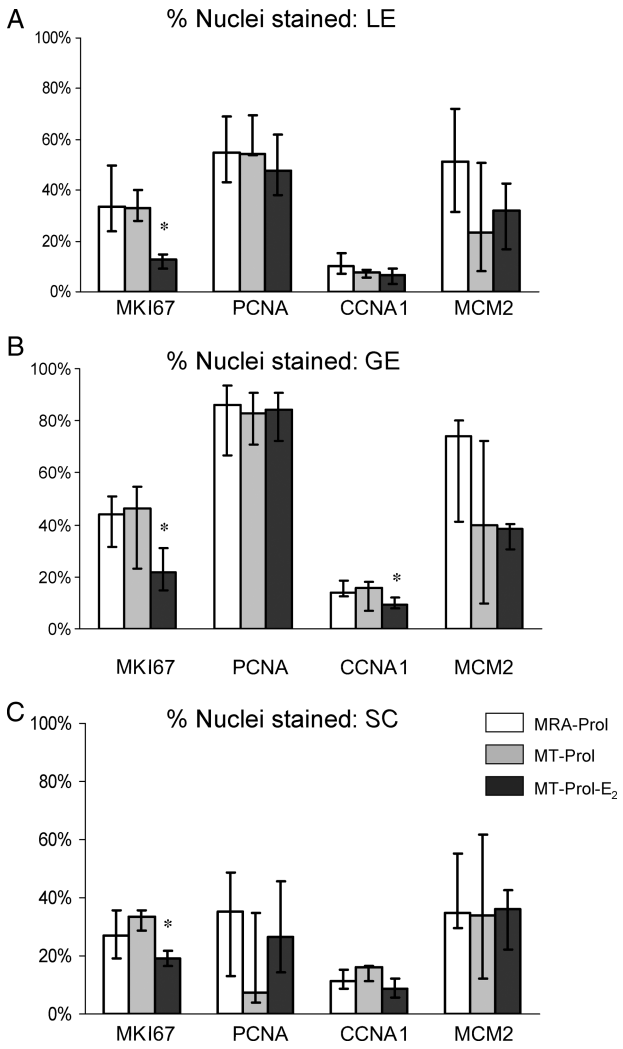

Figure 5. Measurements of the percentage of nuclear protein expression of DNA synthesis markers in lumen (LE; **A**), gland (GE; **B**) and stroma (SC; **C**) compartments of MRA proliferative (MRA-Prol) endometrium compared with E_2-treated (MT-Prol-E_2) and untreated (MT-Prol) MT groups. Data represented as median and IQR (error bars). Solid white, solid gray and solid charcoal bars in key shown represented as MRA-Prol, MT-Prol and MT-Prol-E_2, respectively (sample numbers as designated in Fig. 2). There was a similar nuclear expression of MKI67, PCNA, CCNA1 and MCM2 in all endometrial cell types between MT-Prol and MRA-Prol. However, there was significantly reduced nuclear expression of MKI67 and CCNA1 in different cell types of MT-Prol-E_2 compared with MRA-Prol (*$P < 0.05$).

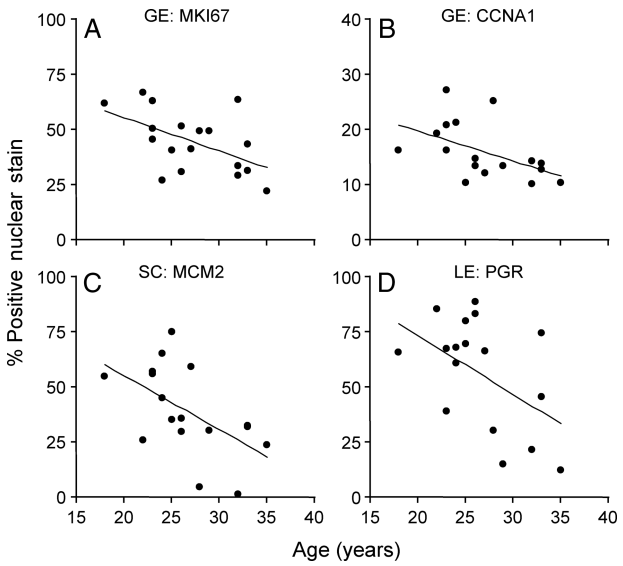

Figure 6. Relationship of the nuclear protein expression of proliferative (**A**–**C**) and steroid hormone receptor (**D**) markers with age in different cell types of proliferative phase endometrium of MRA women. MKI67 (A; $P = 0.023$; $R^2 = 0.281$) and CCNA1 (B; $P = 0.037$; $R^2 = 0.257$) expressions significantly decreased ($P < 0.05$) in the gland (GE) with age as did MCM2 in the stroma (C; SC; $P = 0.022$; $R^2 = 0.303$). PGR expression significantly decreased with age only in the lumen (D; LE: $P = 0.043$; $R^2 = 0.232$).

compared with secretory phase (Shiozawa *et al.*, 1996; Lessey *et al.*, 1988; Press *et al.*, 1988; Hayama *et al.*, 2002; Maldonado *et al.*, 2003). We also observed a small reduction in LE PGR nuclear protein expression across the age range 18–38 years. This finding may have some clinical relevance, in that the efficiency of implantation appears to decline with reproductive ageing in women, and this effect, although small, may be a contributing factor.

Steroid receptor expression was also dependent on cell type, with only ESR1 (Shiozawa *et al.*, 1996; Maldonado *et al.*, 2003) but not PGR (Hayama *et al.*, 2002; Maldonado *et al.*, 2003) protein levels significantly altered by the hormonal milieu in the SC.

MKI67 is expressed in all phases of the cell cycle (except G0) (Gerdes *et al.*, 1984) and is a well-established tool for monitoring cell proliferation in mice (Winking *et al.*, 2004), non-human primates and humans (Brenner *et al.*, 2003). Our MKI67 findings in MRA women agree with those of others (Hayama *et al.*, 2002) and are similar to observations in non-human primate endometrium (Brenner *et al.*, 2003). Similarly, PCNA functions include DNA replication, DNA repair and post-replicative processing (Warbrick, 2000). We found that PCNA was a less reliable marker of human endometrial DNA synthesis than MKI67, with differences only detectable in proliferative phase of GE samples. The disparate protein expression between LE and GE suggests differential PCNA function in these cells. A similar lack of correlation of PCNA expression and DNA synthesis was found in studies evaluating tamoxifen-induced uterine epithelial cell proliferation in the mouse (Zhang *et al.*, 2005).

The binding of the MCM complex consisting of MCM 2–7 to the chromatin is required for the initiation of DNA replication; MCM2 has recently been proposed to be an alternative marker of cell proliferation that is perhaps better than MKI67 (Schrader *et al.*, 2005; Maiorano *et al.*, 2006). We observed that MCM2 protein and mRNA expressions in LE and GE paralleled that of MKI67, with peak levels during the proliferative rather than secretory phase of MRA women, confirming prior findings by others (Kato *et al.*, 2003). In the mouse uterus, reduced MCM2 expression also correlates with progesterone inhibition of epithelial cell proliferation (Pan *et al.*, 2006). Our findings of increased CCNA1 and CCNE1 in the proliferative versus secretory phase of MRA women correlated with E_2-induced changes in the mouse (Tong and Pollard, 1999)

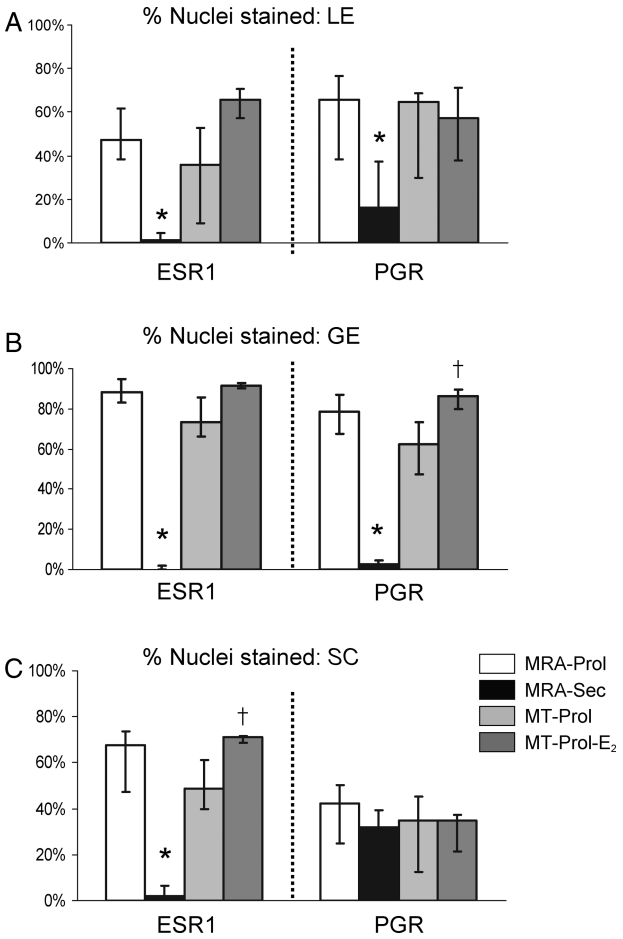

Figure 7. Measurements of the percentage of nuclear protein expression of ESR1 and PGR in lumen (LE; **A**), gland (GE; **B**) and stroma (SC; **C**) compartments of MRA and MT endometrium. Bar designations as in Figure 6 with solid black bar representing MRA-Sec. Data represented as median and IQR (error bars). There were significantly greater nuclear ($^*P < 0.05$) ESR1 and PGR expressions in all cell types (except SC for PGR) of proliferative (MRA-Prol) endometrium compared with that obtained from secretory (MRA-Sec). In older women, only in E_2-treated (MT-Prol-E_2) groups was there a significant increase in stromal ESR1 and glandular PGR compared with MRA-Prol ($^\dagger P < 0.05$). For ESR1, the sample size examined for each group is shown in parenthesis; MRA-Prol ($n = 19$), MRA-Sec ($n = 9$), MT-Prol ($n = 5$) and MT-Prol-E_2 ($n = 4$). That for PGR included: MRA-Prol ($n = 19$), MRA-Sec ($n = 10$), MT-Prol ($n = 5$) and MT-Prol-E_2 ($n = 5$).

but contrasted with the <1% positive nuclear CCNA1 staining of endometrial GE across the human menstrual cycle reported by others (Shiozawa *et al.*, 1996). As it seems unlikely that cells can proliferate in the absence of CCNA1 expression, we attribute our higher rates of positive staining to the normal reproductive status of the participants (all of whom had normal uterine cavities) and relative health of the tissue we removed.

CDKN1B (also known as $p27^{kip1}$) binds and inhibits CCNE/CDK2 and CCNA/CDK2 complexes in the early G1 phase (Sherr and Roberts, 1995). Although CDKN1Bs down-regulation by progesterone is less pronounced in the mouse (Tong and Pollard, 1999), the dramatically higher secretory than proliferative phase nuclear expression in both LE and GE supports data from others (Shiozawa *et al.*, 1998; Dubowy *et al.*, 2003), indicating a major role for CDKN1B in the progesterone-inhibition of E_2-induced epithelial proliferation in human endometrium.

We did not observe any differences between untreated MRA and MT in serum E_2 levels, MKI67, MCM2, G-S cell-cycle marker, ESR1 or PGR expressions in proliferative-phase LE or GE. This implies comparable endometrial sensitivity at physiologic levels of E_2, irrespective of age. However, exogenous E_2-treatment of MT, but not MRA, women was associated with decreased endometrial LE and GE proliferation as shown by MKI67 and a trend towards reduced GE CCNA1 and MCM2. This may reflect systemically, not merely centrally, altered sensitivity to hyperestrogenaemia with ageing (Weiss *et al.*, 2004), which may in turn impact age-related fertility declines (Cano *et al.*, 1995).

When unselected patients were examined in previous infertility studies, both age and ovarian reserve were associated with increased apoptosis in the endometrial GE and SC compartments (Erel *et al.*, 2005). In a separate study, normal volunteers also demonstrated evidence of a reduced secretory endometrial response associated with ageing (Cano *et al.*, 1995). These results suggest that the endometrial abnormalities associated with ageing might be attributed to erratic sex-steroid exposure. However, in women with regular menstrual cycles, we demonstrate evidence for an innate component to endometrial ageing that could possibly influence proliferation and, in turn, the secretory response, well before the onset of the MT. Previous studies suggest that these early changes have limited impact on impairing the secretory response and implantation potential of an ageing endometrium (Levran *et al.*, 1991; Sauer *et al.*, 1992; Abdulla *et al.*, 1993; Flamigni *et al.*, 1993; Meldrum, 1993; Sauer *et al.*, 1993; Check *et al.*, 1994; Sauer *et al.*, 1994; Borini *et al.*, 1996; Sauer *et al.*, 1996; Sauer, 1998). Although the reduced proliferative response and PGR expression we observed were not uniform in all tissue compartments, there was some consistency to the nature of the observations that may explain some of the reduced fecundity that is observed with reproductive ageing in women. Finally, we found nothing to suggest that proliferation increases in any way with ageing nor was there evidence of a role for constitutive activation of the cell-cycle regulatory pathway in the age-related increase in proliferative endometrial disorders.

Limitations of our study that bear mention include possible under-representation of the basalis zone in the MT women due to the use of LCM to uniquely address cyclic changes in mRNA levels of both LE and GE. However, unlike in macaque monkeys (Brenner and Slayden, 2005), the human basalis is considered an inactive proliferative region in human endometrium (Slayden and Brenner, 2004). Additionally, our restriction of sampling to a single time point in the proliferative phase may not have accounted fully for normal variations in follicular phase length and may have missed some of the key cell-cycle events that could have happened prior to the anticipated maximal proliferation at this time.

In summary, we separated endometrium into LE and GE compartments to utilize a molecular approach to reliably and reproducibly detect cyclic cell cycle-related differences in endometrial function while preserving tissue architecture.

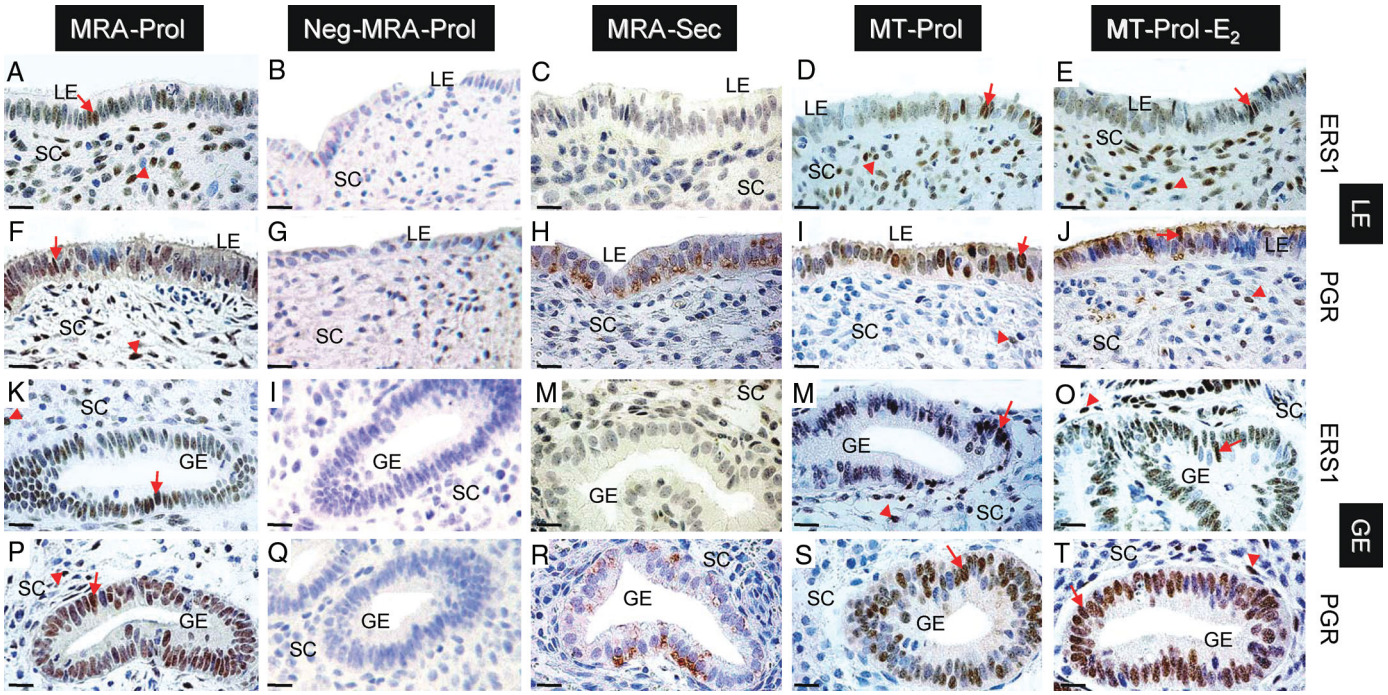

Figure 8. Sex-steroid receptor protein expression in MRA and MT human endometrium. Paraffin sections show representative nuclear ESR1 and PGR IHC staining patterns in gland (GE) (red arrows) and stroma (SC) (red arrowheads) that reflect the results described in Figure 7. ESR1 in lumen (LE) is shown in (**A**–**E**); PGR in LE (**F**–**J**); ESR1 in GE (**k**–**o**); PGR in GE (**P**–**T**).

Using these techniques, we showed that regardless of endogenous or exogenously altered hormonal milieu, the endometrial cell-cycle response is similar in MRA and MT women. However, we also demonstrate evidence for small reductions in the proliferative capacity of endometrial GE and SC cells that antedate the menopause by more than a decade.

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