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human reproduction **ORIGINAL ARTICLE** ***Gynaecology***

Mesenchymal stem/stromal cells in post-menopausal endometrium

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STUDY QUESTION: Does post-menopausal endometrium contain mesenchymal stem/stromal cells (MSC) that have adult stem cell properties and can be prospectively isolated from a biopsy?

SUMMARY ANSWER: Perivascular W5C5$^+$ cells isolated from post-menopausal endometrial biopsies displayed characteristic MSC properties of clonogenicity, multipotency and surface phenotype irrespective of whether the women were or were not pre-treated with estrogen to regenerate the endometrium.

WHAT IS KNOWN ALREADY: Recently MSCs have been identified in human premenopausal endometrium, and can be prospectively isolated using a single marker, W5C5/SUSD2.

STUDY DESIGN, SIZE, DURATION: Endometrial tissue of both the functional and basal layers, from 17 premenopausal (pre-MP) women, 19 post-menopausal (post-MP) women without hormonal treatment and 15 post-menopausal women on estrogen replacement therapy (post-MP+ E_2), was collected through a prospective phase IV clinical trial over 2 years.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Post-menopausal women <65 years of age were treated with or without E_2 for 6–8 weeks prior to tissue collection. Serum E_2 levels were determined by estradiol immunoenzymatic assay. Endometrial tissue was obtained from women by biopsy (curettage) just prior to the hysterectomy. The effect of E_2 on endometrial thickness and glandular and luminal epithelial height was determined using image analysis. Endometrial tissue was dissociated into single cell suspensions and MSC properties were examined in freshly isolated and short-term cultured, magnetic bead-purified W5C5$^+$ cells. MSC properties were assessed using clonogenicity, serial cloning, mesodermal differentiation in adipogenic, chondrogenic, osteogenic and myogenic induction culture media, and surface phenotype analysis by flow cytometry. Estrogen receptor α expression in W5C5$^+$ cells was examined using dual colour immunofluorescence. Vascularity was analysed using CD34 and alpha smooth muscle actin immunostaining and subsequent image analysis.

MAIN RESULTS AND THE ROLE OF CHANCE: A small population of stromal cells with MSC properties was purified with the W5C5 antibody from post-menopausal endometrium, whether atrophic from low circulating estrogen or regenerated from systemic estrogen treatment, similar to premenopausal endometrium. The MSC derived from post-menopausal endometrium treated with or without E_2 fulfilled the minimum MSC criteria: clonogenicity, surface phenotype (CD29$^+$, CD44$^+$, CD73$^+$, CD105$^+$, CD140b$^+$, CD146$^+$) and multipotency. The post-menopausal endometrial MSCs also showed comparable properties to premenopausal eMSC with respect to self-renewal *in vitro* and W5C5 expression. The W5C5$^+$ cells were located perivascularly as expected and did not express estrogen receptor α.

LIMITATIONS, REASONS FOR CAUTION: The properties of the MSC derived from post-menopausal endometrium were evaluated *in vitro* and their *in vivo* tissue reconstitution capacity has not been established as it has for premenopausal endometrial MSC.

WIDER IMPLICATIONS OF THE FINDINGS: The endometrium is an accessible source of MSC obtainable with minimum morbidity that could be used for future clinical applications as a cell-based therapy. This study shows that menopausal women can access their endometrial MSC by a simple biopsy for use in autologous therapies, particularly if their endometrium has been regenerated by short-term E2 treatment, provided they have an intact uterus and are not contraindicated for short-term E2 treatment. Endometrial MSC in post-menopausal women possess key MSC properties and are a promising source of MSC independent of a woman's age.

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Key words: mesenchymal stem cells / mesenchymal stromal cells / post-menopausal / endometrium / W5C5

Introduction

Human mesenchymal stem cells or multipotent stromal cells (MSC) have been identified in almost every adult tissue: bone marrow, adipose tissue, synovial membrane and the endometrium (da Silva Meirelles *et al.*, 2006; Beltrami *et al.*, 2007; Schwab and Gargett, 2007; Crisan *et al.*, 2008). Originally MSC were identified by their adherence to plastic and differentiation into mesodermal lineages: adipocytic, chondrocytic and osteoblastic (Prockop 1997; Pittenger *et al.*, 1999). More recently it has been shown that bone marrow MSC (bmMSC) also differentiate into endodermal and neuroectodermal lineages (Torrente and Polli, 2008; Morikawa *et al.*, 2009). Cultured MSC are highly proliferative with the capacity to produce millions of cells from a single clonogenic cell (Gargett *et al.*, 2009). MSC have characteristic surface markers including CD29, CD44, CD73, CD105, but not haematopoietic cell markers CD34, CD45, CD14, CD11b, CD79α, CD19 and HLA-DR (Dominici *et al.*, 2006; Caplan 2007). The identification of more specific markers, Stro-1, CD146, CD271, has enabled the prospective isolation of MSC from bone marrow (Simmons and Torok-Storb, 1991; Gronthos *et al.*, 2003; Buhring *et al.*, 2007). BmMSC have anti-inflammatory and immunomodulatory properties which make them an attractive source for tissue engineering and regenerative medicine applications (Salem and Thiemermann, 2010; Le Blanc and Mougiakakos, 2012).

Endometrial MSC (eMSC) were recently discovered and characterized in premenopausal endometrium where they are thought to regenerate the stromal vascular component of the functional layer each month (Gargett *et al.*, 2009). Endometrial MSC also possess a high capacity for proliferation, differentiate into mesodermal lineages and express characteristic MSC surface markers, fulfilling the minimal criteria for defining MSC (Dominici *et al.*, 2006). Originally eMSC were prospectively isolated from hysterectomy tissue using co-expression of two markers (CD140b/PDGFRβ and CD146) by FACS sorting (Schwab and Gargett, 2007). Endometrial MSC can now be prospectively isolated from endometrial biopsy tissue using the single marker W5C5 using magnetic bead sorting (Masuda *et al.*, 2012). W5C5 recognizes an epitope of the Sushi Domain containing 2 (SUSD2) molecule (Sivasubramaniyan *et al.*, 2013). Almost all clonogenic endometrial stromal cells are found in the $W5C5^+$ fraction. Similar to $CD140b^+CD146^+$ cells, $W5C5^+$ cells meet the defining criteria for MSC (Masuda *et al.*, 2012). Clonogenic stromal cells have also been identified in post-menopausal endometrium, although the sample size examined has been small ($n = 4$) (Schwab *et al.*, 2005). EMSC are an attractive source of MSC as they can be easily obtained through an office biopsy procedure without anaesthesia or scarring (Gargett *et al.*, 2012; Schuring *et al.*, 2011a) or a simple curettage for many women. Protocols are being developed for culture expansion of eMSC under clinical grade Good Manufacturing Practices (cGMP) conditions, making them an ideal source for future clinical applications, particularly in women's health, where they could be used autologously (Rajaraman *et al.*, 2013).

Clinical conditions for which eMSC could be utilized as a cell-based therapy may affect post-menopausal women, for example pelvic organ prolapse (Hunskaar *et al.*, 2005; Boennelycke *et al.*, 2013; Ulrich *et al.*, 2013a,b). To date however, eMSC have only been characterized in premenopausal women. Due to hormonal depletion, post-menopausal endometrium is thin and atrophic, difficult to biopsy without anaesthesia, and we hypothesized a low yield of eMSC. However, post-menopausal endometrium has significant regenerative potential, particularly when systemic estrogen is administered. A thick functional endometrium can be generated and indeed post-menopausal women in their 60s have borne children via IVF (Paulson *et al.*, 2002).

Therefore, the aim of this study was to determine whether post-menopausal endometrium contains a population of eMSC and to characterize these post-menopausal eMSC for clonogenicity, mesodermal differentiation, self-renewal and surface phenotype. We hypothesized that eMSC could be prospectively isolated from endometrial biopsies of post-menopausal endometrium, that they would be present at similar frequency as in premenopausal endometrium and that they would possess similar properties to premenopausal eMSC.

Materials and Methods

Human tissue and ethical approval

Human endometrial tissue including underlying myometrium was collected from 17 premenopausal (pre-MP) and 19 post-menopausal (post-MP) (longer than 12 months since last period) women who were undergoing hysterectomy and not taking hormones (Table I). We also collected endometrial biopsies from 15 post-menopausal women on oral estrogen replacement therapy (post-MP+E_2). Due to the low endometrial stromal cell yield from atrophic post-menopausal endometrium, a single arm phase IV clinical trial was registered with the Therapeutic Goods Association (CTNRN12610000563066) to treat post-menopausal women with short-term estrogen to regenerate the endometrium and obtain higher cell yields for the collection and analysis of eMSC. The enrolled women ($n = 15$) took oral estrogen replacement therapy (Progynova 2 mg daily for 6–8 weeks) which was ceased 2 days prior to scheduled hysterectomy. Inclusion criteria were at least 12 months since the last period and age <65 years. Exclusion criterion was any condition where systemic estrogen use was contra-indicated, including current/past history of breast cancer, other estrogen responsive tumours, liver adenoma, thromboembolism, undiagnosed vaginal bleeding, uncontrolled hypertension or the use of oral hormone replacement therapy (HRT). The women were assessed for these exclusion

Table I Patient characteristics.

	Pre-MP ($n = 17$)	Post-MP ($n = 19$)	Post-MP+E_2 ($n = 15$)	*P*-value
Age (years)	43 ± 3	60 ± 1	64 ± 3	$P < 0.01$
BMI (kg/m^2)	26.4 ± 1.6	25.4 ± 2.0	25.4 ± 4.3	NS
POP-Q stage*	1.2 ± 0.2	1.6 ± 0.2	1.7 ± 0.2	NS
Serum E_2 (pmol/l)	n.d.	88 ± 9.6	273 ± 58.2	$P < 0.05$

Data are presented as mean (± SEM).
Pre-MP, premenopausal; Post-MP, post-menopausal; Post-MP+E_2, post-menopausal with E_2 treatment; E_2, estrogen; NS, not significant; N.D., not done.
*Reference for POP-Q (Bump *et al.*, 1996).

criteria by a urogynaecologist, A.R. Informed written consent was obtained from all patients in each group and ethics approval was obtained from the Monash Health Human Research and Ethics Committee B and Cabrini HREC.

The biopsies were obtained by curettage just prior to surgical removal of the uterus. The collected tissues ($n = 51$) were used for histological and/or cell culture analysis. Serum samples were also collected to determine the hormonal status of the post-menopausal women ($n = 10$) and determine circulating estrogen levels in the estrogen-treated women ($n = 10$). Endometrial samples ($n = 26$) collected in collection medium (HEPES-buffered DMEM/F12 medium containing 5% fetal calf serum and 5% antibiotics-antimycotics) were immediately transported to the laboratory and processed within 24 h. Samples ($n = 32$) were also frozen in OCT for cryostat sections and fixed for paraffin sections. Blood from Post-MP and Post-MP+E_2 was drawn and centrifuged at 2000 *g* for 20 min at 4°C to collect serum. Estrogen levels were determined by a competitive binding estradiol immunoenzymatic assay performed by Monash Health Pathology Laboratory.

Histology

Tissues collected for histological analysis were fixed in 10% formalin for 24 h, then embedded in paraffin and oriented so that the full thickness endometrium from myometrium to the lumen could be examined (Fig. 1A–C). These were sectioned into 5 μm sections and stained with haematoxylin and eosin (H+E) to determine endometrial thickness.

Immunohistochemistry

To determine the estrogen effect on the luminal and glandular epithelial height, sections from post-menopausal endometrium were stained with the epithelial marker, Cytokeratin 18. To determine the total blood vessel area as a measure of vascularity, sections were immunostained with anti-human CD34, and vessels invested with pericytes and smooth muscle cells were immune-stained with anti-human αSMA (Abberton *et al.*, 1999). Sections underwent dewaxing, rehydrating in graded alcohols and antigen retrieval using citric acid buffer (0.1 M, pH 6.0) by microwaving for 5 min on high power. After cooling to RT and three washes in PBS, endogenous peroxidase was quenched by 3% H_2O_2, followed by a protein block step (Protein Block serum free, ready to use, Dako®, Glostrup, Denmark) for 30 min at RT. The primary antibodies (1:100 for CK18 and CD34, 1:400 for αSMA) and isotype controls at the same concentration (IgG_1, IgG_{2a}) (all from Dako) were incubated overnight at 4°C; sections were then washed and the Envision$^+$ System HRP secondary antibody (Mouse Envision Kit, Dako) was applied for 30 min at RT, respectively, as done previously (Ulrich *et al.*, 2012). Colour was developed with 3,3′-Diaminobenzidine (DAB).

Immunofluorescence

To determine the location of the W5C5$^+$ cells and their estrogen receptor-α status, endometrial tissue was sequentially immunostained with anti-estrogen receptor-α (clone 6F11, Leica Microsystems, Australia) followed by phycoerythrin (PE)-conjugated anti-W5C5 (BioLegend, USA) (Masuda *et al.*, 2012). Frozen sections were cut at 8 μm from OCT embedded tissues, thawed at RT, fixed in 4% paraformaldehyde for 10 min, washed in PBS and treated with 0.2% Triton X-100 in PBS for 15 min. Protein block (Dako®) was applied for 30 min and sections were incubated with anti-estrogen receptor-α (1:100) in PBS for 2 h at RT followed by secondary anti-mouse Alexa Fluor 488 (Life Technologies, Australia). Sections were washed three times in PBS, blocked with mouse IgG for 30 min, incubated with PE-anti-W5C5 (1:100) for 2 h at RT, washed three times with PBS, counter-stained with Hoechst 33258 and imaged on a Nikon C1 confocal microscope.

Image analysis

Three consecutive sections per patient were photographed using the Leica® DMR Microscope at ×5–40 magnification and analysed using ImageJ software. Endometrial thickness was measured in three randomly located regions on each of three H&E sections using the software micrometer and the mean of the nine measurements was obtained for each of the samples examined. These were used to generate means for each of the experimental groups (pre-MP, $n = 8$; post-MP, $n = 10$; post-MP+E_2, $n = 10$). Similarly, luminal and glandular epithelial height was measured on three different locations or gland profiles from three separate sections and the mean of nine measurements from each sample was obtained and used to generate means for the same three experimental groups for both luminal and glandular height.

To assess vascularity, the positive area for CD34 and αSMA stained samples ($n = 8$ for post-MP, $n = 10$ for post- MP+E_2) was analysed using Metamorph® image analysis software (Ulrich *et al.*, 2012). The percentage area was calculated as the positive area detected by Metamorph divided by the total endometrial area from four images obtained from two sections for each sample.

Endometrial stromal cell isolation and culture

Single cells were obtained as published previously (Chan *et al.*, 2004; Schwab and Gargett, 2007) from 8 pre-MP, 13 post-MP and 8 post-MP+E_2 samples. Briefly, the endometrium was finely minced, then dissociated in 5% collagenase I (Worthington Biochemical Corporation, Lakewood, NJ, USA), 40 μg/ml deoxyribonuclease type I (Worthington Biochemical Corporation), and DMEM/F-12 medium containing 15 mM HEPES buffer (Invitrogen, Auckland, New Zealand). Following dissociation, the cells were filtered through a 40 μm cell strainer (BD Biosciences, Durham, NC, USA) to obtain the stromal fraction. Stromal single cell suspensions were layered over Ficoll-Paque PLUS (GE healthcare Bio-Sciences AB, Uppsala, Sweden) and centrifuged to remove red blood cells. The endometrial stromal cells were used fresh or cultured for 1 passage in DMEM medium containing 10% fetal calf serum (Invitrogen), 5% antibiotics-antimycotics and 2 mM glutamine (Invitrogen) to obtain sufficient cell numbers for experiments. Cells were harvested by TrypLE Express (Life Technologies, Auckland, New Zealand) and eMSC were extracted using magnetic beads conjugated to the W5C5 antibody as described previously (Masuda *et al.*, 2012). Briefly, cell suspensions (up to 1×10^7 cells/100 μl) were labelled with the PE-conjugated W5C5 antibody (Biolegend, San Diego, CA, USA) in 0.5% Fetal Calf Serum in PBS (Bead Medium) for 30 min at 4°C followed by three washing steps in PBS, then incubated for 30 min in the dark with the anti-PE antibody-conjugated MACS MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Up to 1×10^8 cells/500 μl were applied to MS columns (Miltenyi

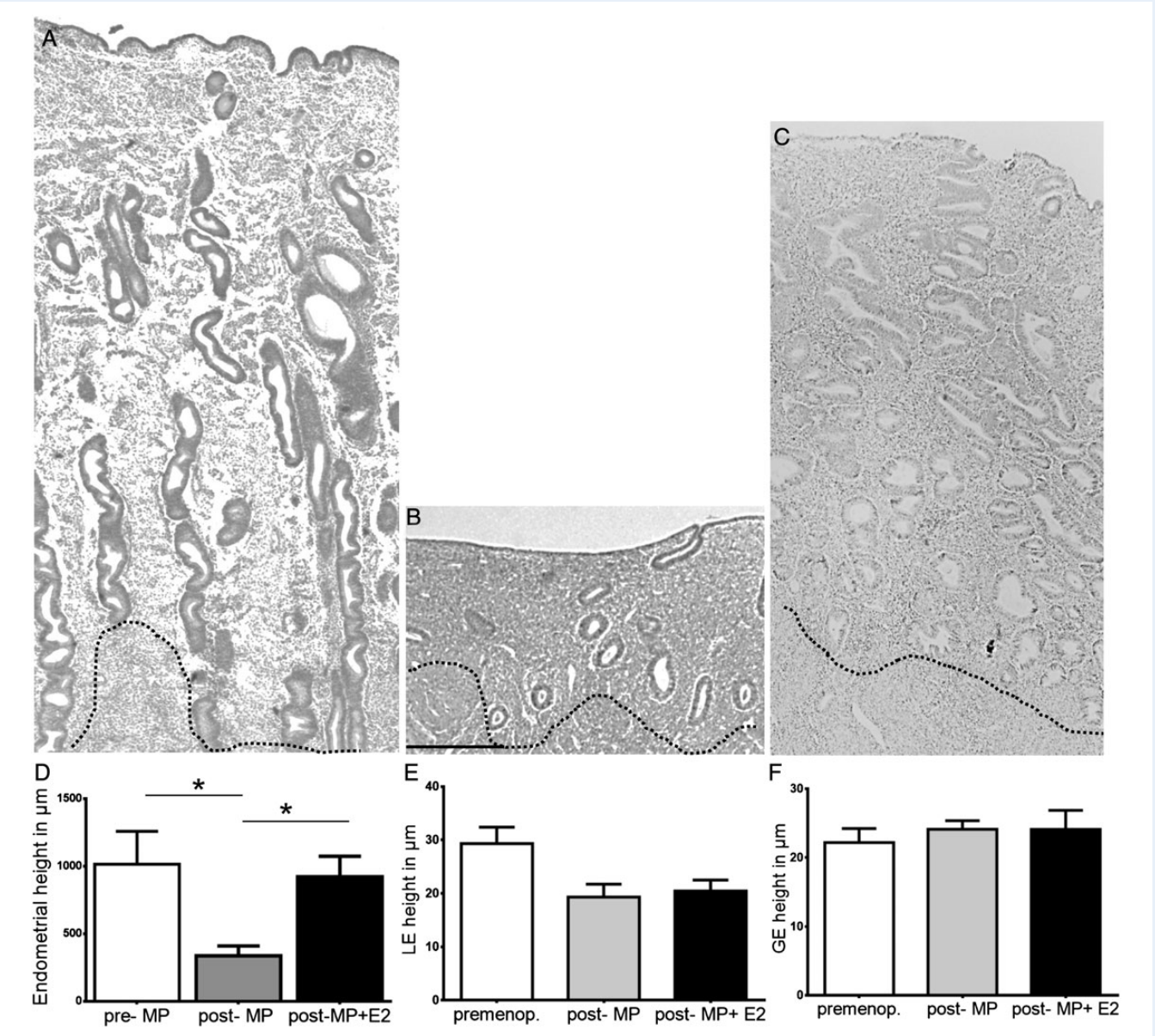

Figure 1 Effect of oral estrogen on post-menopausal endometrium compared with premenopausal endometrium. H+E stained endometrium from hysterectomy tissues in (**A**) pre-MP, (**B**) post-MP, (**C**) post-MP+E_2 groups. The dotted line indicates border between endometrium and myometrium. Scale bar 200 µm. (**D**) Endometrial thickness, (**E**) luminal epithelial (LE) height and (**F**) glandular epithelial (GE) height measured in the basalis layer of pre-MP, post-MP and post-MP+E_2 endometrium. Bars are mean ± SEM of $n = 6$ samples/group. *$P < 0.05$.

Biotec) in a magnetic field, and then the columns were washed with 500 µl Bead Medium three times. The W5C5$^-$ cells passed through the column, while magnetically labelled W5C5$^+$ cells were retained. The columns were removed from the magnetic field and W5C5$^+$ cells were flushed out with 1 ml of Bead Medium. The W5C5$^+$ cells were assayed for cloning efficiency, and the remaining cells were cultured for one more passage for differentiation assays and phenotyping.

MSC functional properties

Clonogenicity was determined by seeding fresh and cultured W5C5$^+$ cells at clonal density (10 and 50 cells/cm^2) on fibronectin-coated 100 mm tissue culture plates (BD Biosciences, San Jose, CA, USA). Cells were incubated at 37°C in 5% CO_2 incubator for at least 2 weeks; DMEM$^+$ media supplemented with 10 ng/ml human fibroblast growth factor 2 (FGF2; Millipore, Billerica, MA, USA) (F-DMEM$^+$) was changed weekly. Colonies were monitored microscopically to ensure they were derived from single cells. Large clones were harvested in cloning rings using TrypLE Express and subcloned twice, at seeding densities of 5–10 cells/cm^2 (Gargett *et al.*, 2009).

For differentiation, P2 W5C5$^+$ cells were cultured in four well plates on coverslips at 1×10^4 cells/cm^2 using specific induction media to obtain adipocytes, osteoblasts and chondrocytes and smooth muscle cells as previously described (Gargett *et al.*, 2009). Controls were cultured in DMEM$^+$ medium. After 4 weeks, adipogenic differentiation was evaluated by detection of lipid accumulation using oil red O staining; osteogenic differentiation was evaluated by histochemical detection of alkaline phosphatase stained osteoblasts;

and myogenic differentiation was evaluated by detection of myofibroblasts and smooth muscle cells using alpha-smooth muscle staining as described previously (Rajaraman *et al.*, 2013). For chondrogenic differentiation, 3–5 × 10^5 cells were cultured as a micromass pellet in a centrifuge tube in chondrogenic differentiation medium for 4 weeks. The pellet was fixed in 10% formalin, embedded in 4% Agar, processed through graded alcohols and xylene, then embedded in paraffin and cut into 5 μm sections. Chondrocyte matrix production was visualized using Alcian blue staining and photographed using a Leica microscope at ×10 magnification (Rajaraman *et al.*, 2013). For each assay, differentiation capacity was scored as 0 (no differentiation), 1 (<than 50% of the cells differentiated) and 2 (>50% of the cells differentiated).

To determine the phenotype of the post-menopausal W5C5$^+$ cells, single-colour flow cytometry on P1 cells was used for known MSC surface phenotype markers (W5C5, CD140b a, PDGFRβ) (R&D Systems, Minneapolis, MN, USA), CD146 (CC9 culture supernatant, kind gift from Prof David Haylock, CSIRO, Clayton, Victoria, Australia), CD29 (BD Biosciences), CD44 (BD Biosciences), CD73 (BD Biosciences), and CD105 (BD Biosciences) as previously described (Masuda *et al.*, 2012). Contaminating cells were analysed using haematopoietic (CD34; BD Biosciences) and myeloid cell makers (CD45; BD Biosciences). Controls were isotype matched IgG used at the same concentration as primary antibodies. A minimum of 5 × 10^4 cells for controls and for surface markers of interest were incubated with individual antibodies in separate tubes for 30 min at 4°C, followed by incubation with a PE-labelled anti-mouse IgG_1 secondary antibody (BD Biosciences). Cells were centrifuged and washed at 4°C with Bench Medium after each incubation and examined in a MoFlo® XDP cell sorter (Beckman Coulter). The initial selection of cells for analysis was based on the forward versus side scatter profile. The percentage of positive cells was based on IgG control setting of gates to <2% positive cells (Masuda *et al.*, 2012) and analysed by Summit Software v5.2.

Statistics

GraphPad Prism v5 was used for statistical analysis. Results are reported as median (range) or mean ± SEM for each group. Since the data were normally distributed (Kolgomorov–Smirnof normality test), one way ANOVA and Holm-Sidak *post hoc* test for pairwise comparisons were undertaken for assessment of differences between groups. *P*-values <0.05 were considered as statistically significant.

Results

The mean age of the patients and other demographic parameters are shown in Table I. The premenopausal women were significantly younger compared with the two post-menopausal groups ($P < 0.01$). Median time since the last menstrual period was 13 (10–20) years for the E_2 treated women and 9 (1–15) for the non-E_2 treated women ($P < 0.05$).

Mean serum E_2 levels for women treated with E_2 were significantly higher than for post-menopausal women without E_2 treatment (Table I).

Evidence of estrogen effects on estrogen-treated post-menopausal endometrium

To demonstrate that post-menopausal endometrium was responsive to E_2 (Progynova) treatment, we examined endometrial thickness and endometrial epithelial cell height. To assess the thickness of the post-menopausal endometrium, we used H+E stained sections (Fig. 1A–C). Pre-MP and post-MP+E_2 endometrium were significantly thicker than post-MP ($P < 0.05$) (Fig. 1D). Systemic estrogen levels also influence the height of endometrial epithelium (Gomes *et al.*, 1997). In CK18 immunostained tissue, there was a trend towards taller luminal epithelium (LE) in the premenopausal women compared with the post-menopausal groups, although this was not significant ($P > 0.05$) (Fig. 1E). Glandular epithelial (GE) height was measured in the basalis layer of pre-MP endometrium since there was no clearly distinguishable functionalis layer in the post-MP groups and previous studies have shown that post-menopausal epithelium has a similar gene expression profile as the basalis of post-menopausal epithelium (Nguyen *et al.*, 2012). GE height was similar in the menopausal tissues and there was no difference between pre-MP and post-menopausal women treated with or without E_2 (Fig. 1F). There was also no significant difference between the GE height of basal glands and those adjacent to the LE (results not shown).

Quantification W5C5$^+$ cells in post-menopausal endometrium

We next measured the proportion of endometrial stromal cells that expressed the eMSC marker, W5C5 in freshly dissociated samples. The mean stromal cell yield from endometrial tissue was 1.6 × 10^6 ± 4.8 × 10^5 ($n = 4$) of estrogen-treated women and 0.7 × 10^5 ± 0.3 × 10^5 ($n = 4$) for the untreated women, per 1 g of tissue. Insufficient cell numbers were obtained from three post-menopausal samples without estrogen treatment after the isolation procedure and could not be used for the further experiments. To determine the percentage of W5C5$^+$ cells present in stromal cells cultured from post-MP endometrium, cells at passage one (P1) were harvested by trypsin, labelled with W5C5 antibodies and passed through a magnetic bead column to select the W5C5$^+$ cells, which were then counted. In post-MP+ E_2 P1 cultures, 3.7 ± 1.8% ($n = 7$) of the cells were W5C5+ cells which compares with 7.7 ± 6.3% ($n = 8$) W5C5+ cells in post-MP cultures ($P = 0.69$).

Surface phenotype of human post-menopausal endometrial W5C5$^+$ cells

Cultured W5C5$^+$ cells were analysed for expression of typical MSC phenotypic markers (Dominici *et al.*, 2006) using flow cytometry (Fig. 2A). The post-menopausal W5C5$^+$ cells expressed MSC markers as shown in Table II without any significant differences between the two post-menopausal groups.

Multilineage differentiation of post-menopausal endometrial W5C5$^+$ cells

We next examined whether the post-menopausal W5C5$^+$ cells could undergo multilineage differentiation, a key MSC property (Dominici *et al.*, 2006). W5C5$^+$ cells derived from post-menopausal endometrium from women treated with ($n = 6$) or without estrogen ($n = 6$) differentiated into adipocytes to a similar extent when cultured in adipogenic induction medium (Fig. 2B, Table III). Similarly, W5C5$^+$ cells derived from women treated with and without E_2 differentiated into chondrocytes producing a cartilaginous-like Alcian Blue stained matrix (Fig. 2C). W5C5$^+$ cells cultured in osteogenic induction medium differentiated into osteocytes, shown by alkaline phosphatase reactivity (Fig. 2D). Similarly W5C5$^+$ cells cultured in myogenic induction medium differentiated into α-SMA-expressing smooth muscle cells (Fig. 2E). There was no difference in the capacity of W5C5$^+$ cells obtained from post-MP or

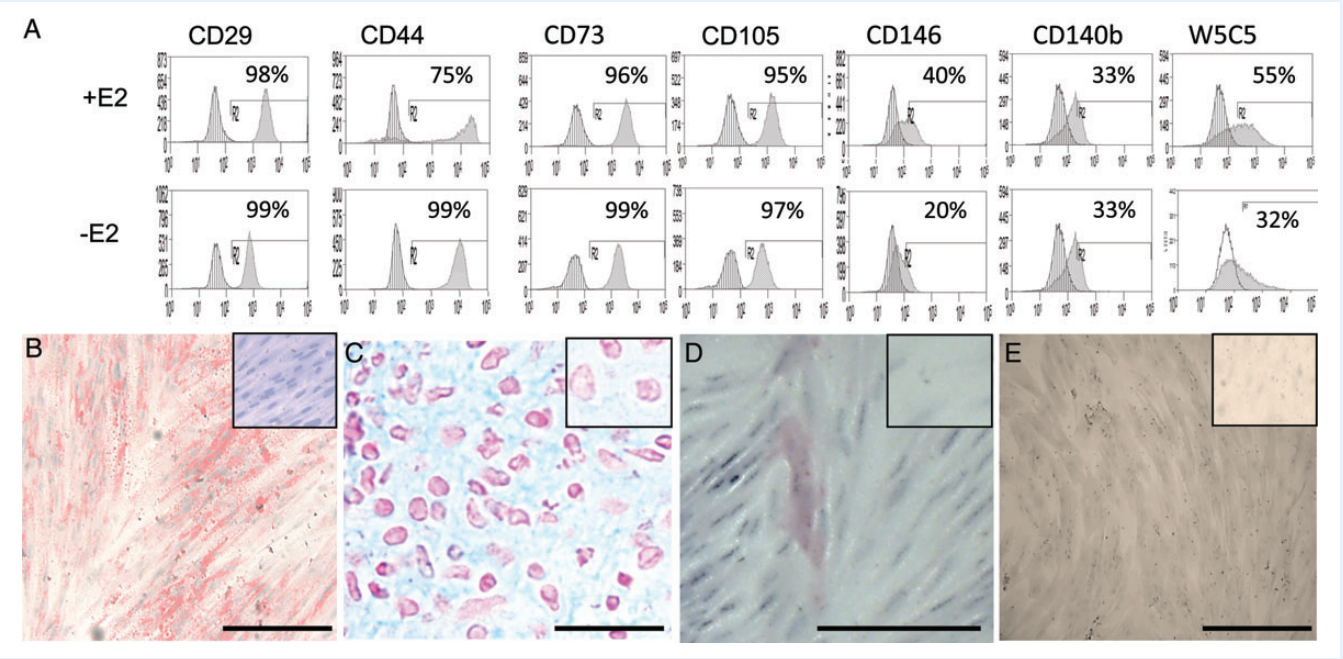

Figure 2 Properties of post-menopausal eMSC. (**A**) Surface phenotype showing representative flow cytometric analysis of P1 cultured post-menopausal W5C5$^+$ cells from a single representative sample from a woman treated with (+E_2) or without (−E_2) oral estrogen for 6–8 weeks. Aggregate data are shown in Table II. Multilineage differentiation of P1 W5C5$^+$ cells from post-MP+E_2 endometrium in various induction media for 4 weeks (**B**) adipocytes stained with Oil Red O, (**C**) chondrocytes (red nuclei) showing production of Alcian Blue stained cartilage-like matrix, (**D**) alkaline phosphatase positive osteoblasts and (**E**) smooth muscle cells immunostained with α-smooth muscle actin. Insets are stained control cultures. Scale bars; B, D and E 100 μm; C 25 μm.

Table II MSC surface marker characteristics of P1 post-menopausal samples.

	Post-MP ($n = 9$)	Post-MP+E_2 ($n = 8$)	*P*-value
CD29	98.7 ± 0.26	96.0 ± 1.6	NS
CD44	94.5 ± 3.5	92.8 ± 2.8	NS
CD73	85.8 ± 12.0	93.3 ± 2.6	NS
CD105	73.3 ± 16.3	83.7 ± 7.4	NS
CD146	27.2 ± 5.6	42.0 ± 5.9	NS
CD140b	32.0 ± 14.1	39.8 ± 14.5	NS
W5C5	35.4 ± 27.2	53.1 ± 27.2	NS
CD34	0.8 ± 0.8	0.1 ± 0.1	NS
CD45	6.1 ± 2.0	1.3 ± 0.6	0.02

Data are from single-colour flow cytometric analysis and presented as mean ± SEM. NS, not significant.

Table III Differentiation score of P1 post-menopausal samples cultured in mesodermal induction media.

	Post-MP ($n = 6$)	Post-MP+ E_2 ($n = 6$)	*P*-value
Adipogenic	1.2 ± 0.3	1.3 ± 0.2	NS
Chondrogenic	1.3 ± 0.3	1.8 ± 0.2	NS
Myogenic	1.6 ± 0.2	2.0 ± 0.0	NS
Osteogenic	1.6 ± 0.2	1.8 ± 0.2	NS

Data are presented as mean ± SEM. NS, not significant.
Score; 0 no differentiation; 1, <50% of the cells showed differentiation; 2, >50% of the cells showed differentiation.

post-MP+E_2 endometrium to undergo multilineage differentiation (Table III).

Clonogenicity and serial cloning of post-menopausal endometrial W5C5$^+$ cells

MSC are clonogenic and we therefore examined the clonogenicity of passage 1 (P1) W5C5$^+$ cells derived from pre- and post-menopausal endometrium (Fig. 3A and B). The mean cloning efficiency for P1 W5C5$^+$ cells from post-MP+ E_2 endometrium was 3.4 ± 0.9%, (n = 6), and comparable to cells from post-MP (3.7 ± 0.4%, $n = 8$) endometrium but statistically lower than those from pre-MP (8.83 ± 0.4, $n = 6$, $P < 0.01$) endometrium (Fig. 3C). The clonogenicity of fresh (P0) pre-MP and post-MP+ E_2 cells was similar with cloning efficiencies of 3.8 ± 0.9% versus 1.8 ± 0.1%, respectively. Both post-MP and post-MP+E_2 W5C5$^+$ cells underwent substantial self-renewal by undergoing serial cloning at least three times (Fig. 3D). The cloning efficiency of secondary (S1) and tertiary (S2) clones was significantly lower in post-menopausal compared with premenopausal samples as shown

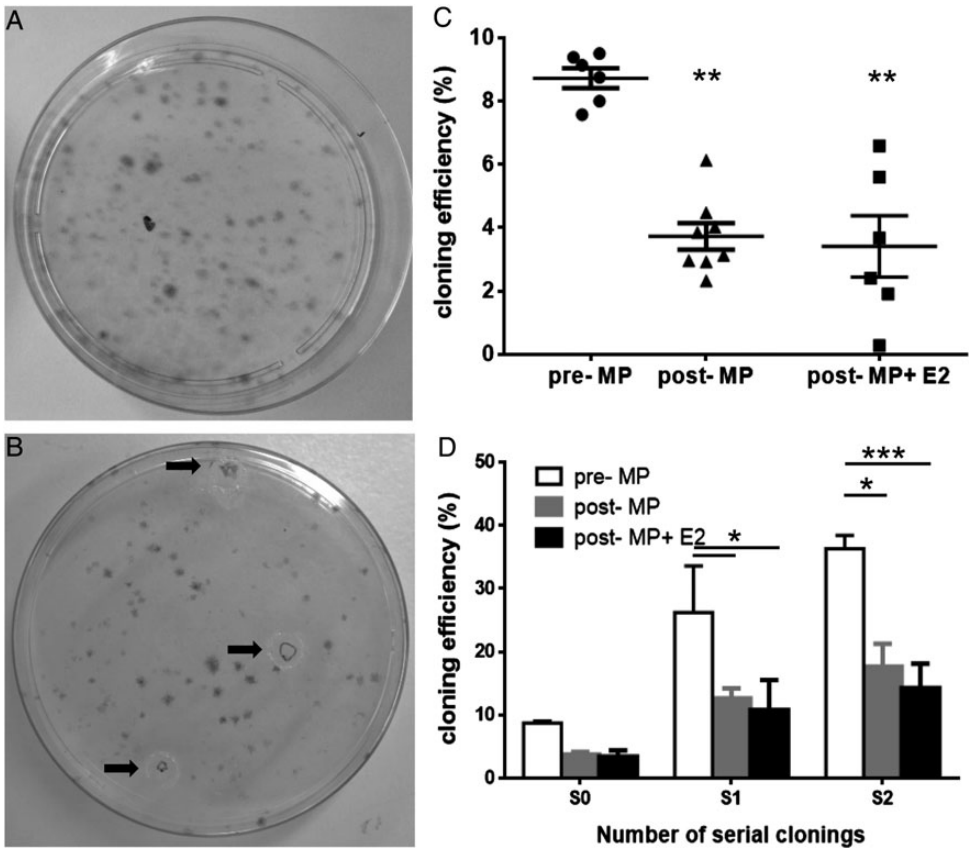

Figure 3 Cloning efficiency and serial cloning analysis for measuring clonogenicity and self-renewal of human endometrial pre- and post-menopausal eMSC. Representative cloning plates of (**A**) post-MP and (**B**) post-MP+E_2 W5C5$^+$ cells. Arrows show individual clones removed for serial cloning. Clonogenicity of (**C**) P1 W5C5$^+$ cells from pre-MP, post-MP and post-MP+E_2 samples and (**D**) at each round of serial cloning. Results are means ± SEM ($n = 6$/group). **Significant difference between pre-MP and post-MP ($P < 0.01$) and post-MP+E_2 (**$P < 0.01$). *$P < 0.05$, ***$P < 0.001$. S0, first cloning; S1, first serial cloning (secondary clones); S2, second serial cloning (tertiary clones).

in Fig. 3D. The cloning efficiency of P1 pre-MP and post-MP+ E_2 W5C5$^+$ was double that of freshly isolated pre-MP cells (results not shown).

Location of W5C5 in post-menopausal endometrium

Since premenopausal eMSC reside in a perivascular location in both functionalis and basalis (Schwab and Gargett, 2007; Masuda *et al.*, 2012), we investigated the localization of the W5C5$^+$ cells in post-menopausal sections by dual-colour immunofluorescence. Post-menopausal W5C5$^+$ cells were similarly identified in a perivascular location in both small and large vessels throughout the endometrium (Fig. 4A and B). Since estrogen drives endometrial growth in post-menopausal women, we examined whether they expressed estrogen receptor-α (ERα). None of the W5C5$^+$ cells expressed ERα, even though ERα stained some glandular epithelial and stromal cells in both post-MP and post-MP+ E_2 tissues. We then looked at the effect of estrogen treatment on vessel density in post-menopausal endometrium. We stained the endometrium with CD34 to mark the endothelial cells (Fig. 5A and B) and detect both capillaries and larger vessels, and αSMA to distinguish larger vessels from capillaries (Fig. 5D and E). We found no significant differences between the post-menopausal groups with or without estrogen treatment (Fig. 5C and F).

Discussion

In this study, we report the first characterization of an endometrial MSC population in post-menopausal endometrium from women treated with or without estrogen for 8 weeks. We show that post-menopausal endometrium, whether atrophic from low circulating estrogen levels or regenerated from short-term systemic estrogen treatment, contains a small population of stromal cells with MSC properties. These post-menopausal eMSC can be obtained by magnetic bead sorting with the W5C5 antibody used to purify eMSC from premenopausal endometrium. We also demonstrated that post-menopausal eMSC can be obtained from a biopsy as is the case for premenopausal eMSC, particularly after 6–8 weeks oral E_2 treatment. As the endometrium is an accessible source of MSC, obtainable with minimum morbidity for potential use in future clinical applications, it was important to know whether post-menopausal women can access their eMSC for autologous cell-based therapies provided they have an intact uterus.

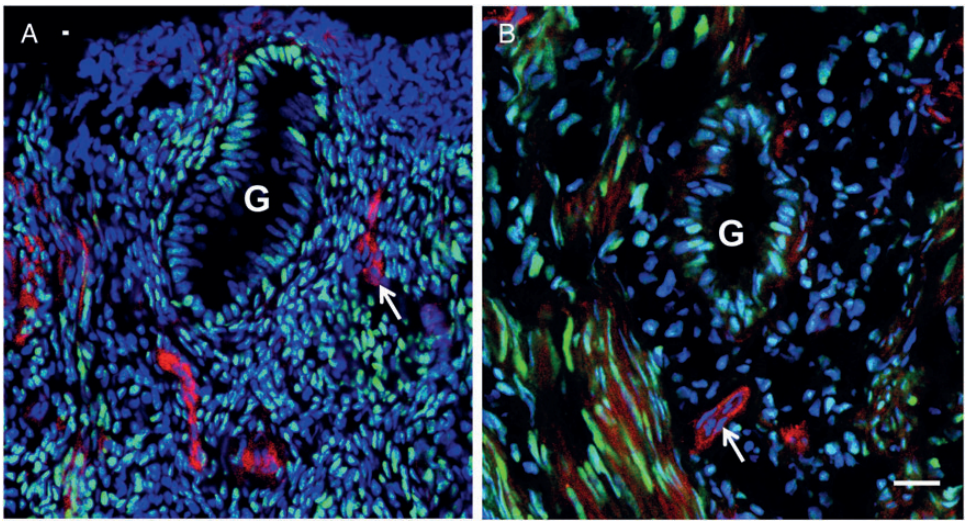

Figure 4 W5C5+ perivascular cells do not express ERα. Dual colour immunofluorescence of (**A**) post-MP and (**B**) post-MP+E_2 endometrial tissue. $W5C5^+$ cells around blood vessels fluoresce red, ERα-positive nuclei fluoresce green. Unstained nuclei are blue (Hoechst stained). Scale bar 20 μm. The arrow indicates representative perivascular $W5C5^+$ cells. G, gland.

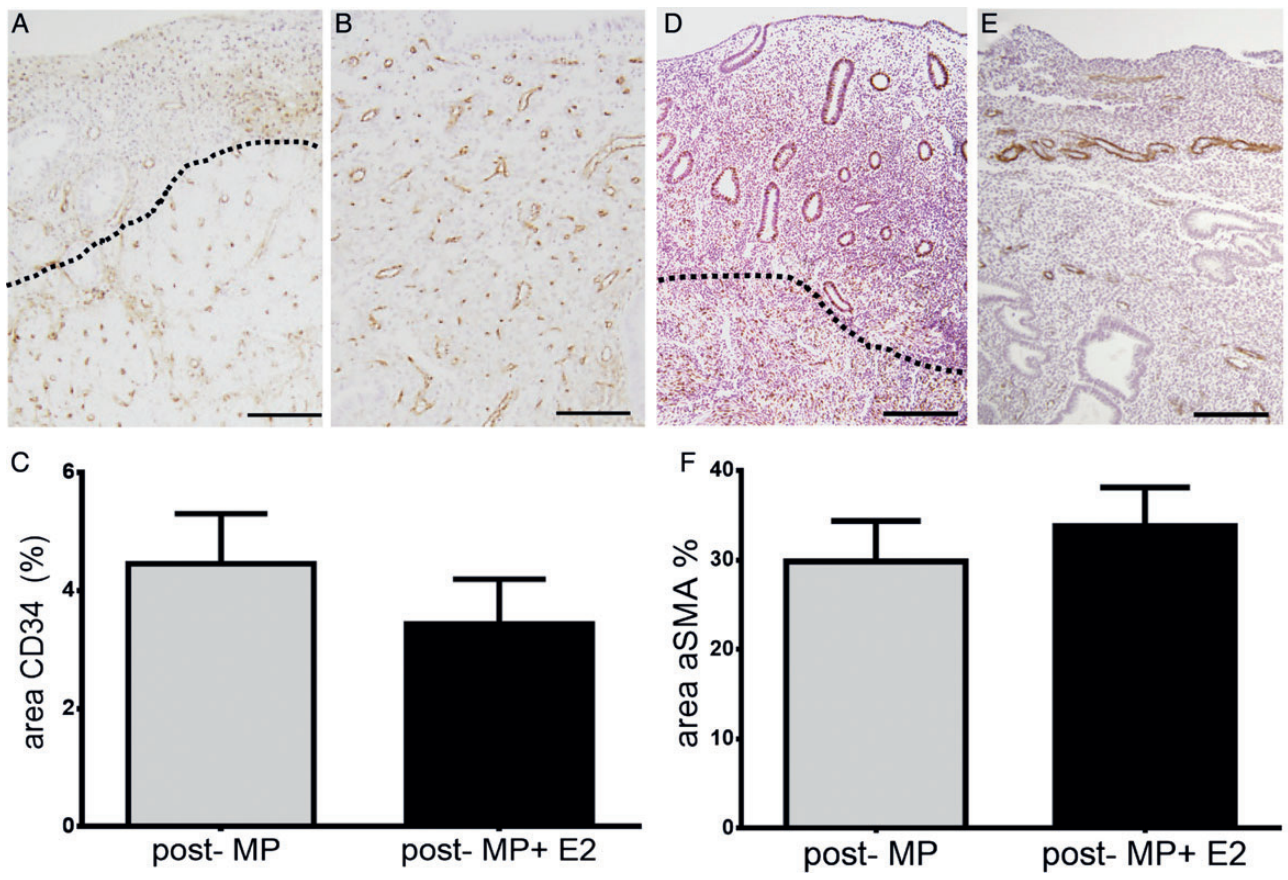

Figure 5 Vessel profiles in post-menopausal endometrium. CD34 immunostained (**A**) post-MP and (**B**) post-MP+E_2 endometrium with (**C**) immunoquantification showing % of area positive for CD34. Dotted lines show endometrial myometrial border. Alpha-SMA immunostained (**D**) post-MP, and (**E**) post-MP+E_2 endometrium counterstained with haematoxylin, with (**F**) immunoquantification showing % of area positive for αSMA. Scale bar in A+B 100 μm, D+E 200 μm. Bars are means ± SEM of $n = 8$ post-MP and $n = 10$ post-MP+E_2.

The eMSC derived from post-menopausal endometrium fulfil the defining MSC criteria: clonogenicity, surface phenotype and multipotency, suggesting that eMSC are retained in the endometrium following menopause. The post-menopausal eMSC also have comparable properties to premenopausal eMSC with respect to self-renewal *in vitro* and W5C5 expression.

We demonstrated that the post-menopausal eMSC derived from women treated with or without E_2 had comparable clonogenicity, albeit at lower levels compared with that of cultured premenopausal eMSC. Due to low cell yields, we could only determine clonogenicity in P1 W5C5$^+$ cells in most of the samples of untreated post-MP endometrium. The clonogenicity (cloning efficiency) in P1 W5C5$^+$ cells was double that of fresh (P0) W5C5$^+$ cells from pre-MP and post-MP+ E_2 indicating selection for clonogenic cells in primary cultures (Masuda *et al.*, 2012).

The surface phenotype of W5C5$^+$ cells was also similar between premenopausal and the two post-menopausal groups suggesting that eMSC remain in post-menopausal endometrium and may be responsible for E_2 mediated regeneration of the stromal vascular components. The eMSC of either estrogen-treated or non-treated post-menopausal endometrium differentiated to a similar degree, but less than that observed for premenopausal eMSC. This together with the lower cloning efficiencies and self-renewal properties suggests that post-menopausal eMSC may lose some potency as they age, similar to MSC from other sources such as bone marrow (Haynesworth *et al.*, 1994). Short-term estrogen treatment appears to be effective for obtaining adequate yields of endometrial cells because post-MP endometrium rapidly regenerated to more than double its original thickness. As demonstrated previously (Ettinger *et al.*, 1997), we found a thicker endometrium in the estradiol-treated women compared with that in post-menopausal controls. The percentage of W5C5$^+$ after magnetic bead selection was similar in the endometrium of E_2 treated and non-treated women suggesting that a defined pool of eMSC is maintained per unit volume of tissue. However the increased absolute volume of endometrial tissue in E_2 treated women allows for greater yields of eMSC, an important consideration for future cell-based therapies. The high degree of variability in the yield of W5C5$^+$ cells in post-MP compared with post-MP+E_2 samples indicates the difficulty in harvesting cells from non-treated women and also suggests that dietary xenoestrogens and other undocumented supplements could also have an influence. Small and large vessel profiles are present in similar numbers in the endometrium of post-menopausal women treated with and without E_2 suggesting that short-term estrogen therapy does not remodel the vasculature, but provides the regenerating 'functionalis' layer with a similar degree of vascularity as atrophic endometrium. Our study also confirms a previously reported lack of difference in vascularity observed between hormone-treated and non-treated premenopausal endometrium as determined by semiquantitative CD34 immunohistochemistry (Hickey *et al.*, 1996). This study also reported a similar vascularity between atrophied and cycling endometrium.

The luminal epithelial height did not differ between the two post-menopausal groups in contrast to previous findings (Gomes *et al.*, 1997). This could be due to the shorter E_2 treatment time in our study, 6–8 weeks versus 3 months in the Gomes *et al.* study, possibly not long enough to remodel the surface epithelium. The glandular epithelial height also did not show significant differences compared with the basalis layer of premenopausal women. These findings suggest that short-term E_2 treatment has little impact on the endometrial epithelium, suggesting that this approach is safe for those women who are not contraindicated for taking E_2.

Short-term oral E_2 rapidly increases endometrial growth suggesting that an ~8-week treatment is sufficient to obtain a reasonably thick endometrium (Ettinger *et al.*, 1997) without inducing endometrial hyperplasia (Furness *et al.*, 2012). It is not quite clear how E_2 regenerates the endometrium since W5C5+ perivascular cells did not express ERα. Similar observations were made in studies of label retaining cells in mice (Chan *et al.*, 2012) and side population and clonogenic cells in human endometrium (Cervello *et al.*, 2011; Schuring *et al.*, 2011b), suggesting that E_2 may act via estrogen receptors on their neighbouring niche cells (Gargett 2007). Our study also showed that eMSC survive in the absence of estrogen and do not require E_2 for trophic support as they were present in a similar proportion in untreated atrophic and E_2 treated post-MP endometrium.

For this study, we only supplemented women without contraindicated medical conditions with 2 mg Progynova daily for 6–8 weeks and found no associated adverse effects. The participants were closely monitored. An initial dose of 1 mg Progynova was insufficient to increase endometrial thickness to yield significant numbers of cells (unpublished observation).

The endometrium is one of the few tissues where MSC can be obtained without anaesthesia, invasive and painful interventions, particularly in parous women. MSC from the endometrium provide an accessible alternate source of MSC for use in cell-based therapies (Ulrich *et al.*, 2013b). It appears that eMSC reside in the endometrium after a woman's fertile years have ceased. We show for the first time that these eMSC can be readily harvested from post-menopausal women using an office-based biopsy, particularly if they are pre-treated with estrogen to regenerate the endometrium to yield sufficient numbers. Similarly, eMSC can be harvested from premenopausal women (Schuring *et al.*, 2011a). The human endometrium is therefore a possible source of MSC independent of a woman's age.

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Authors' roles

D.U.: participation in study design and execution, analysis of data, manuscript writing and critical discussion. K.S.T., J.D., K.S. and A.C.: participation in the study execution, analysis of data and critical discussion. A.R.: participation in study design, manuscript editing and critical discussion. C.E.G.: conception and participation in study design, manuscript editing and critical discussion.

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Conflict of interest

None declared.

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