SSEA-1 isolates human endometrial basal glandular epithelial cells: phenotypic and functional characterization and implications in the pathogenesis of endometriosis

A.J. Valentijn¹, K. Palial¹, H. Al-lamee¹,², N. Tempest¹, J. Drury¹, T. Von Zglinicki³, G. Saretzki³, P. Murray², C.E. Gargett⁴, and D.K. Hapangama¹,*

¹Department of Women's and Children's Health, Institute of Translational Medicine, University of Liverpool, Liverpool Women's Hospital, Crown Street, Liverpool, UK, ²Stem Cell Group, Institute of Translational Medicine, University of Liverpool, Liverpool, UK, ³Institute for Ageing and Health, Newcastle University, Campus for Ageing and Vitality, NE4 5PL, Newcastle upon Tyne and ⁴The Ritchie Centre, Monash Institute of Medical Research and Department of Obstetrics and Gynaecology, Monash University, Melbourne, Australia

*Correspondence address. Tel: +44-151-795-9559; E-mail: dharani.hapangama@liv.ac.uk

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STUDY QUESTION: Can the basal epithelial compartment of the human endometrium be defined by specific markers?

SUMMARY ANSWER: Human endometrial epithelial cells from the basalis express nuclear SOX9 and the cell-surface marker SSEA-1, with some cells expressing nuclear β-catenin. In vitro, primary endometrial epithelial cells enriched for SSEA-1+ show some features expected of the basalis epithelium.

WHAT IS KNOWN ALREADY: The endometrial glands of the functionalis regenerate from the basalis gland stumps following menstruation. Endometriosis is thought to originate from abnormal dislocation of the basalis endometrium. In the highly regenerative intestinal epithelium, SOX9 and nuclear β-catenin are more highly expressed in the intestinal crypt, the stem/progenitor cell region.

STUDY DESIGN, SIZE, DURATION: A large prospective observational study analysing full-thickness human endometrial hysterectomy samples from 115 premenopausal women, 15 post-menopausal women and ectopic endometriotic lesions from 20 women with endometriosis.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Full-thickness endometrium from hysterectomy tissues was analysed by immunohistochemistry for SSEA-1, SOX9 and β-catenin. Primary human endometrial epithelial cells from short-term cultures were sorted into SSEA1+/− fractions with a cell sorter or magnetic beads and analysed for markers of differentiation and pluripotency and telomere lengths (TLs) using qPCR, telomerase activity [telomere repeat amplification protocol (TRAP)] and growth in 3D culture.

MAIN RESULTS AND THE ROLE OF CHANCE: Similar to the intestinal crypt epithelium, human endometrial basal glandular epithelial cells expressed nuclear SOX9 and contained a rare subpopulation of cells with nuclear β-catenin suggestive of an activated Wnt pathway. The embryonic stem cell-surface marker, SSEA-1, also marked the human endometrial basal glandular epithelial cells, and isolated SSEA-1+ epithelial cells grown in monolayer showed significantly higher expression of telomerase activity, longer mean TLs, lower expression of genes for steroid receptors and produced a significantly higher number of endometrial gland-like spheroids in 3D culture compared with SSEA-1− epithelial cells (P = 0.009). Cells in ectopic endometriosis lesions also expressed SSEA-1 and nuclear SOX9, suggesting that the basalis contributes to ectopic lesion formation in endometriosis following retrograde menstrration.

LIMITATIONS, REASONS FOR CAUTION: This is a descriptive study with only short-term culture of the primary human epithelial cells in vitro.

WIDER IMPLICATIONS OF THE FINDINGS: The surface marker SSEA1 enriches for an endometrial epithelial cell subpopulation from the basalis. Since the functional endometrium originates from these cells, it is now possible to study basalis epithelium for stem/progenitor cell activity to extend our current understanding of endometrial biology in health and diseases.
Introduction

The human endometrium is a dynamic tissue undergoing repetitive cycles of regeneration, differentiation and shedding during a woman’s reproductive life. The cyclical transition is under the control of ovarian steroid hormones. Functionally, the endometrium is composed of two layers, the superficial functionalis and the deeper basalis, which serves as a germinal compartment for the monthly regeneration of the functionalis.

The human endometrium and intestinal epithelium are strikingly similar in that they are highly proliferative tissues that rapidly renew, cyclically in the endometrium and constantly in the intestine. The epithelium of both tissues can be divided into two distinct compartments. The luminal epithelium of human endometrium consists of a single layer of columnar epithelium that invaginates into the stroma to form the glands, which extend from the surface through the functionalis and basalis layers to the myometrium. The epithelial progenitor cells are postulated to reside in the basalis (Padykula et al., 1989; Gargett 2007). The intestinal epithelium is arranged in a monolayer along a crypt-villus axis. The crypts of Lieberkuhn represent the proliferative region and contain stem/progenitors that produce daughter cells that migrate upwards from the crypt base to the villus tip and differentiate into enterocytes, goblet and enteroeendocrine cells. The villus represents the differentiated compartment.

The remarkable self-renewal capacity of both the endometrium and intestine is due to the activity of adult stem cells. In vitro genetic lineage tracing identified Lgr5 as a specific marker of the highly proliferative epithelial stem cells in the small intestine and colonic epithelium (Barker et al., 2007). Stem/progenitor cells have been described in the human (Chan et al., 2004; Taylor, 2004, Schwab et al., 2005, Kato et al., 2007; Cervello et al., 2010) and mouse (Chan and Gargett, 2006; Cervello et al., 2007) endometrium by a variety of approaches. The first evidence for the existence of human endometrial stem/progenitor cells was the identification of clonogenic epithelial and stromal cells (Chan et al., 2004). Clonogenicity did not vary with the phase of the cycle or between active (cycling) and inactive post-menopausal endometrium (Schwab et al., 2005). The presence of clonogenic cells in the thin, inactive post-menopausal endometrium (Schwab et al., 2005) together with the fact that it can be regenerated with full functional restoration after treatment with ovarian steroid hormones (Paulson et al., 2002) support the hypothesis that stem/progenitor cells exist in the basalis, and persist beyond menopause (Nguyen et al., 2012). However, the precise location of, or markers to identify, these clonogenic epithelial cells are presently unknown.

The canonical Wnt/β-catenin signalling pathway regulates crucial aspects of cell fate determination. In the absence of Wnt signals, β-catenin is degraded but following Wnt signalling pathway activation, β-catenin is stabilized and translocates to the nucleus where it induces the transcription of target genes (reviewed in Bienz and Clevers, 2000). This pathway is important for the organization of the intestinal epithelium and is obligate for maintaining stemness and the undifferentiated state. Inactivation of β-catenin results in a rapid loss of intestinal epithelial cells that begins in the crypt (van de Wetering et al., 2002; Fevr et al., 2007). Molecules of this important pathway have also been identified, characterized and implicated in the regulation of the menstrual cycle in the human endometrium (Bui et al., 1997; Tulac et al., 2003; Wang et al., 2010). Nei et al. (1999) observed that in endometrial cells, nuclear β-catenin expression was enhanced during the proliferative phase of the menstrual cycle. Moreover, accurate localization and regulated expression of β-catenin is vital for normal endometrial epithelial function (Jeong et al., 2009). Recently, we demonstrated an intact canonical Wnt signalling pathway in epithelial cells from both pre- and post-menopausal endometrium (Nguyen et al., 2012). At the transcript level key Wnt molecules were differentially regulated in post-menopausal compared with premenopausal endometrial epithelium suggesting a functional role for the Wnt pathway in regulating the human endometrial epithelial stem/progenitor cell population.

One of the molecules up-regulated in post-menopausal endometrium was SOX9, a Wnt target (Nguyen et al., 2012). SOX9 is a transcription factor best known for its role in chondrogenesis (Foster et al., 1994; Wagner et al., 1994), male gonad development (Südbeck et al., 1996), neural crest development (Spokony et al., 2002) and in the lower crypt region of the intestinal epithelium (Blache et al., 2004). In the developing intestine SOX9 inhibits Wnt/β-catenin signalling by a feedback loop, thereby influencing cell fate determination (Bastide et al., 2007). In a normal endometrium SOX9 expression is significantly higher in the proliferative phase of the menstrual cycle (Saegusa et al., 2012).

Endometriosis is a common benign gynaecological disease, defined by the presence of endometrial glands and stroma-like tissue outside the uterine cavity. It has been postulated that endometriosis results from abnormal dislocation of basal endometrium, expelled into the pelvic cavity via trans-tubal retrograde menstruation and deposited to give rise to ectopic lesions (Sampson, 1927; Leyendecker et al., 2002). This emphasizes the importance of the basal layer in endometrial biology and the need for markers that define the epithelium in this compartment.

Given the importance of the basalis layer in endometrial regeneration together with the high expression of the SOX9 transcript in post-menopausal endometrium, the expression of SOX9 protein in the endometrium warranted further investigation. We hypothesized that differential expression of SOX9 may have an important role in the epithelial compartments of the endometrium in a manner analogous to the intestine. As SOX9 is a transcription factor located inside the cell, identification of a cell-surface marker that predominantly marks SOX9-expressing cells would facilitate their isolation and characterization. We used immunohistochemistry (IHC) to screen full-thickness healthy
human endometrium from women not on hormonal medications using a panel of human embryonic and adult stem cell markers (#52009; R&D, Oxford, UK) to identify candidate endometrial basal glandular epithelial cell-surface markers and selected the embryonic stem cell marker, SSEA-1, which showed specific expression in glands of the endometrial basalis layer. Therefore, the aim of this study was to further explore our previous finding for SOX9 and phenotypically characterize the epithelial cells of basalis endometrium based on the expression of a cell-surface marker.

Materials and Methods

Human tissue collection:
Collection of human endometrium was approved by Liverpool Adult Ethics committee (REC references: 09/H1005/55 and 11/H1005/4) and 125 human endometrial samples (10 premenopausal and 15 post-menopausal women undergoing hysterectomy who had not been on hormonal treatments in the preceding 3 months) were collected. A wedge of tissue from the lumen to the muscular myometrial layer that included superficial and basal endometrium as well as myometrium was taken from the detached uterus. Ectopic endometriotic lesions were collected from 20 women with active, symptomatic peritoneal endometriosis undergoing excision surgery and matching full thickness, the eutopic endometrium was examined in five women with endometriosis. Further demographic information on patient groups is included in the Supplementary data, Table SI.

Immunohistochemistry

Standard IHC was performed on 3 µm paraffin embedded tissue sections using DAB chromogen as previously described (Hapangama et al., 2010). Immunostaining for all antibodies was analysed with specific reference to the two different epithelial compartments, the functionalis (glands in the upper two-thirds of the endometrium below the luminal epithelium) and the basalis (glands in the lower one-third of the endometrium adjacent to the endo-myometrial junction) in full-thickness endometrial tissue sections. The SSEA-1 and SOX9-expressing epithelial cells were quantified using a modified quickscore (Schissel et al., 2009), β-catenin was analysed at three points in the cells: cell membrane, the cytoplasm and the nucleus as previously described (Nei et al., 1999). Antibodies used for IHC are listed in Table I and the external positive control sections for the antibodies are included in the Supplementary data, Fig. S1.

Immunofluorescence

Immunofluorescence (IF) was performed on 3 µm paraffin embedded sections. Antigen-retrieval conditions and antibody concentrations are listed in Table I. Sections where the application of primary antibody was omitted served as controls. Secondary antibodies were from Sigma-Aldrich, Sigma (FITC anti-goat) and Cell Signalling Technology, Hitchin, UK (Alexa Fluor® 488 and 555 anti-mouse and anti-rabbit) and were used at their recommended concentrations. Slides were mounted in Vectashield with DAPI, a nuclear stain (Vector Laboratories, Peterborough, UK). IF was visualized on a Nikon Eclipse 50i microscope using NIS-Elements F for image capture and Image J and Adobe Photoshop for processing.

Isolation of human endometrial epithelial cells

Freshly harvested endometrial tissue was mechanically and enzymatically digested and separated into stromal and epithelial fractions for flow cytometry or culture (Chan et al., 2004; Gargett et al., 2009). The purity of the epithelial fraction was assessed morphologically and by flow cytometry for the expression of CD9, a glandular epithelial marker and CD13, a stromal marker in normal human endometrium (Kato, 2012) and by IF for cytokeratins (CK7, CK18, pan-CK).

Cell phenotype analysis and sorting

Single-cell suspensions of primary epithelial cultures were labelled with PE anti-human CD13 (#301703; BioLegend, London, UK), FITC anti-human CD9 (#312103; BioLegend) as recommended by the manufacturers and analysed on a FACSCalibur cytometer (BD Biosciences, UK) using Cell Quest Acquisition and Analysis software (CellQuestPro version). For cell sorting an FACSariaII was used and cells were labelled with PE anti-human CD9 (#312105; BioLegend) and Alexa Fluor® 488 anti-mouse/human SSEA-1 (#125609; BioLegend). Data were analysed with FACSdiva software (version 6.1.3). Controls were unlabelled cells and cells labelled with isotype-matched control antibodies. Refer to Table II for antibody specifications. For magnetic bead sorting (MACS), single-cell suspensions were labelled with anti-SSEA-1 (CD15) MicroBeads (Miltenyi Biotec, Surrey, UK) and separated using MACS separation columns (MS columns, Miltenyi Biotec) according to the manufacturer’s instructions. Cell purity was >90% for FACS and >75% for MACS cell sorting.

Ki-67 labelling

Short-term monolayer epithelial cultures were harvested to single cells and labelled with PE anti-human SSEA-1 and then fixed in NBF:PBS (1:1) for 20 min at RT. After washing the cells in PBS, they were permeabilized in 0.1% Triton X-100 in PBS, 5 min at room temperature, washed and then labelled with FITC-Ki-67 (#350507; BioLegend). Cells were also phenotyped for CD9 expression as described above.

SDS-PAGE and immunoblotting

SSEA-1+ and SSEA-1− cells were extracted, and SDS-PAGE and immunoblotting were carried out as previously described (Hapangama et al., 2012). Primary antibodies used included: anti-actin (clone AC-40; Sigma-Aldrich); anti-CD9 (clone 209306; R&D Systems) anti-SSEA-1 (CD15; clone MC-480; R&D Systems); pan-cytokeratin (C2562; Sigma-Aldrich).

Telomere repeat amplification protocol assay

Cell pellets from sorted and cultured cells were lysed, protein content determined (Bradford) and 1 µg protein was loaded into the PCR reaction (30 min telomerase reaction at RT) and 30 PCR cycles according to the manufacturer’s instructions [Roche Diagnostics, Germany, telomere repeat amplification protocol (TRAP) ELISA]. ELISA was performed and the final product measured at 450 nm as previously described (Hapangama et al., 2008). 100 ng Hela protein was used as a positive control, while no protein served as a negative control.

Mean telomere length measurements by real-time PCR

Genomic DNA was extracted from endometrial cells and the TL was measured by quantitative real-time PCR as described previously (Hapangama et al., 2009). Measurements were performed in six to eight technical replicates, with coefficient of variation <4%. Three DNA samples with known TLs were run as internal standards in each batch of samples. The mean TL is expressed in base pairs (bp).

Quantitative real-time PCR and RT–PCR

TRIzol® Reagent (Life Technologies, Paisley, UK) was used to isolate total RNA which was precipitated from samples according to the manufacturer’s
instructions. Total RNA was extracted from SSEA-1 sorted (MACS) cells and reverse transcribed into cDNA using the Superscript III First-Strand Synthesis System (Invitrogen, Paisley, UK). Quantitative-PCR and qPCR were performed as previously described (Fujiwara et al., 2007) using KAPA SYBR FAST qPCR Mix Master 2x (Kapa BioSystems, Anachem Ltd, Bedfordshire, UK) and the Rotor-Gene 3000 centrifugal real-time cycler (Corbett Research, UK). Relative gene expression was calculated and normalized to the reference gene, YWHAZ (Vestergaard et al., 2011). The amplification products were verified using gel electrophoresis. The PCR primers (Sigma-Aldrich, Poole, UK) used are listed in Table III. For 3D culture experiments spheroids from three SSEA-1+ and SSEA-1− independent patient samples were collected into TRIzol Reagent and pooled, final volume per group 1 ml. RNA was extracted and reverse transcribed as described.

3-D epithelial cell cultures in matrigel

Short-term cultured (16–36 h post-plating) epithelial cells from predominantly proliferative phase samples were trypsinized and re-suspended to single-cell suspensions at ~100,000 cells/200 μl undiluted Matrigel (BD Biosciences, Oxford, UK) and diluted serially 2-fold up to ~3000 cells/100 μl: 50 μl of the resulting mixture was plated in duplicate in 24-well tissue culture plates. After allowing the Matrigel to set at 37°C for 15–20 min, DMEM/F12 medium supplemented with insulin-transferrin-selenite (ITS; Invitrogen) and 50 ng/ml EGF (Sigma-Aldrich) was added. Medium was replaced every 3 days and cultures monitored over 14 days. Short-term cultured epithelial cells were also sorted into SSEA-1+ and SSEA-1− fractions and embedded in Matrigel as described.

For IHC and IF, 3-D cultures were fixed in 10% neutral-buffered formalin (NBF) for 30 min, harvested into 1% agarose in PBS and placed in NBF overnight at 4°C, then processed to paraffin wax, 3 μm sections were cut and antigen-retrieval performed (Table II). 3-D morphology and polarity were assessed with antibodies to actin, laminin and E-cadherin (Table II).

Statistical analysis

Statistical package SPSS (PASW 18, IBM, USA) was used. Summary statistics and paired t-test or non-parametric equivalent (Wilcoxon’s signed-rank test) were employed as appropriate to compare the differences in telomerase activity, mean telomere lengths (TLs), gene expression and spheroid formation in 3D culture between the SSEA-1+ and SSEA-1− fractions. The baseline differences in patient demographics and IHC modified quickscores between the different groups defined by menstrual cycle phase was assessed by non-parametric tests (Kruskal–Wallis and Mann–Whitney U-tests). Data are presented as mean ± SD as indicated. Results were considered statistically significant when P < 0.05.

Table I Antibodies used for IHC and IF.

<table>
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<th>Antibody</th>
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<th>Species</th>
<th>Unmasking buffer</th>
<th>Dilution</th>
<th>Supplier</th>
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<td>MC-480</td>
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<td>Tris–EDTA</td>
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<td>1:200 (IF) 1:600 (IHC)</td>
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<td>β-catenin</td>
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<td>Mouse</td>
<td>Tris–EDTA</td>
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<td>Laminin</td>
<td>—</td>
<td>Rabbit</td>
<td>Citrate</td>
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<td>Cytokeratin 18</td>
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<td>Cytokeratin 7</td>
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<tr>
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Table II Antibodies used for flow cytometry.

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<tr>
<td>PE anti-human CD13</td>
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<tr>
<td>FITC anti-human CD9</td>
<td>H19a</td>
<td>Mouse IgG1</td>
<td>BioLegend</td>
</tr>
<tr>
<td>PE anti-mouse/human CD15 (SSEA-1)</td>
<td>MC-480</td>
<td>Mouse IgM</td>
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<tr>
<td>Alexa Fluor® 488 anti-human Ki-67</td>
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<td>PE mouse IgM isotype control</td>
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FITC, fluorescein isothiocyanate; PE, phycoerythrin.
Table III  Primer sequences for quantitative real-time PCR.

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<td></td>
<td>Anti-sense</td>
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<td>Sense</td>
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<td>Sense</td>
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<td></td>
<td>Anti-sense</td>
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<tr>
<td></td>
<td>Anti-sense</td>
<td>5’GATGGTGTGATGGATGCAC3’</td>
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Results

Characterization of endometrial glandular epithelium for expression of SOX9, β-catenin and SSEA-1

We investigated the location of SOX9 protein in the human endometrium at a cellular level by IHC. SOX9 expression was largely confined to the basal epithelial cells throughout the cycle. There were significantly greater number of epithelial cells expressing SOX9 in the basalis (46.2–52.3%) over the functionalis (8–12.1%) glands (n = 30; P < 0.0001, Fig. 1A and B). Post-menopausal endometria (n = 15) showed the highest modified quickscores with over 75% epithelial cells expressing nuclear SOX9 (P = 0.001, Fig. 1A and B).

Cell membrane staining for β-catenin in the endometrium was observed in both the functionals and the basalis epithelium (Fig. 1A); cytoplasmic accumulation of β-catenin and nuclear β-catenin was only observed in the basalis, with nuclear β-catenin only in occasional epithelial cells (3.05 ± 2.9%, n = 30) of basal glands suggestive of an active Wnt pathway (Fig. 1A and C).

As SOX9 is a protein located inside the cells we sought to characterize the basal glandular epithelial cells with a cell-surface marker. SSEA-1 fulfilled this criterion primarily labelling epithelial cells of the basalis (Fig. 1A). In premenopausal endometrium, intense basal glandular SSEA-1 staining was seen in the early proliferative phase of the menstrual cycle, with the strongest staining in basal glands with decreasing reactivity in the superficial glands (Fig. 1B). The reduced levels of SSEA-1 staining of the functional glands was most obvious during the secretory phase (quickscors for basalis, 8.7 versus 0.93 for functionals, P = 0.0001, Fig. 1A and B). Less than 1% of the glandular cells showed SSEA-1 immunoreactivity in the secretory phase functionals compared with over 60% in the basalis. Whereas the entire surface of the cell expressed SSEA-1 in epithelial cells of the basalis and post-menopausal glands, the SSEA-1 staining in cells of the superficial functionals glands was mainly restricted to the apical membrane. Stromal cells did not show immunoreactivity to SSEA-1, although there were scattered intravascular cells positive for SSEA-1.

By examining sequential sections, we demonstrated that nuclear SOX9 and β-catenin expression was largely confined to the SSEA-1 expressing basal epithelial cells (Fig. 1). IHC staining for β-catenin in a type I endometrial cancer, our positive control, shows strong nuclear β-catenin accumulation (Fig. 1C).

Histological staining of the human endometrium provided a snapshot of protein expression and so we progressed to primary cultures of endometrial epithelial cells to further characterize the properties of SSEA-1-expressing cells.

Characterization of short-term cultured human endometrial SSEA-1⁺ and SSEA-1⁻ epithelial cells

In our short-term monolayer cultures of isolated human epithelial cells from full-thickness normal endometrial samples 83.1 ± 1.5% (n = 10) of cells expressed CD9 and 23.7 ± 3.3% expressed SSEA-1 (Fig. 2A). They were also positive for cytokeratin 7 and 18 (Fig. 2B). Stromal contamination of the cultures was routinely <20% as assessed by CD13 expression (data not shown). These cultures were subsequently used to enrich for SSEA-1⁺ cells by MACS. Immunoblotting for SSEA-1 confirmed enrichment in epithelial cells, expressing the markers CD9 and cytokeratin (Fig. 2B).

Given that SSEA-1 primarily labelled epithelial cells of the basalis, which is postulated to have differential responsiveness to ovarian hormones from functionals glands, we analysed the estrogen receptor α (ERα) and progesterone receptor (PR) gene expression of these cells. There was significantly reduced expression of both receptors in the MACS-sorted SSEA-1⁺ epithelial cells from short-term monolayer cultures (Fig. 2C).

SSEA-1⁺ epithelial cells show high telomerase activity, longer mean TLs and cycle less frequently

As telomerase activity in the human endometrium is localized to the glandular epithelial cells (Tanaka et al., 1998) we were interested in whether there were differences between SSEA-1⁻ and SSEA-1⁺ epithelial cells. Short-term cultures of glandular epithelium subjected to FACS were analysed for telomerase activity by TRAP. CD9⁺/SSEA-1⁻ cells isolated by flow cytometry sorting (Fig. 3A) showed significantly higher telomerase activity than CD9⁻/SSEA-1⁻ cells (Fig. 3B). The mean TLs of the FAC-sorted CD9⁺/SSEA-1⁻ cells were also significantly higher than the CD9⁻/SSEA-1⁻ cells (408 ± 663 versus 339 ± 503 bp, n = 3 pairs, Wilcoxon’s signed-rank test, P = 0.04) (Fig. 3C), suggesting that their replicative lifespan will exceed that of the SSEA-1⁻ cells and that they might be less differentiated.
Short-term cultures of endometrial epithelial cells were dual labelled for SSEA-1 and Ki-67 and analysed by FACS to assess their proliferative capacity. CD9 was expressed by >85% of these cells. There was a 2-fold reduction in Ki-67 expression in the SSEA-1+ cells compared with the SSEA-1− cells, suggesting that the former cycle less frequently. Immunostaining endometrium for Ki-67 showed fewer epithelial glands positive for Ki-67 in the basalis compared with the functionalis (Fig. 3F).

**Figure 1.** SOX9, β-catenin and SSEA-1 expression follows an increasing gradient from the functionalis to basalis. (A) Representative photomicrographs illustrating the distribution of immunoreactive SOX9+, β-catenin and SSEA-1+ cells in the sequential full-thickness normal human endometrium across the menstrual cycle and in post-menopausal women. Brown nuclear SOX9, brown membranous, cytoplasmic and nuclear β-catenin and brown plasma membrane SSEA-1 immunostaining of epithelial cells of full-thickness low-power (X4) endometrial tissue sections. Representative high-power (×40) micrographs of the functionalis and basalis layers of premenopausal endometrium, and in post-menopausal endometrium are also shown. External positive controls for SSEA1 (human kidney); SOX9 (human tonsil) and hepatocellular carcinoma (nuclear β-catenin) controls were used (Supplementary data, Fig. S1). Scale bars ¼ 500 μM in top panels (×4) and ¼50 μM in all other panels (×40). (B) Semi-quantitative quickscores (mean ± SD) for SOX9 and SSEA1 staining (accounting for intensity and proportions of positive cells per gland in at least 10 high-power fields) in glandular epithelium of basalis \( n = 15 \) proliferative phase (PROL), \( n = 15 \) in secretory phase (SEC) and functionalis \( n = 15 \) PROL, \( n = 15 \) SEC, and post-menopausal glands \( n = 15 \). * \( P < 0.001 \), ** \( P < 0.0001 \). (C) Brown nuclear β-catenin (arrows) was observed in 3.1 ± 2.9% of basal glandular cells (10 high-power fields per section, \( n = 30 \) ) of the normal endometrium and in the positive control (endometrial adenocarcinoma) tissues. PM, post-menopausal.
Isolated SSEA-1\(^+\) epithelial cells differentiate into endometrial gland-like, single-lumen spheroids in 3D culture

Singly dispersed, short-term cultured endometrial epithelial cells were grown in 3D Matrigel culture to recapitulate the architecture characteristic of glandular epithelial cells in vivo. Under these 3D-culturing conditions epithelial cells produced cyst-like spheroids with a hollow lumen over time (Fig. 4B–E). After 3–4 days in 3D-culture two types of spheroids, 50–100 μm in diameter, were observed: those with apico-basal polarity (Fig. 4C) and those without (Fig. 4B). Some of the non-polarized spheroids continued to proliferate and expand in culture prior to developing a polarized phenotype, typically on Days 10–14 (Fig. 4E).

After 10–14 days in culture these polarized spheroids showed morphology characteristic of glandular epithelium in vivo such as basal laminin and apical actin indicating correct positioning of the actin cytoskeleton.
E-cadherin was confined to the lateral sides of epithelial cells at the site of adherens junctions (Fig. 4E and F). The non-polarized spheroids comprised mostly SSEA-1-expressing cells and upon developing a polarized-phenotype SSEA-1 immunoreactivity was lost, suggesting a hierarchical lineage where SSEA-1 is presumably associated with a less-differentiated state. Interestingly, when confronted with a 2D environment at the interface between the Matrigel and medium (Fig. 4G) or Matrigel and plastic (Fig. 4H), these spheroids produced a monolayer of epithelial cells. Almost all spheroids expressed ER beta (ERβ) and occasional cells of the polarized spheroids expressed ERα and PR (results not shown).

Spheroid development is associated with SSEA-1 and stem cell marker expression

The primary endometrial epithelial cells enriched for SSEA-1 by MACS produced significantly more spheroids in 3D culture than the corresponding SSEA-1-depleted fraction suggesting that an endometrial epithelial progenitor resides within this population (Fig. 5A). We speculated that stem/progenitor cells might initiate spheroid production and that continued 3D culture might favour their differentiation by recapitulating the stem cell niche and that spheroid forming cells would be found in the SSEA-1+ enriched cultures. In support of this we observed an augmented expression of the pluripotency markers NANOG and OCT4 in a pool of three independent 3D cultures (Fig. 5C). Taken together the 3D data suggest that the SSEA-1+ population has the ability to form spheroids that differentiate in vitro into endometrial gland-like structures and possibly the surface/luminal epithelium.

Prominent expression of SSEA-1 and nuclear SOX9 in ectopic lesions from patients with endometriosis

Agreeing with the hypothesis that the shed basalis contributes to the formation of endometrial deposits, all examined active, peritoneal endometriotic deposits from 20 women contained cytokeratin-expressing epithelial cells with positive immunoreactivity for nuclear SOX9, SSEA-1 and membranous β-catenin (Fig. 6A). Some ectopic epithelial cells also expressed cytoplasmic/nuclear β-catenin. In five matching ectopic and eutopic full-thickness endometrial samples collected during the secretory phase, similar SOX9 and SSEA-1 expression was
observed in the ectopic epithelial cells and eutopic basalis epithelium (Fig. 6B).

**Discussion**

This study has demonstrated that SOX9 and cytoplasmic/nuclear β-catenin are more prominent in the basalis epithelium, where endometrial epithelial adult stem/progenitor cells are proposed to exist than in the more differentiated functionalis epithelium of the human endometrium. This pattern of expression of SOX9 and β-catenin is analogous to the intestine where there is increasing expression of both proteins moving from the differentiated compartment of the villus to the stem/progenitor compartment of the crypt (van de Wetering et al., 2002; Blache et al., 2004). Moreover, we show that the expression pattern for SSEA-1 is strikingly similar to that of nuclear SOX9, the strongest expressing SSEA-1+ epithelial cells being located in the basalis of cycling endometrium and in thin basalis-like post-menopausal endometria. SSEA-1 and SOX9 were also expressed by ectopic peritoneal deposits, supporting the notion that shed basalis contributes to the formation of ectopic endometriotic lesions. Characterization of endometrial basal epithelium...
short-term-cultured SSEA-1+ cells showed these cells expressed higher telomerase activity and had longer TLs, lower expression of the differentiation markers, PR and ERα and proliferate less frequently. This suggests a less-differentiated cell type consistent with the expected phenotype of the basalis. Moreover, in 3D culture epithelial cells enriched for SSEA-1 produced more gland-like spheroids and this was associated with increased expression of two pluripotency markers.

SSEA-1, an antigenic epitope defined as Lewis X carbohydrate, is expressed by preimplantation mouse embryos, teratocarcinoma stem cells and mouse embryonic stem cells (Knowles et al., 1978; Solter and Knowles, 1978; Fox et al., 1981; Knowles et al., 1982). Its expression on human embryonic stem cells is associated with differentiation. In human endometrium we found that SSEA-1 is predominantly expressed in the basal glandular epithelial cells, the postulated location of epithelial stem/progenitor cells. That its expression and pattern of expression change from the basalis to the functionalis suggests that it is associated with cellular hierarchy. The function of SSEA-1 in the endometrium is unknown but may be associated with cell adhesion, migration and capacity to differentiate. SSEA-1 may also function to bind and modulate growth factors such as FGF and Wnt-1 (Capela and Temple, 2006) both of which are involved in stem cell maintenance and differentiation. Thus, considering the prominent expression of SSEA-1 in the basalis epithelium, it is conceivable that these cells are a component of the endometrial-specific epithelial stem/progenitor cell niche.

In the intestine, activated Wnt/β-catenin signalling occurs in proliferating epithelial cells with diminished activity in differentiated cells (van de Wetering et al., 2002). Such a role for Wnt/β-catenin has been implicated in the endometrium. During the proliferative phase of the menstrual cycle estrogen induces Wnt/β-catenin signalling, whereas progestagens counteract it (Nei et al., 1999). Wnt/β-catenin signalling in the intestinal epithelium follows a decreasing gradient from the stem cell niche in the crypt to the more differentiated compartment of the villus (van de Wetering et al., 2002). In the endometrium cytoplasmic accumulation/nuclear β-catenin was observed in the basalis; nuclear β-catenin was restricted to the occasional basal glandular epithelial cells in normal premenopausal women and in the basalis-like post-menopausal glands, suggestive of an active Wnt pathway.
The transcript for SOX9, a downstream Wnt target, was shown to be up-regulated in post-menopausal endometrium compared with premenopausal suggesting that it is primarily expressed in basalis epithelium (Nguyen et al., 2012). We observed nuclear SOX9 largely confined to the basal epithelia which was also highly expressed in post-menopausal endometrium. Nuclear SOX9 has also been observed in the normal endometrium with expression confined to epithelial cells where it is significantly higher in proliferative than in secretory stages (Saegusa et al., 2012). A role for SOX9 in modulating proliferation through the Wnt/β-catenin pathway is emerging, with SOX9 acting in a feedback loop to repress Wnt signalling, thereby keeping proliferation under tight regulatory control (Akiyama et al., 2004; Blache et al., 2004; Bastide et al., 2007). Furthermore, in the intestinal epithelium, nuclear SOX9 expression is restricted to cells at the lower half of the crypt where its role is to repress CDX2 and MUC2, two genes associated with differentiation (Blache et al., 2004). Nuclear SOX9, coincident with SSEA-1, in the basal glands of premenopausal endometrium could function in a similar manner maintaining the SSEA-1 cells in a less-differentiated state. The high level of nuclear SOX9 observed in the post-menopausal endometrial epithelial cells might also function as a checkpoint to prevent

**Figure 6.** SSEA-1 in endometriosis. (A) IHC for SOX9, β-catenin, SSEA-1 and cytokeratin 18 in active peritoneal endometriotic lesions. Representative photomicrographs of ectopic, active red, peritoneal endometriotic deposits from symptomatic women (n = 20). Brown positive immunoreactivity was seen for nuclear SOX9; cytoplasmic and nuclear β-catenin; surface SSEA-1+ and cytokeratin 18 in representative sequential sections of an ectopic lesion. Scale bar 50 μm. (B) The expression pattern of basal epithelial markers SOX9 and SSEA1 in eutopic secretory phase endometrial functionalis, basalis and in a matched ectopic endometriotic lesion from the same woman. The expression pattern of SOX9 and SSEA-1 in the ectopic lesions collected during the secretory phase of the cycle was similar to the basalis layer of the matching eutopic endometrium.
hyperplasia as loss of SOX9 in the intestinal epithelium leads to hyperplasia (Bastide et al., 2007). The up-regulation of SOX9 in post-menopausal endometrium might result from the associated hypo-estrogenic state as the SOX9 transcript in the endometrium is down-regulated by estrogen (Talbi et al., 2006).

SSEA-1+ appears to define epithelial cells of the basalis which have a less-differentiated phenotype. In support of this, the transcripts for ERα and PR were significantly lower in SSEA-1+ epithelial cells compared with SSEA-1− epithelial cells consistent with the view that the expression of ERα and PR are associated with a more differentiated endometrial epithelial phenotype (Chan et al., 2012; Gargett et al., 2012). Additionally, SSEA-1+ cells have higher telomerase activity, longer TLs and cycle less frequently. Telomerase activity is a feature of the highly regenerative endometrium (Tanaka et al., 1998) and intestine (Schepers et al., 2011). Epithelial glandular cells are the source of the telomerase activity whose activity changes according to the menstrual cycle (Tanaka et al., 1998). Stem/progenitor cells have been proposed to harbour longest telomeres in a tissue compartment (Flores et al., 2008). SSEA-1+ cells representing the basal glandular epithelia had higher telomerase activity and longer TLs than SSEA-1− cells, which predominate in the functionalis. Ki-67 protein is present during all active phases of the cell cycle (G1, S, G2, M) and is associated with cell proliferation. Lower expression of Ki-67 in the SSEA-1+ cells suggests that they cycle less frequently and this in part might explain the longer telomeres. Lower proliferation in the SSEA-1+ cells is consistent with the basalis epithelial compartment having lower proliferation rates compared with the functionalis (Ferency et al., 1979; von Rango et al., 1998). In the context of endometrial regeneration of the functionalis from the basalis, longer telomeres in the SSEA-1+ epithelial cells from the basalis might extend the lifespan of this highly regenerative compartment by limiting telomere erosion (Saratzki, 2010). In the intestine telomerase activity is associated with cells of the crypt and is absent in the differentiated epithelial cells of the villus (Schepers et al., 2011).

Functionally, the capacity of endometrial epithelial cells to form gland-like spheroids in 3D culture resides within the SSEA-1+ population. The migration of cells from the polarized spheroids to cover the surface as a monolayer appears to recapitulate the re-epithelialization of the endometrial surface of the denuded endometrium following menstrual shedding in vivo (Ludwig and Spornitz, 1991). The identity of stem/progenitor cells for the endometrial epithelium remains unclear. Our finding that epithelial cells enriched for SSEA-1+ form spheroids in 3D that can give rise to luminal-like epithelium raises the intriguing possibility that a stem/progenitor is located in the SSEA-1+ endometrial basal epithelial population. In support, two pluripotency-associated genes were required to define this compartment of the endometrium and any other epithelial subtypes that may exist. Nonetheless, SSEA-1+ cells repre- sent the basal epithelial compartment or the stem cell niche, which play an important role in homing stem cells. Clearly, future work needs to examine these possibilities further and confirm the significance of our results in examining the pathogenesis of endometriosis where basalis is expected to play a prominent role. Further examination of isolated SSEA-1+ cells from the eutopic endometrium from women with and without endometriosis is now possible to identify any disease-specific therapeutic and diagnostic targets.

In conclusion SSEA-1 appears to be a marker suitable for the enrichment of basal epithelial cells. These cells possessed features associated with a less-differentiated phenotype, suggesting that SSEA-1 expression represents immature endometrial epithelial cells. The relatively uniform expression of SOX9 in the basalis suggests that it might have an important role in the homeostasis of this compartment. Clearly, additional markers are required to define this compartment of the endometrium and any other epithelial subtypes that may exist. Nonetheless, SSEA-1+ cells represent a useful starting point. For example, we are currently undertaking further studies characterizing the SSEA-1+ cells in terms of their specific gene expression profile.

Furthermore, given the striking similarities between the endometrium and intestine, application of techniques used in intestinal biology to endometrial biology might also further our understanding of endometrial biology in health and diseases.

**Supplementary data**

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

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**Authors’ roles**

D.K.H. obtained the ethical approval, and conceived the study design. D.K.H. and A.J.V. formulated experiments, analysed and interpreted data, produced figures and produced the first draft. The samples were collected by D.K.H., K.P. and H.A.-L. Experiments were carried out and data collected by A.J.V., D.K.H., J.D., H.A.-L., P.M., K.P., N.T., G.S. and T.V.Z. D.K.H., C.G., T.V.Z. and G.S. were involved in obtaining funding, C.G. and P.M. provided advice on experiments, and were involved in data interpretation, and revising the manuscript critically for important intellectual content. All authors had final approval of the submitted version.

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Conflict of interest
All authors have no conflict of interest to declare.

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