Introduction

It has been suggested that all tissues with a connective tissue or stromal component may contain a population of mesenchymal or stromal stem cells responsible for lifetime tissue regeneration and remodelling (Vaaninen, 2005). Human endometrium also contains a substantial vascularized stroma, which regenerates far more than most other tissues under normal physiological conditions. The upper functionalis layer is shed at menstruation, followed by regeneration initiated by cells in the lower basalis layer (Padykula, 1991). Therefore, we hypothesized that stromal stem/progenitor cells reside in human endometrium (Gargett, 2006, 2007). This was supported by studies which demonstrated the existence of colony-forming (Chan et al., 2004; Schwab et al., 2005) and side-population positive (Kato et al., 2007) stromal cells in human endometrium. Mesenchymal or stromal stem cells (MSC) have been identified in several stroma-containing tissues, including bone marrow, synovial fluid, dental pulp, adipose tissue, cord blood and skeletal muscle (Gronthos et al., 2000; Minguell et al., 2001; Romanov et al., 2003). They were originally identified in bone marrow as haemopoietic support cells, but are now regarded as a separate stem cell population. MSC are defined by their properties of adherence to plastic, clonogenic activity, extended proliferation, and differentiation into the mesenchymal lineages, adipocytes, osteoblasts, chondrocytes and muscle cells (Bianco et al., 2001; Minguell et al., 2001; La Russa et al., 2002; Vaananen, 2005). However, it is their remarkable plasticity which has garnered much interest within the scientific community, since MSC are able to differentiate into cells of a different phenotype than their tissue of origin (Ferrari et al., 1998; Kopen et al., 1999; Herzog et al., 2003; Ortiz et al., 2003; Grove et al., 2004). Therefore, MSC have huge potential in the development of future treatment modalities. 

Adult stem cells, including MSC, are notoriously difficult to purify, since there are no stem cell-specific markers. Various markers or combinations of markers have been used to isolate MSC; however, none was able to isolate a pure MSC population. Hence, the precise phenotypic identity of MSC...
remains unknown. The identification of stem cell-specific markers for their prospective isolation is crucial for future tissue engineering or cell-based therapies to become a reality. Markers which have been used to partially purify MSC include STRO-1, CD133, CD90 and CD146 (Simmons and Tokor-Storb, 1991; Guerrierio et al., 1997; Shi and Gronthos, 2003; Tondreau et al., 2005). STRO-1, an antibody which binds to a trypsin-insensitive epitope, successfully isolated all fibroblast colony-forming units (CFU) from bone marrow, with a cloning efficiency of 0.02%, and STRO-1⁺ CFU differentiated into fibroblasts, smooth muscle cells, adipocytes (Simmons and Tokor-Storb, 1991), osteoblasts (Granthos et al., 1994; Encina et al., 1999; Gronthos et al., 1999) and chondrocytes (Dennis et al., 2002). CD133 has also been used to enrich for haematopoietic stem cells (HSC), neural stem cells and prostatic epithelial stem cells also been used to enrich for haematopoietic stem cells (HSC), neural stem cells and prostatic epithelial stem cells (Simmons and Torok-Storb, 1991), osteoblasts (Gronthos et al., 2005), reinforcing the need to validate any putative stem cell markers and immune cells. Also, previous studies have shown that colony-forming endometrial stromal cells are unlikely to support clonal growth (Chan et al., 2004; Schwab et al., 2005), reinforcing the need to validate any putative stem cell marker expression with functional assays (Gargett, 2007). The aims of this study were to screen potential stem cell markers for the prospective isolation of human endometrial stromal CFU, and to determine the location of cells expressing these markers in human endometrium. This study used a colony-forming assay as a screening test for identifying potential markers of endometrial stromal stem/progenitor cells.

Materials and Methods

Human tissues

Human endometrium (n = 54) was obtained from hysterectomy samples collected from reproductive aged women (mean 42.6 ± 0.8 years, range 31–52 years) who had not taken exogenous hormones for 3 months prior to surgery. Seventeen samples were from proliferative phase endometrium, 24 were collected at secretory phase, 1 each at interval and menses, 4 had hyperplasia, 2 were inactive, 1 was poorly developed and 1 was atrophic. Full thickness endometrium attached to 5 mm myometrium was either collected in bench medium (BM) containing HEPES-buffered Dulbecco modified Eagle medium/Hams F-12 (DMEM/F-12; Invitrogen, Carlsbad, CA, USA), 1% antibiotic antimycotic solution (final concentrations: 100 mg/ml penicillin G sodium, 100 mg/ml streptomycin sulphate, 0.25 mg/ml amphotericin B; Invitrogen), and 5% newborn calf serum (CSL, Parkville, Australia), stored at 4°C, and processed within 2–18 h, or frozen in OCT Tissue Tek (Sakura Finetek Co., Tokyo, Japan) on dry ice and stored at −80°C until required.

Ethics approval was obtained from the Monash Medical Centre Human Research and Ethics Committee. Informed written consent was obtained from each patient. Menstrual cycle stage, assessed by histological examination according to well-established criteria (Noyes et al., 1975), was obtained from pathology reports.

Preparation of single-cell suspensions of human endometrial stromal cells

Human endometrial tissue was digested to single-cell suspensions using Collagenase type 3 (300 µg/ml; Worthington Biochemical Corporation, Freehold, NJ, USA) and mechanical digestion as previously described (Chan et al., 2004). Red blood cells were removed using Ficol-Paque density gradient centrifugation (Pharma Biotechnology, Uppsala, Sweden). Purified stromal cell suspensions were then obtained by removing epithelial cells and leukocytes using magnetic Dynabeads (Dynal Biotech, Oslo, Norway) coated with specific antibodies to EpCAM and CD45, respectively (Chan et al., 2004). Less than 5% epithelial cell and leukocyte contamination remained following beading.

Flow cytometric analysis/FACS of purified human endometrial stromal cells

Fresh purified endometrial stromal cell suspensions (1 × 10⁷ cells/ml) were incubated with antibodies against either STRO-1 (1 µg/ml, clone STRO-1; R&D Systems, Minneapolis, MN, USA) or mouse IgM isotype control (Caltag, Burlingame, CA, USA), PE-conjugated CD133 (5.5 µg/ml, clone AC141; Miltenyi Biotec, Auburn, CA, USA) or PE-conjugated mouse IgG1 control (Caltag), FITC-conjugated anti-CD90 (1 µg/ml, clone 5E10) or FITC-conjugated mouse IgG1 control (both Becton Dickinson, Bedford, MA, USA), CD146 (supematant, 500 µl/ml, clone CC9) or IgG2a (supematant) (both donated by P. Simmons, University of Texas Health Science Center at Houston, Houston, TX, USA) for 45 min at 4°C. Tubes incubated with STRO-1 or CD146 and their controls were subsequently incubated with FITC-conjugated goat anti-mouse IgM (10 µg/ml; Southern Biotech, Birmingham, AL, USA) or PE-conjugated sheep anti-mouse IgG(F(ab')₂ fragments) (10 µl/ml; Chemicon Australia, Melbourne, Australia), respectively, for 30 min at 4°C. Cells were further incubated with APC-conjugated anti-CD45 (10 µg/ml; Caltag) for 30 min at 4°C, and resuspended in 5% fetal calf serum.
phosphate buffered saline (PBS) for flow cytometric analysis using a MoFlo flow cytometer and Cyclops SUMMIT software (Version 3.1; Dako Cytomation Inc., Fort Collins, CO, USA). Cells were selected for FACS sorting by electronic gating according to their forward versus side scatter profile (Fig. 1A) and lack of CD45 expression (Fig. 1B). For those markers where a discrete positive population was not observed (STRO-1, CD133 and CD146), the top 1–2% of cells with the highest fluorescence intensity, and the bottom 50% of cells with the lowest fluorescence intensities (Figs 1C, 2A and 4A) were sorted as enriched and depleted populations, respectively, and subjected to colony-forming assays. Typically, on this instrument using these conditions, 98% purity and 95% viability are obtained for endometrial cell suspensions.

**In vitro colony-forming assay**

Enriched and depleted FACS sorted populations of endometrial stromal cells were seeded in triplicate at clonal density, 50 cells/cm², into gelatin-coated 60 mm Petri dishes (Becton Dickinson) and cultured in bicarbonate-buffered DMEM/F-12 medium containing 10% FCS, 2 mM glutamine (Invitrogen) and antibiotic-antimycotic for 15 days, as previously described (Chan et al., 2004). Medium was changed every 6–7 days and colonies were monitored to ensure they were derived from single cells. Cultures were terminated at 15 days and stained with 0.5% Toluidine Blue. Clusters $\geq$50 cells were counted and colony-forming ability calculated by dividing the average number of colonies per plate by the number of cells seeded, and multiplied by 100 to obtain a percentage (Chan et al., 2004).

**Immunohistochemistry**

Frozen sections of human endometrium (5 μm) were fixed in either buffered formal acetone for 30 s at 4°C for STRO-1 and CD146 staining, or acetone for 5 min at room temperature (RT) for CD133 and CD90 staining. Sections were then sequentially incubated with 0.3% hydrogen peroxide (Orion Laboratories, Welshpool, Australia) and protein blocking agent (Thermo Scientific, Pittsburgh, PA, USA) for 10 min each at RT. Primary and isotype control antibodies were diluted in 0.1% BSA/PBS, and incubated for 1 h at 37°C. Antibodies used were raised against STRO-1 (10 μg/ml; IgM), CD133 (22 μg/ml; IgG1), CD90 (5 μg/ml; IgG1) and CD146 (undiluted supernatant; IgG2a). Sections were then incubated for 15 min with Dako LSAB+ biotinylated secondary antibody (Dako, Carpinteria, CA, USA) for CD133 and CD90 staining, or for 1 h with biotin-conjugated anti-IgM or -IgG2a (both 40 μg/ml) for STRO-1 and CD146 staining, respectively, followed by Dako LSAB+ streptavidin horse-radish peroxidase conjugate (Dako). Staining was visualized using AEC chromogen (Zymed, San Francisco, CA, USA) for 5 min at RT. Cells

Figure 1: STRO-1 does not enrich for human endometrial stromal CFU. (A) Fresh endometrial stromal cells were analysed by flow cytometry, and selected by gating on their forward versus side scatter. (B) Leukocytes were excluded from analysis by electronic gating. (C) Single parameter histogram for STRO-1-FITC fluorescence showing a small population of positive cells ($n = 13$). Populations were sorted using the two gates shown (left for negative expression, right for top 1–2% positive expression). Grey line, isotype control. (D) Percentage of STRO-1$^+$ stromal cells from proliferative and secretory phase endometrial stromal cell suspensions. Bar, median. Representative perivascular staining of STRO-1 from proliferative (E) and secretory (F) phase endometrium. Inset, representative section stained with isotype-matched control. (G) Proportion of CFU within FACS sorted and magnetic bead isolated STRO-1$^+$ and STRO-1$^-$ stromal cell populations. Results from the same sample are joined by a line. Representative colonies initiated by STRO-1$^+$ (H) and STRO-1$^-$ (I) stromal cells. Scale bar, 200 μm. g, epithelial gland; *, blood vessel.
were counterstained with Mayer’s haematoxylin (Amber Scientific, Midvale, Australia) for 30 s, washed with tap H2O, followed by PBS, and aqueous mounted. Sections were examined under a Zeiss microscope (Axioskop; Carl Zeiss, Oberkochen, Germany) and images were captured by a digital video camera (Fujix; Fuji, Tokyo, Japan).

**Statistical analysis**

Colony-forming capacity was analysed using GraphPad Prism software (version 4.01; GraphPad Software, San Diego, CA, USA). Data were tested for homogeneity of variance using Bartlett test and was found significant, and therefore non-parametric tests were used. Paired Wilcoxon signed rank test was performed to determine statistical significance between enriched and depleted populations. Data are presented as mean ± SEM. Results were considered statistically significant when \( P < 0.05 \).

**Results**

The expression of four markers, STRO-1, CD133, CD90 and CD146, was examined on fresh human endometrial stromal cells by flow cytometry using a two-colour protocol, in order to identify markers for the prospective isolation of human endometrial stromal stem/progenitor cells. Stromal cells were selected for analysis on the basis of their forward versus side scatter profile (Fig. 1A) to exclude dead cells, debris and red blood cells, and any residual CD45⁺ leukocytes (usually 5% remain after CD45 beading) were excluded in the CD45 gate (Fig. 1B). Cells were FACS sorted on the basis of positive or negative marker expression, and cloning assays were performed as a screening test to determine the utility of each potential stem cell marker to enrich for endometrial stromal CFU. The location of putative human endometrial stromal stem/progenitor cell niche is currently unknown. Therefore, frozen full thickness human endometrial tissue from throughout the menstrual cycle was immunostained for each marker in order to further characterize the endometrium and identify a potential stromal stem/progenitor cell niche.

**STRO-1 does not enrich for human endometrial stromal CFU**

STRO-1 was examined first, since it is the most common marker used to prospectively isolate bone marrow MSC. STRO-1 was expressed on \( 2.1 \pm 0.6\% \) (\( n = 13 \)) of human endometrial CD45⁻ stromal cells (Fig. 1C), although at low
levels. This did not vary during the menstrual cycle: $2.1 \pm 0.9\%$ ($n = 7$) and $2.1 \pm 1.0\%$ ($n = 6$), for proliferative and secretory phase endometrium, respectively (Fig. 1D). There was substantial variation between samples; however, this did not appear to mask any true differences ($P = 1.0$). STRO-1 was also expressed at similar levels in both atrophic ($n = 1$) and inactive endometrium ($n = 1$), and for one or two endometrial hyperplasia samples examined. STRO-1 was expressed on endothelial cells and on the stroma around blood vessels (Fig. 1E and F). The degree of perivascular staining varied, from very few cells (Fig. 1E) to whole surrounding layers of cells around blood vessels (Fig. 1F), which may be due to the type of vessel and the amount of surrounding smooth muscle cells. The intensity or pattern of the STRO-1 staining did not vary across the cycle or between endometrial layers.

STRO-1$^+$ and STRO-1$^-$ cells were initially isolated by FACS sorting ($n = 3$), which identified similar stromal CFU activity in the STRO-1$^+$ and STRO-1$^-$ populations, $0.2 \pm 0.1\%$ and $0.3 \pm 0.03\%$, respectively ($P = 0.7$). As this was unexpected, we used an alternative method to verify the FACS results. Anti-IgM magnetic beads were used to isolate the STRO-1$^+$ cells ($n = 2$) from fresh stromal cell suspensions. However, the trend was the same; $0.24\%$ and $2.3\%$ for STRO-1$^+$ cells and $0.54\%$ and $3.3\%$ for STRO-1$^-$ cells for

Figure 3: CD90 enriches for human endometrial stromal CFU.
(A) Single parameter histogram for CD90-FITC showing percentage of CD90$^-$ and CD90$^+$ cells ($n = 19$). Populations were sorted using the gates shown into CD90$^-$ and CD90$^+$ expression. Grey line, isotype control. (B) Percentage of CD90$^-$ and CD90$^+$ stromal cells from proliferative (Pro) and secretory (Sec) phase endometrial stromal cell suspensions. Bar, median. (C) Proportion of CFU from FACS sorted CD90$^-$ and CD90$^+$ stromal cell populations. Results from the same sample are joined by a line. CD90 differentially stained the basalis and functionalis stroma (D and G). Weaker stromal cell staining with stronger perivascular staining was observed in the basalis (E and H), compared with the strong stromal cell staining in the functionalis (F and I). This was consistent for both proliferative (D–F) and secretory (G–I) phase endometrium. Inset, representative section stained with isotype-matched control. Representative colonies initiated by CD90$^-$ (J) and CD90$^+$ (K) stromal cells. Scale bar, 200 μm. g, epithelial gland; *, blood vessel
the two samples, respectively. Overall, STRO-1\(^+\) cells were not enriched for stromal CFUs with no difference detected between STRO-1\(^+\) (0.6 \pm 0.4\%) and STRO-1\(^-\) (0.9 \pm 0.6\%) populations (\(n = 5, P = 0.1875\), Fig. 1G). Similarly, no differences were detected in abnormal endometrial samples; an endometrial hyperplasia and an inactive sample contained less CFUs in the STRO-1\(^+\) fraction compared with STRO-1\(^-\) fraction, although one atrophic endometrial sample showed greater CFUs present in the STRO-1\(^+\) fraction compared with STRO-1\(^-\) fraction. The proportion present in proliferative phase endometrium may have been altered by one outlier. Similar percentages of CD45\(^-\)CD133\(^+\) stromal cells were also detected in all abnormal endometrial samples examined: inactive, 4.9\% and 6.0\% (\(n = 2\)), and hyperplasia, 7.3\% (\(n = 1\)). Surprisingly, CD133 did not immunostain the endometrial stroma or blood vessels, but rather glandular and luminal epithelium were immunoreactive (Fig. 2D–F).

**CD133 does not enrich for human endometrial stromal CFUs**

CD133, a marker present on a subset of HSC and epithelial stem cell populations, was expressed on 5.4 \pm 1.7\% (\(n = 13\)) of human CD45\(^-\) endometrial stromal cells (Fig. 2A). Similar to STRO-1, the percentage of CD133\(^+\) stromal cells did not vary across the menstrual cycle, with 5.1 \pm 3.9\% (\(n = 5\)) detected in proliferative phase, 5.9 \pm 1.9\% (\(n = 7, P = 0.2677\)) in the secretory phase (Fig. 2B) and 4.0\% (\(n = 1\)) in the menstrual phase (not shown). The proportion present in proliferative phase endometrium may have been altered by one outlier. Similar percentages of CD45\(^-\)CD133\(^+\) stromal cells were also detected in all abnormal endometrial samples examined: inactive, 4.9\% and 6.0\% (\(n = 2\)), and hyperplasia, 7.3\% (\(n = 1\)). Surprisingly, CD133 did not immunostain the endometrial stroma or blood vessels, but rather glandular and luminal epithelium were immunoreactive (Fig. 2D–F). Substantial staining variation was observed within individual sections of endometrium (Fig. 2F), although this did not appear to be basalis:functionalis or menstrual cycle-dependent. In accord with the immunohistochemical staining, FACS sorted CD133\(^+\) stromal cells contained substantially less CFUs compared with CD133\(^-\) cells, 0.2 \pm 0.1\% and 3.7 \pm 1.7\%, respectively (\(n = 5, P = 0.0625\), Fig. 2C). Smaller colonies were formed by CD133\(^+\) (Fig. 2G) compared with CD133\(^-\) (Fig. 2H) cells.
CD90 as a potential marker for human endometrial stromal CFU

The majority of human endometrial CD45− stromal cells expressed the fibroblast marker, CD90, 87.5 ± 2.9% (n = 19) (Fernandez-Shaw et al., 1992). Interestingly, CD90 segregated endometrial stromal cells into two distinct populations, CD90lo and CD90hi expressing cells, 12.5 ± 2.2% and 74.5 ± 3.4%, respectively (n = 19, Fig. 3A). The CD90lo population always contained significantly fewer cells than the CD90hi population (P < 0.0001). There were no significant differences for CD90lo (P = 0.3969) and CD90hi (P = 0.1893) between proliferative and secretory phase samples (Fig. 3B). CD90 immunostained all the stroma of the human endometrium, including the fibroblasts, perivascular and endothelial cells, but not the epithelial cells (Fig. 3D and G). Weak and strong CD90 staining was observed, which correlated with the CD90lo and CD90hi populations identified by flow cytometry, respectively. Weaker stromal cell staining was observed in the basalis stroma (Fig. 3E and H), whereas stronger staining was evident in the functionalis stroma (Fig. 3F and I) and around the blood vessels in the basalis. The staining was consistent for both proliferative (Fig. 3D–F) and secretory (Fig. 3G–I) phase endometrium, verifying CD90 as a marker that distinguishes basalis and functionalis stroma (Koumas et al., 2001).

Endometrial stromal cells from different patient samples were sorted into CD90hi and CD90lo populations, and then subjected to cloning assays. The majority of the endometrial stromal CFU were found in the CD90hi population (4.9 ± 4.6%) rather than the CD90lo population (2.7 ± 2.6%, n = 4, P = 0.125), although this trend was not statistically significant, most likely due to the variation observed between patient samples (Fig. 3C). Similar-sized colonies were observed for both CD90lo (Fig. 3J) and CD90hi (Fig. 3K) stromal cell populations. Since the majority of human endometrial stromal cells are CD90hi, another marker is required for use with CD90 to prospectively isolate a more purified population of human endometrial stromal CFU.

CD146 enriches for human endometrial stromal CFU

CD146 is an endothelial cell, perivascular cell and MSC marker. We hypothesize that human endometrial stromal stem/progenitor cells reside in a perivascular location, similar to other MSC. CD146 was strongly expressed on 9.4 ± 2.4% of fresh endometrial stromal cells (n = 12, Fig. 4A), which was higher in secretory phase (11.0 ± 3.0%, n = 7) compared with proliferative phase endometrium (5.8 ± 0.7%, n = 3, Fig. 4B), although not statistically significant (P = 0.5167). However, this may be due to the low numbers of proliferative samples analysed (n = 3). An endometrial sample at interval phase contained 8.3% CD146+ cells, perhaps supporting an increase in CD146+ stromal cells from proliferative to secretory endometrium. CD146 stained all endothelial cells of all blood vessels and perivascular cells of some vessels of the human endometrium (Fig. 4C–D). This was consistent for proliferative (Fig. 4C) and secretory (Fig. 4D) phase endometrium, and for basalis and functionalis layers.

CD146+ and CD146− cells were FACS sorted and subjected to cloning assays. All samples showed that the CD146+ cells (Fig. 4F) had a significantly greater colony forming capacity than the CD146− cells (Fig. 4G), 3.5 ± 1.3% and 1.8 ± 0.8%, respectively (n = 7, P = 0.0156) (Fig. 4E). The CD146+ population also produced a significantly greater number of large colonies compared with the CD146− population, 0.052 ± 0.01% and 0.01 ± 0.006%, respectively (P = 0.0156). In summary, the clonal analysis of CD146+ and CD146− cells supports CD146 as a marker enriching for endometrial stromal CFU.

Discussion

This study has used a combination of flow cytometry, immunohistochemistry, FACS sorting and CFU assays as a strategy to screen human endometrial stromal cells for individual markers that will identify and enrich for candidate stromal stem/progenitor cells. Using this approach, we identified CD90hi and CD146, as individual markers with potential value and two well-known MSC markers, CD133 and STRO-1, of little value for prospectively isolating human endometrial stromal stem/progenitor cells. CD146 and CD90 are the first individual markers of endometrial stromal CFU to be validated functionally, and their perivascular location supports the concept that endometrial stromal CFU may be a MSC-like cell.

CD90 as a potential marker for human endometrial stromal CFU

CD90 is one of the key MSC phenotypic markers routinely used in conjunction with CD29, CD44, CD73 and CD105 to characterize putative MSC populations isolated from various tissues (In’t Anker et al., 2004; Tsai et al., 2004; Dominici et al., 2006; Ma et al., 2006). CD90 has been used in combination with CD34+CD38−HLA-DR− to significantly enrich fetal bone marrow cells for colony-forming stromal progenitors to 1 per 20 cells (Guerriero et al., 1997). When combined with negative markers, CD45 and CD14, CD90-expressing cells comprised 49% of bone marrow stromal CFU (Boirot et al., 2005). This study used CD90 in combination with CD45 as a negative marker and showed a trend to enrichment for CFU in the CD90hi stromal cell population. Thus, the combination of CD90 with other markers may increase its value for identifying and isolating purer populations of endometrial stromal stem/progenitor cells.

CD90 differentially stained the basalis and functionalis stroma, further confirming an earlier study (Koumas et al., 2001). We hypothesized that human endometrial stromal stem/progenitor cells reside in the basalis, to avoid their loss during menstruation. Since the CD90hi population contained more colony-forming cells than the CD90lo population, this study supports a basalis perivascular location of endometrial stromal stem/progenitor cells. However, this cannot be confirmed until a true differential basalis and functionalis marker is identified. A perivascular location seems ideal for resident...
endometrial stromal stem/progenitor cells to receive signals from endothelial cells or the blood stream, to initiate stromal and vascular regeneration each month (Gargett, 2007). This is in accord with other perivascularly located MSC populations from the bone marrow and dental pulp (Shi and Gronthos, 2003).

**CD146 enriches for human endometrial stromal CFU**

CD146 is a marker of endothelial and smooth muscle cells (Filshie et al., 1998). Although its use has been limited to bone marrow and dental pulp MSC (Filshie et al., 1998; Shi and Gronthos, 2003), CD146 has been used to divide the STRO-1⁰ fraction of bone marrow cells into two populations, with CD146⁺STRO-1⁻ producing a 9-fold increase in CFU compared with the CD146⁻STRO-1⁺ fraction (Filshie et al., 1998). In addition, the CD146⁺ population accounted for 96% of the dental pulp CFU population compared with STRO-1, which only contained 82% of CFU (Shi and Gronthos, 2003), suggesting that CD146 is the more important marker in identifying dental pulp MSC. STRO-1 and CD146 co-localize in the bone marrow on large blood vessels and in dental pulp on the outer blood vessel wall (Shi and Gronthos, 2003). The enrichment of human endometrial stromal CFU by CD146 provides the strongest evidence for a perivascular location for human endometrial stromal stem/progenitor cells, and concurs with the CD90 findings for a potential location in the basalis. It also suggests that endometrial stromal stem/progenitor cells are similarly located to other MSC (Shi and Gronthos, 2003). Recently, the co-expression of CD146 and platelet-derived growth factor receptor β was used to prospectively isolate MSC-like cells from human endometrium exhibiting stem cell properties of colony-forming activity, multilineage differentiation and expression of key MSC markers (Schwab and Gargett, 2007), validating the use of CD146 to prospectively isolate endometrial stromal stem/progenitor cells. These two markers also co-localized on perivascular cells of the endometrium (Schwab and Gargett, 2007).

**STRO-1 does not enrich for human endometrial stromal CFU**

STRO-1 is the most commonly used marker for prospective isolation of bone marrow MSC (Simmons and Torok-Storb, 1991; Gronthos et al., 1994; Encina et al., 1999; Dennis et al., 2002; Gronthos et al., 2003; Stewart et al., 2003), despite conflicting reports on its expression on bone marrow MSC (Colter et al., 2000, 2001). It is also expressed on adipose tissue, trabecular bone and periodontal ligament MSC (Zuk et al., 2002; Song et al., 2005; Nagatomo et al., 2006). Despite being expressed on perivascular cells in human endometrium, STRO-1⁺ cells did not enrich for endometrial stromal CFU, limiting its potential for prospectively isolating human endometrial stromal stem/progenitor cells. However, this suggests that endometrial stromal CFU are located on the microvasculature, which is STRO-1 negative but CD146 positive, similar to dental pulp (Shi and Gronthos, 2003). Although STRO-1 expression is known to be up-regulated in culture (Simmons and Torok-Storb, 1991; Stewart et al., 2003), it does not appear to be up-regulated in cultured human endometrial stromal cells (Naqvi and Gargett, unpublished data). STRO-1 expression in the bone marrow is restricted to blood vessel walls, similar to human endometrium, and perineurium surrounding nerve bundles (Shi and Gronthos, 2003). STRO-1 staining around nerve fibres was not assessed in this study, but should be investigated, as it may be important for gynaecological diseases such as endometriosis where nerves are present in the functionalis endometrium of women with endometriosis, but not in women without endometriosis (Tokushige et al., 2006). Perhaps, the STRO-1 staining of perivascular cells in human endometrium is incidental to the stem/progenitor cell population.

**CD133 does not enrich for human endometrial stromal CFU**

CD133 is well known as an HSC marker, binding to a novel cell surface antigen present on a CD34⁺ subset of human HSC. Recently CD133 was used to prospectively isolate MSC from MPB and umbilical cord blood (UCB), and this population demonstrated high proliferative potential, Oct-4 expression and multilineage differentiation into adipocytes, osteocytes, chondrocytes and neuronal/glial cells (Tondreau et al., 2005). A large proportion of the CD133⁺ cell population were CD34⁺ (Tondreau et al., 2005), which most probably contained HSC since MSC are CD34⁺, suggesting that CD133 was able to isolate both HSC and MSC from those tissues. Similar to our studies, MSC in human placenta were CD133⁺, although CD133 mRNA was detected in most (7/9) samples, suggesting CD133 may be a trypsin-sensitive epitope (Fukuchi et al., 2004). Cells with HSC phenotype were recently identified in human endometrium (CD34⁺CD45⁻) which co-expressed lymphoid lineage antigens (CD7 and CD56) (Lynch et al., 2007), but CD133 expression on the CD34⁺CD45⁻ cells was not investigated, nor were any functional stem cell assays undertaken. Therefore, it remains unknown whether CD133⁺ HSC are present in human endometrium. CD133 stained the epithelial cells of the human endometrium. Similarly, CD133 has been documented as staining several epithelia including embryonic epithelium from neural tube, gut and kidney (Miraglia et al., 1997; Corbeil et al., 1999; Dennis et al., 2000, 2001). It is also expressed on osteocytes, chondrocytes and neuronal/glial cells (Tondreau et al., 2005), which most probably isolated from human endometrium may contain HSC, which were unable to form colonies in adherent culture and thus gave a low CFU activity. CD133⁺ cells should be cultured in methyl cellulose to determine whether this marker isolates HSC from human endometrium.

The findings of this study provide impetus for the discovery of better marker(s) to prospectively isolate putative endometrial stromal stem/progenitor cells. This study identifies potential stem cell/progenitor cell markers that when combined with other markers, or analysed with side population (SP) cells (Kato et al., 2007), may further enrich stem/progenitor cell populations. The data obtained also validate the strategy of combining flow cytometry and immunohistochemistry analysis when screening potential stem cell markers, providing a framework for further studies. For example, it identified that CD133, used to identify circulating MSC, does not immunostain human endometrial stromal cells and yet isolates a small population of non-CFU-forming cells in the stroma. An
alternative method to isolate endometrial stromal cells based on CD13 expression (Kato et al., 2007) may further improve stromal cell purity, and could be analysed simultaneously with potential stem cell markers using flow cytometry. The combined use of immunohistochemistry and flow cytometry will also identify markers with enzyme-sensitive epitopes, which will not be robust markers for prospective isolation of putative stem cell populations. This strategy could also be applied to future studies to assess potential markers of endometrial epithelial stem/progenitor cells for their prospective isolation. There are several technical issues worthy of future consideration. FACS sorting may adversely affect the clonogenicity of freshly isolated human cells since we have observed a 3-fold decrease in CFU activity for the sorted cells compared with non-sorted stromal cells using similar seeding densities (Schwab and Gargett, unpublished observations). This indicates that the cloning efficiencies reported herein for both FACS sorted enriched and depleted fractions for the four markers are an underestimation, although both fractions are likely to be similarly affected. The CFU assay employed by this study identifies both stem/progenitor cells and transit amplifying cells, so further defining stem cell properties are also required to validate marker expression (Gargett, 2007). Substantial variation between patient samples was observed, similar to our original cloning studies on human endometrial epithelial and stromal cells (Chan et al., 2004; Schwab et al., 2005). We found it most helpful to report individual data for both enriched and depleted fractions to indicate whether a marker was providing consistent enrichment. Thus, increased CFU were observed for CD90hi versus CD90lo stromal cells in all samples examined, but there was no such trend in the samples tested for STRO-1+ versus STRO-1− cells.

In conclusion, this is the first study to investigate STRO-1, CD133, CD90 and CD146 as potential markers of human endometrial stromal CFU. CD146 was identified as a potential marker enriching for endometrial stromal CFU, and CD90hi stromal cells. CD133 as negative markers of endometrial stromal CFU validates the importance of undertaking functional stem cell assays to demonstrate the utility of the stem cell markers for isolating desired stem/progenitor cell populations (Gargett, 2007). This study supports a perivascular location for endometrial stromal colony-forming cells, perhaps near the microvasculature, and also provides evidence that human endometrial stromal CFU may be endometrial stromal stem/progenitor cells.

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