

# Co-expression of two perivascular cell markers isolates mesenchymal stem-like cells from human endometrium

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**BACKGROUND:** Human endometrium has immense regenerative capacity, growing ~5 mm in 7 days every month. We have previously identified a small population of colony-forming endometrial stromal cells which we hypothesize are mesenchymal stem cells (MSC). The aim of this study was to determine if the co-expression of two perivascular cell markers, CD146 and platelet-derived growth factor-receptor  $\beta$  (PDGF-R $\beta$ ), will prospectively isolate endometrial stromal cells which exhibit MSC properties, and determine their location in human endometrium. **METHODS:** Single cell suspensions of human endometrial stromal cells were fluorescence activated cell sorting (FACS) sorted into CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> and CD146<sup>+</sup>PDGF-R $\beta$ <sup>-</sup> populations and analysed for colony-forming ability, *in vitro* differentiation and expression of typical MSC markers. Full thickness human endometrial sections were co-stained for CD146 and PDGF-R $\beta$ . **RESULTS:** FACS stromal CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> stromal cells (1.5% of sorted population) were enriched for colony-forming cells compared with CD146<sup>+</sup>PDGF-R $\beta$ <sup>-</sup> cells ( $7.7 \pm 1.7$  versus  $0.7 \pm 0.2\%$   $P < 0.0001$ ), and also underwent differentiation into adipogenic, osteogenic, myogenic and chondrogenic lineages. They expressed MSC phenotypic surface markers and were located near blood vessels. **CONCLUSION:** This study shows that human endometrium contains a small population of MSC-like cells that may be responsible for its cyclical growth, and may provide a readily available source of MSC for tissue engineering applications.

**Keywords:** human endometrium; mesenchymal stem cells; cell surface markers; *in vitro* differentiation; clonal assays

## Introduction

Mesenchymal stem cells (MSC) have been identified in several human tissues including bone marrow, dental pulp, adipose tissue, synovial membrane, umbilical cord blood and amniotic fluid (Vaananen, 2005). Key features of MSC are their colony-forming ability, self-renewal and multipotency since they can be differentiated into mesoderm-derived lineages including adipogenic, chondrogenic, osteogenic and myogenic *in vitro* (Pittenger *et al.*, 1999; Colter *et al.*, 2000; Zuk *et al.*, 2002). The most exciting property of MSC is their *in vivo* differentiation capacity, as they can repair or induce repair of damaged muscles and ischemic heart muscle, and survive and differentiate into astrocytes in the brain (Grove *et al.*, 2004). Thus MSC have immense therapeutic potential. However, the next step for the use of MSC in cell-based therapies is the development of reliable and stringent methods for prospective isolation of fresh MSC directly from their source tissues. In most studies, MSC were obtained non-selectively by attachment to plastic culture dishes and subsequent expansion in culture. However, since the properties of MSCs may

be altered by culture conditions (Boquest *et al.*, 2005), there is currently a major research effort to identify specific MSC markers for their prospective isolation. Typical surface markers of culture expanded MSC (e.g. CD105, CD29) are also expressed by many other cell types and therefore unsuitable (Conget and Minguell, 1999; Dominici *et al.*, 2006). Several markers have been used individually or in combination for prospective isolation of MSC from bone marrow, including stromal precursor cell marker STRO-1, CD105, CD146 (or MUC18) and low affinity nerve growth factor receptor (LNGFR) (Quirici *et al.*, 2002; Campioni *et al.*, 2003; Shi and Gronthos, 2003). Bone marrow MSC-like cells have also been enriched using combinations of STRO-1 with platelet-derived growth factor-receptor  $\alpha$  (PDGF-R $\alpha$ ), epidermal growth factor-receptor, insulin-like growth factor-1 receptor or nerve growth factor receptor (Gronthos and Simmons, 1995). However, there is no consensus on the best method to prospectively isolate MSC from the various source tissues. For future tissue engineering purposes, highly purified

populations of fresh MSC will be required. This is currently unavailable.

Another issue regarding MSC concerns their identity. It has been suggested that MSC may be similar to vascular smooth muscle cells (SMC), pericytes or endothelial-like cells since MSC progeny express several vascular SMC markers and are contractile (Perkins and Fleischman, 1990; Bianco *et al.*, 2001; Kinner *et al.*, 2002; Short *et al.*, 2003). In addition, studies have found that pericytes possess similar characteristics and phenotype as MSC (Tintut *et al.*, 2003). Also pericytes and MSC both respond to PDGF-BB, which is involved in recruiting pericytes, as well as supporting bone marrow MSC colony-forming ability (Gronthos and Simmons, 1995; Hirschi and D'Amore, 1996). Bone marrow and dental pulp MSC are localized to the perivascular region in their respective tissues supporting their pericyte or SMC identity (Shi and Gronthos, 2003). Adipose tissue MSC are also derived from the stromal vascular fraction (Zuk *et al.*, 2001).

The human endometrium is the highly regenerative mucosal lining of the uterus; a dynamic tissue which grows 4–7 mm within 4–10 days every menstrual cycle (McLennan and Rydell, 1965). The human endometrium is comprised of epithelial-lined glands surrounded by a supportive stroma. Each month, the upper two-thirds that forms the functionalis layer is shed at menstruation and regenerates from the remaining basalis layer under the control of fluctuating plasma sex steroid hormone levels (Padykula, 1991). The human endometrium is the only adult tissue that contains a substantial stroma that regularly regenerates under normal physiological conditions. We hypothesized that the human endometrial basalis layer would contain a MSC population responsible for monthly stromal and vascular regeneration (Gargett, 2007). We have previously identified a small population of colony-forming stromal cells in human endometrium with a colony-forming capacity of 1.25% (Chan *et al.*, 2004; Schwab *et al.*, 2005). However, only 0.02% endometrial stromal cells produce large colonies, which we hypothesize are MSC (Chan *et al.*, 2004; Schwab *et al.*, 2005). More recently, a BrdU label-retaining stromal cell population has been identified in mouse endometrium, further supporting the presence of MSC in this highly regenerative tissue (Chan and Gargett, 2006; Cervello *et al.*, 2007; Szotek *et al.*, 2007). Therefore the human endometrium may provide an additional source of MSC for cell-based therapies.

There are no known markers of endometrial MSC-like cells. Our studies identified that PDGF-BB is necessary for endometrial stromal cell colony formation (Chan *et al.*, 2004; Schwab *et al.*, 2005). We reasoned that colony-forming endometrial stromal cells express PDGF-receptor  $\beta$  (CD140b; PDGF-R $\beta$ ), a receptor also expressed by pericytes (Short *et al.*, 2003). Colonies initiated by human endometrial stromal cells also expressed  $\alpha$  smooth muscle actin ( $\alpha$ SMA) (Chan *et al.*, 2004), suggesting that colony-forming endometrial stromal cells may be similar to bone marrow and adipose tissue MSC. Another perivascular and endothelial cell marker, CD146, a member of the immunoglobulin superfamily, has been used to enrich for MSC in bone marrow and dental pulp (Shi and Gronthos, 2003). In this study, we

demonstrate that the co-expression of perivascular cell markers, CD146 and PDGF-R $\beta$ , enables prospective isolation of multipotent endometrial MSC-like cells that are located perivascularly in human endometrium. The identification of these two markers for prospective isolation of MSC from human endometrium provides a readily available alternative source of MSC for future tissue engineering applications such as the development of constructs with scaffolds for use in pelvic floor prolapse surgery (Gargett and Chan, 2006).

## Materials and Methods

### Human tissues

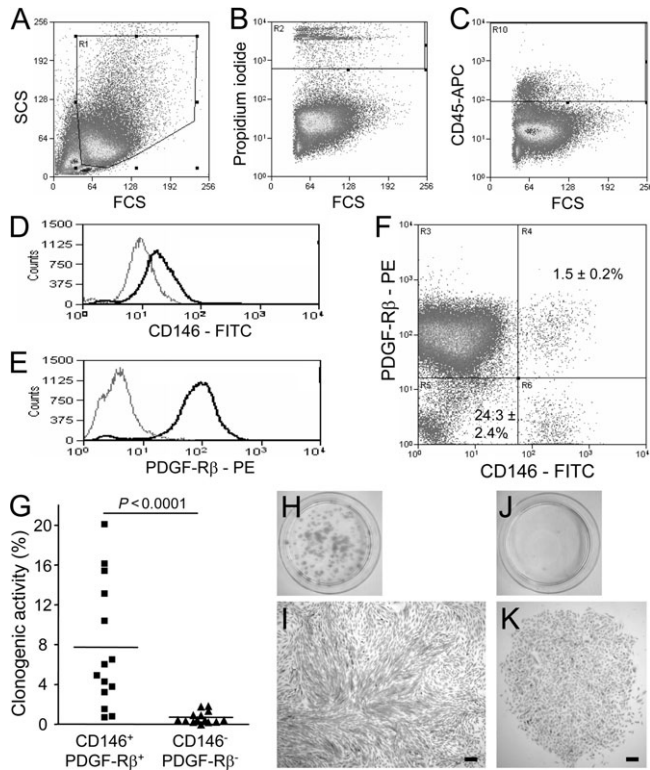
Human endometrial tissue ( $n = 17$ ), including underlying myometrium, was collected from ovulating women aged 31–49 years undergoing hysterectomy for non-endometrial pathologies, who had not taken exogenous hormones for 3 months before surgery. Informed written consent was obtained from each patient and ethics approval was obtained from the Monash Medical Centre Human Research and Ethics Committee. Menstrual cycle stage was assessed according to well-established histologic criteria for the normal menstrual cycle (Noyes *et al.*, 1975) by experienced histopathologists. This study used 13 proliferative and 4 secretory phase samples.

### Preparation of single cell suspensions of human endometrial stromal cells

Endometrial tissue including basalis was scraped off the underlying myometrium and dissociated to single cells using enzymatic and mechanical digestion as previously described (Chan *et al.*, 2004). A thin layer of myometrium at the endometrial–myometrial interface was also removed and partially digested to dislodge any remaining endometrial tissue, but not the myometrium. Purified single stromal cell suspensions were obtained by negative selection using magnetic Dynabeads (Dynal Biotech, Oslo, Norway) coated with specific antibodies to remove epithelial cells (BerEP4) and leukocytes (CD45) (Chan *et al.*, 2004).

### FACS using CD146 and PDGF-R $\beta$

Fresh purified endometrial stromal cell suspensions ( $1 \times 10^7$  cells/ml) were labelled with CD146 antibody [CC9; supernatant (Filshie *et al.*, 1998), IgG<sub>2a</sub>; donated by P. Simmons, Peter MacCallum Cancer Centre, Melbourne, Australia], PDGF-R $\beta$  (20  $\mu$ g/ml, clone PR72 112, IgG<sub>1</sub>; R&D Systems, Minneapolis, MN, USA) or isotype matched controls, followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG<sub>2a</sub> (50  $\mu$ g/ml) and phycoerythrin (PE)-conjugated anti-mouse IgG<sub>1</sub> (10  $\mu$ g/ml) (both, Becton Dickinson, Bedford, MA, USA). Cells were then incubated with allophycocyanin (APC)-conjugated anti-CD45 (10  $\mu$ g/ml; Caltag Laboratories, Burlingame, CA, USA), resuspended in 5% fetal calf serum/phosphate-buffered saline (FCS/PBS) containing propidium iodide (PI, 10  $\mu$ g/ml; Sigma-Aldrich, St. Louis, MO, USA) and fluorescence activated cell sorting (FACS) sorted on a MoFlo flow cytometer using Cyclops SUMMIT software (Version 3.1; Cytomation Inc., Fort Collins, CO, USA). The majority of cells were selected for analysis based on forward versus side scatter profile (Fig. 1A), dead cells (PI<sup>+</sup>, Fig. 1B) and leukocytes (CD45<sup>+</sup>, Fig. 1C) were excluded by electronic gating, and remaining cells analysed for CD146-FITC and PDGF-R $\beta$ -PE expression for FACS sorting of CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> and CD146<sup>+</sup>PDGF-R $\beta$ <sup>−</sup> populations.



**Figure 1:** Co-expression of CD146 and PDGF-R $\beta$  isolates human endometrial stromal cells with colony-forming ability. Freshly isolated human endometrial stromal cells were analysed by flow cytometry for expression of cell surface markers. Viable cells were selected by their forward versus side scatter profile (A), and dead cells and leukocytes were removed by electronic gating using PI (B) and CD45-APC (C), respectively. Single parameter histograms for CD146-FITC (D) and PDGF-R $\beta$ -PE (E) ( $n = 17$ ). Grey line indicates background fluorescence with isotype matched IgG control. (F) Representative dual parameter fluorescence histogram showing co-staining of CD146 and PDGF-R $\beta$  ( $n = 17$ ). (G) Scatterplot showing colony-forming ability of the two sorted cell populations—CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> and CD146<sup>-</sup>PDGF-R $\beta$ <sup>-</sup> ( $n = 14$ ,  $P < 0.0001$ , Mann–Whitney test), bar represents the mean. Representative culture dishes showing distribution of colonies from (H) CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> and (J) CD146<sup>-</sup>PDGF-R $\beta$ <sup>-</sup> sorted cell populations. A typical colony initiated by a (I) CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> and a (K) CD146<sup>-</sup>PDGF-R $\beta$ <sup>-</sup> cell. Scale bar, 200  $\mu$ m

#### In vitro colony-forming assay

FACS sorted populations of endometrial stromal cells were seeded in triplicate at clonal density, 50 cells/cm<sup>2</sup>, into gelatin-coated 60 mm Petri dishes (Becton Dickinson) and cultured in serum medium (SM) containing bicarbonate-buffered DMEM/F-12 medium (Invitrogen, Auckland, New Zealand) with 10% FCS (CSL Limited, Melbourne, Vicotia, Australia), 2 mM glutamine (Invitrogen) and antibiotic-antimycotic (Invitrogen) (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 the colony-forming ability determined (Chan *et al.*, 2004).

#### In vitro differentiation

FACS sorted populations of endometrial stromal cells were cultured in SM at clonal density ( $< 100$  cells/cm<sup>2</sup>) to enable only those CD146<sup>+</sup>

PDGF-R $\beta$ <sup>+</sup> cells with colony-forming capacity to grow. The resultant colonies were pooled and passed twice to increase cell numbers, and then incubated with adipogenic, osteogenic and myogenic differentiation induction media for 4 weeks as described (Pittenger *et al.*, 1999; Zuk *et al.*, 2002). For *in vitro* chondrogenic differentiation,  $3 \times 10^5$  cells were cultured as a pelleted micromass in a centrifuge tube in chondrogenic differentiating media for 4 weeks. Cells were also cultured concurrently for 4 weeks in 1% serum-containing medium as an undifferentiated control. To assess differentiation cells were harvested for RNA or immunostained with an alkaline phosphatase kit (Sigma-Aldrich), Oil Red O, alcian blue or immunohistochemistry using antibodies for  $\alpha$ SMA (3.6  $\mu$ g/ml, clone 1A4; Dako, Glostrup, Denmark) and smooth muscle myosin heavy chain (MHC; 1:400, clone hSM-V; Sigma-Aldrich) for osteogenic, adipogenic, chondrogenic and myogenic differentiation, respectively. Stained cells were examined under a Zeiss microscope (Axioskop; Carl Zeiss, Oberkochen, Germany) and images were captured using a digital video camera (Fujix; Fuji, Tokyo, Japan).

Differentiated cells were also assessed for expression of lineage specific genes using RT-PCR. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA), genomic DNA contamination was removed using RNase-free DNase (Promega, Madison, WI, USA) and reverse transcribed with AMV reverse transcriptase (Roche, Penzberg, Germany). cDNA was amplified using GoTaq Green Master Mix (Promega) in a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). Primer sequences used are shown in Supplementary Table S1. Primer concentrations used were 0.15  $\mu$ M. After amplification, reactions were analysed by 1.5% agarose gel electrophoresis, and visualized with ethidium bromide staining. The sequence of each product was confirmed using automated sequencing.

#### Phenotypic analysis

FACS sorted CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> human endometrial stromal cells were cultured to confluence in SM and analysed for typical MSC markers by flow cytometry. Cells were incubated with antibodies against CD29 (1  $\mu$ g/ml, clone mAb 13, rat IgG<sub>2a</sub>; Becton Dickinson), CD44 (1  $\mu$ g/ml, clone G44-26, mouse IgG<sub>2b</sub>; Becton Dickinson), CD73 (20  $\mu$ g/ml, clone AD2, mouse IgG<sub>1</sub>; Becton Dickinson), CD105 (10  $\mu$ g/ml, clone 266, mouse IgG<sub>1</sub>; Becton Dickinson), STRO-1 (1  $\mu$ g/ml, clone STRO-1, mouse IgM; R&D Systems), CD146 (as above) and PDGF-R $\beta$  (as above), followed by either FITC-conjugated goat anti-mouse IgM (10  $\mu$ g/ml; Southern Biotech, Birmingham, AL, USA), Alexa Fluor 488-conjugated chicken anti-rat IgG (10  $\mu$ g/ml; Molecular Probes, Eugene, OR, USA) or PE-conjugated sheep anti-mouse Ig F(ab')<sub>2</sub> fragment (10  $\mu$ l/ml; Chemicon Australia, Melbourne, Australia) depending on antibody isotype. Some cells were incubated directly with PE-Cy5.5 conjugated anti-CD34 (50  $\mu$ l/ml, clone 581, mouse IgG<sub>1</sub>; Southern Biotech), APC-conjugated anti-CD45 (as above) or FITC-conjugated anti-CD90 (1  $\mu$ g/ml, clone 5E10, mouse IgG<sub>1</sub>; Becton Dickinson). Isotype matched controls were included for each antibody.

Following antibody incubations, cells were incubated with PI and cells analysed by flow cytometry as described above.

#### Immunohistochemistry

For single immunostaining, full thickness human endometrium obtained from hysterectomy was frozen in OCT Tissue Tek (Sakura Finetek Co., Tokyo, Japan) on dry ice and stored at  $-80^{\circ}\text{C}$  until required. 5  $\mu$ m endometrial sections were fixed in acetone for 30 s, and then incubated with 0.3% hydrogen peroxide, followed by protein blocker PBA for 10 min each. Primary antibodies, CD146 (neat), PDGF-R $\beta$  (10  $\mu$ g/ml) or isotype matched control antibodies

(IgG<sub>2a</sub> and IgG<sub>1</sub>, respectively) diluted in 0.1% bovine serum albumin (BSA)/PBS, were incubated for 1 h at 37°C. Sections were incubated for 15 min with Dako labelled Streptavidin-Biotin (LSAB) system + biotinylated secondary antibody (Dako) for PDGF-R $\beta$  staining, or for 1 h with biotin-conjugated rat anti-mouse IgG<sub>2a</sub> (40  $\mu$ g/ml; Becton Dickinson) for CD146 staining. Sections were washed, and then incubated for 15 min with Dako LSAB+ streptavidin horseradish peroxidase conjugate followed by 5 min with chromogens, AEC (red Zymed, San Francisco, CA, USA) or DAB (brown, Sigma-Aldrich), counterstained with Mayer's haematoxylin (Amber Scientific, Midvale, Australia), and examined under a Zeiss microscope and images captured.

### Dual immunofluorescence

For double immunofluorescent staining, frozen sections of full thickness human endometrium were incubated with CD146 and PDGF-R $\beta$  primary antibodies simultaneously, followed by an incubation with biotin-conjugated rat anti-mouse IgG<sub>2a</sub> for 1 h. Sections were then incubated with both Alexa Fluor 488-conjugated streptavidin (2  $\mu$ g/ml; Molecular Probes) and Alexa Fluor 568-conjugated goat anti-mouse IgG<sub>1</sub> (4  $\mu$ g/ml; Molecular Probes) for 30 min protected from light. Sections were washed, counterstained with Hoechst 33 258 (4  $\mu$ g/ml; Molecular Probes) and mounted using a fluorescent mounting medium (Dako). Dual immunofluorescence was detected using a Leica confocal microscope and images were captured with Leica confocal software (version 2.5; Leica, Heerbrugg, Switzerland).

### Statistical analysis

Colony-forming capacity was analysed using GraphPad PRISM software (version 4.01; GraphPad Software Inc., 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. Mann-Whitney test was performed to determine statistical significance between CD146<sup>-</sup>PDGF-R $\beta$ <sup>-</sup> and CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> cloning efficiencies. Data are presented as mean  $\pm$  SEM. Results were considered statistically significant when  $P < 0.05$ .

## Results

### Perivascular marker expression on freshly isolated human endometrial stromal cells

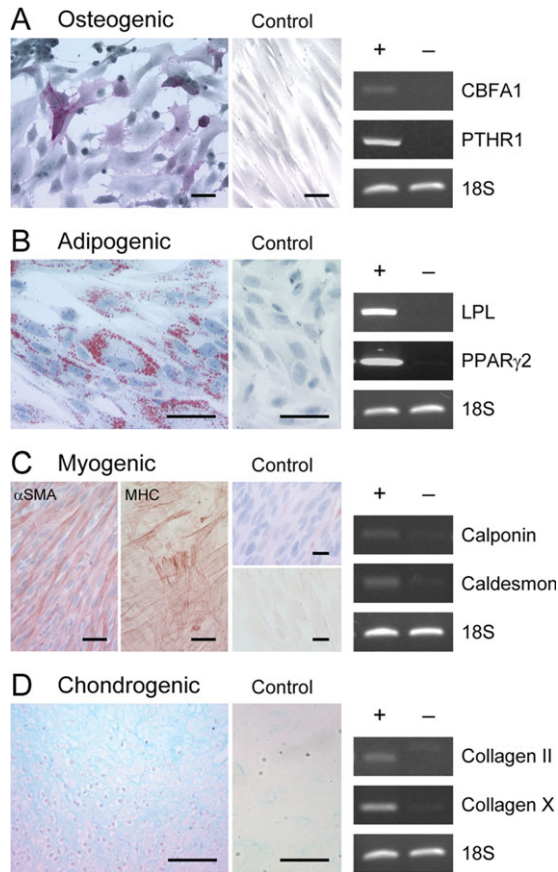
CD146 is a perivascular and endothelial cell marker previously used to identify bone marrow MSC (Shi and Gronthos, 2003), and PDGF-R $\beta$  is an established perivascular/pericyte marker (Hirschi and D'Amore, 1996). To determine the utility of these two perivascular cell markers to prospectively isolate MSC-like cells from human endometrium, freshly isolated and purified stromal cells were analysed by flow cytometry using a four-colour protocol. Dead cells (PI<sup>+</sup>, Fig. 1B) and leukocytes (CD45<sup>+</sup>, Fig. 1C) were gated and removed from analysis to further purify the stromal cells. Only a small population of human endometrial stromal cells expressed CD146 ( $3.1 \pm 0.3\%$ ,  $n = 17$ , Fig. 1D), whereas the majority expressed PDGF-R $\beta$  ( $71.6 \pm 2.6\%$ ,  $n = 17$ , Fig. 1E), consistent with the fibroblast phenotype of endometrial stromal cells. Further flow cytometric analysis showed four discrete populations of endometrial stromal cells (Fig. 1F), and identified a small population co-expressing CD146 and PDGF-R $\beta$  ( $1.5 \pm 0.2\%$ ,  $n = 17$ ). A substantial number of cells within endometrial stroma did not express either marker ( $24.3 \pm 2.4\%$ ,  $n = 17$ ), and

$1.5 \pm 0.2\%$  ( $n = 17$ ) were considered endothelial cells as they only expressed CD146.

### CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> FACS sorted endometrial stromal cells exhibit MSC-like activity

Having identified a well separated CD146 and PDGF-R $\beta$  co-expressing population, the CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> and CD146<sup>-</sup>PDGF-R $\beta$ <sup>-</sup> populations were FACS sorted and investigated for MSC attributes using functional assays; colony-forming capacity and multilineage differentiation. The CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> FACS sorted endometrial stromal cells produced significantly more colonies than the CD146<sup>-</sup>PDGF-R $\beta$ <sup>-</sup> cells, with colony-forming capacities of  $7.7 \pm 1.7$  and  $0.7 \pm 0.2\%$ , respectively ( $n = 14$ ,  $P < 0.0001$ , Fig. 1G). Of the 14 samples FACS sorted, 12 were from proliferative and 2 were from secretory phase endometrium. Since there were only two secretory phase samples and considerable variation between proliferative phase samples, it was difficult to determine if the magnitude of enrichment was affected by cycle stage. Nevertheless, there was a >15-fold enrichment of colony-forming endometrial stromal cells, as illustrated by two representative cloning plates (Fig. 1H and J). Colonies initiated by CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> stromal cells were typically larger and densely packed comprising cells with a high nuclear:cytoplasmic ratio compared with CD146<sup>-</sup>PDGF-R $\beta$ <sup>-</sup> cell clones (Fig. 1I and K, respectively).

Colonies of CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> endometrial stromal cells were pooled and expanded in culture, and then exposed to osteogenic, adipogenic, myogenic and chondrogenic induction media ( $n = 4$ , separate samples for each type of differentiation). Following incubation with osteogenic differentiation-inducing media, CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> cells stained positively for alkaline phosphatase, and expressed the critical early stage osteogenic transcription factor, core binding factor alpha 1 (CBFA1), and late stage osteogenic lineage specific marker, parathyroid hormone receptor type 1 (PTH1R) (Fig. 2A). The majority of CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> cells underwent adipogenic differentiation as visualized by Oil Red O staining of lipid droplets (Fig. 2B). Two early stage adipocyte lineage markers, lipoprotein lipase (LPL) and peroxisome proliferator-activated receptor  $\gamma$ 2 (PPAR $\gamma$ 2), were also expressed by the differentiated cells, but not when cultured in control medium. Passaged CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> endometrial stromal cells subjected to myogenic differentiation media immunostained strongly for early stage SMC marker,  $\alpha$ SMA, and late stage marker, smooth muscle MHC, a specific marker of contractile SMC (Fig. 2C), compared with cells cultured in control medium. RT-PCR analysis demonstrated expression of early and mid-stage SMC markers, caldesmon and calponin, respectively, in the myogenic-differentiated cells (Fig. 2C). Slight expression of these two markers was observed in the undifferentiated cells, which has also been observed in adipose-derived MSC (Rodriguez *et al.*, 2006). For chondrogenic differentiation, CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> cells were cultured as a micromass pellet which formed alcian blue stained spheroids containing cells reminiscent of chondrocytes surrounded by cartilaginous matrix (Fig. 2D). Cells cultured in 1% serum media failed to maintain a pellet structure

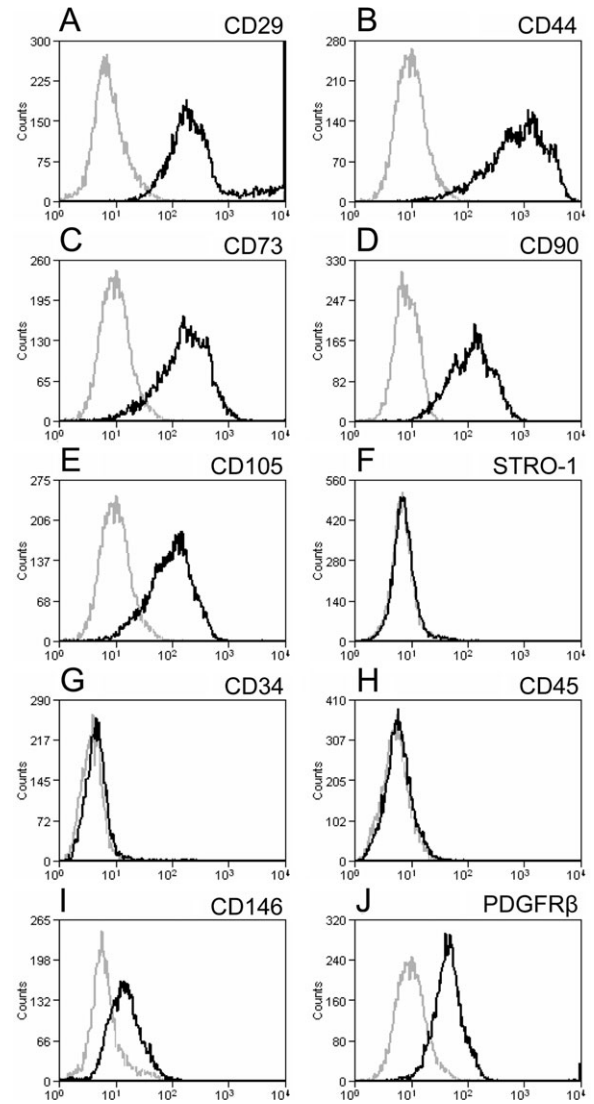


**Figure 2:** *In vitro* differentiation of FACS sorted and cultured CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> human endometrial stromal cells

Cells were cultured as monolayers or micromass pellets (chondrogenic) for 4 weeks in differentiation induction media for osteogenic, adipogenic, myogenic and chondrogenic lineages. (A) Osteogenic differentiated cells stained with alkaline phosphatase. Cells incubated with (+) and without (–) differentiation induction media expressed lineage specific genes, CBFA1 and PTHR1 as analysed by RT–PCR. (B) Adipogenic differentiation visualized by Oil Red O staining and RT–PCR analysis for adipocyte gene LPL and PPAR $\gamma$ 2. (C)  $\alpha$ SMA and smooth muscle-specific MHC protein in myogenic differentiated cells, and RT–PCR analysis for SMC genes calponin and caldesmon. (D) Paraffin embedded section of a micromass pellet cultured in chondrogenic induction medium and stained with alcian blue, and RT–PCR analysis for collagen type II and type X. (A–D) Representative data from a single sample representative of  $n = 4$  patient samples examined. Representative undifferentiated cells (control) stained with alkaline phosphatase, Oil Red O, alcian blue and SMC marker immunohistochemistry are also shown. 18S mRNA was the internal control. Scale bar, 50  $\mu$ m

in culture and could not be stained with alcian blue. RT–PCR analysis showed expression of early stage chondrogenic marker, collagen type II, and late stage chondrocyte marker, collagen type X, by the differentiated, but not undifferentiated control cells (Fig. 2D). None of the cells cultured in control medium stained in any histological or immunohistochemical differentiation assay for any of the lineages. Thus, FACS sorted CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> endometrial stromal cells demonstrated multipotentiality *in vitro*, similar to other MSC.

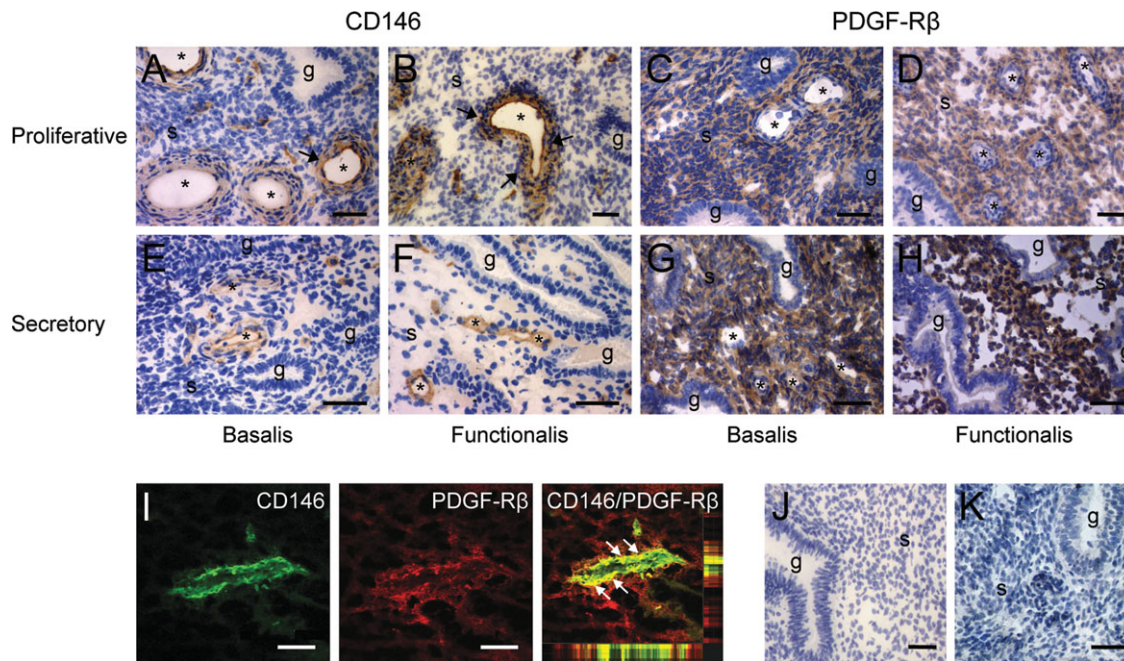
**Phenotype of endometrial CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> stromal cells**  
FACS sorted and cultured CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> cells were then examined for expression of typical MSC phenotypic markers.



**Figure 3:** Phenotyping of FACS sorted and cultured CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> human endometrial stromal cells for typical MSC phenotypic markers ( $n = 3$ )

Single parameter histograms for individual MSC markers (A–F), MSC exclusion markers (G, H), CD146 (I) and PDGF-R $\beta$  (J). Grey line indicates background fluorescence obtained with isotype control IgG or IgM

The majority of the cells showed strong expression levels for CD29, CD44, CD73, CD90 and CD105 by flow cytometry (Fig. 3A–E). Endometrial CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> cells expressed very little to no STRO-1 ( $0.25 \pm 0.2\%$ ,  $n = 3$ , Fig. 3F). The CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> cells were also negative for the haematopoietic stem cell marker CD34 (Fig. 3G), CD45 (Fig. 3H) and endothelial cell marker CD31 (Schwab and Gargett, unpublished data). A small percentage of CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> cells maintained expression of CD146 in culture ( $10.3 \pm 6.5\%$ ,  $n = 3$ , Fig. 3I), suggesting this marker is lost on more mature progeny, or is only expressed on MSC-like cells. In contrast, the majority of cells maintained PDGF-R $\beta$  expression ( $62.6 \pm 15.9\%$ ,  $n = 3$ , Fig. 3J) after 3–4 weeks in culture. These data suggest that cultured endometrial CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> share a similar phenotype to cultured MSC from bone marrow and adipose tissue.



**Figure 4:** Localization of CD146 and PDGF-Rβ in human endometrium

Full thickness human endometrial sections were single or double immunostained for CD146 and PDGF-Rβ. All endothelial cells and some perivascular (arrow) cells expressed CD146 (A, B, E, F). Surface expression of PDGF-Rβ on endometrial stromal cells and perivascular cells (C, D, G, H). Sections were stained from proliferative (A–D) and secretory (E–H) phase of the menstrual cycle to determine cyclical variation for basal (A, C, E, G) and functionalis (B, D, F, H) layers. (I) Co-localization (white arrow) of CD146 and PDGF-Rβ was found in venules and possibly capillaries in the functionalis layer perivascular stroma. The *x/z* and *y/z* planes are shown on the far right and underneath the merged images demonstrating co-localization of the two surface markers. (J, K) Isotype negative controls for CD146 and PDGF-Rβ, respectively. Scalebar, 50 μm. g, epithelial glands; s, stroma; \*, blood vessel

#### Localization of CD146<sup>+</sup>PDGF-Rβ<sup>+</sup> in human endometrium

Human endometrial tissue obtained from hysterectomy was then examined for the localization of CD146 and PDGF-Rβ by immunohistochemistry. CD146 membrane staining was localized to endothelial cells of capillaries (Fig. 4F), venules (Fig. 4B), arterioles (Fig. 4A) and spiral arterioles in both functionalis and basalis layers of human endometrium, and perivascular cells of some of these vessels (Fig. 4A, B, E, F). No CD146 immunostaining was observed on circulating cells within the vessels. Strong PDGF-Rβ membrane staining was observed on endometrial stromal cells, but not endothelial or epithelial cells (Fig. 4C, D, G, H). On some vessels, the perivascular cells in intimate contact with endothelial cells did not stain for PDGF-Rβ (Fig. 4D). Stronger perivascular staining was observed in some samples, particularly around arterioles (Fig. 4G, H). These immunostaining observations correlated with the flow cytometry data (Fig. 1D–F). Since many markers demonstrate cyclical changes in human endometrium in parallel with plasma sex steroid hormone fluctuations, we examined sections taken from proliferative and secretory stages of the menstrual cycle. The staining patterns were mostly consistent for both markers at all stages of the menstrual cycle. Confocal microscopy co-localized CD146 (green) and PDGF-Rβ (red) to the perivascular cells around venules and arterioles in the functionalis (Fig. 4I) and basalis layers, respectively. These data suggest that the CD146<sup>+</sup>PDGF-Rβ<sup>+</sup> cells are located perivascularly in human endometrium.

#### Discussion

##### Prospective isolation of CD146<sup>+</sup>PDGF-Rβ<sup>+</sup> endometrial MSC-like cells

The major finding of this study was the identification of two perivascular markers, CD146 and PDGF-Rβ, which enabled the prospective isolation of MSC-like cells from human endometrial tissue. The small population of fresh endometrial stromal cells co-expressing both markers was enriched 15-fold for colony-forming cells compared with CD146<sup>−</sup>PDGF-Rβ<sup>−</sup> cells, expressed key MSC phenotypic markers and was multipotent, differentiating into four mesenchymal lineages *in vitro*. This is the first study to show that a subset of endometrial stromal cells differentiate into cells of adipogenic, osteogenic, myogenic and chondrogenic cell lineages. CD146<sup>+</sup>PDGF-Rβ<sup>+</sup> stromal cells were localized to the perivascular region of some blood vessels in both the functionalis and basalis layers of human endometrium. Since this subset of CD146<sup>+</sup>PDGF-Rβ<sup>+</sup> endometrial stromal cells exhibit typical properties of bone marrow and adipose tissue MSC, such as colony-forming ability, multipotency, MSC marker expression and perivascular location, we suggest that they are MSC.

##### Identification of markers for prospective isolation of MSC-like cells

This is the first study to use CD146 and PDGF-Rβ in combination to prospectively isolate MSC. The combination of CD146 and PDGF-Rβ substantially enriched for endometrial

MSC-like cells, resulting in a colony-forming capacity of 8% for CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> stromal cells. This is a 6-fold increase over unfractionated endometrial stromal cells (Chan *et al.*, 2004; Schwab *et al.*, 2005), and a 400-fold enrichment of large colony-forming stromal cells that have capacity to undergo multilineage differentiation (Gargett *et al.*, 2005). Previous studies using various markers to prospectively isolate MSC from other tissues have shown varying levels of enrichment, from as little as 2–100-fold. Markers which have been used to prospectively isolate MSC include STRO-1 (Gronthos and Simmons, 1995; Shi and Gronthos, 2003), CD63 (HOP-26) (Joyner *et al.*, 1997; Stewart *et al.*, 2003), CD105 (Campioni *et al.*, 2003; Boiret *et al.*, 2005), CD49a ( $\alpha$ 1-integrin) (Deschaseaux and Charbord, 2000; Stewart *et al.*, 2003), LNGFR (Quirici *et al.*, 2002) and vascular cell adhesion molecule (Jones *et al.*, 2006). A recent study showed LNGFR enriches 40-fold for colony-forming bone marrow MSC, and when combined with PDGF-R $\beta$  produced a 45-fold enrichment (Buhring *et al.*, 2007). Despite the addition of PDGF-R $\beta$  marginally increasing the enrichment of bone marrow MSC, all the colony-forming cells were contained within the PDGF-R $\beta$ <sup>+</sup> fraction, validating it as a marker for prospective isolation of MSC (Buhring *et al.*, 2007). It will be important to examine LNGFR together with PDGF-R $\beta$  in human endometrium. Currently, there is no consensus on the most effective marker or combination of markers for prospective isolation of MSC, since very few markers have been compared across different source tissues. Cultured endometrial MSC-like cells express bone marrow and adipose tissue MSC phenotypic markers. On the basis of the present study and those of Shi and Gronthos (2003) and Buhring *et al.* (2007), it is likely that the combination of CD146 and PDGF-R $\beta$  would be suitable for the prospective isolation of other MSC.

It has been suggested that MSCs may be pericytes, and that pericytes have MSC activity (Perkins and Fleischman, 1990; Doherty *et al.*, 1998; Bianco *et al.*, 2001; Kinner *et al.*, 2002; Short *et al.*, 2003; Tintut *et al.*, 2003; Brachvogel *et al.*, 2005), however, the lack of pericyte-specific markers has hampered definitive investigations. Perivascular cell markers used for prospective isolation of MSC include CD146 and 3G5, where CD146 enriches for bone marrow and dental pulp MSC, although 3G5, a pericyte marker, was only expressed in low levels on colony-forming bone marrow and dental pulp cells (Shi and Gronthos, 2003). STRO-1, the most commonly used marker for prospective isolation of MSC, is a perivascular cell marker, staining the perivascular regions in bone marrow and dental pulp, as well as circulating MSC (Shi and Gronthos, 2003). However, it is unlikely that endometrial CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> cells are circulating stromal cells, since most clonogenic stromal cells are STRO-1<sup>−</sup> (Schwab and Gargett, unpublished data). Annexin A5, a perivascular cell marker, has also been shown to identify MSC-like cells in the mouse bone marrow (Brachvogel *et al.*, 2005). The perivascular staining by CD146 and PDGF-R $\beta$  supports the possibility that MSC are pericytes. A study of mouse endometrium provides further evidence that MSC are perivascular, identifying stromal label-retaining

cells, putative MSC, to a perivascular location (Chan and Gargett, 2006). These cells were also  $\alpha$ SMA positive, further supporting a perivascular cell identity (Chan and Gargett, 2006). Similarly, the present study has demonstrated that CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> endometrial MSC-like cells share a perivascular location with MSC from bone marrow, adipose tissue and dental pulp MSC.

The percentage of colony-forming mesenchymal cells present in human endometrial stroma appears greater than in bone marrow or dental pulp, with a baseline colony-forming capacity of 1.2% (Chan *et al.*, 2004; Schwab *et al.*, 2005), compared with 0.1–0.01% for dental pulp and bone marrow (Shi and Gronthos, 2003). However, bone marrow contains a large number of contaminating blood-forming mononuclear cells affecting the true percentage of MSC identified. Human endometrium also contains a significant number of CD45<sup>+</sup> cells which vary in number during the menstrual cycle, however, their removal from our analysis contributed to the relatively high cloning efficiencies observed for endometrial stromal cells. Since the percentage of stromal cells present in bone marrow is not clear, it is difficult to compare the relative proportion of MSC in these tissues. However, the baseline colony-forming ability of endometrial stromal cells is comparable to most enriched bone marrow MSC populations (Deschaseaux and Charbord, 2000; Shi and Gronthos, 2003; Buhring *et al.*, 2007). The further enrichment of colony-forming endometrial MSC-like cells on the basis of CD146 and PDGF-R $\beta$  co-expression suggests that the reservoir of MSC in human endometrium may be similar to bone marrow or dental pulp. Endometrium may therefore be an attractive source of MSC-like cells for tissue engineering purposes, obtainable with no extra morbidity than that required for bone marrow or adipose tissue.

### ***The role of MSC-like cells in endometrial proliferation***

This study extends previous observations that small populations of colony-forming epithelial and stromal cells are present in human endometrium (Chan *et al.*, 2004; Schwab *et al.*, 2005) and provides further evidence for the existence of adult stem/progenitor cells in this highly regenerative tissue. Approximately half a centimetre of human endometrial stroma grows each menstrual cycle, and it is possible that endometrial MSC-like cells play an important role in this cyclical regeneration (Gargett, 2007). The present study showed CD146 and PDGF-R $\beta$  co-localization in the functionalis as well as the basalis layer of human endometrium, implying that some CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> cells may be shed at menstruation. Since only 8% of these cells exhibit this colony-forming capacity, it is not known whether they are preferentially located in the basalis or functionalis layers of the endometrium. The MSC-like cells identified in this study may play a role in the pathogenesis of gynaecological disease, such as endometriosis and adenomyosis, due to inappropriate shedding of stem cells or alterations in the stem cell niche (Gargett and Chan, 2006; Gargett, 2007). Further characterization of these newly identified endometrial MSC-like cells will result in an

improved understanding of endometrial physiology and gynaecological disease.

Very little is known about MSC-like cells in human endometrium, including their origin. They may be derived from remnant fetal stem cells, which persist after uterine development (Snyder and Loring, 2005), or from bone marrow MSC, which may migrate into the endometrium every menstrual cycle (Gargett and Chan, 2006; Du and Taylor, 2007; Gargett, 2007). The latter is supported by the demonstration of significant donor–host chimerism within the endometrium of women who received single antigen HLA-mismatched bone marrow transplants (Taylor, 2004). However, it is not clear whether this chimerism is representative of normal endometrial physiology or resulted from depletion of the endogenous endometrial MSC population by pre-transplant conditioning. Further studies in mice have demonstrated the presence, <0.01%, of bone marrow-derived stem cells contributing to mouse endometrium (Du and Taylor, 2007). However, this small number of cells would seem insufficient to produce the substantial endometrial regeneration which occurs every cycle, thus resident endometrial stem cells are a more likely source for this regeneration. Further studies are required to establish the role bone marrow-derived cells play in endometrial regeneration and their interactions with resident endometrial stem cells.

The findings of this study are significant in the field of endometrial biology and MSC characterization, however, there are several technical issues worthy of future consideration. The differentiation assays conducted in this study used pooled clonally-derived CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> cells, rather than single expanded cell clones, and thus did not demonstrate the true potential of a single CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> cell to undergo multilineage differentiation. Despite this limitation, differentiation was observed in the majority of the cells cultured in each of the induction media, suggesting that all cells derived from single pooled clonogenic CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> cells have capacity to differentiate into four mesenchymal lineages. Nonetheless, differentiation of single CD146<sup>+</sup>PDGF-R $\beta$ <sup>+</sup> colonies is warranted to confirm this finding. It would also be worth investigating the stem cell properties of the CD146<sup>−</sup>PDGF-R $\beta$ <sup>+</sup> and CD146<sup>+</sup>PDGF-R $\beta$ <sup>−</sup> populations of cells identified in the flow cytometry histograms. This would confirm the validity of using these two perivascular markers for the prospective isolation of MSC from endometrial and other tissues.

This is the first study to prospectively isolate a small subpopulation of stromal cells from human endometrium with MSC properties of colony-forming ability and multipotency. These cells express typical MSC markers and are found in a perivascular location in the basalis endometrial layer, as well as in the upper functionalis layer which is shed. The ability to prospectively isolate MSC-like cells from this highly regenerative tissue provides an opportunity to characterize these cells further. It also provides an alternative readily available autologous source of MSC for possible use in future cell-based therapies and tissue engineering applications such as the development of tissue constructs with biological scaffolds for use in pelvic floor prolapse repair surgery (Gargett and Chan, 2006).

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## Supplementary material

Supplementary material is available at <http://humrep.oxfordjournals.org/>.

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