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Endometrial stem/progenitor cells: the first 10 years

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BACKGROUND: The existence of stem/progenitor cells in the endometrium was postulated many years ago, but the first functional evidence was only published in 2004. The identification of rare epithelial and stromal populations of clonogenic cells in human endometrium has opened an active area of research on endometrial stem/progenitor cells in the subsequent 10 years.

METHODS: The published literature was searched using the PubMed database with the search terms 'endometrial stem cells and menstrual blood stem cells' until December 2014.

RESULTS: Endometrial epithelial stem/progenitor cells have been identified as clonogenic cells in human and as label-retaining or $CD44^{+}$ cells in mouse endometrium, but their characterization has been modest. In contrast, endometrial mesenchymal stem/stromal cells (MSCs) have been well characterized and show similar properties to bone marrow MSCs. Specific markers for their enrichment have been identified, $CD146^{+}$ $PDGFR\beta^{+}$ (platelet-derived growth factor receptor beta) and $SUSD2^{+}$ (sushi domain containing-2), which detected their perivascular location and likely pericyte identity in endometrial basalis and functionalis vessels. Transcriptomics and secretomics of $SUSD2^{+}$ cells confirm their perivascular phenotype. Stromal fibroblasts cultured from endometrial tissue or menstrual blood also have some MSC characteristics and demonstrate broad multilineage differentiation potential for mesodermal, endodermal and ectodermal lineages, indicating their plasticity. Side population (SP) cells are a mixed population, although predominantly vascular cells, which exhibit adult stem cell properties, including tissue reconstitution. There is some evidence that bone marrow cells contribute a small population of endometrial epithelial and stromal cells. The discovery of specific markers for endometrial stem/progenitor cells has enabled the examination of their role in endometrial proliferative disorders, including endometriosis, adenomyosis and Asherman's syndrome. Endometrial MSCs (eMSCs) and menstrual blood stromal fibroblasts are an attractive source of MSCs for regenerative medicine because of their relative ease of acquisition with minimal morbidity. Their homologous and non-homologous use as autologous and allogeneic cells for therapeutic purposes is currently being assessed in preclinical animal models of pelvic organ prolapse and phase I/II clinical trials for cardiac failure. eMSCs and stromal fibroblasts also exhibit non-stem cell-associated immunomodulatory and anti-inflammatory properties, further emphasizing their desirable properties for cell-based therapies.

CONCLUSIONS: Much has been learnt about endometrial stem/progenitor cells in the 10 years since their discovery, although several unresolved issues remain. These include rationalizing the terminology and diagnostic characteristics used for distinguishing perivascular stem/progenitor cells from stromal fibroblasts, which also have considerable differentiation potential. The hierarchical relationship between clonogenic epithelial progenitor cells, endometrial and decidual SP cells, $CD146^{+}PDGFR\text{-}\beta^{+}$ and $SUSD2^{+}$ cells and menstrual blood stromal fibroblasts still needs to be resolved. Developing more genetic animal models for investigating the role of endometrial stem/progenitor cells in endometrial disorders is required, as well as elucidating which bone marrow cells contribute to endometrial tissue. Deep sequencing and epigenetic profiling of enriched populations of endometrial stem/progenitor cells and their differentiated progeny at the population and single-cell level will shed new light on the regulation and function of endometrial stem/progenitor cells.

Key words: endometrium / endometrial stem cells / mesenchymal stem cells / regenerative medicine / epithelial progenitor cells / endometriosis / adenomyosis / menstrual blood / sushi domain containing-2 / immunomodulation

Introduction

It is 10 years since the first evidence for the existence of adult stem cell populations in the endometrium was published. In this study, rare clonogenic cells or colony-forming units (CFUs) were identified in purified populations of human endometrial epithelial and stromal cells isolated from hysterectomy tissue (Chan *et al.*, 2004). Concurrently, it was reported that some epithelial and stromal cells in human endometrium of HLA-antigen-mismatched bone marrow transplant recipients were of donor origin (Taylor, 2004). Subsequently, label-retaining cells (LRCs) were identified in mouse endometrium (Chan and Gargett, 2006). This early direct evidence of stem/progenitor cells in the endometrium was then summarized in the first comprehensive review on endometrial stem/progenitor cells published in *Human Reproduction Update* (Gargett, 2007). Later in 2007, a second publication on murine endometrial LRCs confirmed and extended the original findings (Cervelló *et al.*, 2007). The 2007 *Human Reproduction Update* review also provided a blueprint on how to identify stem/progenitor populations in tissues and organs not previously characterized for adult stem cell activity, focusing on functional assays used in other organs. These included CFU activity, self-renewal, differentiation, proliferative potential, label retention and tissue reconstitution assays. It pointed out the importance of linking stem cell markers to functional stem cell activity. It also summarized the indirect evidence for stem/progenitor cells in the highly regenerative human endometrium gleaned from the literature.

In this comprehensive review, we summarize the progress that has been made on the identification and characterization of endometrial stem/progenitor cells in both human and mouse models since this last review. We will focus on the identity and *in vivo* location of the stem/progenitor cells as specific markers and approaches that have now been identified for their purification, particularly for the mesenchymal stem/stromal cell (MSC) population. Specific markers also allow 'omics' characterization of endometrial stem/progenitor cell populations. The role of bone marrow-derived and endogenous stem/progenitor cells in endometrial proliferative disorders, including endometriosis, adenomyosis, thin dysfunctional endometrium and Asherman's syndrome, will also be covered. The review will also describe the use of the endometrial MSCs (eMSCs) as potential cell-based therapies for several women's health and other diseases. Finally, we will raise unresolved issues facing the field, particularly the similarities and differences between eMSCs and endometrial stromal fibroblasts and the identity of bone marrow-derived cells involved in endometrial function.

Methods

The published literature was searched using the PubMed database with the search terms 'endometrial stem cells and menstrual blood stem cells' until December 2014. Only original articles in English were included. The review includes human, mouse and domestic animal studies.

Identity of stem/progenitor cells in human endometrium

The immense regenerative capacity of human endometrium and its bilayer structure, in which the upper functionalis is shed at menses and regenerates from the remaining basalis in the subsequent cycle (Padykula *et al.*, 1984; Padykula, 1991; Spencer *et al.*, 2005; Jabbour *et al.*, 2006), has been the motivation for investigators to identify and characterize endometrial stem/progenitor cell populations. The endometrium comprises luminal and glandular epithelial cells and a substantial vascularized stroma; hence, a number of laboratories have focused on identifying epithelial and mesenchymal stem/progenitor cells (Gargett *et al.*, 2012).

Epithelial progenitor cells

Endometrial glands lined with a pseudostratified columnar epithelium extend from the luminal epithelium to the endometrial/myometrial junction. During menses, the basal component of the glands remains in the basalis layer and epithelial cells re-epithelialize the exposed surface and then proliferate to regenerate the new functionalis under the influence of rising estrogen levels (Gargett *et al.*, 2012). It was hypothesized that remaining glands of the basalis contained the epithelial progenitor cell population (reviewed in Gargett, 2007). To date, epithelial progenitor cells have been identified as CFUs in cell suspensions derived from hysterectomy tissue, which includes the basalis (Chan *et al.*, 2004; Schwab *et al.*, 2005; Gargett *et al.*, 2009) and they are present in the side population (SP) (see SP cells). The large, single-cell-derived epithelial CFU, comprising 0.08% of epithelial cells serially cloned at least three times, a measure of self-renewal *in vitro*, underwent 34 population doublings, indicating high proliferative potential, and differentiated into large gland-like structures in three-dimensional (3D) culture (Gargett *et al.*, 2009). The 3D cultures included a stromal feeder layer, which likely provided epithelial progenitor cell niche factors that promote differentiation and morphogenesis. To date, there are no publications identifying specific markers that isolate epithelial progenitor cells in human endometrium with these stem cell properties.

There has been progress in identifying a marker of endometrial basalis epithelium, the postulated location of endometrial epithelial stem/progenitor cells (Fig. 1D). Stage-specific embryonic antigen 1 (SSEA-1, or CD15), a Lewis X epitope of a glycoprotein expressed on differentiating human embryonic stem (hES) cells and human neutrophils (Wright and Andrews, 2009), was expressed most strongly in the basalis glands of endometrial tissue from hysterectomy samples of cycling women (Valentijn *et al.*, 2013). SSEA-1 was also strongly expressed in the glandular epithelium of postmenopausal endometrium, which has gene profiles similar to basalis epithelium of cycling women (Gaide Chevronnay *et al.*, 2009; Nguyen *et al.*, 2012). The stem cell activity of human endometrial SSEA-1$^+$ cells has not yet been examined. However, cultured SSEA-1$^+$ endometrial epithelial cells had greater telomerase activity and longer telomeres and were more quiescent with lower proliferation rates than SSEA-1$^-$ epithelial cells, features of progenitor cell populations. They also formed spheroids in 3D culture and differentiated into spheres with polarized epithelium. SSEA-1$^+$ cells expressed lower levels of estrogen receptor-α (ESR1) and progesterone receptor (PR) when compared with the SSEA-1$^-$ cells (Valentijn *et al.*, 2013), suggesting a less differentiated cell phenotype and reliance on growth factors released from ESR1-expressing niche cells to mediate estrogen-induced proliferative signals. In contrast, ESR1 is detected in basalis glands of normal endometrium throughout the menstrual cycle, whereas functionalis expression is restricted to the proliferative stage (Leyendecker *et al.*, 2002). This suggests that human endometrial epithelial progenitor cells will be a subset of the SSEA-1$^+$ population that may reside in the functionalis abutting the basalis. The surface marker LGR5 (leucine-rich repeat-containing G-protein-coupled receptor 5), which identifies rapidly cycling murine intestinal epithelial stem cells (Barker *et al.*, 2007), was located on rare epithelial cells in human endometrium in the lower functionalis near the basalis (Gil-Sanchis *et al.*, 2013). LGR5 was dynamically expressed in the endometrium and was negatively regulated by estrogen and progesterone in mice (Krusche *et al.*, 2007; Sun *et al.*, 2009). Whether LGR5 and SSEA-1 will be markers for endometrial epithelial progenitor cells awaits assessment using stem cell assays.

Multipotent MSCs

MSCs were originally identified in bone marrow cultures as clonogenic fibroblasts (CFU-F). It became apparent that plastic adherent bone marrow cultures comprised MSCs as well as fibroblasts, and to reflect this heterogeneity, they were recently renamed multipotent mesenchymal stromal cells and the MSC acronym was retained (Table I). MSCs have been identified in adipose tissue (Zuk *et al.*, 2002), endometrium (Schwab and Gargett, 2007; Gargett *et al.*, 2009) and many other organs over the last decade (Crisan *et al.*, 2008). The defining features of bone marrow MSCs (bmMSCs) are plastic adherence, clonogenicity, multilineage differentiation into bone and marrow lineages (osteocytes, chondrocytes, adipocytes) *in vitro* and a surface phenotype (CD29$^+$, CD44$^+$, CD73$^+$, CD90$^+$, CD105$^+$, CD146$^+$, CD31$^-$, CD34$^-$ and CD45$^-$) distinguishing them from haematopoietic stem cells (HSCs) also resident in marrow (Dominici *et al.*, 2006). Clonogenic endometrial cells from human (Gargett *et al.*, 2009; Cervelló *et al.*, 2010) and porcine

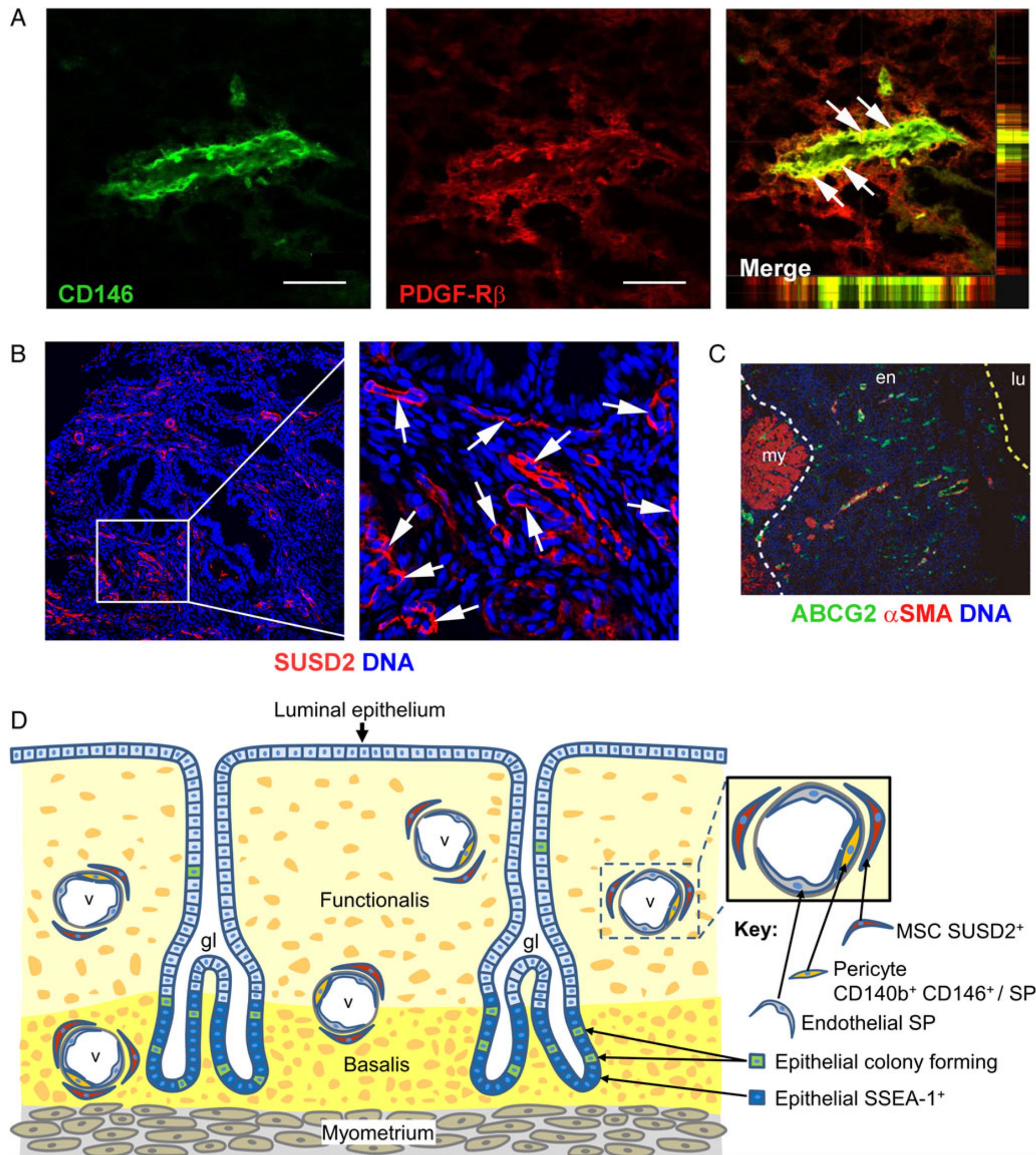

Figure 1 Localization of human endometrial mesenchymal stem cells. (**A**–**C**) Immunofluorescence images of human endometrium showing perivascular identity of human eMSCs. (A) Co-localization (white arrows) of CD146 and platelet-derived growth factor receptor beta (PDGF-Rβ) in pericytes of venules and possibly capillaries in the functionalis 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. (B) Perivascular SUSD2 expression (white arrows). (C) ATP-binding cassette, subfamily G member 2 (ABCG2) and αSMA co-staining showing perivascular and endothelial identity of SP cells. The white dotted lines indicate the junction between the endometrium (en) and myometrium (my) and yellow dotted line indicates the luminal surface (lu) of the uterine epithelium. (**D**) Schematic showing location of stem/progenitor cells identified in the human endometrium. Epithelial progenitor cells are postulated to be a subpopulation of cells located in the base of the glands in the basalis, identified by SSEA-1. Sushi domain containing-2$^+$ (SUSD2$^+$) eMSCs are perivascular cells. eMSC co-expressing CD146 and PDGFRβ/CD140b are most likely pericytes, as they are located adjacent to endothelial cells in vessels (v) in both the basalis and the functionalis. SP cells are a heterogeneous population comprising CD31$^+$ endothelial cells and CD140b$^+$CD146$^+$ pericytes. Scale bar in (A) = 50 μm. (A) Reprinted with permissions from Schwab and Gargett (2007). (C) Reprinted with permissions from Masuda *et al.* (2010). (D) Adapted from Gurung *et al.* (2015).

(Miernik and Karasinski, 2012) species and the human CD146$^+$ PDGFR-β$^+$ and SUSD2$^+$ [sushi domain containing-2 (previously W5C5$^+$)] or endometrial stromal subpopulations (Schwab and Gargett, 2007; Masuda *et al.*, 2012; Ulrich *et al.*, 2014c) exhibit the same *in vitro* properties as bmMSCs. Cultured fibroblasts from the endometrium (stromal cells), bone marrow and many organs also exhibit

Table I Glossary of cell types.

Cell type	Definition
Bone marrow mesenchymal stem/stromal cells (bmMSCs)	Multipotent, highly proliferative, self-renewing adult stromal stem cells found in the bone marrow that display immunomodulatory properties. Plastic adherent cultures are heterogeneous and contain perivascular cells and stromal fibroblasts
Endometrial MSCs (eMSCs)	Multipotent, highly proliferative, self-renewing adult stromal stem cells found in a perivascular location in the endometrium and distinct from endometrial stromal fibroblasts
Endometrial regenerative cell (ERC)	A collective term for MSC and stromal cells isolated from menstrual blood that are highly proliferative and multipotent (see Table IV for acronyms)
Human embryonic stem (hES) cells	Pluripotent stem cells derived from the inner cell mass of a blastocyst, able to differentiate into cells of all three germ layers
Haematopoietic stem cells (HSCs)	Multipotent, self-renewing non-plastic adherent stem cells that reside in the bone marrow and are responsible for producing all blood cell types
Induced pluripotent stem (iPS) cells	A pluripotent stem cell produced from an adult cell through reprogramming by introduction of pluripotency genes or transcription factors
Label retaining cells (LRCs)	A quiescent stem-like cell that retains a DNA label over a longer period of time than more mature cells
Multipotent mesenchymal stem/stromal cells (MSCs)	A stromal cell that exhibits characteristics of clonogenicity, multipotency and self-renewal and is responsible for tissue maintenance
Main population (MP) cells	A cell that does not efflux the Hoechst dye or a non-SP cell. Differentiated cells likely derived from an SP cell
Progenitor or transit-amplifying cells	A cell that has less potential than a stem cell, i.e. undergoes less differentiation and reduced proliferative capacity
Side population (SP) cells	A cell that is able to efflux the Hoechst dye, through expression of the ABCG2 transporter that pumps organic molecules out of the cell. A property of stem cells
Stem cell niche	The microenvironment in which stem cells are found, comprising niche cells and extracellular matrix, which directly or indirectly interact with the stem cells to control cell fate decisions regarding proliferation, self-renewal and differentiation
Stromal fibroblasts (fibroblasts)	Main component of stromal or connective tissue. A non-stem cell which lacks clonogenicity but can differentiate into mesodermal lineages and expresses common phenotypic cell surface markers. Gene profiling and RNA sequencing show that endometrial stromal fibroblasts are closely related but distinct from endometrial MSCs
SUSD2 (W5C5)	A cell surface marker that enriches for endometrial and bone marrow MSCs, also known as W5C5 and detected by the W5C5 antibody

these classic bmMSC properties *in vitro*, prompting MSC biologists to question the utility of these defining features (Hematti, 2012; Bianco *et al.*, 2013; Phinney and Sensebé, 2013). Rather, the ability for a single cell to generate heterotropic bone or bone marrow organs (ossicles) *in vivo* is now considered the definition of bmMSC (Sacchetti *et al.*, 2007; Bianco *et al.*, 2013). The analogous definition for eMSCs would be the generation of a vascularized stroma with the capacity to differentiate into decidualized stroma when transplanted into an animal at the single-cell level. This has not been achieved; however, clonally derived, purified SUSD2$^+$ eMSCs produced endometrial stroma and incorporated into renal parenchymal blood vessels when xenografted under the kidney capsule of immunocompromised NSG mice (Masuda *et al.*, 2012). Likewise, endometrial stromal SP cells with some bmMSC *in vitro* properties produced stroma in subrenal xenografts (Masuda *et al.*, 2010; Cervelló *et al.*, 2011). In contrast, human endometrial stromal cell (fibroblast) cultures have more limited differentiation capacity *in vitro*, usually into single bone, marrow or non-mesodermal lineages (Wolff *et al.*, 2007; Dimitrov *et al.*, 2008).

Markers of eMSCs

Specific markers or combinations of markers have been identified for eMSCs (Gargett and Masuda, 2010; Lv *et al.*, 2014a) (Table II). The CD146$^+$PDGFR-β^+ subpopulation comprises 1.5% of endometrial stromal cells (Schwab and Gargett, 2007). Almost all stromal CFUs with *in vitro* bmMSC properties are found in the CD146$^+$PDGFR-β^+ CD45$^-$ fraction (CD45 excludes leukocytes). This marker set identified an *in vivo* perivascular location for eMSCs in both the functionalis and basalis of human endometrium (Fig. 1A), indicating that the CD146^{+-} PDGFR-β^+ subpopulation can be harvested from endometrial biopsy samples (Schüring *et al.*, 2011; Spitzer *et al.*, 2012) and will be shed in menstrual blood (Gargett and Masuda, 2010). Similarly, bone marrow and many other MSCs have a perivascular location *in vivo* (Shi and Gronthos, 2003; Sacchetti *et al.*, 2007; Crisan *et al.*, 2008). STRO-1, the most widely used single bmMSC marker, is also expressed by endothelial and perivascular cells in human endometrium, but failed to enrich for stromal CFUs (Schwab *et al.*, 2008). Screening endometrial cell suspensions with a panel of perivascular markers identified a single marker, SUSD2, for isolating clonogenic eMSC from human endometrium (Masuda *et al.*, 2012) (Fig. 1B). This marker, sometimes referred to as W5C5, recognizes the sushi domain containing-2 (SUSD2) antigen (Sivasubramaniyan *et al.*, 2013). Purification with SUSD2 antibody-labelled magnetic beads is less damaging to the cells increasing the yield of eMSCs compared with flow cytometry sorting using the CD146^{+-} PDGFR-β^+ markers, indicating the utility of magnetic bead sorting over flow cytometry sorting (Schwab and Gargett, 2007; Masuda *et al.*, 2012). The relationship between SUSD2$^+$ eMSC and existing markers of eMSCs was explored by flow cytometry (Masuda *et al.*, 2012). Most

Table II Surface marker phenotype of human endometrial marker-enriched mesenchymal stem cells and stromal cell populations.

Cell type investigated	Marker expression										Other markers investigated	References
	CD29	CD44	CD73	CD90	CD105	PDGFRβ	CD146	CD31	CD34	CD45		
CD146^{+} PDGFRβ^{+} endometrial stromal cells	+	+	+	+	+				−	−	STRO-1^{-}	Schwab and Gargett (2007)
SUSD2^{+} endometrial stromal cells	+	+	+	+	+	+	+	−		−	CD117^{+} STRO-1^{+}	Masuda *et al.* (2012)
PMP SUSD2^{+} endometrial stromal cells	+	+	+		+	+	+					Ulrich *et al.* (2014c)
Briefly cultured endometrial SP cells									−	−	CD9^{-} CD13^{-}	Kato *et al.* (2007)
Endometrial SP cells					+		+	+	+		CD13^{+} CD49f^{+} EMA^{+}	Tsuji *et al.* (2008)
Endometrial stromal SP cells			+	+				−	−	−	CD9^{+} VM^{+} CD133^{-} STRO-1^{-} ESR1^{-} PR^{-}	Cervelló *et al.* (2010, 2011)
Endometrial SP cells				+	+		+	+	+		CD10^{+} CD144^{+} CD326^{+} CD133^{-} SUSD2^{+}	Masuda *et al.* (2010), Miyazaki *et al.* (2012)
Endometrial stromal colonies	+		+	+					−	−	CD14^{-} CD19^{-} CD56/16^{-} HLA-DR^{-}	Dimitrov *et al.* (2008)
Endometrial stromal colonies	+	+	+	+	+	+	+	−	−	−		Gargett *et al.* (2009)
Endometrial stromal colonies	+	+			+						CD81^{+}	Li *et al.* (2010)
Endometrial stromal colonies			+	+	+		+		−		CD14^{-}	Schüring *et al.* (2011)
Endometrial stromal colonies				+	+		+	−	−	−		Ai *et al.* (2012)
Endometrial large stromal colonies			+	+			+		−	−	VM^{+} CK^{-}	Yang *et al.* (2014)
Passaged endometrial stromal cells		+		+	+		+	−	−	−	CD133^{-}	Ebrahimi-Barough *et al.* (2013)
Passaged endometrial stromal cells				+		+	+	−		−		Santamaria *et al.* (2011)
Passaged endometrial stromal cells				+		+	+	−		−	αSMA^{+}(5%)	Wolff *et al.* (2011)
Passaged endometrial stromal cells	+			+					−	−	CK^{-}	Wang *et al.* (2012b)

PDGFRβ^{+}, platelet-derived growth factor receptor beta; αSMA, alpha smooth muscle actin; CK, cytokeratin; EMA, epithelial membrane antigen; ESR1, estrogen receptor alpha; PMP, postmenopausal; PR, progesterone receptor; VM, vimentin.

SUSD2$^+$ cells expressed PDGFR-β, whereas all SUSD2$^+$CD146$^+$ cells were positive for PDGFR-β. These SUSD2$^+$CD146$^+$ cells generated more CFUs than the CD146$^+$PDGFR-β$^+$ subpopulation, highly expressed SUSD2 (SUSD2^{hi}) and were increased in proliferative endometrium, suggesting their role in growth of the endometrial functionalis stroma. The surface phenotype of SUSD2$^+$ endometrial stromal cells indicates that they are predominantly CD90$^+$ (93%) perivascular cells, which are CD90^{hi} in the human endometrium (Schwab *et al.*, 2008). SUSD2$^+$ cells also express Stro-1 (60%) (Masuda *et al.*, 2012).

Other less well-known markers of human eMSCs include MSC antigen-1 (MSCA-1), which is suitable for prospective isolation of bmMSCs (Sobiesiak *et al.*, 2010; Lv *et al.*, 2014a). The MSCA-1 is the ectoenzyme, tissue non-specific alkaline phosphatase also expressed on hES and on endometrial CD146$^+$ cells. It has less value for prospective isolation of eMSC as MSCA-1 is also expressed on the apical surface of endometrial glandular epithelial cells (Sobiesiak *et al.*, 2010).

SUSD2$^+$ cells were also identified in the atrophic and estrogen-treated postmenopausal endometrium (Ulrich *et al.*, 2014c). Although stromal CFUs were found in the SUSD2$^+$ subpopulation, the percentage was less than that in the premenopausal endometrium and the capacity for mesodermal *in vitro* differentiation was less in ageing eMSCs. Perivascular eMSCs do not express ESR1, unlike the stromal fibroblast population, showing further differences between the two populations (Ulrich *et al.*, 2014c). Gene profiling of freshly isolated CD146$^+$PDGFR-β$^+$ eMSCs and CD146$^-$PDGFR-β$^+$ endometrial stromal cells showed 762 differentially expressed genes with 374 upregulated in eMSCs and 384 downregulated, and by implication the latter were upregulated in stromal fibroblasts (Spitzer *et al.*, 2012). These results show that endometrial perivascular cells are a population distinct from stromal fibroblasts. The identification of specific markers of eMSCs enables their prospective isolation using magnetic bead or flow cytometry sorting for further characterization and for potential use in cell-based therapies.

SP cells

Adult stem cells have been identified in many organs by their ability to rapidly efflux Hoechst 33342 DNA binding dye (Table I). These cells are identified by a discrete SP using dual colour flow cytometry (Goodell *et al.*, 1997; Challen and Little, 2006) and can be sorted for further characterization. Human endometrium contains up to 5% SP cells in freshly isolated (Tsuji *et al.*, 2008; Cervelló *et al.*, 2010; Masuda *et al.*, 2010) and short-term cultured (Kato *et al.*, 2007) human endometrial cells (Table II). The SP number varies considerably among subjects, although higher percentages were found in proliferative (Tsuji *et al.*, 2008; Masuda *et al.*, 2010) and menstrual (Kato *et al.*, 2007) stages. Immunostaining with the SP marker, ABCG2, labelled endothelial cells lining blood vessels in both the functionalis and the basalis (Fig. 1C) (Tsuji *et al.*, 2008; Masuda *et al.*, 2010). Flow cytometric analysis indicated that the SP comprises a mixed cell population: CD31$^+$ endothelial cells (51%), CD326$^+$ (EpCAM) epithelial cells (27%) and CD10$^+$ or PDGFR-β$^+$ stromal cells (10–14%) (Table II) (Miyazaki *et al.*, 2012). The main population (MP), which does not efflux Hoechst, has an epithelial and stromal composition similar to the SP, but significantly fewer CD31$^+$ endothelial cells. Although the SP is enriched for the CD146$^+$ PDGFR-β$^+$ perivascular eMSC population compared with the MP, SUSD2$^+$ cells are equally distributed between the SP (11%) and the MP (14%) (Miyazaki *et al.*, 2012). Epithelial and stromal SPs have typical *in vitro* characteristics of MSCs (Table III) and telomerase activity at a level intermediate between embryonic stem cells and mature cells (Cervelló *et al.*, 2010). Similar to SUSD2$^+$ eMSCs, the endometrial SP does not express ESR1 or PR (Cervelló *et al.*, 2010; Masuda *et al.*, 2010), but expresses ESR2 in keeping with their endothelial predominance. Freshly sorted human endometrial SP cells were quiescent (85% in the G0 phase of the cell cycle), a typical feature of adult stem cells, and showed little clonogenic growth in culture. Cultured SP cells were primarily in G1 and G1/M/S phases and showed enhanced clonogenicity (Tsuji *et al.*, 2008).

The SP reconstitutes endometrial tissue *in vivo* when transplanted underneath the kidney capsule of immunocompromised mice (Table III) (Masuda *et al.*, 2010; Cervelló *et al.*, 2011). Lentiviral labelling with the red fluorescent protein Tandem Tomato (TdTom) has enabled tracing of xenografted SP and MP cells *in vivo* (Miyazaki *et al.*, 2012). Non-labelled, unfractionated endometrial cell suspensions were mixed with the SP or MP to provide niche cells for the transplanted cells. The image analysis revealed that the SP contributed significantly more TdTom$^+$ vimentin$^+$ stroma, TdTom$^+$ cytokeratin$^+$ epithelium and TdTom$^+$ CD31$^+$ endothelial cells in the transplants when compared with the xenografted MP (Miyazaki *et al.*, 2012), suggesting that the SP was enriched for the progenitor populations of these three lineages. However, it does not indicate whether a single stem/progenitor cell in the SP can differentiate into these lineages. These features and the marker profiles of the SP suggest that vascular and perivascular cells are enriched in the SP and are the main contributors to their tissue reconstitution capacity.

Differentiation of eMSCs

Several studies have demonstrated the differentiation potential of various eMSC populations and of cultured endometrial stromal fibroblasts (Table III). Most have focused on mesodermal differentiation, particularly bone marrow lineages to satisfy the minimal criteria for MSC status (Dominici *et al.*, 2006). Both clonogenic (Gargett *et al.*, 2009), CD146$^+$PDGFR-β$^+$ (Schwab and Gargett, 2007) and SUSD2$^+$ (Masuda *et al.*, 2012) stromal cells exhibited trilineage differentiation into adipocyte, osteoblast and chondrocyte lineages *in vitro*. They also differentiated into smooth muscle cells and fibroblasts (Masuda *et al.*, 2012; Su *et al.*, 2014). Endometrial SP cells differentiated into adipocyte and osteoblast lineages (Cervelló *et al.*, 2010, 2011), whereas some studies have shown single lineage differentiation for clonogenic or CD146$^+$ PDGFR-β$^+$ cells (Dimitrov *et al.*, 2008; Spitzer *et al.*, 2012).

Endometrial stromal fibroblast cell differentiation

Cultured endometrial stromal fibroblasts undergo mesodermal lineage differentiation, usually to single lineages and to a lesser extent than purified eMSC or clonogenic populations (Table III). Cell types produced include chondrocytes (Wolff *et al.*, 2007) and adipocytes (Ai *et al.*, 2012; Ebrahimi-Barough *et al.*, 2013). Bovine endometrial stromal fibroblasts showed osteogenic lineage differentiation (Donofrio *et al.*, 2008). Cultured human endometrial stromal fibroblasts also differentiated into a haematopoietic lineage generating CD41a$^+$ and CD42b$^+$ polyploid megakaryocytes that released platelets *in vitro*, indicating the plasticity of cultured endometrial stromal fibroblasts (Wang *et al.*, 2012b). It is clear that endometrial stromal fibroblasts have broad mesodermal multipotency.

Table III ***In vitro* and *in vivo* differentiation of human endometrial marker-enriched mesenchymal stem cells and stromal cell populations.**

Cell type investigated	Adi	Ost	Chon	Myo	Neu	Functional differentiation studies	References
CD146$^+$ PDGF-Rβ^+ endometrial stromal cells	H, R	H, R	H, R	I, R			Schwab and Gargett (2007)
CD146$^+$ PDGFRβ^+ endometrial stromal cells	H						Spitzer *et al.* (2012)
SUSD2$^+$ endometrial stromal cells	H, R	H, R	H, R	I, R		*In vitro*: angiogenic—CD31$^+$; *in vivo*: SUSD2$^+$ cells produced endometrial stromal-like tissue under the kidney capsule of NSG mice	Masuda *et al.* (2012)
SUSD2$^+$ endometrial stromal cells				H, I		*In vitro*: PA+G scaffold—myogenic−Masson's trichrome, SM22α+ SM-MHC$^+$; fibroblastic—Masson's trichrome, COL-1$^+$ Tn-C$^+$	Su *et al.* (2014)
PMP SUSD2$^+$ endometrial stromal cells	H	H	H	I			Ulrich *et al.* (2014c)
Briefly cultured endometrial SP cells						*In vitro*: epithelial SP—CD9$^+$ E-cadherin$^+$ gland structures on Matrigel;Stromal SP—CD9$^+$ VM$^+$ clusters on Matrigel	Kato *et al.* (2007)
SP endometrial cells						*In vitro*: decidualization—*Prolactin*$^+$ *IGFRB-1*$^+$, secrete prolactin	Tsuji *et al.* (2008)
SP endometrial stromal and epithelial cells	H, R	I, R				*In vivo*: regenerated human endometrium when stromal SP cells or cell lines were transplanted into NOD-SCID mice	Cervelló *et al.* (2010, 2011)
SP endometrial cells						*In vivo*: ESP with/without EMP transplanted under kidney capsule of OVX NOG mice reconstituted VM$^+$ CD13$^+$ stroma and CK$^+$ glands with human CD31$^+$ αSMA$^+$ vessels	Masuda *et al.* (2010), Miyazaki *et al.* (2012)
Passaged endometrial stromal colonies	H						Dimitrov *et al.* (2008)
Endometrial stromal and epithelial colonies	H, R	H, R	H, R	I, R		*In vitro*: large epithelial CFU—CK$^+$ gland-like structures in 3D Matrigel	Gargett *et al.* (2009)
Endometrial stromal colonies	H, R	H, R	H, R		I, R	*In vitro*: pancreatic-lineage cells—insulin-secreting, resistant to oxidative stress and IL-1β-induced apoptosis; *in vivo*: restored INS production in STZ-treated SCID mice	Li *et al.* (2010)
Endometrial stromal colonies	H, R		H				Schüring *et al.* (2011)
Endometrial stromal colonies	H, R						Ai *et al.* (2012)
Passaged endometrial large stromal colonies	H	H				*In vitro*: hepatogenic—CK8$^+$ Albumin$^+$, demonstrated urea synthesis, ammonia removal and glycogen storage (PAS$^+$)	Yang *et al.* (2014)
First passage endometrial stromal cells			H, I				Wolff *et al.* (2007)
Passaged endometrial stromal cells						*In vitro*: pancreatic β-like cells—*PDX1*, *PAX4*, *GLUT2*, *INS*, produced INS in response to glucose; *in vivo*: restored INS production in STZ-treated SCID mice	Santamaria *et al.* (2011)
Passaged endometrial stromal cells					I	*In vitro*: neurogenic—barium-sensitive K channels; *in vivo*: in a PD mouse model, stromal cells migrated to lesion site, differentiated and produced dopamine	Wolff *et al.* (2011)
Passaged endometrial stromal cells						*In vitro*: CD41a$^+$ CD42b$^+$ megakaryocytes releasing CD62p$^+$ functional platelets that bound fibrinogen after thrombin stimulation	Wang *et al.* (2012b)
Passaged endometrial stromal cells					I	*In vitro*: oligodendrocyte progenitors—A2B5, Nestin, O4, Olig2, PDGFRα, *SOX10*	Ebrahimi-Barough *et al.* (2013)

Adi, adipocyte; Ost, osteocyte; Chon, chondrocyte; Myo, smooth muscle myocyte; Neu, neural; AFP, alpha-fetoprotein; CFU, colony-forming unit; CK, cytokeratin; COL-1, collagen 1; EMP, endometrial main population cells; ESP, endometrial side population cells; *GLUT1*, glucose transporter 1; H, histology stain; I, immunohistochemistry; INS, insulin; NSG/NOG, NOD/SCID/IL-2Rγchainnull; OVX, ovariectomized; PA+G, polyamide and gelatin-composite meshes; PAS, periodic acid-Schiff; PD, 1-methyl 4-phenyl 1,2,3,6-tetrahydro pyridine-induced animal model of Parkinson's disease; *PAX4*, paired box 4; *PDX1*, pancreatic and duodenal homeobox 1; PMP, postmenopausal; R, mRNA expression; RedFluc, red-emitting firefly luciferase; SP, side population; STZ, streptozotocin; TdTom, Tandem Tomato; TH, tyrosine hydroxylase; Tn-C, tenascin-C; VM, vimentin.

Endometrial stromal fibroblasts also show differentiation potential across embryonic lineage boundaries (Table III). They differentiated into endodermal pancreatic lineages *in vitro* and *in vivo* (Li *et al.*, 2010; Santamaria *et al.*, 2011). Passaged endometrial stromal fibroblasts (Santamaria *et al.*, 2011) or 3D spheroid cultures (Li *et al.*, 2010) produced differentiated cells that secreted insulin and expressed β-cell pancreatic genes. Glucagon-producing cells were also generated (Li *et al.*, 2010). Xenografting these differentiated cells into an immunocompromised mouse model

of diabetes reduced hyperglycaemia, and human insulin was detected in the mouse serum (Li *et al.*, 2010). These studies indicate that *in vitro* differentiated endometrial stromal fibroblasts have relevant functional properties *in vivo*. Clonogenic eMSCs also differentiated into a hepatocyte-like lineage in a four-step *in vitro* hepatogenic differentiation protocol (Yang *et al.*, 2014). The differentiated cells generated urea and metabolized ammonia.

Cultured endometrial stromal fibroblasts have been differentiated into an ectodermal lineage; dopaminergic neuron-like cells expressing neural stem cell markers and tyrosine hydroxylase, the rate-limiting enzyme for dopamine synthesis (Wolff *et al.*, 2011). Only the differentiated cells produced potassium currents. In a mouse model of Parkinson's disease, cultured human endometrial stromal fibroblasts transplanted directly into the striatum migrated to the lesioned substantia nigra and either differentiated into dopamine-secreting neurons or promoted endogenous neuronal function, partially restoring dopamine levels. Stromal fibroblasts also differentiated into oligodendrocyte progenitor cells, the myelinating cells of the central nervous system, and expressed oligodendrocyte progenitor cell proteins (Ebrahimi-Barough *et al.*, 2013). Whether the perivascular eMSC population demonstrates ectodermal lineage differentiation is unknown.

Differentiation of eMSCs into decidual cells

Decidualization is the physiological differentiation pathway of eMSCs around the spiral arterioles and the subepithelial stroma. Progesterone mediates decidualization, a process critically important in establishing the feto-maternal interface of pregnancy. The relative roles of cultured perivascular SUSD2$^+$ eMSCs and SUSD2$^-$ endometrial stromal fibroblasts were examined by RNA sequencing, following decidualization induction *in vitro* (Murakami *et al.*, 2014). Despite the SUSD2$^-$ cells upregulating SUSD2 expression in culture, the two cell types retained distinct gene expression profiles, with the SUSD2$^+$ cells enriched in novel and known endometrial perivascular signature genes. In the undifferentiated state, SUSD2$^+$-derived decidual cells produced lower levels of inflammatory mediators and certain chemokines when compared with the SUSD2$^-$ stromal fibroblasts. An even greater divergence in the secretomes of the two cell types was observed upon decidual differentiation. Decidualized SUSD2$^+$ cells were the major source of several cytokines, including an 18-fold greater production of leukaemia inhibitory factor and a 43-fold increase in chemokine (C-C motif) ligand 7 when compared with 4–5-fold increases in the differentiated SUSD2$^-$ cells (Murakami *et al.*, 2014). This differential molecular response in decidualizing SUSD2$^+$ and SUSD2$^-$ cells further emphasizes the differences between perivascular eMSCs and stromal fibroblasts.

Profiling eMSCs and stromal fibroblast populations

Specific markers that purify endometrial stem/progenitor cell populations enable the identification of gene expression signatures. Gene profiling of three freshly isolated cell populations sorted from CD146 and PDGFR-β co-labelled cells confirmed that the CD146$^+$ PDGFR-β$^+$ population was clonogenic, perivascularly located, differentiated into adipocytes and expressed pericyte markers and genes associated with angiogenesis and vasculogenesis (Spitzer *et al.*, 2012). The CD146$^+$ PDGFR-β$^+$ eMSC selectively expressed high levels of the SUSD2 gene (Wells *et al.*, 2013), confirming flow cytometry data (Masuda *et al.*, 2012). They also expressed genes involved in steroid hormone and hypoxia responses, inflammation, immunomodulation and cell communication, emphasizing their role in tissue homeostasis and immune tolerance required for embryo implantation and placental development. Increased expression of Notch, Hedgehog, insulin-like growth factor (IGF), transforming growth factor β (TGFβ) and G-protein-coupled receptor signalling pathway genes suggest stem cell function in self-renewal and differentiation. The gene profile of CD146$^+$PDGFR-β$^+$ eMSCs clustered with CD146$^-$PDGFR-β$^+$ endometrial fibroblasts, but was distinct from CD146$^+$PDGFR-β$^-$ endothelial cells, indicating that their main differentiated lineage is the endometrial stromal fibroblast (Spitzer *et al.*, 2012). RNAseq analysis comparing cultured SUSD2$^+$ and SUSD2$^-$ cells confirmed the pericyte phenotype of eMSCs and a role for Notch in regulating SUSD2 expression (Murakami *et al.*, 2014). The secretome of *in vitro* decidualized SUSD2$^+$ cells revealed greater production of chemokines and inflammatory modulators, compared with decidualized SUSD2$^-$ stromal fibroblasts. This suggests that the SUSD2$^+$ perivascular cells establish a specific chemokine microenvironment around the endometrial vasculature, likely crucial in establishing early pregnancy through recruitment of leukocyte populations to the materno-foetal interface for mediating maternal immune tolerance and promoting trophoblast invasion of the spiral arterioles (Murakami *et al.*, 2014).

Gene profiling of cultured epithelial and stromal SP cells generated a gene signature showing considerable overlap of differentially regulated genes, suggesting a common gene signature and possibly a single stem/progenitor cell phenotype (Cervelló *et al.*, 2010). However, the lack of purity of the epithelial and stromal SP cultures may have contributed to this similar gene signature. Several common endometrial SP genes were differentially expressed compared with bmMSCs, including interleukin-1B (*IL-1B*), growth differentiation factor 15, von Willebrand factor (VWF), matrix metalloproteinase 3, colony stimulating factor 2, intercellular adhesion molecule *1* (Wang *et al.*, 2012a; Gaafar *et al.*, 2014) exemplifying the uniqueness of MSCs derived from different sources.

Gene profiling of bmMSCs differentiated towards endometrial decidual cells by a cyclic AMP analogue showed that the culture process generates a distinct gene expression pattern (Aghajanova *et al.*, 2010). Among the upregulated genes of cAMP-regulated, decidualized bmMSCs were the typical decidual markers *IGFBP1* and *prolactin*, correlating with observed phenotypic changes. Several c-AMP-regulated genes with roles in endometrial function were also upregulated, including *IGF1*, inhibin βA, vascular endothelial growth factor (VEGF) A, *pappalysin* and *parathyroid hormone-like hormone*. Comparison of c-AMP-treated bmMSCs with cultured human endometrial stromal fibroblasts revealed 20 common genes, all of which are involved in the endometrial function. Identification of a gene signature for bmMSCs and eMSCs distinct from endometrial stromal fibroblasts is important in determining the role of endogenous or bone marrow-derived MSCs in endometrial regeneration and differentiation (Aghajanova *et al.*, 2010; Spitzer *et al.*, 2012).

Profiling studies confirmed the similarity between cultured endometrial stromal fibroblasts from hysterectomy tissue and menstrual blood and skin fibroblasts (Wang *et al.*, 2012a). Similarly, there was a core gene signature common to bmMSCs and cultured endometrial stromal fibroblasts, although there were also distinct differences, particularly in inflammatory, immunomodulatory and angiogenesis genes (Wang *et al.*, 2012a; Gaafar *et al.*, 2014). Although these two studies compared bmMSCs with menstrual blood or endometrial stromal fibroblasts, only two overlapping gene expression differences were observed: *ITGA10* (integrin-α10)

and *VCAM1* (vascular cell adhesion molecule 1—CD106), both downregulated in menstrual blood and endometrial stromal fibroblasts compared with bmMSCs. Lack of correlation between these similar studies may be due to the different platforms used for gene profiling, as one used focused arrays (Gaafar *et al.*, 2014) and the other whole-genome microarrays (Wang *et al.*, 2012a).

Stem/progenitor cells in endometrial decidua

Human endometrial stroma terminally differentiates into the decidua during the mid-late secretory stage of the menstrual cycle. Decidualization commences in the perivascular cells of the spiral arterioles and spreads to the subepithelial stroma. The decidua of pregnancy may therefore harbour a subpopulation of undifferentiated MSCs related to eMSCs (Kyurkchiev *et al.*, 2010). Indeed, clonogenic SP cells were identified in the first trimester decidua (Tsuji *et al.*, 2008; Guo *et al.*, 2010; Wang *et al.*, 2013) comprising 0.03–1.4% of cells, a lower abundance than their endometrial counterparts. SP cells sorted from short-term cultured human decidual cells expressed neither CD31 (endothelial marker) nor CD146 (MSC marker) (Wang *et al.*, 2013), but differentiated into endothelial cells *in vitro* and induced neovascularization following intramascular injection in a mouse ischaemic hind limb injury model, rescuing the limb (Wang *et al.*, 2013). This $CD31^{-}CD146^{-}$ SP proliferated more rapidly than MP cells when cultured in 0.2% serum-containing media supplemented with either epidermal growth factor (EGF) or fibroblast growth factor 2 (FGF2), similar to clonogenic eMSCs (Chan *et al.*, 2004). They also proliferated in IGF-1- and VEGF-containing media (Wang *et al.*, 2013). In contrast, short-term cultured decidual SP cells required IL-6, stem cell factor and thrombopoietin for growth in serum-free (SF) medium (Guo *et al.*, 2010). The clonogenic cells appeared more heterogeneous than endometrial clones and differentiated into prolactin-staining cells following treatment with cAMP. Confocal analysis of the decidua parietalis using specific markers for purifying bmMSC or eMSC demonstrated a vascular niche for decidua MSCs (Castrechini *et al.*, 2012). STRO-1 co-localized with vWF (endothelial marker), in agreement with recent reports that STRO-1 was an endothelial marker in adipose tissue arterioles and capillaries (Lin *et al.*, 2011). In contrast, CD146 was perivascular with partial overlap with vWF.

Cultured stromal fibroblasts from first trimester and term decidua demonstrated characteristic MSC properties: clonogenicity (2–18%), mesodermal lineage differentiation and surface marker phenotype (Dimitrov *et al.*, 2010; Castrechini *et al.*, 2012). Cultured placental decidua basalis stromal fibroblasts differentiated into pancreatic cells *in vitro* when transfected with a microRNA involved in pancreas development (Shaer *et al.*, 2014), indicating that decidua basalis stromal fibroblasts are equally as capable of differentiating across germ lineage boundaries as endometrial stromal fibroblasts.

Decidual SP cells and stromal fibroblasts respond to sex steroid hormones. Both estrogen and progesterone dose dependently stimulated greater proliferation and migration of the decidual $CD31^{-}CD146^{-}$ cells *in vitro* than MP cells (Wang *et al.*, 2013). High concentrations of progesterone (7–30 μM) upregulated HLA-G on a small proportion (5.3%) of decidual stromal fibroblasts, suggesting that they may function in immunomodulation of the implanting embryo (Ivanova-Todorova *et al.*, 2009). It is unknown whether this HLA-G-expressing subpopulation are decidual perivascular MSCs. Nor is it known if the perivascular decidual stromal cells or HLA-G-expressing cells are $SUSD2^{+}$. More studies are required using specific markers and profiling technologies to determine the relationship among eMSCs, decidual MSCs, endometrial stromal fibroblasts and decidual stromal fibroblasts.

Endometrial stem/progenitor cells in menstrual blood

The markers used to enrich for eMSCs (co-expression of CD146 and PDGFRβ or SUSD2) revealed their perivascular location in both the basalis and the functionalis of human endometrium, indicating that eMSCs would be shed in menstrual blood (Gargett and Masuda, 2010). Several laboratories have identified and characterized an MSC-like population in menstrual blood. Generally, menstrual blood was collected in menstrual cups (Koks *et al.*, 1999) and cultured directly onto plastic culture dishes in a manner similar to bmMSCs; similarly, they comprise a mix of eMSCs and stromal fibroblasts. One group used c-KIT (CD117) to further purify the cultured cells (Patel *et al.*, 2008). CD117 is induced during culture as freshly isolated endometrial stromal fibroblasts are $CD117^{-}$, but cultured $SUSD2^{+}$ cells are $CD117^{+}$ (Masuda *et al.*, 2012).

Cells cultured from menstrual blood have been given various names (Table IV): endometrial regenerative cells (ERCs) (Meng *et al.*, 2007), endometrial menstrual MSCs (Patel *et al.*, 2008), menstrual blood MSCs (mbMSCs) (Gargett and Masuda, 2010), endometrial decidual tissue MSCs (EDT-MSCs) (Rossignoli *et al.*, 2013), menstrual blood-derived MSCs (MMSCs) (Hida *et al.*, 2008) and menstrual blood progenitor cells (MBPCs) (Wu *et al.*, 2014b). In this review, cultured menstrual blood cells will be referred to as ERCs, a term that encompasses both the stromal fibroblast and MSC composition of these isolates. Epithelial cells were not generally observed in cultured menstrual blood, because they were not present, were overlooked or had been overgrown by the stromal fibroblast populations (Musina *et al.*, 2008), suggesting that epithelial progenitors are more likely located in the basalis and not normally shed during menstruation (Gargett and Masuda, 2010).

Several recent reviews summarize the properties of ERCs and their potential for cellular therapies (Ulrich *et al.*, 2013; Khoury *et al.*, 2014). ERCs cultured from menstrual blood are clonogenic (Musina *et al.*, 2008), highly proliferative, with a short population doubling time of 20 h (Meng *et al.*, 2007; Patel *et al.*, 2008; Rossignoli *et al.*, 2013; Wu *et al.*, 2014b) and underwent 25–30 population doublings (Hida *et al.*, 2008; Rossignoli *et al.*, 2013). Clonogenic ERCs retained a stable karyotype for 68 passages (Meng *et al.*, 2007; Wu *et al.*, 2014b).

Markers of menstrual blood ERCs

Cultured ERCs express telomerase reverse transcriptase (hTERT) and demonstrate telomerase activity, as well as typical MSC phenotypic markers (Table IV), but like eMSCs, they do not express the specific bmMSC marker STRO-1 (Cui *et al.*, 2007; Meng *et al.*, 2007; Hida *et al.*, 2008; Patel *et al.*, 2008; Khanmohammadi *et al.*, 2014). Pluripotency marker expression has been demonstrated in ERC, including OCT-4 (Patel *et al.*, 2008; Borlongan *et al.*, 2010; Darzi *et al.*, 2012; Wu *et al.*, 2014b), SSEA-4 (Patel *et al.*, 2008; Rossignoli *et al.*, 2013) and NANOG (Borlongan *et al.*, 2010). However, these markers were not found consistently on ERC and OCT4 was cytoplasmic by

Table IV **Surface marker phenotype of human menstrual blood stem/progenitor cell and stromal cell populations.**

Menstrual blood stem cell name	Acronym	Cell type investigated	Marker expression								Other markers investigated	References
			CD29	CD44	CD73	CD90	CD105	OCT4	CD34	CD45		
Endometrial decidual tissue MSC	EDT-MSC	Adherent MB cells			+	+					$CD146^{+}$ $SSEA\text{-}4^{+}$ (1–19.4%)	Rossignoli *et al.* (2013)
Endometrial regenerative cells	ERC	Created ERC cell lines from MB	+	+	+	+	+	+	−	−	$CD59^{+}$ $hTERT^{+}$ $MMPs^{+}$ $CD133^{-}$ $NANOG^{-}$ $SSEA\text{-}4^{-}$ $STRO\text{-}1^{-}$	Meng *et al.* (2007)
Menstrual blood MSC	mbMSC	Adherent MB cells		+		+	+		−	−		Musina *et al.* (2008)
Menstrual blood-derived mesenchymal cells	MMC	Adherent MB cells	+	+		+					$CD55^{+}$ $CD59^{+}$ $CD166^{+}$	Hida *et al.* (2008)
		Passaged adherent MB cells	−		+	+	+		−	−	$CD13^{+}$ $CD54^{+}$ $CD146^{+}$ $CD166^{+}$ $CD14^{-}$ $CD16^{-}$ $CD19^{-}$ $HLA\text{-}DR^{-}$	Sugawara *et al.* (2014)
Menstrual blood progenitor cells	MBPC	Adherent MB cells						+			$CD117^{-}$ $SSEA\text{-}4^{-}$	Wu *et al.* (2014b)
Menstrual blood-derived (stem) cells		Passaged (P6–P9) adherent MB cells						+			$CXCR4^{+}$ $NANOG^{+}$ $SSEA^{+}$	Borlongan *et al.* (2010)
Menstrual stromal stem cells	MenSC	P5 adherent MB cells selected for C-KIT	+	+		+	+	+	−	−	$CD9^{+}$ $CD49f^{+}$ $CD166^{+}$ $C\text{-}KIT^{+}$ $CXCR4^{+}$ $MHC\text{-}I^{+}$ $SSEA\text{-}4^{+}$ $CD38^{-}$ $CD133^{-}$ $MHC\text{-}II^{-}$ LIN^{-}	Patel *et al.* (2008)
		From S-Evans Biosciences (China)			+	+	+		−		$CD14^{-}$ $CD19^{-}$ $CD35^{-}$ $HLA\text{-}DR^{-}$	Hu *et al.* (2014)
		Passaged adherent MB cells	+	+	+		+	+	−	−	$CD9^{+}$ $CD10^{+}$ $CD146^{+}$ $CD38^{-}$ $CD133^{-}$ $C\text{-}KIT^{-}$ $STRO\text{-}1^{-}$	Darzi *et al.* (2012), Kazemnejad *et al.* (2012), Khanjani *et al.* (2014), Khanmohammadi *et al.* (2014)

MB, menstrual blood; MHC, major histocompatibility complex; MMP, matrix metalloproteinase.

Table V *In vitro* and *in vivo* differentiation of human menstrual blood stem/progenitor cells and stromal cell populations.

Cell type investigated	Adi	Ost	Cho	Myo	Neu	Functional differentiation studies	References
Adherent MB cells						*In vivo*: DMD mouse model—improvement via fusion with host myocytes	Cui *et al.* (2007)
Created ERC cell lines from MB	I	H		I	I	*In vitro*: angiogenic—$CD34^+$ $CD62^+$; hepatocytic—$Albumin^+$; pancreatic—$Insulin^+$; respiratory epithelial—proSP-C^+; cardiogenic—Troponin I^+	Meng *et al.* (2007)
Adherent MB cells						*In vitro*: cardiogenic via co-culture—troponin-I^+ AP^+, beating MMCs*In vivo*: improvement of cardiac function following MI after MMC Tx	Hida *et al.* (2008)
Adherent MB cells	H	H					Musina *et al.* (2008)
P5 adherent MB cells selected for C-KIT	H	H	H		I, R	*In vitro*: cardiogenic—$Actin^+$ $Troponin^+$ Connexin 43^+ *ANP*$^+$ *Mef2C*$^+$	Patel *et al.* (2008)
Passaged adherent MB cells						*In vivo*: ERC inhibited glioma volume by inhibition of angiogenesis in Sprague-Dawley rat model	Han *et al.* (2009)
Passaged adherent MB cells						*In vivo*: ERC Tx into four MS patients—no immune/ adverse effects at 1 year	Zhong *et al.* (2009)
Passaged (P6–P9) adherent MB cells						*In vitro*: OGD-exposed rat neuron co-culture—reduced cell death*In vivo*: Tx into rat stroke model—motor/neurological improvements	Borlongan *et al.* (2010)
Adherent MB cells						*In vitro*: cardiogenic via co-culture—Troponin-I^+, beating MMCs	Ikegami *et al.* (2010)
Passaged adherent MB cells	H, R	H, R	I		I, R	*In vitro*: hepatocytes—PAS, $Glycogen^+$ ALB^+ CK-18^+ *TAT*$^+$; chondrocytes on 3D nanofibrous scaffold—collagen type II^+ AB^+ $sGAG^+$	Darzi *et al.* (2012), Kazemnejad *et al.* (2012), Khanjani *et al.* (2014), Azedi *et al.* (2014), Khanmohammadi *et al.* (2014)
Adherent MB cells	H	H, R	H			*In vitro*: adipogenic, osteogenic and chondrogenic lineages	Rossignoli *et al.* (2013)
MenSCs from S-Evans Biosciences (Hangzhou, China)						*In vitro*: MenSC co-cultured with NP cells from intravertebral disc tissue expressed NP markers and ECM accumulation	Hu *et al.* (2014)
Passaged adherent MB cells						*In vitro*: decidualization—$Prolactin^+$ $IGFBP1^+$	Sugawara *et al.* (2014)
Adherent MB cells	H	H	H			*In vivo*: IV MBPC ameliorated diabetic symptoms in a T1DM murine model through differentiation of endogenous progenitor cells	Wu *et al.* (2014b)

ALB, albumin; ANP, atrial natriuretic peptide; AP, action potential; DMD, Duchenne muscular dystrophy; ECM, extracellular matrix; ERC, endometrial regenerative cells; H, histology stain; I, immunohistochemistry; IV, intravenous; Mef2C, myocyte enhancer factor 2C; MI, myocardial infarction; MMC/MenSC, menstrual blood stem cells; MS, multiple sclerosis; NP, nucleus pulposus; OGD, oxygen glucose deprivation; PAS, periodic acid-Schiff; proSP-C, ProSurfactant protein C; R, mRNA expression; sGAG, sulphated glycosaminoglycan; T1DM, type 1 diabetes mellitus; TAT1, TAT amino acid transporter 1; Tx, transplantation.

immunofluorescence (Borlongan *et al.*, 2010), indicating that the ERCs are not truly pluripotent.

Differentiation of menstrual blood ERCs

ERCs have broad *in vitro* differentiation capacity (Table V) and, under appropriate conditions, differentiated into typical mesodermal lineages: adipogenic, chondrogenic, osteogenic (Meng *et al.*, 2007; Patel *et al.*, 2008; Darzi *et al.*, 2012; Rossignoli *et al.*, 2013) and skeletal and cardiac muscle (Cui *et al.*, 2007; Hida *et al.*, 2008). Compared with bmMSCs, mesodermal differentiation was less robust for ERCs; however, greater differentiation was achieved for the adipogenic lineage using retinoic acid (Khanmohammadi *et al.*, 2014), the osteogenic lineage with human platelet releasate (Darzi *et al.*, 2012) and cardiomyogenic lineage in SF medium containing thyroxine and insulin (Ikegami *et al.*, 2010). Co-culture of ERCs with mouse foetal cardiomyocytes generated spontaneously beating cells, showing striations and expressing cardiac-specific Troponin I (Hida *et al.*, 2008; Khanmohammadi *et al.*, 2014). ERCs co-cultured with human nucleus pulposus cells in 2% oxygen differentiated into nucleus pulposus-like cells (Hu *et al.*, 2014). Cultured ERCs, bmMSCs and amnion-derived MSCs differentiated into decidua-like cells expressing prolactin and IGFBP1 when induced by a cAMP analogue, but not estrogen and/or progesterone, despite expressing *ESR1* and *PR* (Sugawara *et al.*, 2014). This suggests that decidualized cells originally cultured from menstrual blood de-differentiated during culture expansion and required a strong decidual stimulus to redifferentiate.

ERCs also differentiated into several neural lineages *in vitro*, similar to endometrial stromal fibroblasts (Patel *et al.*, 2008; Azedi *et al.*, 2014). In SF medium, a small proportion of cultured ERCs and bmMSCs generated neurosphere-like structures, which upon dissociation produced further neurospheres derived from single cells (Azedi *et al.*, 2014).

Retinoic acid and PDGF induced these secondary neurospheres to differentiate into glial cells, although to a lesser extent than bmMSCs. In another co-culture model, ERCs protected primary rat neurons from oxygen and glucose deprivation-induced loss of viability (Borlongan *et al.*, 2010). These ERCs, delivered either intravenously (4×10^6 cells) or intracerebrally (4×10^5 cells), into a rat stroke model survived and increased the survival of host cells in the ischaemic penumbra after 14 days, improving behavioural and histological scores compared with vehicle controls.

Similar to endometrial stromal fibroblasts, ERCs differentiated into endodermal lineages *in vitro* and *in vivo*. ERCs differentiated into hepatocyte-like cells (Khanjani *et al.*, 2014), expressing hepatocyte genes and proteins, secreted albumin and accumulated glycogen in greater quantities than similarly differentiated bmMSC. In a type I diabetes mellitus mouse model, intravenously administered ERCs (3×10^5 cells) migrated to the damaged pancreas and promoted the differentiation of endogenous endocrine progenitors into functional β-cells, reversing hyperglycaemia (Wu *et al.*, 2014b). Dye-labelled ERCs were found in the pancreas, lungs and liver 3 days after transplantation and were still detectable in the pancreas after 14 days. Immunofluorescence co-localization studies showed that the ERC did not differentiate into pancreatic progenitors or insulin-producing β-cells, nor was human insulin detected in the mouse serum. Several pancreatic developmental genes and mature β-cell genes were sequentially upregulated in ERC-transplanted mice compared with vehicle controls. ERCs were more potent in reversing hyperglycaemia in this type I diabetes mouse model than bmMSCs or umbilical cord MSCs, indicating their potential for cell-based therapies.

Identity and regulation of stem/progenitor cells in mouse endometrium

In the absence of specific markers for identifying mouse endometrial stem/progenitor cells, label retention was initially used to characterize their phenotype and *in vivo* location.

LRCs

The quiescent or slow-cycling phenotype of many quiescent adult stem cells allows their identification by label retention assays in mice (Table I). The thymidine analogue bromodeoxyuridine (BrdU) is typically delivered as a pulse during development or remodelling and incorporates into the DNA of actively dividing cells. A chase period follows, when actively dividing cells dilute the label below detectable levels while quiescent and slow-cycling cells retain detectable label. The timing of the initial pulse and the length of the chase are critical variables in determining which cells incorporate and retain detectable label (Gargett *et al.*, 2007). Several labelling and chase regimes have been used to identify LRC in the stroma and epithelium of mouse endometrium (Chan and Gargett, 2006; Cervelló *et al.*, 2007; Chan *et al.*, 2012; Patterson and Pru, 2013; Cao *et al.*, 2014) (Table VI). A transgene-based label retention system was also used in the murine female reproductive tract, with labelling initiated by antibiotic-inducible expression of green fluorescent protein (GFP)-labelled histones (H2B-GFP) (Wang *et al.*, 2012c; Patterson and Pru, 2013).

Table VI Summary of label retention papers published 2006–2014.

Label	Pulse	Chase	LRC present after chase	Comments	References
BrdU	PND 3–5P	Up to 12 weeks	L, G, S	Epithelial LRCs are Esr1$^-$	Chan and Gargett (2006)
	PND 19–21	Up to 10 weeks	L, S	Stromal LRCs are Esr1$^{+/-}$, αSMA$^+$, Sca-1^{-a}	
BrdU	PND 3–5	8–10 weeks	S	Some LRCs express Oct-4 and c-Kit	Cervelló *et al.* (2007)
BrdU	Adult, model of menstrual breakdown and repair	4.5–8.5 days	L, G	Glandular epithelial LRCs proliferate following epithelial repair	Kaitu'u-Lino *et al.* (2010)
BrdU	PND 3–5	4 and 8 weeks	L, S	Epithelial LRCs proliferate after estrogen LRCs initiate epithelial proliferation in prepubertal endometrium 12% of stromal LRCs proliferate after estrogen	Chan *et al.* (2012)
BrdU	PND 19–22	Up to 11 weeks	L, S	Luminal epithelial LRCs at 5 weeks chase Stromal LRCs persist through pregnancy and proliferate postpartum Stromal LRCs express CD140b (46%), CD146 (2%), CD44 (24%), CD90 (45%), Sall4 (34%), Sca-1 (72%); ABCG2$^-$	Cao *et al.* (2014)
H2B-GFP	Adult cycling	Up to 47 weeks	G, S	Endometrial epithelial LRCs lost within 4 weeks Long-term epithelial LRCs in distal oviduct Long-term epithelial LRCs are Esr1$^-$, CD44$^-$, Sca-1$^-$, Lgr5$^-$, c-Kit$^-$	Wang *et al.* (2012c)
H2B-GFP	ED 13.5–PND 21 PND 21–40	Up to 47 weeks 8 months	L, G, S G	Endometrial LRCs are short-lived (<5 weeks) Long-term epithelial LRCs in distal oviduct and endocervical transition zone Epithelial LRCs persist	Patterson and Pru (2013)

ED, embryonic day; PND, postnatal day; L, luminal epithelial; G, glandular epithelial; S, stromal.
[a]About16% stromal LRCs are Esr1$^+$ and 84% Esr1$^-$.

Stromal LRCs

Postnatal (days 3–5) or prepubertal (days 19–21) administration of BrdU provides a window to label developmentally active stem/progenitor cells expected to reside in the endometrium. Stromal LRCs produced by this protocol were detectable after a chase in excess of 9 weeks (Chan and Gargett, 2006; Cervelló *et al.*, 2007) (Table VI). Label retention studies using the H2B-GFP system during embryonic, early postnatal development and adulthood also produced stromal LRCs after a 3–8-week chase (Wang *et al.*, 2012c; Patterson and Pru, 2013). Stromal LRCs from postnatal or prepubertal BrdU labelling were detected at the endometrial–myometrial junction, beneath the luminal epithelium, or in a perivascular location near CD31^{+} endothelial cells (Chan and Gargett, 2006). LRCs did not express CD45, demonstrating that they were not infiltrating leukocytes (Chan and Gargett, 2006). Stromal LRCs expressed the stem cell markers Oct-4, c-Kit (Cervelló *et al.*, 2007), CD140b, CD146, CD44, CD90 and Sall4 (Chan and Gargett, 2006; Cao *et al.*, 2014). Sca1 was absent from postnatal-derived LRCs but expressed in prepubertal-derived LRCs (Chan and Gargett, 2006; Cao *et al.*, 2014). Postnatal-derived LRCs in the perivascular zone expressed α-smooth muscle actin (αSMA), suggesting that they are perivascular cells or pericytes (Chan and Gargett, 2006). A small proportion (16%) of stromal postnatal or prepubertal-derived LRCs expressed Esr1, the predominant ESR involved in estrogen-mediated endometrial regeneration. In summary, stromal LRCs in postnatal and prepubertal models are heterogeneous populations, and further investigation is required to determine whether subpopulations of LRCs are the MSCs of the mouse endometrium. Examining LRCs in mouse models of endometrial regeneration may identify which subpopulation of stromal LRCs functions in generating new stromal vascular tissue (see later section).

Epithelial LRCs

Epithelial LRCs in postnatal and prepubertal models are absent or very rare after a 3–4-week chase (Table VI) (Chan and Gargett, 2006; Cervelló *et al.*, 2007; Patterson and Pru, 2013). The shorter persistence of epithelial LRCs is due to higher rates of epithelial cell proliferation, particularly under the influence of estrogen once estrous cycling begins at ~4 weeks of age (Chan and Gargett, 2006). Epithelial LRCs were predominantly in the luminal rather than glandular epithelium, reflecting the higher turnover of luminal epithelium during development that facilitates labelling and subsequent dilution (Chan and Gargett, 2006). These luminal epithelial LRCs did not express Esr1, unlike most non-labelled epithelial cells. Epithelial LRCs do, however, proliferate in response to estrogen, pointing to an indirect effect mediated via neighbouring Esr1^{+} cells (Chan and Gargett, 2006; Chan *et al.*, 2012). Glandular epithelial LRCs were rare in postnatal or prepubertal models and have not been characterized in detail.

H2B-GFP labelling spanning embryonic development to postnatal day 21 yielded highly persistent epithelial LRCs (9–13-week chase) in the distal oviduct and endocervical transition zone, but not endometrium (Wang *et al.*, 2012c). Peripubertal H2B-GFP labelling (postnatal days 21–40) gave rise to glandular LRCs that persisted for 8 months and through several pregnancies, further emphasizing differences between glandular and luminal LRCs (Patterson and Pru, 2013). In contrast, H2B-GFP labelling in adult cycling mice did not produce long-term glandular LRC in the endometrium, suggesting that the peripubertal phase is a unique developmental window when some glandular epithelial development is permanently completed. Long-term epithelial H2B-GFP LRCs were reported in the distal oviduct after labelling of adult cycling mice (Wang *et al.*, 2012c).

The LRC approach does not definitively identify stem/progenitor populations. It does, however, provide insight into patterns of development, rates of cell turnover and reactivation during endometrial regeneration and repair. LRC experiments highlight the higher turnover of luminal epithelium, relative to glandular epithelium and the stromal compartment. These observations suggest that luminal epithelium may be replenished from glandular epithelial or a stromal stem/progenitor population, but this is currently unclear (Kaitu'u-Lino *et al.*, 2010; Huang *et al.*, 2012; Patterson and Pru, 2013). The location of many stromal LRCs directly under the luminal epithelium (Chan and Gargett, 2006) may represent a snapshot of the 'mesenchymal-to-epithelial transition' (MET) believed to occur in the endometrium (Huang *et al.*, 2012; Patterson and Pru, 2013). The perivascular location of other stromal LRCs (Kaitu'u-Lino *et al.*, 2012) suggests a link to the perivascularly located human eMSCs. Unfortunately, the functional properties of BrdU-LRC have been impossible to assess directly because BrdU detection assays require fixation and treatment that kills the tissue being examined. This limitation is circumvented by the use of the transgenic H2B-GFP system, which allows the isolation of living LRCs. This transgenic system has only recently been used in the study of LRCs in the female reproductive tract (Wang *et al.*, 2012c; Patterson and Pru, 2013) and is compatible with *in vitro* and *in vivo* assays that could clarify the identity and potential of quiescent putative mouse endometrial stem/progenitor populations.

SP cells

SP cells have been identified in murine postpartum but not in the normal cycling endometrium (Hu *et al.*, 2010). The postpartum endometrial SP was enriched in clonogenic cells, which expressed Esr1 and tended to differentiate on exposure to estrogen in culture (Hu *et al.*, 2010). However, unlike human endometrial SP, the mouse endometrial SP was not enriched for endothelial cells and its exact identity remains unclear.

CD44 as an epithelial progenitor marker in mouse endometrium

Compared with the human, cell surface markers for stem/progenitor cells are less well characterized in the mouse endometrium. CD44 is a transmembrane protein expressed on many cell types, including HSCs, MSCs and cancer stem cells (Zöller, 2011). In the mouse endometrium, CD44-expressing epithelial cells constituted an epithelial progenitor population, which lacked Esr1 or PR (Janzen *et al.*, 2013). This epithelial population survived hormonal deprivation, possibly due to Wnt pathway activation. CD44^{+} epithelial cells generated more gland-like structures than CD44^{-} cells in a tissue reconstitution assay in immunocompromised mice. CD44^{+} cells were also proliferative, suggesting that they are distinct from slow-cycling epithelial LRCs.

Role of endometrial stem/progenitor cells in endometrial regeneration

The human endometrium not only regenerates each month as part of the menstrual cycle, but also following parturition, almost complete

resection and in postmenopausal women taking estrogen-based hormone replacement therapy (Gargett *et al.*, 2012).

Tissue reconstituting cells regenerate human endometrium

Unfractionated single-cell suspensions of endometrium cells from hysterectomy tissue have regenerated endometrial tissue following xenografting beneath the kidney capsule of severely immunocompromised NOG mice lacking T, B and natural killer (NK) cells (Masuda *et al.*, 2007b). The uterine cells organized into endometrial and myometrial tissue layers, comprising cytokeratin$^+$CD9$^+$ glandular structures, CD10$^+$CD13$^+$ stroma and αSMA myometrial-like tissue. The endometrial xenografts responded to cyclical estrogen and progesterone administration, mimicking the human menstrual cycle in ovariectomized-recipient mice. Estrogen stimulated epithelial and stromal proliferation, whereas progesterone induced tortuous glands and decidualized the stroma. When progesterone was withdrawn, large blood-filled cysts formed, suggestive of menstruation. Cells labelled with a lentiviral luciferase vector prior to xenografting enabled non-invasive bioluminescence imaging during hormonally induced 'menstrual' cycles, showing growth and regression of the human endometrial tissue generated *in vivo* (Masuda *et al.*, 2007b; Maruyama *et al.*, 2010).

This xenograft model has been used to examine the tissue reconstitution activity of candidate endometrial stem/progenitor cell populations, including SP cells. As noted in the SP cells section, xenografting the SP, but not the MP alone, generated endometrium when transplanted under the kidney capsule of immunocompromised mice (Cervelló *et al.*, 2010; Masuda *et al.*, 2010). Freshly isolated SP cells generated vasculature and migrating ERβ$^+$ (ESR2) endothelial cells, comprising 80% of the grafts. Stromal and glandular components comprised 13 and 8%, respectively. Clonally derived human endometrial epithelial and stromal SP cells also generated endometrial tissue when xenografted into immunocompromised mice (Cervelló *et al.*, 2010). These xenografts immunostained for stroma (vimentin) and epithelium (CD9), but not for endothelium (CD31). Organized endometrial glands were not easily distinguished, possibly due to prolonged clonal culture of the SP prior to xenografting. The regenerated endometrial tissue did not express ESR1, but some cells expressed PR. These studies indicate that endometrial cell subpopulations with stem/progenitor cell activity regenerate endometrial tissue *in vivo*. As indicated earlier, further research is required to more precisely identify the cell type(s) in the transplanted SPs that generate human endometrial tissue (Gargett and Ye, 2012).

Regeneration of postmenopausal endometrium

The atrophic endometrium of postmenopausal women regenerates to a thickness similar to premenopausal endometrium by the administration of estradiol valerate for 8 weeks (Ettinger *et al.*, 1997; Ulrich *et al.*, 2014c). Clonogenic SUSD2$^+$ eMSCs have been identified in the regenerated endometrium, with similar self-renewal and multipotency to premenopausal eMSCs (Ulrich *et al.*, 2014c). Comparable vascular densities of endometrium from postmenopausal women treated with or without estrogen suggested that perivascular SUSD2$^+$ cells have a role in the stromal vascular regeneration of postmenopausal endometrium. Although SSEA-1, the marker for basalis epithelium, is present on all postmenopausal epithelial cells, its role in regenerating the glandular and luminal epithelium is unknown (Valentijn *et al.*, 2013). Similarly, clonogenic epithelial cells or SP cells have not yet been identified in postmenopausal endometrium, nor has their role in regenerating epithelial tissue been investigated.

Mouse models of endometrial repair and regeneration

Although the mouse estrus cycle does not involve menstruation and regeneration of a functionalis, mice undergo up to 80 estrus cycles and/or produce 8–10 litters during reproductive life, indicating that repair and regeneration are important features of the endometrial mucosa (Gargett *et al.*, 2012). The BrdU label retention system was utilized to determine the role of LRCs in models of endometrial epithelial repair following a menstruation-like event (Kaitu'u-Lino *et al.*, 2010, 2012), estrogen-induced endometrial regeneration in ovariectomized mice (Chan *et al.*, 2012) and in postpartum repair and regeneration (Cao *et al.*, 2014) (Table VI).

Epithelial LRCs were only observed in the glands in a modified mouse model of menstrual breakdown and epithelial repair, incorporating a BrdU pulse in adult mice during estrogen priming and a short 7–9-day chase during progesterone-mediated differentiation and rapid epithelial re-epithelialization (Kaitu'u-Lino *et al.*, 2010). During endometrial shedding, the luminal epithelium and the subsequent estrogen-independent epithelial repair rapidly lost the BrdU label, whereas the glandular epithelium remained quiescent. Following complete re-epithelialization, >30% of the glandular LRCs proliferated, indicating a role for glandular LRCs following endometrial epithelial repair. In longitudinal endometrial profiles, these glands were located close to the endometrial–myometrial junction, similar to the basalis in humans. These glands showed features of basalis epithelium, relative quiescence as shown by lower BrdU pulse labelling, slower BrdU dilution and higher Esr1 expression, compared with luminal epithelium. A subpopulation of these glandular epithelial LRCs may be the candidate epithelial progenitors of the mouse endometrium rather than the luminal LRCs identified in pulse-labelled neonatal mice. However, the chase period was insufficient to fully dilute the label in the glands, which can be achieved by hormonal manipulation to simulate estrus cycling following re-epithelialization. Perivascular LRCs were also identified in this model, which may have a role in considerable remodelling of the stromal vascular fraction during decidualization, breakdown and epithelial repair (Kaitu'u-Lino *et al.*, 2012).

A functional role for mouse endometrial LRCs in driving endometrial regeneration was demonstrated in an estrogen replacement model using postnatal pulse labelling in subsequently ovariectomized mice (Chan *et al.*, 2012). In this kinetic study, endometrial LRCs of 4-week chased peripubertal mice localized to the luminal epithelium. Eight hours following estrogen replacement, these LRCs initiated epithelial cell proliferation, indicating their role in driving tissue regeneration. In contrast, in postnatal-labelled, 8-week chased adult mice, both luminal epithelial LRCs and non-LRCs initiated proliferation 2 h after estrogen treatment. This suggests that neonatally labelled luminal epithelial LRCs play a larger role in estrogen-induced proliferation in the peripubertal endometrium relative to the adult cycling endometrium (Chan *et al.*, 2012). A subpopulation of perivascular stromal LRCs (12%) also proliferated in response to estrogen stimulation, but the stromal proliferative response was modest (Chan and Gargett, 2006; Chan *et al.*, 2012). These findings suggests that a minority of stromal LRCs in the 8-week chase of postnatal-labelled mice are likely the

stem/progenitor cells and that longer chase periods are required to identify them (Gargett et al., 2012).

Prepubertal (postnatal days 19–22) BrdU labelling allows the chase period to extend into pregnancy and the postpartum period, phases of major endometrial remodelling, repair and regeneration. Endometrial stromal LRC numbers were maintained during pregnancy but decreased in the postpartum period coinciding with repair, regeneration and increased levels of proliferation (Cao et al., 2014). On postpartum day 1, there was a peak of proliferating stromal LRCs in the endometrium, suggesting a role in postpartum repair. In contrast, there was no evidence that distal oviduct and endocervical junctional zone (JZ) and long-term glandular LRCs identified by H2B-GFP labelling participated in endometrial repair and regeneration following endometrial shedding or in the postpartum period (Patterson and Pru, 2013). However, distal oviduct H2B-GFP LRCs form spheroids *in vitro* and have differentiation capacity (Wang et al., 2012c), suggesting that they may be reserve cells with potential to contribute to endometrial repair and/or regeneration, possibly under circumstances yet to be experimentally tested.

Genetic lineage tracing has provided evidence that a stromal subpopulation derived from anti-Mullerian hormone (AMH) receptor type II expressing cells contributed to epithelial repair and regeneration via MET (Huang et al., 2012; Patterson et al., 2013). A study of cellular dynamics and gene expression in an experimental model of endometrial re-epithelialization supports the importance of MET (Cousins et al., 2014); however, the exact stromal cell type involved remains to be determined.

In summary, the LRC approach has identified small populations of epithelial and stromal cells with label retention properties, some of which appear to participate in endometrial remodelling and regenerative events. However, functional studies of living LRCs using transgenic labels, such as H2B-GFP, lineage tracing and additional models, and stem cell assays are required to elucidate the role of quiescent putative epithelial and stromal stem/progenitor populations in the murine endometrium.

Role of bone marrow-derived cells in endometrial regeneration

Bone marrow has been proposed as a source of cells that cross lineage barriers to differentiate into specialized cell types of several organs including the endometrium (Krause et al., 2001; Du and Taylor, 2009). Bone marrow comprises small populations of haematopoietic, mesenchymal and endothelial stem/progenitor cells and large numbers of myeloid cells at various stages of differentiation. Several reports suggest that bone marrow gives rise to endometrial stromal, epithelial and endothelial cells. These studies used donor-specific markers to identify cells from transplanted bone marrow in the endometrium of recipient patients, mice and baboons. As the endometrium is an immunologically active tissue that recruits bone marrow-derived immune (myeloid and lymphoid) cells, the identity of bone marrow-derived endometrial cells needs to be carefully verified by the absence of immune cell markers and/or the presence of endometrial cell-specific markers to distinguish between a stem cell and immune cell origin.

Human studies

The first study to report bone marrow-derived endometrial cells examined endometrium from patients who had received HLA-mismatched bone marrow transplants (Taylor, 2004). Donor-derived cells were detected by reverse transcriptase–polymerase chain reaction and immunostaining for their HLA type and their phenotype determined by location and morphology. Bone marrow-derived cells accounted for up to 48% of the epithelial cells and 52% stromal cells. Subsequent studies using the Y-chromosome as a marker of bone marrow-derived cells in the endometrium of patients receiving sex-mismatched bone marrow transplants also reported contributions to epithelia and stroma, albeit at more modest levels of $<10\%$ (Ikoma et al., 2009; Cervelló et al., 2012). In keeping with reports of bone marrow-derived endothelial progenitors, bone marrow-derived endometrial endothelial cells have also been reported (Mints et al., 2008). However, bone marrow-derived cells did not contribute to the endometrial SP of putative stem/progenitor cells in the human endometrium (Cervelló et al., 2012). Thus, the question still remains whether the bone marrow-derived cells incorporating into the human endometrium are stem cells or immune cells.

Mouse models

Mouse models have also been used to examine the role of bone marrow-derived cells in endometrial regeneration. Bone marrow transplantation studies, using conditioning to ablate host marrow stem cells and allow haematopoietic engraftment, reported donor bone marrow-derived stromal, epithelial and endothelial cells in the mouse endometrium (Bratincsák et al., 2007; Du and Taylor, 2007; Mints et al., 2008; Du et al., 2012; Morelli et al., 2013). Factors driving the formation of bone marrow-derived endometrial cells include estrogen, which increased the incorporation of bone marrow-derived endothelial progenitors into uterine vasculature (Masuda et al., 2007a). Hormone-driven endometrial cycling showed no detectable effect on rates of bone marrow cell integration into endometrial stroma and epithelium (Du et al., 2012), arguing against a proposed role for bone marrow-derived cells in cyclic regeneration. However, uterine ischaemia or trauma approximately doubled the rates of stromal engraftment, without increasing epithelial engraftment (Du et al., 2012; Alawadhi et al., 2014). These observations suggest a role for bone marrow-derived stromal cells in repairing endometrial injury, rather than normal cyclic regeneration. Exposure to cigarette smoke decreases the recruitment of both stromal and epithelial bone marrow-derived cells, a finding linked to both infertility and reduced rates of endometriosis in smokers (Zhou et al., 2011). An important aspect of this type of study is the ability to clearly distinguish endometrial cells from bone marrow derived-leukocytes through co-immunolocalization and confocal microscopy. Several micrographs of uterine CD45 immunostaining in some studies of bone marrow-derived endometrial cells (Du et al., 2012; Alawadhi et al., 2014) are unusually devoid of $CD45^{+}$ cells, suggesting a low detection rate for leukocytes, possibly leading to misclassification of bone marrow-derived leukocytes as stromal and epithelial cells.

Most studies of bone marrow-derived endometrial cells have not addressed the type of bone marrow cells contributing to the endometrium. Protocols used have been typically designed to facilitate engraftment of HSCs. HSCs have been widely reported to give rise to non-haematopoietic lineages in other tissues (Krause et al., 2001), but this has been disputed (Wagers and Weissman, 2004). In support of HSCs as the source of bone marrow-derived endometrial cells, transgenic mouse models tracing the $CD45^{+}$ bone marrow haematopoietic lineage demonstrated the existence of bone marrow-derived endometrial epithelium in a small number of animals (Bratincsák et al., 2007). In contrast,

human patients and baboons transplanted with mobilized and purified HSCs failed to exhibit evidence of HSC-derived endometrial stroma (Wolff *et al*., 2013). Also arguing against HSC-derived contributions to the endometrium is the finding that granulocyte-colony stimulating factor, which mobilizes bone marrow HSCs, reduced the engraftment of bone marrow-derived stromal cells in the endometrium (Du *et al*., 2012). It was proposed that bmMSC, a population not mobilized by granulocyte-colony stimulating factor, rather than HSC, generated endometrial stroma. Interpretation of these studies is further complicated by the finding of a resident population of uterine haemangioblasts that generate HSCs and vascular cells in the murine endometrium (Sun *et al*., 2010).

Challenges in defining the role of bone marrow-derived cells in endometrial regeneration

The concept of bone marrow as a source of transdifferentiating cells responsible for cellular replacement has been investigated in many organs including lung, liver, kidney, intestine and skin (Krause *et al*., 2001). Studies of these organs tell a cautionary tale relevant to ongoing work on bone marrow-derived cells in endometrial repair and regeneration. Initial enthusiasm for the concept of bone marrow transdifferentiation (Petersen *et al*., 1999; Krause *et al*., 2001; Gupta *et al*., 2002) was tempered by subsequent reports that genuine transdifferentiation of bone marrow-derived cells is either very rare or cannot be detected (Wagers *et al*., 2002; Duffield *et al*., 2005; Kotton *et al*., 2005). Cell fusion, artefactual marker staining and overlaying leukocytes are commonly cited as sources of cells that may be misinterpreted as transdifferentiated cells (Wang *et al*., 2003; Duffield and Bonventre, 2005). During the ongoing debate on bone marrow cell transdifferentiation, a common emerging theme is that rigorous technical analysis and multiple markers must be used to definitively identify and determine the phenotype of candidate bone marrow-derived cells in any organ (Duffield and Bonventre, 2005; Kassmer and Krause, 2010). The existence of bone marrow-derived endometrial cells has yet to receive the level of scrutiny applied to many other organs.

Generating endometrial epithelial-like tissue from hES cells

In contrast to adult stem/progenitor cells, pluripotent hES cells are available in large numbers, and many protocols have been developed for their differentiation into clinically relevant cell types (Trounson, 2006), including endometrial cells (Song *et al*., 2015). Induced pluripotent stem (iPS) cells (Table I) offer a more attractive source of pluripotent cells to generate cells for tissue repair as they can be derived from the patient's own cells, overcoming immunological barriers associated with hES cell derivatives (Takahashi *et al*., 2007). Endometrial cells are an attractive source of cells for reprogramming into iPS cells as they express elevated levels of pluripotent factors and more efficiently generate iPS cells when compared with conventional cells (Park *et al*., 2011). Human endometrial epithelial-like tissue has been derived from hES cells by recapitulating the stages of reproductive tract development using a two-stage strategy (Ye *et al*., 2011). GFP-tagged hES cells were initially partially differentiated to intermediate and lateral plate mesoderm *in vitro* using bone morphogenetic protein 4 and activin A during embryoid body formation. This was followed by further differentiation *in vivo* by induction with neonatal mouse uterine mesenchyme as a tissue recombinant and transplanted into immune-compromised NSG mice. Differentiation during this 9-week process was monitored by assessing transcripts and protein for specific mesodermal differentiation markers in the differentiating embryoid bodies, Müllerian duct markers (LIM homeobox protein 1, LIM1; paired box 2, PAX2; homeobox A10, HOXA10) in early xenografts and endometrial epithelial markers (ovarian cancer-related tumour marker, CA125; ESR1, β-tubulin on ciliated cells) in late harvest xenografts (Ye *et al*., 2011, 2012). These endometrial gland-like profiles were functional and proliferated in response to exogenous estrogen and upregulated glycodelin A. The ability of uterine stroma to direct the differentiation of hES cell-derived mesodermal derivatives could be used to differentiate endometrial stromal cell-derived iPS cells into endometrial epithelial cells for potential use in tissue engineering applications to regenerate endometrial tissue in Asherman's syndrome (Gargett and Ye, 2012).

Endometrial stem/progenitor cells in disorders of endometrial proliferation

As adult stem cells have a key role in maintaining tissue homeostasis, it is likely that their function is aberrant in benign gynaecological disease associated with altered endometrial proliferation. Stem/progenitor cells are regulated by the stem cell niche, which may also have roles in the development and progression of endometriosis, adenomyosis, thin dysfunctional endometrium and Asherman's syndrome (Table VII).

Endometriosis

Endometriosis is characterized by the growth of endometrial tissue outside the uterine cavity (Giudice and Kao, 2004; Viganó *et al*., 2004). The most widely accepted theory for the pathogenesis of endometriosis is that retrograde menstruation deposits viable endometrial fragments into the pelvic cavity which attach to and invade the peritoneal mesothelium to establish ectopic growth of endometrial tissue (Sampson, 1927). Despite most women experiencing retrograde menstruation, it is not known why only 6–10% of the reproductive age women develop endometriosis (Halme *et al*., 1984). Following the discovery of endometrial stem/progenitor cells, Sampson's hypothesis was extended to address this disparity. It is proposed that endometrial stem/progenitor cells with associated niche cells are abnormally shed during menses, where they gain access to the peritoneal cavity by retrograde menstruation and establish ectopic implants, causing endometriosis (Starzinski-Powitz *et al*., 2001; Leyendecker *et al*., 2002; Gargett, 2007; Sasson and Taylor, 2008; Gargett and Masuda, 2010). It was also proposed that endometriosis lesions initiated by endometrial stem/progenitor cells would be more severe and invasive than lesions initiated by more differentiated transit-amplifying cells, explaining the different grades of endometriosis (Gargett, 2007). Alternatively, endometrial stem/progenitor cells, with yet to be identified intrinsic abnormalities, such as carrying one of the endometriosis susceptibility alleles (Nyholt *et al*., 2012), may have increased propensity to implant and establish an ectopic colony. Normal endometrial stem/progenitor cells may also implant more readily on an abnormal peritoneal mesothelium (Gargett and Chan, 2006; Gargett, 2007). It was also hypothesized that endometrial stem/progenitor cells may be involved in the pathogenesis of premenarcheal

Table VII Endometrial diseases in which endometrial stem/progenitor cells may play a role.

Endometrial disease	Description
Adenomyosis	A benign disease involving extensive growth and invasion of basalis endometrial tissue into the uterine myometrium with associated smooth muscle hyperplasia, resulting in an enlarged uterus and painful, heavy or prolonged periods
Asherman's syndrome and intrauterine adhesions (IUAs)	An acquired uterine condition characterized by complete obliteration of the endometrium with fibrotic intrauterine adhesions (IUAs) causing amenorrhea and infertility. IUA is a less severe condition involving partial replacement of the endometrium with fibrous tissue, causing hypomenorrhea, infertility and pregnancy loss. It results from trauma to the basalis endometrium following dilation and curettage (D&C) due to miscarriage, abortion or retained placenta in a setting of low estrogen and/or infection
Endometriosis	A benign disease affecting reproductive aged women in whom endometrial tissue grows outside the uterine cavity, most often in the pelvic cavity, around/on the ovaries and in the rectovaginal septum, resulting in inflammation, infertility and severe pelvic pain
Thin dysfunctional endometrium	Endometrial tissue that does not respond to estrogen stimulation and fails to reach at least 7 mm in thickness necessary for embryo implantation and maintenance of an ongoing pregnancy

and adolescent endometriosis through retrograde neonatal uterine bleeding due to maternal progesterone withdrawal at birth (Brosens and Benagiano, 2013; Brosens *et al.*, 2013; Gargett *et al.*, 2014). Endometrial stem/progenitor cells, together with niche cells, would remain dormant beneath the peritoneum until rising estrogen levels triggered at menarche activate them to initiate clonal growths of ectopic endometrium and establish early onset endometriosis prior to menstruation (Fig. 2).

No direct evidence for the role of endometrial stem/progenitor cells in the pathogenesis of endometriosis has yet been reported. Several studies support a role for shedding of endometrial epithelial progenitor cells from the basalis in women with endometriosis. Fragments of basalis endometrium were identified more often in menstrual blood of women with than without endometriosis (Leyendecker *et al.*, 2002). SSEA-1, a marker of endometrial basalis epithelial cells, was found in endometriotic lesions (Valentijn *et al.*, 2013). The monoclonality of ectopic endometrial epithelial cells (Jimbo *et al.*, 1997; Tamura *et al.*, 1998; Wu *et al.*, 2003) and identification of DNA losses/genomic imbalances demonstrating clonal proliferation (Silveira *et al.*, 2012) in endometriotic lesions suggest the involvement of progenitor cells in the pathogenesis of endometriosis. Stem cell genes and proteins OCT4, SOX2, NANOG, Musashi and C-KIT have been observed in endometriotic lesions (Götte *et al.*, 2008; Forte *et al.*, 2009; Song *et al.*, 2014). Cultured ectopic, clonally derived eMSCs expressed *OCT-4* and demonstrated MSC phenotypic surface markers (Kao *et al.*, 2011). Together, these studies suggest that women with endometriosis shed basalis endometrium at menstruation, increasing the likelihood of endometrial epithelial progenitor cells gaining access to the peritoneal cavity to initiate and develop into endometriotic lesions. However, these findings do not explain a role for eMSCs, which are present in both functionalis and basalis.

Recent functional human studies identified stem/progenitor cells in ectopic endometriotic lesions, suggesting their potential role. Similar to eutopic endometrial stem/progenitor cells, endometriotic epithelial and stromal CFUs were observed, and these serially cloned two to three times (Chan *et al.*, 2011). Ectopic stromal CFUs were multipotent (Chan *et al.*, 2011; Kao *et al.*, 2011) and underwent more than 25 population doublings before senescence (Kao *et al.*, 2011; Moggio *et al.*, 2012), similar to eutopic stromal CFUs (Gargett *et al.*, 2009). Ectopic endometriotic stromal cell lines differentiated into cytokeratin- and E-cadherin-expressing epithelial cells in culture (Moggio *et al.*, 2012). Ectopic MSCs demonstrated greater migration and invasion than eutopic MSCs with increased angiogenesis and invasion into surrounding tissue in a scaffold transplantation mouse model (Kao *et al.*, 2011). Together, these studies support the presence of endometrial stem/progenitor cells in ectopic endometriotic lesions. The peritoneal fluid of menstruating women with and without endometriosis contained similar numbers of endometrial cells (Bokor *et al.*, 2009), although markers used to identify them were not specific and also immunostained resident peritoneal mesothelial cells. As endometrial stromal cells rapidly attach to peritoneal mesothelial cells *in vitro* (Lucidi *et al.*, 2005), their concentration in peritoneal fluid may not accurately represent those retrogradely shed during menstruation. The presence of endometrial stem/progenitor cells in peritoneal fluid has not yet been reported, which is crucial for identifying their role in the pathogenesis of endometriosis. The identification of the specific eMSC marker SUSD2 will enable their identification in shedding endometrium and peritoneal fluid. However, markers are currently lacking for endometrial epithelial progenitor cells.

Endometriosis models in mice, rats, baboons and marmosets (Grümmer, 2006) exist, but these have focused on pathophysiological mechanisms involved in the development of disease, rather than the involvement of stem/progenitor cells, although bone marrow-derived cells may have contributed to the progression of established endometriosis lesions (Taylor, 2004). However, it can be inferred that endometrial stem/progenitor cells are likely responsible for lesion formation in animal models, in which menstrual debris was transplanted into the peritoneal cavity (Greaves *et al.*, 2014) or endometrial cells/tissues were injected subcutaneously (Wang *et al.*, 2014). Genetic labelling of endometrial stem/progenitor cells with fluorescent tags is needed to exploit these animal models for cell tracking to determine their role in ectopic lesion formation. This approach was used to label all endometrial cells with adenovirus encoding red fluorescent protein (Wang *et al.*, 2014) or recombinant lentivirus conferring yellow fluorescent protein or click beetle red-emitting luciferase (Masuda *et al.*, 2007b) for non-invasive monitoring of cell xenografts over several menstrual-like cycles. These animal models need further adapting to monitor labelled endometrial stem/progenitor cells, transplanted into the peritoneal cavity and subjected to a similar hormonal regime as the mouse model of menstruation (Brasted *et al.*, 2003) for several cycles to examine the role of endometrial stem/progenitor cells in the initiation and progression of endometriosis.

Adenomyosis

Adenomyosis is considered a dichotomous disease characterized by thickening and disruption of the endo-myometrial JZ structure

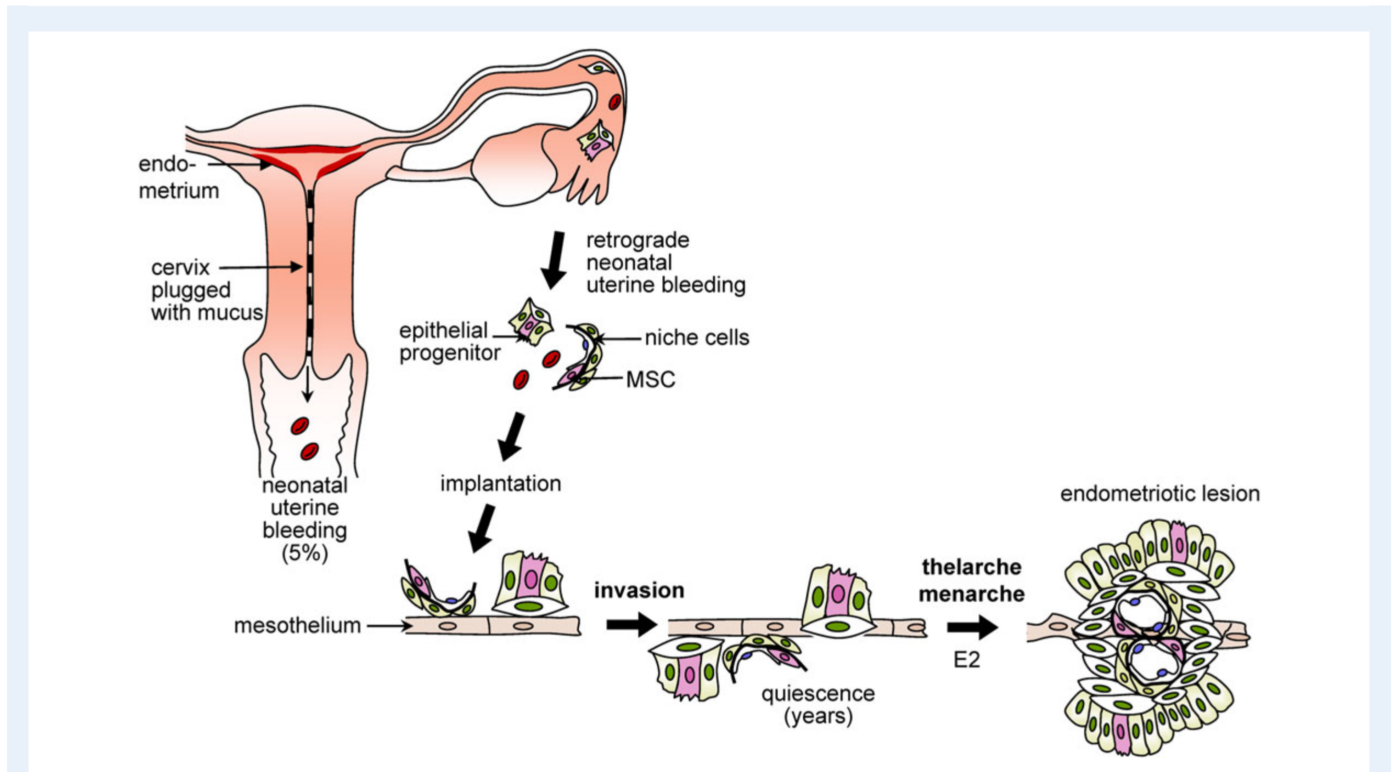

Figure 2 Schematic describing the hypothesis that endometrial stem/progenitor cells shed in neonatal uterine bleeding may play a role in early onset endometriosis. Neonatal uterine bleeding occurs in 5% of neonates. It is hypothesized that retrograde neonatal bleeding occurs because thick mucus obstructs the long neonatal cervix. Fragments of shed endometrial tissue are postulated to contain an endometrial epithelial progenitor cell (pink) and a perivascular MSC (pink) together with niche cells. These rapidly adhere to the neonatal mesothelium, invade and/or become contiguous with the mesothelial lining where they remain quiescent for ~10 years. Rising estrogen (E2) levels associated with thelarche and menarche reactivate the stem/progenitor cells to initiate growth of endometriosis lesions on the surface of or below the peritoneal mesothelium, resulting in early onset endometriosis. Reprinted with permissions from Gargett *et al.* (2014).

(Benagiano *et al.*, 2014). Little is known about the pathophysiology of adenomyosis, which mainly affects parous women. Theories suggest that chronic microtrauma to the JZ from chronic peristaltic myometrial contractions causes repeated cycles of tissue injury and repair (Leyendecker *et al.*, 2009). A vicious cycle is established in which local estrogen production promotes uterine hyperperistalsis and further autotraumatization, allowing basal endometrial glands and stroma to penetrate the myometrium and proliferate to form pockets of adenomyosis within the uterine muscle. Tissue injury typically activates adult stem cells, which may establish ectopic endometrial lesions through disruption of endometrial stem/progenitor cell niches (Gargett, 2007). Abnormal differentiation of eMSCs may be responsible for the smooth muscle hyperplasia (Gargett, 2007). In support of this concept, stromal cells cultured from adenomyotic tissue differentiated into typical mesodermal lineages and expressed MSC surface markers (Chen *et al.*, 2010). Whether adenomyotic stromal cells exhibit MSC properties of clonogenicity and self-renewal or contain a subpopulation of perivascular $CD146^+PDGFR\beta^+$ or $SUSD2^+$ or SP cells is unknown. Gene expression profiling identified differences between adenomyotic and normal endometrial stromal cells (Chen *et al.*, 2010), particularly COX-2 overexpression. COX-2 has a role in the tissue injury repair cycle and local estrogen production (Leyendecker *et al.*, 2009). In an attempt to identify endometrial stem cells in adenomyotic tissue, immunostaining with an adult stem cell marker, Musashi-1, was undertaken (Chen *et al.*, 2014). However, most epithelial cells and a proportion of stromal cells were Musashi-1^+, in contrast to observations of rare immunostained cells in the normal endometrium (Götte *et al.*, 2008). Given this discrepancy and that Musashi-1^+ endometrial stromal cells in eutopic and ectopic adenomyosis do not appear in a perivascular location, it seems unlikely that Musashi-1-expressing cells are endometrial stem/progenitor cells in adenomyosis. It also indicates that functional stem cell assays should accompany studies examining stem cell marker expression. More research is required to establish a role for endometrial stem/progenitor cells in the initiation and progression of adenomyosis.

Thin endometrium and Asherman's syndrome

Dysfunctional endometrial stem/progenitor cells may be responsible for the inability of some women to generate a sufficiently thick endometrium (>7–8 mm) to support embryo implantation (Yu *et al.*, 2008). Thin dysfunctional endometrium unresponsive to estrogen stimulation is particularly challenging in IVF clinics. In Asherman's syndrome, defined as the complete obliteration of the uterine cavity with adhesions, we hypothesize a complete loss of endometrial stem/progenitor cells. Intrauterine adhesions (IUAs) represent a continuum between Asherman's syndrome and thin dysfunctional endometrium, where we postulate that there are insufficient endometrial stem/progenitor cells, which may or

may not be dysfunctional, residing in the pockets of remaining endometrial tissue (Gargett and Ye, 2012). Between 2 and 22% of infertile women have Asherman's syndrome or IUA (Yu et al., 2008; Panayotidis et al., 2009). Trauma to the endometrial basalis and JZ from termination of pregnancy, spontaneous miscarriage and postpartum curettage in a setting of low circulating estrogen levels hinder endometrial regeneration. Any remaining endometrial stem/progenitor cells survive a low estrogen environment, but their niche cells require an estrogen-rich milieu to activate them. When infection and inflammation also occur, the inflammatory products may damage endometrial stem/progenitor cells, critically reducing their numbers and limiting their capacity to regenerate sufficient endometrium (Gargett and Ye, 2012).

A mouse model of Asherman's syndrome has been developed using a needle to traumatize the lumen of both uterine horns (Alawadhi et al., 2014). Immediately following the damage, unfractionated male mouse bone marrow cells or saline was administered intravenously and the mice were examined 3 months later. Histological analysis showed reduced fibrosis and pregnancies with normal litter sizes in 90% of the bone marrow-transplanted animals, compared with 30% of saline-treated mice. A small number (0.1%) of non-leukocyte ($CD45^{-}$) Y $chromosome^{+}$ cells were detected in the endometrium of the transplanted mice, but the original identity of the incorporated cells is unknown. The effective repair of damaged endometrium by this small number of cells suggests either an immediate systemic cytokine effect or an indirect activation of endogenous endometrial stem/progenitor cells or their niches.

In a rat model of Asherman's syndrome in which trichloroacetic acid was instilled into the uterine lumen, cultured male rat adipose-derived MSCs were injected into one uterine horn and then intraperitoneally at 5 day intervals. Histological assessment of the endometrium revealed that 4–6% of the stroma was BrdU-labelled cells (Kilic et al., 2014). MSCs alone increased vascular and cellular proliferation and VEGF immunostaining, but had no effect on inflammation or fibrosis. When co-administered with oral estrogen, the MSCs also reduced fibrosis. In contrast to systemic delivery, local injection of allogeneic MSCs to the injured endometrium enabled a proportion to incorporate into the tissue with apparent promotion of tissue regeneration, although neither tissue architecture nor endometrial function in supporting implantation or pregnancy was assessed.

Several case studies have reported the instillation of fresh $CD34^{+}$ or cultured autologous bone marrow cells into the uterine cavity or subendometrial region of women with Asherman's syndrome or IUA (Nagori et al., 2011; Singh et al., 2014). Very moderate increases in endometrial thickness and evidence of menstruation or pregnancy were reported. However, these must be viewed with caution as there were no control subjects; a lack of detail on bone marrow cell preparation and the increase in endometrial thickness was generally below that required for successful embryo implantation (Gargett and Healy, 2011). Other sources of cells should also be considered in further development of a stem cell approach to treating thin dysfunctional endometrium, for example, allogeneic endometrial stem/progenitor cells or autologous endometrial cells derived from iPS cells. Endometrial-like tissue has been generated from hESCs (Ye et al., 2011), and iPS cells are easily created from human endometrial stromal cells (Park et al., 2011) or shed menstrual blood cells due to their plasticity and regenerative capacity (de Carvalho Rodrigues et al., 2012; Li et al., 2013). Further development of the animal model is required to determine the mechanism of action before cell-based therapies for Asherman's syndrome or thin dysfunctional endometrium are trialled in humans.

Clinical use of endometrial and mbMSCs for regenerative medicine

The ease with which endometrial tissue can be obtained by Pipelle biopsy without anaesthetic in comparison to the collection of bone marrow aspirates or adipose tissue liposuctions makes it an attractive source of MSCs for regenerative medicine (Ulrich et al., 2013). Menstrual blood is an even easier source for collection of MSC-like cells, although greater attention is required to ensure sterility of the cell product and methods for the purification of eMSCs from this source have not been refined.

Non-homologous use of menstrual blood stromal cells (ERCs)

The potential of cultured menstrual blood ERCs as a regenerative medicine therapy for a range of allogeneic non-homologous applications has been examined in several preclinical animal models. In a rat model, cell sheet technology was used to patch an infarcted heart, resulting in ERC incorporation and differentiation into cardiomyocytes, and improved cardiac function (Hida et al., 2008). Similarly, ERC transdifferentiated into skeletal muscle myocytes by fusion in a Duchenne muscular dystrophy mouse model (Cui et al., 2007). In a rat stroke model, cultured ERCs sorted for CD117 expression showed neuroprotection by differentiating into neuronal cells, improving motor and neurologic impairments (Borlongan et al., 2010). Cultured ERCs have reversed ovarian damage in a cyclophosphamide-induced mouse model of premature ovarian failure (Liu et al., 2014). In this model, locally injected ERC labelled with a fluorescent DiO dye showed greater retention than DiO-labelled human fibroblasts (source unknown) in the ovaries after 14 days. The DiO-labelled cells also expressed ovarian markers, AMH, FSH receptor, inhibin-α and -β. cDNA profiling revealed greater similarity in gene expression with human ovaries for mice treated with ERCs compared with human fibroblasts. Ovarian weight increased and function improved as judged by normalized circulating estradiol and FSH levels in ERC-treated mice. There were also fewer atretic follicles and more normal follicles. These studies indicate that ERCs have superior retention and reparative action than fibroblasts (presumably dermal) when delivered locally to injured ovaries.

Tissue engineering principles are being developed using ERC for eventual clinical translation. ERCs cultured on polycaprolactone nanofibres showed greater proliferative capacity and ability to differentiate into chondrocytes than bmMSCs (Kazemnejad et al., 2012). In evaluating ERCs for a potential cardiac tissue engineering application, a greater ability was observed to penetrate silk fibroin scaffolds and proliferate than bmMSCs (Rahimi et al., 2014). Further development of these tissue engineering constructs and their evaluation *in vivo* are warranted for ERCs, given their greater proliferation rates and easier acquisition than other sources of MSCs. Summarized in a recent review (Ulrich et al., 2013) are several case reports on the treatment of patients with various disorders using systemically delivered, cultured ERCs. In addition, a phase 2 clinical trial, the RECOVER-ERC launched by Medistem for treating congestive cardiac failure (Bockeria et al., 2013), is also detailed. Although there have been no side effects reported, the clinical data have not yet been published. The potential of menstrual blood stromal fibroblasts for regenerative medicine purposes looks promising

for non-homologous use. However, more research is required to compare local versus systemic delivery and to determine the mechanism of action by using genetic labelling to track the cells more accurately in appropriate preclinical animal models to generate sufficient confidence for translating this potential into the clinic.

Autologous use of eMSCs as a therapy for pelvic organ prolapse

Pelvic organ prolapse (POP) is a major hidden disease burden for women, a legacy of vaginal birth for 50% of postmenopausal parous women (Nygaard *et al.*, 2008). POP is the herniation of the pelvic organs into the vagina causing urinary and faecal incontinence, voiding and sexual dysfunction. Many women (19%) have a lifetime risk of undergoing reconstructive surgery for POP (Smith *et al.*, 2010; Wu *et al.*, 2014a) and ~30% require additional operations due to failure of native tissue surgery or from significant adverse events associated with the use of vaginal mesh (Olsen *et al.*, 1997; FDA, 2011). It has been proposed that autologous eMSCs used together with new mesh designs matching the biomechanical properties of vaginal tissue (Ulrich *et al.*, 2012; Edwards *et al.*, 2013) in a tissue engineering construct may improve surgical outcomes of vaginal mesh surgery for POP (Gargett, 2006; Ulrich *et al.*, 2013). In a proof-of-principle experiment, culture-expanded SUSD2$^+$ DiO-labelled eMSC seeded on new mesh (polyamide/gelatin composite, 2.5×10^5 cells) improved tissue integration of the mesh and the biomechanical outcomes of the tissue/mesh complex after 90 days of implantation in a fascial defect wound of immunocompromised nude rats, despite surviving at the site for only 14 days (Ulrich *et al.*, 2014a). The eMSC promoted early neovascularization around the implanted mesh (7 days). An early inflammatory response was also observed around the mesh with eMSCs, characterized by increased numbers of M1 macrophages, but by 30 days these had differentiated into the M2 wound-healing phenotype and at 90 days the chronic macrophage response was significantly reduced. Similar quantities of new tissue collagen were deposited whether or not eMSCs were present, but more physiological, crimped collagen was observed in the mesh with eMSCs (Edwards *et al.*, 2015). This led to a more compliant, less stiff mesh/tissue complex than mesh alone, addressing one problem associated with the use of clinical polypropylene mesh (FDA, 2011). This promising result in a heterologous small animal model of POP repair surgery has led to the development of an autologous large animal, ovine preclinical model of POP using ovine eMSCs (Ulrich *et al.*, 2014b). This animal model is required to determine whether the mechanism of action of locally delivered eMSCs is due to paracrine release of factors promoting wound repair or whether they differentiate and incorporate into the dermis and smooth muscles of the vaginal wall (Atala, 2009; von Bahr *et al.*, 2012). *In vitro*, TGFβ1 and platelet-derived growth factor BB (PDGF-BB) induced differentiation of SUSD2$^+$ eMSCs seeded on the polyamide/gelatin meshes into SUSD2$^-$ smooth muscle cells expressing SM22α and smooth muscle myosin heavy chain, intermediate and late smooth muscle cell differentiation markers (Su *et al.*, 2014). Connective tissue growth factor also differentiated eMSCs into collagen-producing fibroblasts. It remains to be determined whether autologous eMSCs delivered on the new mesh will undergo differentiation to these tissue-forming cells *in vivo*.

Clinical good manufacturing practice production of eMSCs

To prepare eMSCs or ERCs for cell-based therapies, it is necessary to incorporate the principles of Good Manufacturing Practice guidelines into the isolation and culture expansion process to ensure that the cells produced are of desired quality, safety and efficacy for each batch (Eaker *et al.*, 2013; Hunsberger *et al.*, 2015). This requires the removal of all animal products (xeno-free, XF) used in the dissociation of tissue, cell purification, cryopreservation and in culture expansion protocols. In particular, bovine foetal calf serum needs to be replaced in the development of optimized SF culture medium, an area of active development for MSCs from other sources. Initial steps towards the optimization and scale-up or scale-out culture of CD146$^+$PDGFRβ$^+$ eMSCs examining commercial and in-house XF and SF media formulations found that eMSC attachment and proliferation were optimal on a fibronectin matrix in an in-house Dulbecco's modified Eagle's medium/F-12 SF medium containing FGF2 and EGF in physiological hypoxia (5% oxygen) (Rajaraman *et al.*, 2013). eMSCs cultured under these optimized conditions retained their MSC properties. The specific markers used to enrich eMSCs (PDGFRβ, CD146 and SUSD2) were more informative in discriminating between the various media and matrix combinations tested than the classic MSC markers (CD29, CD44, CD73 and CD105), pointing to the value of more specific perivascular markers. Further research is needed to determine the optimal XF conditions for eMSC and ERC culture expansion.

Immunomodulatory properties of eMSCs and ERCs and potential therapeutic use

In addition to their progenitor properties, MSCs have anti-inflammatory and immunomodulatory properties, regulating both the innate and adaptive immune systems, that have been exploited clinically (Le Blanc and Mougiakakos, 2012; Bianco *et al.*, 2013). Tissue fibroblasts also have these anti-inflammatory and immunomodulatory properties (Haniffa *et al.*, 2009; Bianco *et al.*, 2013), and this is why cultures of unfractionated adherent bone marrow cells containing predominantly stromal fibroblasts have been effective in many studies and clinical trials.

In a proinflammatory environment, resident MSCs exposed to the inflammatory cytokine tumour necrosis factor-α and/or interferon-γ are activated to secrete anti-inflammatory mediators (Krampera, 2011). These mediators include IL-10, prostaglandin E2, TGFβ, HLA-G, indoleamine oxidase and nitric oxide, which together with cell–cell interaction suppress dendritic, T, B and NK cell activation (Le Blanc and Mougiakakos, 2012). The low level of expression of major histocompatibility complex II and co-stimulatory molecules confers MSCs with low alloreactivity, enabling them to be used for allogeneic transplantation. MSCs can also switch macrophages from an inflammatory M1 to a reparative M2 phenotype (Krampera, 2011).

Although no *in vitro* studies have examined the immunoregulatory function of human eMSCs or ERCs, transcriptional profiling of fresh CD146$^+$PDGFRβ$^+$ and cultured SUSD2$^+$ and SUSD2$^-$ cells revealed that eMSCs expressed inflammatory and immunomodulatory genes (Spitzer *et al.*, 2012; Murakami *et al.*, 2014). The SUSD2$^-$ stromal fibroblasts secreted more chemokines and inflammatory mediators than SUSD2$^+$ eMSCs, but both cell types secreted similar levels of many cytokines and chemokines including the Th-2-associated IL-4 and anti-inflammatory IL-10 (Murakami *et al.*, 2014). In contrast, *in vivo* studies

showed that eMSCs and ERCs have anti-inflammatory and immunomodulatory properties. Locally delivered eMSCs reduced the chronic inflammatory response to implanted synthetic mesh in a nude rat fascial wound repair model and promoted the switch of M1 to M2 macrophages, resulting in improved biocompatibility compared with controls (Ulrich et al., 2014a). Cultured endometrial stromal cells and ERCs suppressed neuroinflammation in an autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis (Peron et al., 2012) and bowel inflammation in a colitis model (Lv et al., 2014b), respectively. In the multiple sclerosis model, endometrial stromal fibroblasts were delivered intraperitoneally 24 h prior to EAE induction. In the colitis model, ERCs were systemically delivered several times after disease induction. Both models showed improved clinical symptoms and histological scores, reduced leukocyte infiltrates, lower inflammatory cytokine transcription in the damaged organs and upregulated anti-inflammatory cytokine transcripts in the spleens. There were also fewer active T cells, cytotoxic T cells and dendritic cells and more regulatory T cells in the spleens of animals receiving cells. In these models, the eMSCs, endometrial stromal fibroblasts and ERCs acted in a paracrine manner to suppress inflammation, with the latter two cell types also downregulating immune responses. These findings concur with many observations reported for MSCs derived from other sources, suggesting that eMSC-like cells have similar anti-inflammatory and immunoregulatory properties.

Unresolved issues in endometrial stem cell research

A key issue facing the endometrial stem/progenitor cell field is the use of numerous terms to describe the various stem/progenitor cell types (Tables I, II and IV). A common nomenclature recognizing the species, type of cell and its source is needed. It will be important that any nomenclature fits with that used in the broader stem cell discipline. This particularly applies to the universal definition of MSC (Dominici et al., 2006), which is increasingly recognized as inadequate for defining MSC from tissues other than bone marrow (Bianco et al., 2013). The nomenclature will need to reflect the different cell types studied, perivascular cells versus the endometrial stromal fibroblast, clonogenic versus non-clonogenic stromal fibroblasts, SP versus MP and those identified with specific markers enriching for clonogenic, self-renewing and multipotent progenitors versus unfractionated multipotent stromal fibroblasts.

Although it is apparent that bone marrow-derived cells incorporate in low numbers into the endometrium, the nature of these cells is currently unknown. Careful studies using transgenic tools to track cells are needed. Together with known markers of endometrial stem/progenitor cells and those to be identified in future will enable mechanistic studies to more precisely determine the role of bone marrow-derived cells in normal endometrial physiology and in endometrial diseases.

The hierarchical relationship among the clonogenic epithelial progenitor cells, endometrial and decidual SP cells, CD146^{+}PDGFR-β^{+} and SUSD2^{+} cells and menstrual blood ERCs is not fully known. This requires more defining markers or gene signatures and determination of the *in vivo* activity for the various cell types. As these data are generated, it may be possible to determine whether MET links the various stem/progenitor cell types and whether this process occurs during endometrial regeneration.

Future perspectives

Identification of specific markers for eMSCs enables profiling of these stem/progenitor cells and their differentiated progeny to identify their signatures. Comparison with bmMSCs, adipose MSCs and placental MSCs also becomes possible. This work has commenced and already shows differences between the perivascular-derived MSCs and the stromal fibroblast and their decidualized derivatives (Spitzer et al., 2012; Murakami et al., 2014). RNA sequencing will uncover regulatory pathways involved in the endometrial stem/progenitor cell function and their differentiation pathways allowing comparisons between cells from normal and dysfunctional endometrium and ectopic endometrial lesions of adenomyosis and endometriosis. Epigenetics governs cellular phenotype, particularly during cellular differentiation. Interrogation of the epigenetic profiles of endometrial stem/progenitor cells and stromal fibroblasts will uncover regulatory pathways, governing the function of these cell types. Similarly, once markers for endometrial epithelial progenitors are determined, their chromatin state can be determined and compared between well-characterized cell phenotypes, shedding light on the regulation of the adult stem cell state and its differentiation. These advances in sequencing technologies are matched by the availability of single-cell analysis using Fluidigm® technologies, enabling investigations into cell heterogeneity. For example, SUSD2 has a wide range of expressions on individual cells, but the function and phenotype of low versus high expression are currently unknown (unpublished), but could be determined using single-cell analysis and deep sequencing or epigenetic profiling.

eMSCs and menstrual blood ERCs are showing promise for cell-based therapies for gynaecological disease and non-homologous use. The properties of eMSCs are favourable for further development in regenerative medicine applications as they are relatively easy to obtain compared with the current commonly used sources. Regenerative medicine frequently requires biomaterials and scaffolds to deliver cells to the injured site. These can be fabricated (Edwards et al., 2013) or can be derived from decellularized uterine matrix for endometrial applications (Miyazaki and Maruyama, 2014), the latter with potential application for Asherman's syndrome or IUA. Once epithelial progenitor markers are identified, these and the MSCs could be used to seed these decellularized matrices.

Concluding remarks

In the 10-year history of endometrial stem/progenitor cells, substantial progress has been made in developing assays for their evaluation and for identifying specific markers and characterizing these populations. The *in vivo* identity of eMSC and SP cells has been determined, and examination of their role in endometriosis and adenomyosis has commenced. The ease with which eMSCs and ERCs can be obtained makes them attractive candidates for cell-based therapies for gynaecological disease and other regenerative medicine applications.

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Authors' roles

C.E.G. was involved in conception and design, acquisition of data, analysis and interpretation of data, writing the article and revising it critically for

important intellectual content plus gave final approval of the version to be published. K.E.S. provided data acquisition, analysis and interpretation of data, drafting the article and gave final approval of the version to be published. J.A.D. was involved in acquisition of data, analysis and interpretation of data, writing the article and revising it critically for important intellectual content plus gave final approval of the version to be published.

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Conflict of interest

The authors have nothing to declare.

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