The role of stem cells in uterine involution

Madelyn K Spooner¹, Yasser Y Lenis^{1,3}, Rachel Watson², Daniela Jaimes⁴ and Amanda L Patterson^{1,2}

¹Division of Animal Sciences, University of Missouri, Columbia, Missouri, USA, ²Department of Obstetrics, Gynecology and Women's Health, University of Missouri, Columbia, Missouri, USA, ³Department of Animal Sciences, Faculty of Agricultural Sciences, National University of Colombia, Palmira, Colombia and ⁴Faculty of Agricultural Sciences, University of Applied and Environmental Sciences U.D.C.A, Bogota, Colombia

Correspondence should be addressed to A L Patterson; Email: Pattersonama@missouri.edu

Abstract

Uterine remodeling during pregnancy and repair postpartum are fundamental to the successful propagation of eutherian species. The most drastic remodeling occurs in species with invasively implanting embryos, including humans and mice. During embryo implantation, embryonic trophoblasts breach the epithelium, penetrating into the stroma. Stromal cell decidualization, which is critical for the establishment and maintenance of early pregnancy, occurs throughout the implantation site. Trophoblasts further invade into and remodel uterine spiral arteries, which is necessary for placental formation. The uterus increases in size up to 24-fold, which is largely attributed to myometrial expansion. Uterine changes that occur during pregnancy must then be resolved postpartum. Following parturition, the uterus repairs the remodeled tissue in the process of uterine involution. During involution, the majority of the endometrium is regenerated to replace the tissue that is shed postpartum. The myometrium returns to the pre-gravid state which is thought to occur through apoptosis and autophagy of smooth muscle cells. Although we understand the general process of postpartum uterine involution, the detailed mechanisms, particularly the role of putative stem cells, are poorly understood. This review discusses the evidence for the existence of epithelial, stromal and myometrial stem cells and their role in uterine involution. Gaps in knowledge and areas for future research are also considered. Studies of both postpartum and menstrual uterine repair, which likely involve similar mechanisms, are described under the broad definition of uterine involution. Although the primary focus of this review is human, mouse models are discussed to provide additional information.

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Overview of uterine physiology

In the simplest of terms, pregnancy and postpartum involution can be likened to normal physiological damage and repair processes, respectively. During pregnancy, the uterus undergoes dramatic remodeling events including invasion by embryonic trophoblasts, stromal cell decidualization and myometrial hyperplasia and hypertrophy, among others. Although these events are physiologically normal and necessary for pregnancy, they result in physiological 'damage' and loss of many cell types that require regeneration and repair postpartum. Likewise, the menstrual cycle in women is a series of physiological remodeling and repair events, which likely shares similarities with those that occur during pregnancy and postpartum involution. It has long been proposed that due to the extensive remodeling that the uterus undergoes, it likely contains stem cells that function to repopulate the various cell types of the uterus (Gargett 2007). In this section, a brief description of the uterine histoarchitecture of the human and mouse is given. This is followed by discussion of

the major events in the menstrual (and estrous) cycle, pregnancy and uterine involution (both menstrual and postpartum). The primary focus of this review is human uterine physiology, however comparisons with mice are included to provide additional insight into uterine remodeling and regeneration.

Uterine histoarchitecture

Uterine involution has mostly been studied either by histological evaluation of uterine tissue during menstrual repair in humans or using pregnancy and menses-like mouse models. Histologically, the uteri of humans and mice are similar being comprised of three distinguishable tissue layers: (1) the outer-most perimetrium, or serosa, which is contiguous with the broad ligament, (2) the smooth muscle layer, termed the myometrium and (3) the inner-most endometrium (Fig. 1A). In humans, the endometrium is subdivided into the transient functional layer (functionalis; inner two-thirds), which is shed and regenerated throughout the menstrual cycle and the germinal basal layer (basalis; lower one-

Figure 1 Uterine histoarchitecture and hypothesized mechanisms of uterine involution. (A) Human and mouse histology images show myometrial (Myo) and endometrial (Endo) layers of the uterus. The human endometrium is segmented into the functionalis and basalis, indicated by dashed lines in cartoon/histology images. Both mouse and human uteri contain luminal and glandular epithelial cells (LE and GE, respectively) stromal cells, and myometrial cells. (B) Key of cartoon cells depicted in A and C. (C) Proposed mechanisms of uterine involution include: 1) replication of residual glandular and luminal epithelial cells that escaped desquamation, 2) Endogenous epithelial, stromal and myometrial stem cells, 3) Mesenchymal-epithelial transition (MET) of stromal cells into epithelial cells, and 4) Exogenous bone marrow-derived cells.

third) (Fig. 1A, Human). Mice have similar layers to the functionalis and basalis; however, the ‘functional layer’ is not shed and regenerated during the estrous cycle but only after pregnancy. The endometrium is predominantly comprised of epithelial and stromal (mesenchymal) cells. The functionalis, which is nearest to the lumen, is lined by a monolayer of luminal epithelium (LE) that also invaginates to form epithelial-lined glands (glandular epithelium, GE). These glands are embedded within the stroma and extend into the basalis down to the myometrium. The myometrium is comprised primarily of smooth muscle and fibroblast cells. In the mouse, but not in the human, the myometrium consists of two distinct layers, the inner circular and outer longitudinal muscularis (Fig. 1A, Mouse). Both the endometrium and myometrium are inundated with blood vessels, lymphatics and immune cells.

Uterine remodeling during the menstrual and estrous cycles

The menstrual cycle is standardized to 28 days and proceeds in three phases: menses, proliferative and secretory. Ovulation occurs on day (D) 14 and demarcates the proliferative and secretory phases, which correspond to the estrogen (E_2) dominant

follicular phase and progesterone (P_4) dominant luteal phase, respectively, of the ovarian cycle. The first day of menses (menstrual bleeding/shedding) marks the beginning (D1) of the menstrual cycle and occurs due to steroid hormone withdrawal following the demise of the corpus luteum (luteolysis). During menses, desquamation occurs: epithelial, stromal and endothelial cells of the functionalis undergo apoptosis leading to shedding and expulsion of the tissue (Jabbour *et al.* 2006). With loss of the functionalis, only a small proportion (~2 mm) of the endometrium, the basalis, remains. Regeneration of the functionalis occurs simultaneously with degeneration (Ferenczy 1976a, Ludwig & Spornitz 1991). Re-epithelialization begins on D2 during active menstruation and is complete by D5–6 (Ferenczy 1976a, Ludwig & Spornitz 1991). On D6–7 (proliferative phase), substantial stromal proliferation begins (Ludwig & Spornitz 1991), increasing the overall thickness of the endometrium from 1–4 mm to 11 mm just prior to ovulation (Nalaboff *et al.* 2001). In addition to stromal growth, both GE and LE proliferation continues along with angiogenesis under E_2 stimulation. On approximately D14, ovulation occurs initiating the secretory phase. Cellular changes in this stage under P_4 influence include epithelial differentiation, particularly GE, stromal cell decidualization and infiltration

of immune cells (late secretory). Of importance is decidualization of perivascular stromal cells. Decidualization is the morphological and functional differentiation of stromal cells into decidual cells to provide support for an embryo. The process begins with a brief increase in mitotic activity of perivascular stromal cells around D22–23 (Gellersen & Brosens 2014) resulting in minimal polyploidization (~1% of stromal cells) (Qi *et al.* 2015). This is followed by transition of stromal cells from elongated, fibroblast-like mesenchymal cells into rounded, secretory epithelial-like cells. Changes indicative of epithelial-like transformation include expression of E-cadherin and cytokeratin, increased cellular area and circularity, enlarged and rounded nuclei, dense membrane-bound secretory granules and cytoplasmic accumulation of glycogen and lipid droplets (Gellersen & Brosens 2014, Pan-Castillo *et al.* 2018). Decidualization occurs in preparation for an embryo and is critical for establishment and maintenance of early pregnancy. Finally, at the end of the secretory phase, in the absence of pregnancy, endometrial shedding ensues and the menstrual cycle repeats.

The estrous cycle comprises four phases: proestrus, estrus, metestrus and diestrus. Proestrus and estrus correspond to the ovarian follicular phase and are dominated by E_2 , whereas metestrus and diestrus are dominated by P_4 and correspond to the luteal phase. In mice the cycle duration is 4–5 days, and in contrast to the menstrual cycle, D1 begins on the day following ovulation which coincides with behavioral estrus (Bertolin & Murphy 2014). LE proliferation is relatively comparable across the cycle however, apoptosis is significantly higher in metestrus (Wood *et al.* 2007) resulting in a two- to four-fold higher cell number in proestrus and estrus (Evans *et al.* 1990). Stromal cell proliferation is highest in estrus, with a moderate amount in the other three stages, and apoptosis is highest in metestrus. Interestingly, changes in the GE do not follow the same pattern as the LE. GE proliferation is lowest in estrus and highest in proestrus and metestrus, with low levels of apoptosis throughout the cycle except in estrus. Overall uterine width is positively correlated with E_2 and negatively correlated with P_4 (Wood *et al.* 2007). In the absence of copulation, the ovarian corpora lutea regress rapidly and the next cycle begins.

The menstrual and estrous cycles are both characterized by stages of differential cellular activity including proliferation, differentiation, degeneration and regeneration which are regulated by ovarian derived E_2 and P_4 . However, a major distinction between the menstrual and estrous cycles is the extent of endometrial remodeling that occurs. The greatest amount of remodeling occurs during menstruation in which the functionalis is shed and expelled from the body thus requiring extensive regeneration of the endometrium. However, in estrous cycling species such as mice, the

endometrium is not shed, rather the tissue goes through cycles of mild resorption and growth (Wood *et al.* 2007).

Regarding the myometrium, much less is known about the cellular changes that occur throughout the menstrual cycle. Up until the early 2000s, the myometrium was considered a quiescent tissue outside of pregnancy, composed predominantly of differentiated smooth muscle cells (Teixeira *et al.* 2008). However, the myometrium is a dynamic tissue, responsive to various stimuli including E_2 , P_4 and a milieu of paracrine factors (Burroughs *et al.* 2000, Taniguchi *et al.* 2001, Liu *et al.* 2013). During the menstrual cycle, myometrial proliferation is greatest in the secretory phase, whereas there are low levels of relatively consistent apoptosis throughout (Wu *et al.* 2000). This shows that, although less than the endometrium, there is in fact cellular turnover in the myometrium across the menstrual cycle.

Pregnancy remodeling and postpartum repair (species comparison)

During pregnancy, embryo implantation, stromal cell decidualization and placentation are critically important events in human (and murine) pregnancy that result in a substantial amount of uterine remodeling. In human implantation, embryonic trophoblasts invade the endometrium in between adjoining uterine LE cells (intrusion) with the goal of degrading the basement membrane and contacting the underlying stroma (Bischof & Campana 1996). Comparatively in mice, the LE is also displaced by intrusion, as well as protrusion and entosis by trophoblasts. Additionally, LE opposite the invading trophoblast undergoes apoptosis (Li *et al.* 2015). In women, stromal cell decidualization expands beyond the perivascular stromal cells that differentiated in the secretory phase of the menstrual cycle, to those adjacent to the embryo (reviewed in: Abrahamssohn & Zorn 1993, Ramathal *et al.* 2010). In mice, decidualization does not occur during the estrous cycle and is therefore only initiated by an implanting embryo. Trophoblast cells continue to invade the decidualized stroma to the myometrium and remodel uterine spiral arteries, which is critical for formation of the placenta. As pregnancy progresses, in women in particular, the uterus increases in weight approximately 24-fold, which is largely attributed to expansion of the myometrium by hyperplasia and hypertrophy (Johansson 1984). The human hemochorial placenta represents the most intimate association between the fetal membranes and maternal circulation and as such is also the most invasive form of placentation and involves substantial uterine remodeling (reviewed in: Rosenfeld 2007, Senger 2012). Mice also have a hemochorial placenta, however, major anatomical differences with the human placenta include: (1) trophoblast invasion stops at the myometrium in mice but continues into the muscle in women, (2) labyrinth (mouse) vs chorionic villi (human) structure and (3)

number of trophoblast cell layers: monochorial (human) vs trichorial (mouse) (Schmidt *et al.* 2015). Following parturition and expulsion of the placenta in both species, the uterus exists in a degenerated state and all cellular components of the endometrium and myometrium are remodeled and/or repaired. Similar to menstrual regeneration, re-epithelialization of the endometrium precedes stromal expansion. This is likely because in the degenerated state, the endometrium is essentially an extensive wound and rapid re-epithelialization is needed to prevent infection. Historical observations in women show that the epithelium covers the non-placental site of the endometrium by D7 postpartum and re-epithelialization of the entire endometrium is complete by approximately D14 (Sharman 1953). This is then followed by stromal expansion. In mice, the majority of information on endometrial repair comes from induced decidualization models (also termed menses-like models, described in detail subsequent). Similar to humans, epithelialization commences first within 24 h after the initiation of decidual breakdown/shedding and is proceeded by stromal expansion (Brasted *et al.* 2003). Repair is complete within 48 h. The myometrium having expanded several-fold due to cellular hyperplasia and hypertrophy, regresses to near its pre-gravid size, which is thought to include apoptosis, proliferation (Shynlova *et al.* 2009) and autophagy (Hsu *et al.* 2014) based on mouse studies. Uterine involution following parturition occurs relatively quickly allowing the uterus to be receptive to another embryo in 40–45 days in women and 3–4 days in mice.

Menstrual and postpartum uterine involution, specifically regeneration of lost or terminally differentiated cells (epithelial, stromal, myometrial), is highly dynamic and poorly understood. Proposed mechanisms of uterine regeneration include: (1) replication of residual GE and LE cells that escaped desquamation; (2) epithelial, stromal and myometrial stem cells; (3) mesenchymal–epithelial transition (MET) of stromal cells; and (4) infiltration and differentiation of bone marrow (BM)-derived cells into endometrial stromal and epithelial cells (Fig. 1C).

Evidence for uterine stem cells and their role in involution

Adult stem cells play an important role in tissue homeostasis and regeneration in many tissues (Blanpain & Fuchs 2009, Biteau *et al.* 2011). Stem cells have the unique ability to self-renew, generate lineage-specific daughter cells, maintain long-term proliferative capacity and restore function to damaged tissue. They are also relatively quiescent compared to more lineage committed cells. Common methods used to enrich putative stem cells (Fig. 2) include expression of stem cell markers (Table 1), Hoechst dye exclusion (side population (SP) analysis) and label retention (Bianco *et al.* 2008, Vemuri

et al. 2011). These putative stem cells must then be tested in a series of *in vitro* (colony/spheroid formation, lineage differentiation, long-term proliferation) and *in vivo* (lineage tracing, label retention) experiments to assess their stem cell qualities, the 'gold standard' of which is engraftment into the tissue of origin (i.e. orthotopic transplantation) and restoration of function to damaged tissue (van Os *et al.* 2004, Garry *et al.* 2009). Subsequent are discussions of the current evidence in support of the presence of uterine stem cells in women (summarized in Table 1). Following that are sections that describe mouse models used to further assess putative stem cells in uterine involution.

Human endometrial mesenchymal stem cells (eMSCs)

The first report of putative eMSCs (i.e. stromal stem cells) was published in 2004 (Chan *et al.* 2004). Using *in vitro* colony formation, distinct small and large colonies were observed that formed from single human endometrial stromal cells plated at a density of 300 cells/cm² (Chan *et al.* 2004). An average of 1.25% of stromal cells formed colonies with significantly greater colony forming efficiency (CE) of small colonies (1.23%) compared to large (0.02%). Although more small colonies formed on initial seeding (Chan *et al.* 2004), large colonies had greater serial propagation/cloning potential (Gargett *et al.* 2009). This suggests that small colonies may represent more differentiated transit amplifying (TA) cells with limited propagation potential whereas large colonies may be enriched for stem/progenitor cells. Clonogenic stromal cells were further characterized and enriched for using surface markers including melanoma cell adhesion molecule (MCAM; CD146), platelet-derived growth factor receptor-beta (PDGFRβ; CD140b) (Schwab & Gargett 2007, Schwab *et al.* 2008) and sushi domain containing 2 (SUSD2; clone W5C5) (Masuda *et al.* 2012). The osteogenic, myogenic, chondrogenic and adipogenic lineage differentiation of colony forming CD146⁺CD140b⁺, and SUSD2⁺ cells was also shown (Schwab & Gargett 2007, Dimitrov *et al.* 2008, Schwab *et al.* 2008, Gargett *et al.* 2009, Schuring *et al.* 2011, Masuda *et al.* 2012, Spitzer *et al.* 2012, Fayazi *et al.* 2015). Further, CD146⁺CD140b⁺ and SUSD2⁺ cells were both located in the perivascular space (Cousins *et al.* 2018), which is a proposed MSC niche in multiple organs (Crisan *et al.* 2008). There was some overlap in expression of CD146/CD140b with SUSD2 (Masuda *et al.* 2012), however it is unclear if these markers identify the same or different populations of stem/progenitor-like cells.

Hoechst dye exclusion was used to obtain side population (SP) cells that were enriched for stem-like cells (Cervello *et al.* 2010). This cell-sorting procedure exploits the propensity of stem cells to efflux Hoechst dye because of relative overexpression of ATP transporters (Goodell *et al.* 1996). SP cells could form colonies

Figure 2 Diagram of common stem cell assays. Cells are typically isolated by various flow cytometry techniques to enrich for putative stem cells. The isolated cells are then tested for stem cell function in using *in vitro* and *in vivo* assays.

and differentiate *in vitro* and expressed various stem cell markers. Unfortunately, this method poses some caveats. First, Hoechst dye is cytotoxic and can impact downstream assays making results difficult to interpret. Second, the SP, although enriched for stem-like cells, is a heterogeneous population. It may contain stromal, epithelial and endothelial stem-like cells. Therefore, purifying each cell type requires additional methods such as surface marker selection, which is often not performed.

In 2007, a mouse xenograft model was developed using human cells to study endometrial function (Masuda *et al.* 2007). Following injection of dissociated

human endometrial cells under the renal capsule of immunodeficient mice and hormone treatment, endometrial-like tissue was formed that comprised of stroma and glands. Progesterone receptor (PGR) was upregulated upon E_2 treatment and the stroma expressed the decidual marker prolactin (PRL) with combination of E_2 and P_4 treatment. Moreover, menstrual-like changes could be induced with cyclic administration of $E_2 + P_4$ followed by P_4 withdrawal. A hemorrhagic cyst was formed and there appeared to be epithelial and stromal cell shedding. This xenograft model has since been used as an *in vivo* stem cell assay to assess endometrial reconstitution by putative stem cells including SUSD2⁺

Table 1 Summary of proposed markers used to identify putative uterine stem cells in human and mouse.

Stem cell/ proposed markers	Reference
Human	
eEpSCs	
SSEA-1	Valentijn <i>et al.</i> (2013)
N-cadherin	Nguyen <i>et al.</i> (2017)
β -catenin, SOX9	Nguyen <i>et al.</i> (2012, 2017), Tempest <i>et al.</i> (2018)
LGR5	Gil-Sanchis <i>et al.</i> (2013)
myoMSCs	
OCT4	Ono <i>et al.</i> (2010)
CD44, Stro-1	Mas <i>et al.</i> (2015)
CD140b, CD146, SUSD2	Patterson <i>et al.</i> (2020)
CD34, CD49f	Ono <i>et al.</i> (2015)
eMSCs	
MCAM (CD146), PDGFR β (CD140b)	Schwab & Gargett (2007), Schwab <i>et al.</i> (2008)
SUSD2 (clone W5C5)	Masuda <i>et al.</i> (2012)
MenSCs	
OCT4	Meng <i>et al.</i> (2007), Nikoo <i>et al.</i> (2014), Patel <i>et al.</i> (2008)
SOX2, Nanog	Liu <i>et al.</i> (2018), Sun <i>et al.</i> (2019)
C-MyC	Liu <i>et al.</i> (2018)
SSEA-4	Liu <i>et al.</i> (2018), Patel <i>et al.</i> (2008), Rossignoli <i>et al.</i> (2013), Sun <i>et al.</i> (2019)
Mouse	
eEpSCs	
AXIN2	Syed <i>et al.</i> (2020)
CD44	Janzen <i>et al.</i> (2013)
mTert	Cousins <i>et al.</i> (2019), Deane <i>et al.</i> (2016)
LGR5	Seishima <i>et al.</i> (2019)
myoMSCs	
OCT4, CD44	Brakta <i>et al.</i> (2018), Mas <i>et al.</i> (2017)*
Stro-1	Mas <i>et al.</i> (2017)*
CD140b, CD146	Patterson <i>et al.</i> (2018)

*Reference shows proposed markers also found in rat model.

eEpSCs, endometrial epithelial stem cells; eMSCs, endometrial mesenchymal stem cells; MenSCs, menstrual blood stem cells; myoMSCs, myometrial mesenchymal stem cells.

(Masuda *et al.* 2012) and SP enriched (Cervello *et al.* 2010, 2011, Masuda *et al.* 2010, Miyazaki *et al.* 2012) cells. However, it has not been used to study putative stem cells in menstrual repair. Although this renal-graft model can be used to provide valuable information about endometrial cell activity, to date, the 'gold standard' stem cell assay, which is transplantation into the tissue of origin (i.e. the endometrium), has not been performed. Transplantation of putative stem cells into the endometrium is necessary to determine their ability to contribute to endometrial function, including derivation of endometrial specific daughter cells that function in decidualization, embryo implantation and postpartum regeneration/repair.

Regarding the characteristics of putative eMSCs across the menstrual cycle, there do not appear to be significant differences in colony forming efficiency (Schwab *et al.* 2005, 2008) or expression of CD146 and CD140b (Schwab & Gargett 2007) based on cycle stage. These results are not surprising considering that putative eMSCs express relatively low levels of estrogen receptor alpha (ESR1) and progesterone receptor (PGR). Therefore, regulation of these cells is likely through paracrine signaling by the niche rather than direct hormone action. Interestingly, although E₂ is required for

epithelial proliferation during the proliferative phase of the cycle, re-epithelialization during menstruation occurs at a time of relatively low E₂ levels (Ferenczy 1976a,b). Additionally, it was demonstrated that endometrial regeneration occurred following menstruation in human endometrial explants in ovariectomized mice in the absence of exogenous hormones (Matsuura-Sawada *et al.* 2005).

Human menstrual blood stem cells (MenSC)

Originally termed endometrial regenerative cells (ERC), putative menstrual blood-derived stem cells (MenSCs) were first observed in 2007 (Meng *et al.* 2007). Found to be derived from shed deciduous endometrial tissue (Rossignoli *et al.* 2013, Liu *et al.* 2018), these cells displayed the spindle-like morphology characteristic of fibroblasts (Mou *et al.* 2013, Rossignoli *et al.* 2013, Du *et al.* 2016, Faramarzi *et al.* 2016, Liu *et al.* 2018, Zheng *et al.* 2018). Through flow cytometry and immunofluorescence, it was demonstrated that putative MenSCs expresses various MSC markers but do not express hematopoietic, endothelial or immune activation markers (Patel *et al.* 2008, Mou *et al.* 2013, Rossignoli *et al.* 2013, Nikoo *et al.* 2014, Du *et al.* 2016, Faramarzi

et al. 2016, Ren *et al.* 2016, Liu *et al.* 2018, Zheng *et al.* 2018, Sun *et al.* 2019). Although epithelial cells are shed along with mesenchymal cells, an epithelial stem-like population has not been isolated from menstrual blood. Reports show variable expression of pluripotency markers like OCT4, SOX2, Nanog, c-MyC and SSEA-4 by putative MenSCs (Meng *et al.* 2007, Patel *et al.* 2008, Rossignoli *et al.* 2013, Nikoo *et al.* 2014, Liu *et al.* 2018, Sun *et al.* 2019), with expression of SSEA-4 showing some of the greatest discrepancies between labs. This suggests a heterogeneous population of putative MenSCs, which requires further refinement.

Putative MenSCs have high proliferative capacity with no abnormalities identified by karyotyping following long-term passaging, thus indicating low mutagenesis (Meng *et al.* 2007, Patel *et al.* 2008, Rossignoli *et al.* 2013, Liu *et al.* 2018, Zheng *et al.* 2018). Interestingly, putative MenSCs have a higher CE (~14%) (Rossignoli *et al.* 2013) compared to reports of putative eMSCs. This may indicate that menstrual blood contains a mixture of stem, progenitor and transit amplifying cells which are all capable of forming primary colonies. Serial cloning may refine this population and give a more accurate estimation of stem cells. Many studies have demonstrated the *in vitro* capacity of putative MenSCs to differentiate into a variety of cell lineages: adipogenic, osteogenic, chondrogenic, neurogenic, cardiogenic, hepatogenic, pancreatogenic, myogenic, endothelial and pulmonary epithelia (Meng *et al.* 2007, Patel *et al.* 2008, Mou *et al.* 2013, Rossignoli *et al.* 2013, Du *et al.* 2016, Ren *et al.* 2016, Liu *et al.* 2018, Sun *et al.* 2019). Putative MenSCs also reformed endometrial-like tissue after s.c. grafting in immunocompromised mice and responded to hormone treatment as seen by expression of ESR1 and PGR (Zheng *et al.* 2018). When injected in the tail vein of immunocompromised mice, putative MenSCs tended to localize to the liver, lung and spleen and were not tumorigenic (Liu *et al.* 2018). Because MenSCs can reside in the liver following tail vein injection, evaluating their ability to regenerate hepatic tissue using a hepatogenic lineage seems promising. Indeed, transplantation of MenSC-derived hepatocyte-like cells following 2/3 partial hepatectomy in mice showed partial restoration of liver function (Mou *et al.* 2013). Therefore, providing the ease of collection and multi-lineage differentiation, researchers are beginning to realize the capabilities and uses of this putative stem cell population in regenerative medicine. Although these cells show promising therapeutic potential, more research is needed to fully characterize them and assess their regenerative capabilities.

Human endometrial epithelial stem cells (eEpSC)

Studies suggest the existence of endometrial epithelial stem cells (eEpSC) on the basis of *in vitro* colony formation, differentiation and long-term proliferation (Chan *et al.*

2004, Kato *et al.* 2007, Gargett *et al.* 2009, Nguyen *et al.* 2017) and *in vivo* reconstitution of endometrial epithelial-like tissue using the mouse renal-graft model (Masuda *et al.* 2007, Cervello *et al.* 2010, Miyazaki *et al.* 2012, Janzen *et al.* 2013). Uniquely, epithelial cells are capable of forming 3D colonies, termed spheroids or organoids. Organoids can be generated from individual epithelial cells and differentiate into the two primary endometrial cell types, ciliated and secretory (Boretto *et al.* 2017, Turco *et al.* 2017). Single-cell sequencing of endometrial epithelial organoids revealed a putative stem cell population (Fitzgerald *et al.* 2019). This population was the most predominant of all cell types when cultured in the absence of hormones. Upon hormone treatment, the presumptive stem-like population decreased and differentiated cell types increased. This may reveal a stem cell maintenance condition in hormone-free medium and differentiation of stem-like cells upon hormone induction. Whether this is an effect of culture or mimics *in vivo* regulation, remains to be determined. Interestingly, the proportion of cells expressing putative stem cell markers, *in vivo*, appeared to be higher in the hormone-deplete, post-menopausal endometrium compared to pre-menopausal (Valentijn *et al.* 2013, Nguyen *et al.* 2017). Together, these data may indicate that putative eEpSCs are more abundant in the absence of ovarian hormones and differentiate with hormone stimulus.

To date, there is no consensus on markers of putative eEpSCs, which has hindered further characterization and functional assessment of these cells. Proposed markers include the surface proteins SSEA-1 (Valentijn *et al.* 2013), N-cadherin (Nguyen *et al.* 2017), and LGR5 (Gil-Sanchis *et al.* 2013, Cervello *et al.* 2017, Tempest *et al.* 2018) and the transcription factors β -catenin and SOX9 (Nguyen *et al.* 2012, Valentijn *et al.* 2013, Nguyen *et al.* 2017, Tempest *et al.* 2018). N-Cadherin⁺ and SSEA-1⁺ cells were predominantly located in the basalis layer and showed higher *in vitro* CE and spheroid formation, respectively, compared to negative cells (Valentijn *et al.* 2013, Nguyen *et al.* 2017). SSEA1⁺ cells had reduced expression of *ESR1* and *PGR* mRNA compared to SSEA1⁻ cells, whereas most N-cadherin⁺ cells appeared to express *ESR1* by immunofluorescence. SSEA1⁺ cells expressed SOX9 but N-cadherin⁺ cells rarely did and co-expression of SSEA1 and N-cadherin was infrequent. These reports suggest two distinct populations of cells with *in vitro* stem/progenitor activity that may be indicative of cells at different stages of differentiation. LGR5, a known intestinal epithelial stem cell marker, was also investigated as an eEpSC marker. By IHC, LGR5 was expressed in random epithelial, stromal and perivascular cells (Gil-Sanchis *et al.* 2013) but by *in situ* hybridization was localized exclusively to the epithelium in a cycle-dependent manner (Tempest *et al.* 2018). When isolated and characterized, LGR5⁺ cells did not reconstruct endometrial-like tissue but rather

had a phenotype more consistent with hematopoietic-derived cells (Cervello *et al.* 2017). Therefore, LGR5 is unlikely to be a useful eEpSC marker in women. Telomerase activity (Valentijn *et al.* 2015) and SP analysis (Cervello *et al.* 2010, Masuda *et al.* 2010) also revealed putative eEpSCs based on CE and spheroid formation, and *in vivo* epithelium reconstruction in mouse renal or s.c. xenografts were used to assess stem-like activity in endometrial epithelium. However, as with putative eMSCs, epithelial cells have not been transplanted into the endometrium to more definitively assess stem cell characteristics and function.

Human myometrial mesenchymal stem cells (myoMSC)

The majority of studies on putative myoMSCs were conducted to identify stem/progenitor cells in fibroids, benign uterine smooth muscle tumors, compared with normal myometrium. Investigators have identified putative myoMSCs using many of the aforementioned methods including surface markers (Mas *et al.* 2015, Ono *et al.* 2015, Patterson *et al.* 2020) and SP analysis (Ono *et al.* 2007, Chang *et al.* 2010) followed by *in vitro* and *in vivo* stem cell assays. In the first report, SP analysis was used to enrich cells with osteogenic and adipogenic differentiation potential. Similar to eMSCs, these cells expressed low levels of *ESR1* and *PGR* (Ono *et al.* 2007). More importantly, SP cells were engrafted into the myometrium of immunocompromised mice and appeared to differentiate into α -SMA expressing myocytes. Although main population (MP) control cells were also able to engraft, they rarely expressed alpha-smooth muscle actin (α -SMA), suggesting they did not contain lineage-differentiating stem/progenitor cells. To date, there is no consensus on markers to isolate putative myoMSCs. OCT4 (*POU5F1* gene) was expressed at relatively low levels in myometrium but was enriched for in the SP compared to the MP (Ono *et al.* 2010). Known as a pluripotency marker and expressed in embryonic stem cells, OCT4 may serve as a candidate marker of human MyoMSCs, however, this has yet to be tested. Other candidate markers include CD34/CD49f (Ono *et al.* 2015), CD44/Stro-1 (Mas *et al.* 2015) and CD140b/CD146 or SUSD2 (Patterson *et al.* 2020). Compared to control cells (CD34⁺CD49f⁺), CD34⁺CD49⁺ myometrial cells were enriched for SP cells, formed more colonies and had greater osteogenic and adipogenic differentiation potential *in vitro* (Ono *et al.* 2015). Of note, CD34⁺CD49f⁺ cells showed higher myometrial engraftment in immunocompromised mice, which was further enhanced with pregnancy. Stro1⁺CD44⁺ cells expressed stem cell markers (e.g. OCT4 and NANOG) and showed adipogenic, osteogenic and chondrogenic differentiation compared to non-inducing conditions, however, they did not show significantly higher CE *in vitro* (Mas *et al.* 2015). Stro1⁺CD44⁺ cells were engrafted

under the renal capsule of immunocompromised mice and appeared to reconstitute some myometrial-like tissue based on actin and collagen expression, however, the contribution of human-derived cells was difficult to ascertain based on the cell tracker method used (Molday Ion Rhodamine B). Additionally, Stro1⁺CD44⁺ cells were not compared with control cells to determine differences in engraftment potential (Mas *et al.* 2015). Lastly, the markers used to enrich for putative eMSCs, CD146/CD140b co-expression or SUSD2, were shown to enrich for putative myoMSCs (Patterson *et al.* 2020). CD146⁺CD140b⁺ and SUSD2⁺ cells were located in the perivascular region similar to their location in the endometrium and had higher CE compared to control cells (CD146⁺CD140b⁺ or SUSD2⁺). Interestingly, SUSD2⁺ cells not only showed osteogenic and adipogenic differentiation but also appeared to undergo decidualization *in vitro* similar to endometrial stromal cells. This study suggests a possible common MSC for the endometrial stroma and myometrium (Patterson *et al.* 2020). However, further research is needed to assess the *in vivo* potential of SUSD2⁺ myometrial-derived cells to decidualize and contribute to uterine function in a stem/progenitor cell capacity. Although there is compelling evidence for human myometrial stem/progenitor cells, there is inconsistency in isolation methods and a lack of in-depth studies to assess *in vivo* function of these cells.

Murine models of uterine stem cells

The literature discussed previously provides a solid foundation for the existence of uterine stem cells in women, however, technical and ethical constraints prevent *in vivo* studies of these cells in uterine involution. However, through the use of mouse models invaluable insight into uterine repair mechanisms has been garnered. Mice and humans share many similarities in pregnancy and postpartum uterine involution. In both species, embryo implantation is invasive, breaching the luminal epithelium, the stroma decidualizes and a hemochorial placenta is formed. Postpartum, the uterus undergoes extensive remodeling including stromal and epithelial regeneration and myometrial reduction. Mice do not menstruate; however, a menses-like mouse model was developed, which mimics the events of human menstruation (Finn & Pope 1984, Brasted *et al.* 2003). Women menstruate, in part, because stromal cells near the spiral arteries decidualize in the late secretory phase of the menstrual cycle in preparation for pregnancy (Gellersen & Brosens 2014). In mice, decidualization is induced by an implanting embryo. However, it can be induced by scratching the LE or infusing oil into the uterine lumen in a hormonally primed mouse. The scratch/oil causes epithelial cell death which initiates the decidual cascade in stromal cells. Upon removal of P₄ stimulus, decidual tissue will breakdown which is manifested as menstrual-like shedding. Concomitant with decidual

degeneration is endometrial repair/regeneration akin to menstrual shedding and regeneration. Using mouse models, intricate studies can be performed that provide more in-depth understanding of uterine involution *in vivo*. This includes additional stem cell assays such as label retention and lineage tracing, which are discussed subsequently and summarized in Fig. 2.

Label-retention

Stem cells divide relatively infrequently and therefore are proposed to retain label, such as bromodeoxyuridine (BrdU), while other more frequently dividing cells will lose label over time. Label-retaining cells (LRC) are then identified and assessed for additional stem cell properties. BrdU label retention has been used to identify slow cycling LRCs as potential stem/progenitor cells in mouse endometrium (Chan & Gargett 2006, Cervello *et al.* 2007, Kaitu'u-Lino *et al.* 2010, 2012, Chan *et al.* 2012, Cao *et al.* 2015) and myometrium (Szotek *et al.* 2007). Stromal LRCs were localized primarily to the sub-luminal stroma, near blood vessels (perivascular) and at the stromal-myometrial boarder (analogous to the human basalis). This pattern remained consistent following pregnancy and postpartum uterine involution, although the percentage of LRCs was greatly reduced (Cao *et al.* 2015). Similarly, stromal LRCs were mostly perivascular and at the stromal-myometrial border during menses-like endometrial regeneration (Kaitu'u-Lino *et al.* 2012). The perivascular location of murine stromal LRCs corroborates data in human that putative eMSCs are also perivascular (Spitzer *et al.* 2012). Epithelial LRCs were predominantly found in the LE and to a lesser extent in the GE in nulliparous mice (Chan & Gargett 2006b), however, label was rapidly diluted in the LE but retained in the GE during menses-like regeneration (Kaitu'u-Lino *et al.* 2010).

Using a transgenic mouse LRC model (H2Bj-GFP), LRCs were identified in the distal oviduct epithelium but not in the endometrium after labeling cells in adult mice for 7 days (Wang *et al.* 2012). The oviduct LRCs formed spheroids and based on differentiation *in vitro*, were proposed to be the stem/progenitor cells for the endometrial epithelium. In another study, LRCs were also found in the distal oviduct epithelium as well as the endocervical transition zone after embryonic thru prepubertal labeling (Patterson & Pru 2013). However, these cells did not appear to participate in menses-like endometrial regeneration. Rather, when the label period was adjusted to the peripubertal period, long-term LRCs were found in the endometrial GE. These studies clearly illustrate how labeling under different physiological circumstances impacts which cells retain label. Recently, myometrial LRCs were characterized using the H2Bj-GFP transgenic labeling model (Patterson *et al.* 2018). The brightest LRCs expressed the putative human stromal and myometrial MSC markers, CD140b

and CD146, were perivascular, and preferentially proliferated during menses-like uterine involution. Similar to putative human uterine stem cells, mouse uterine LRCs expressed little-to-no ESR1 and PGR (Chan & Gargett 2006b, Chan *et al.* 2012, Wang *et al.* 2012, Patterson & Pru 2013, Patterson *et al.* 2018).

An important caveat of LRC models is that cells undergoing terminal differentiation can also become labeled and if long-lived will be identified as LRCs. Therefore, follow up assays are critical to determine stem-cell activity of LRCs. To date, only one study has assessed the stem-like activity of epithelial LRCs by sphere formation (Wang *et al.* 2012). Stem cell activity of myometrial and stromal LRCs has yet to be assessed *in vitro* and no uterine LRCs have been transplanted to reconstitute uterine tissue *in vivo*.

Lineage tracing and marker expression

Using real-time, lineage tracing and ablation techniques, LGR5 was investigated as an eEpSC marker in mice (Seishima *et al.* 2019). LGR5 was expressed in the LE and budding GE during adenogenesis and became increasingly more restricted to the glands in prepubertal mice. In the adult, LGR5 was expressed sporadically in the LE and GE and almost exclusively during diestrus. The stem-like activity of LGR5⁺ cells was examined using a lineage tracing model (*Lgr5-2A-CreERT2; R26-tdTomato*) to indelibly label *Lgr5* expressing cells and their progeny. When tracing was initiated during adenogenesis, labeling both LE and budding GE cells, LGR5⁺ cells contributed to growth and maintenance of the LE and GE in the adult. However, when tracing began after adenogenesis, labeling predominantly GE, LGR5⁺ cells only contributed to the GE in the adult but not the LE. Finally, if LGR5⁺ cells were traced beginning in the adult during diestrus, label was diminished by 2 weeks and undetectable after 1 year. These results suggest that prior to adenogenesis, LGR5 marks stem-like cells for both the LE and GE but shortly after, a delineation occurs and LGR5 only marks GE stem-like cells and in the adult does not serve as a marker for either. Importantly, ablation of LGR5⁺ cells during adenogenesis resulted in a significant reduction in the number of glands formed and present in the adult, indicating their requirement for adenogenesis (Seishima *et al.* 2019). Because of the dynamic expression of LGR5 during postnatal adenogenesis, it would be interesting to see if LGR5 is upregulated during postpartum adenogenesis and re-epithelialization and could serve as a marker of stem cells in adult epithelial regeneration.

Two independent groups used an inducible, clonal, lineage-tracing model to evaluate the presence and activity of putative eEpSCs. By using an epithelial specific promoter, *Krt19* (Jin 2019) or *Pax8* (Syed *et al.* 2020), and low dose of the inducing agent, small

subsets of LE and GE cells were labeled (fluorescent or LacZ) and their lineages traced over time. In both models, there was a progressive increase in the number of labeled cells throughout the epithelium suggesting they originated from individually labeled stem-like cells (Jin 2019, Syed *et al.* 2020). In a similar study, Confetti mice that expressed multiple fluorescent proteins stochastically were used to identify clones of epithelial cells that likely originated from individually labeled *Pax8*⁺ cells (Fu *et al.* 2020). These epithelial markers, however, are not restricted to putative stem cells but are also expressed in differentiated cells and in fact, abundantly throughout the endometrial epithelium (Fu *et al.* 2020, Syed *et al.* 2020). Therefore, more specific markers were used to assess clonal expansion of labeled cells as an indicator of stem cell activity. It has long been proposed that eEpSCs reside in endometrial glands (Gargett 2007). FOXA2 is a well-accepted GE marker (Jeong *et al.* 2010, Filant & Spencer 2013) and AXIN2 (Syed *et al.* 2020) was shown to be restricted to the GE in the adult predominantly at the base of the glands nearest the myometrium. Again, using a low dose to induce label, a small proportion of GE cells expressing AXIN2 became labeled, allowing subsequent daughter cells to be tracked (Syed *et al.* 2020). The label appeared to spread from the base of the glands toward the lumen and after 3 months nearly all GE cells were labeled suggesting that individual AXIN2⁺ stem-like cells were responsible for GE expansion during the estrous cycle. However, the extent of contribution of AXIN2⁺ cells to the LE is unclear. Even though the LE proliferated every 3 days, labeled LE cells were not observed until after 3 months (Syed *et al.* 2020). One explanation could be that the low initial labeling of cells requires more time for labeled cells to reach the LE from the base of the GE and replace non-labeled cells. However, even with extensive initial labeling and after approximately 17 estrous cycles (2.5 months), there appeared to be little-to-no contribution to the LE. Additionally, following six pregnancies, there appeared to be only a few patches of LE cells derived from the labeled AXIN2⁺ GE cells. In agreement with these results are those obtained from FOXA2-inducible lineage tracing, where FOXA2⁺ GE were reported to not contribute to the LE (Jin 2019), however, this model was not tested during postpartum repair. Although AXIN2⁺ GE cells may represent the stem/progenitor cell population for the GE, the identity of eEpSCs responsible for LE regeneration remains to be determined.

CD44 (Janzen *et al.* 2013) and mTert (mouse telomerase) (Deane *et al.* 2016, Cousins *et al.* 2019) have also been investigated as possible markers of endometrial stem/progenitor cells in mouse models. Using an *in vivo* renal-graft mouse model, hormonally deprived epithelial cells were serially transplanted in limiting dilution and as few as 100 transplanted epithelial cells were able to form gland-like structures (Janzen *et al.* 2013). These cells

had increased expression of *Cd44* and *Axin2*. CD44 and ITGA6, which marks the epithelial basement membrane, were used to isolate CD44⁺ITGA6⁺ epithelial cells. Using serial xenografting, 0.92% of CD44⁺ITGA6⁺ cells had regeneration potential compared to 0.03% of control cells (Janzen *et al.* 2013). In other studies, real-time expression of mTert was evaluated in the endometrium using *mTert-GFP* mice (Deane *et al.* 2016, Cousins *et al.* 2019). Expression was observed at very low percentages in several cell types within the endometrium, including immune, stromal, epithelial and endothelial (Deane *et al.* 2016). Consistent with other reports of putative stem/progenitor cells, no difference was found across the estrous cycle. Although some mTert-GFP⁺ GE cells co-stained for CD44, some stromal and immune cells also expressed CD44, indicating lack of specificity. mTert-GFP⁺ cells did not retain BrdU label or proliferate during endometrial maintenance (e.g. estrous turnover) (Deane *et al.* 2016). During menses-like repair in mice, the majority of mTert-GFP⁺ cells were immune cells and <0.1% of epithelial cells were mTert-GFP⁺ (Cousins *et al.* 2019). Very rare mTert-GFP⁺ cells co-localized with the proliferation marker, Ki-67. It was suggested that mTert may mark highly quiescent stem-like cells that upon activation undergo asymmetric cell division to generate highly proliferative transit amplifying cells (Cousins *et al.* 2019). It still remains to be determined to what extent mTert⁺ endometrial cells contribute to postpartum uterine involution. CD44, in combination with Stro1 (CD44⁺Stro1⁺), was also suggested as a marker for putative myoMSCs in rodent models. Rat CD44⁺Stro1⁺ myometrial cells expressed the pluripotency marker *Oct4* and low levels of ESR1 and PGR suggesting their undifferentiated state (Mas *et al.* 2017). Similarly, uteri from *Oct4-GFP* mice contained GFP myometrial cells that co-expressed CD44 (Brakta *et al.* 2018). Rodent cells expressing CD44/Stro1/OCT4 require further testing as putative myoMSCs including analysis of stem cell activity (e.g. colony formation and lineage differentiation *in vitro* and *in vivo*, etc.) and function in uterine involution.

The aforementioned studies (summarized in Table 1) have provided valuable insight into uterine repair using mouse models. But it is important to note some of the caveats. Label retention in itself does not definitively delineate stem cells from non-stem cells, and thus far, the stem cell activity of uterine LRCs has not been validated. Also, it is difficult to extrapolate the information learned from LRC studies to humans because there is no comparable assay that can be performed in women. Therefore, the LRC technique should be used as a steppingstone to further characterize uterine stem cells, such as a means to identify surface markers that may be useful in humans. However, it is cautioned that mouse and human markers may not correlate. LRG5 is a good example of a putative neonatal stem/progenitor marker in mice (Seishima *et al.* 2019) but not in adult women (Cervello *et al.* 2017).

Bone marrow-derived cells

There are two potential sources of stem cells for the uterus: intra-uterine (endogenous uterine stem cells) and extra-uterine (Fig. 1). The previous sections were devoted to discussion of the evidence for endogenous uterine stem/progenitor cells in women and mice. In this section, a possible exogenous source will be discussed for both species.

In humans and mice, the presence of extra-uterine, bone marrow (BM)-derived cells was reported in the endometrium of transplant recipients (Taylor 2004, Du & Taylor 2007, Ikoma *et al.* 2009, Morelli *et al.* 2013, Gil-Sanchis *et al.* 2015, Tal *et al.* 2019). It is well-known that immune cells, which are derived from the BM, reside within all compartments of the uterus (Zhou *et al.* 2018, Lee *et al.* 2015), however, it is controversial whether these cells give rise to functional endometrial cells (Ong *et al.* 2018) or rather provide support to resident endometrial cells. Some reports suggest that non-immune BM-derived cells, presumably MSCs, engraft in the endometrium and become stromal, GE and LE cells by transdifferentiation (Taylor 2004, Du & Taylor 2007, Ikoma *et al.* 2009, Cervelló *et al.* 2012, Morelli *et al.* 2013, Gil-Sanchis *et al.* 2015, Tal *et al.* 2019). This theory is controversial and it is disputed if, and how, these BM-derived cells contribute to the endometrium in regards to regeneration, maintenance and function (Cervelló *et al.* 2012, Wolff *et al.* 2013, Ong *et al.* 2018).

The first report of BM-derived endometrial cells was conducted in 2004 (Taylor 2004). Uterine tissues were obtained from women who had previously received BM transplants, following radiation and chemotherapy (Taylor 2004). Donor-derived cells were identified in the endometrium of recipients and composed 0.2–48% of the epithelium and 0.3–52% of the stroma (Taylor 2004). In a similar study, 0.6–8.4% of epithelial cells and 8.2–9.8% of stromal cells were derived from donor BM (Ikoma *et al.* 2009). It is thought that BM-MSCs are the cells that engraft in the endometrium. However, Cervelló *et al.* (2012) concluded that engrafted BM-derived cells do not contribute to the endometrial SP and therefore are unlikely to contribute to the tissue in a stem cell capacity. Furthermore, clonal expansion of BM-derived cells, a hallmark of stem cell engraftment, has only been documented in the original study (Taylor 2004) and has not been replicated. In both women and mouse models, only sporadic BM-derived cells have been verified to express epithelial or stromal cell markers while also being negative for the pan-leukocyte marker, CD45 (Taylor 2004, Du & Taylor 2007, Ikoma *et al.* 2009, Cervelló *et al.* 2012, Morelli *et al.* 2013, Gil-Sanchis *et al.* 2015). Thus, these studies concluded that although most BM-derived cells were immune cells, a few became epithelial or stromal cells. In contrast, another study used refined immunofluorescent and flow cytometry techniques in mice and reported that all engrafted BM-derived cells

were immune cells based on marker expression and were therefore not epithelial or stromal cells (Ong *et al.* 2018). Another recent study used an inducible saturation labeling strategy in mice to investigate intra- vs extra-uterine sources of cells responsible for epithelial maintenance and regeneration postpartum. By labeling the majority of the epithelium (~90%), the contribution of non-labeled extra-uterine cells was evaluated by calculating the percentage of labeled epithelial cells over time. The percentage of labeled epithelial cells remained relatively unchanged, suggesting that an un-labeled extra-uterine cell (e.g. BM) did not produce endometrial epithelial cells (Syed *et al.* 2020).

Studies on the function of purported BM-derived endometrial epithelial or stromal cells during pregnancy and uterine involution are lacking. BM-derived cells improved fertility in a mouse model of Asherman's syndrome (intra-uterine adhesions) (Alawadhi *et al.* 2014), but only rare BM-derived epithelial- and stromal-like cells were identified (0.14 and 0.55%, respectively). Although pregnancy rates were improved, their direct function in pregnancy was not assessed (Alawadhi *et al.* 2014). The only study that has investigated the role of BM-derived cells in pregnancy used tail vein BM transplantation following chemotherapy treatment (Tal *et al.* 2019). Depending on the day of pregnancy, the percentage of BM-derived cells in the endometrium ranged from 10 to 24%, with 72–95% being CD45⁺ leukocytes. Although other immune cell markers were used to assess different leukocyte populations that were CD45⁺, they were not used in combination. Therefore, the population of immune cells may have been underrepresented. Moreover, no BM-derived epithelial cells were identified. Putative BM-derived stromal cells were assessed based on expression of PGR and DPRP (decidual-prolactin related protein). However, PGR may be expressed by various types of leukocytes (Shah *et al.* 2019), and BM-derived cells were concentrated in the mesometrial pole of the uterus whereas DPRP is typically concentrated in the anti-mesometrial side (Rasmussen *et al.* 1997, Candeloro & Zorn 2007). The clonal expansion, longevity and contribution to subsequent pregnancies of these BM-derived putative stromal cells were not evaluated (Tal *et al.* 2019).

The evidence surrounding BM-derived cells in endometrial function is thus far circumstantial. BM-derived cells are being investigated for their ability to treat endometrial conditions including Asherman's syndrome in women (Santamaria *et al.* 2016, Singh *et al.* 2014). It will therefore be important to gain a more complete understanding of the existence and function of non-immune, BM-derived cells in the uterus.

Mesenchymal–epithelial transition

In the past decade, mesenchymal–epithelial transition (MET) has become an emerging hypothesis as a

mechanism of endometrial re-epithelialization. Broadly, cellular transdifferentiation is the conversion of cells from one differentiated cell type to another, examples being MET or the reciprocal EMT (Lamouille *et al.* 2014). Of note, the occurrence of cellular transdifferentiation in the uterus was first described in embryonic development 60 years ago (Gruenwald 1959) and the MET hypothesis in adult re-epithelialization was first proposed in 1967 (Baggish *et al.* 1967). More recently using electron microscopy, the cellular and morphological changes during menstruation were evaluated (Garry *et al.* 2009). In the early stages of repair, single and small islands of new epithelial cells were observed that were frequently isolated from, and did not appear to arise from, protruding glandular stumps (Garry *et al.* 2009). These new cells were morphologically distinct from the more mature glandular cells; they appeared low and cuboidal in shape, smooth and lacked microvilli. As repair progressed, the new cells eventually fused with the more mature gland cells to form a continuous epithelial lining. The new epithelial cells, which formed in isolated singlets or small clusters, were suggested to originate from the stroma presumably by MET. Studies in mouse models also support the mechanism of MET in endometrial re-epithelialization (Huang *et al.* 2012, Patterson *et al.* 2013, Cousins *et al.* 2014, Yin *et al.* 2019). Mesenchyme-specific Cre driving promoters were used to induce reporter expression (e.g. EYFP or LacZ) to indelibly mark mesenchymal cells and their progeny. With this approach, reporter-positive epithelial cells were identified following postpartum and menses-like endometrial re-epithelialization suggesting that stromal cells underwent MET to produce epithelial cells (Huang *et al.* 2012, Patterson *et al.* 2013, Yin *et al.* 2019). Interestingly, presumptive mesenchymal/epithelial transitional cells (vimentin/cytokeratin co-expressing) were initially located at the stromal-myometrial boarder, a proposed mesenchymal stem cell niche for the stroma (Chan & Gargett 2006a, Cervello *et al.* 2007) and moved toward the lumen as regeneration progressed (Patterson *et al.* 2013). This may suggest a role for stromal stem/progenitor cells in MET. In support of this, mouse CD34⁺KLF4⁺ putative eMSCs enhanced epithelial regeneration presumably through MET (Yin *et al.* 2019), and human CD146⁺ putative eMSCs spontaneously differentiated into epithelial-like cells when cultured in a 3D-scaffold on top of myometrial cells (Fayazi *et al.* 2017). Collectively these studies provide evidence for MET, possibly by eMSCs, as a mechanism of endometrial re-epithelialization. However, this mechanism was recently challenged. Discussed previously, a saturation labeling technique was used in mice to investigate extra-uterine BM cell contribution to endometrial epithelial turnover/regeneration. Because the percentage of labeled epithelial cells remained relatively unchanged, this not only suggested that un-labeled BM-derived cells did not contribute to the endometrial epithelium but

also that unlabeled stromal cells also did not (Syed *et al.* 2020). Furthermore, MET was refuted on the suggestion that the Cre reporter mouse models used previously were not mesenchyme-specific (Ghosh *et al.* 2020). Clearly, more studies need to be conducted to confirm MET as a mechanism of re-epithelialization and determine if eMSCs are the source. It also remains to be determined if MET-derived (i.e. stromal-derived) epithelial cells are functional endometrial epithelial cells.

Conclusions and future perspectives

The uterus in women is a highly dynamic organ that withstands repeated physiological damage and repair events during the menstrual cycle and pregnancy/postpartum. The extensive regeneration and rapid repair achieved suggests a role for stem/progenitor cells. The evidence for the existence of stem/progenitor cells is compelling however, the true identity, characteristics and functions of these putative cells have yet to be fully elucidated. This is due in part to the technical and ethical constraints surrounding human research. Although mouse models have provided invaluable clues into human uterine involution, caution must be placed on translation from mouse to human. Further, a suitable *in vitro* model that recapitulates the events of menstrual and postpartum involution, particularly cellular regeneration, has not been established.

A recent review nicely highlights the advances in development of *in vitro* models of the human endometrium (Fitzgerald *et al.* 2020). Readers are directed to this review for a detailed discussion; however, some important points are described here. First, improvements have been made in endometrial epithelial cell culture to more accurately mimic *in vivo* histoarchitecture. Of note, is incorporation of extracellular matrix components, typically using Matrigel, treatment with chemically defined media and formation of 3D organoids. These organoids are genetically stable, can be propagated long-term, express molecular signatures of LE and GE and are hormonally responsive (Boretto *et al.* 2017, Turco *et al.* 2017). Moreover, organoids can be derived from single cells suggesting the presence of stem cells, which is further supported by scRNA-Seq data revealing a potential stem/progenitor cell population (Fitzgerald *et al.* 2019). Beyond epithelial culture alone, attempts have been made to incorporate stromal cells, which is critical for recapitulating the *in vivo* endometrial environment. Advancements have been made using 3D porous scaffolds (Abbas *et al.* 2020), PEG hydrogels (Cook *et al.* 2017, Valdez *et al.* 2017) and scaffold-free, self-assembling environments (Wiwatpanit *et al.* 2020), to co-culture stromal and epithelial cells with varying degrees of success. Considerations include scaffold or gel components, matrix stiffness, ability of cells to properly orient and media conditions to support both

cell types. Moreover, efforts to incorporate myometrial cells into these models have not been made, which will be important for a complete understanding of uterine involution. However, as progress continues in this field, it is anticipated that a suitable model will be developed that will facilitate research in human endometrial remodeling/repair and advance the identification and characterization of stem cells and their role in uterine involution.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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