REPRODUCTION

Focus on Stem Cells in Human Reproduction

Human uterine stem/progenitor cells: their possible role in uterine physiology and pathology

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Abstract

The human uterus mainly consists of the endometrium and the outer smooth muscle layer termed the myometrium. The uterus harbours the exceptional and remarkable regenerative ability responsible for cyclical regeneration and remodelling throughout the reproductive life. The uterus must swiftly and cooperatively enlarge to hold the growing foetus during pregnancy. Furthermore, the endometrium, in particular the functionalis layer, must also regenerate, differentiate and regress with each menstrual cycle under hormonal control. Endometrial regeneration from the basal layer is thought to contribute to replacement of the functionalis layer followed by its slough off during menses and parturition. These morphological and functional features of human endometrium can be reproduced in murine models in which severely immunodeficient mice are xenotransplanted with dispersed human endometrial cells under the kidney capsule. The uterine myometrium possesses the similar plasticity of the endometrium. This is demonstrated by multiple cycles of pregnancy-induced enlargement and regression after parturition. It is likely that regeneration and remodelling in the female reproductive tract are achieved presumably through endometrial and myometrial stem cell systems. Recent evidence now supports the existence of these stem cell systems in humans. Here, we will review our current understanding of uterine stem/progenitor cells. We also propose a novel hypothetical model in which stem cell activities explain the physiological remodelling and regeneration of the human uterus and the pathogenesis of gynaecological diseases such as endometriosis.

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Introduction

During the menstrual cycle, the human endometrium, which consists of the functionalis and basalis layers, undergoes proliferation, differentiation, tissue break-down and shedding (menstruation) under the influence of ovarian steroid hormones. These menstruation-associated cyclical changes can repeat throughout a woman's reproductive life. The rise in ovarian progesterone after ovulation leads to remodelling and differentiation of the oestrogen-primed endometrium. When pregnancy is not achieved, the production of progesterone and oestrogen from the ovary discontinues. As a consequence, the progesterone withdrawal induces tissue breakdown and shedding of the endometrium, in particular the differentiated functionalis layer. The endometrium is believed to regrow and regenerate from the basal layer, which is primarily driven by oestrogen. The reconstitution of the functional layer plays a critical role in the development of a tissue prepared for implantation or for menstruation (Maruyama & Yoshimura 2008).

Not only must the endometrium regenerate with each menstrual cycle, the uterus must also rapidly enlarge to accommodate the developing foetus. Pregnancy-induced expansion of the human uterus, which is mainly composed of myometrial cells, can be repeated multiple times throughout a woman's reproductive life. Based on these features, adult stem cell system(s) has long been believed to be critical for the regeneration and remodelling properties of the female reproductive tract. Increasing bodies of evidence substantiate the idea that adult stem cells analogous to findings in non-reproductive organs are present in human and mouse female reproductive tracts (Gargett 2007, Ono *et al.* 2008, Maruyama 2010). In this review, we briefly address the current paradigm regarding stem/progenitor cells and the regeneration potential of the human uterus, together with a possible pathogenesis of uterine-derived diseases. This review article will also describe our recently developed experimental murine model system. We also propose a novel model which explains how eutopic and ectopic endometria can regenerate from putative endometrial stem/progenitor cells.

This paper is one of four papers that form part of a special Focus Issue section on Stem Cells in Human Reproduction. The Guest Editor for this section was C E Gargett, Clayton, Australia.

Stem/progenitor cells in human endometrium

Adult stem cells (also termed somatic stem cells or tissue-specific stem cells) are found in an undifferentiated state throughout the whole body after embryonic development. They are able to self-renew through indefinite and/or asymmetric cell division, generating committed cells that reconstitute the belonging organ and tissue. In this context, all of the cell types of the organ from which they originate are thought to have potentials of giving rise to an entire organ from a few cells (van der Kooy & Weiss 2000). Thus, adult stem cells play a critical role in replenishment and regeneration of dying cells and damaged tissues, thereby contributing to the structural and functional maintenance of the organs and tissues. The hierarchy of adult stem cell differentiation is illustrated in Fig. 1. Maintenance of the stem cell population requires cellular self-renewal, i.e. the capacity to generate identical daughter cells (Fig. 1). Alternatively, stem cells can undergo asymmetric division, producing an identical daughter cell and a more differentiated daughter or symmetric division producing two daughter stem cells or two transit amplifying progenitors (Fig. 1). Although neither progenitors nor precursors are called stem cells, it is often practically difficult to distinguish adult stem cells from their progenitor/precursor cells. Indeed, for instance, a small subset of haematopoietic progenitor cells have been termed haematopoietic stem cells (HSCs; Martinez-Agosto et al. 2007).

The most stringent criteria in defining a HSC, perhaps the best described stem cell population in mammals, include 1) multipotency; 2) asymmetric cell division; 3) quiescence; 4) slow self-renewal; 5) undifferentiated state; and 6) in vivo reconstitution of HSC system with long-term repopulation (Martinez-Agosto et al. 2007).
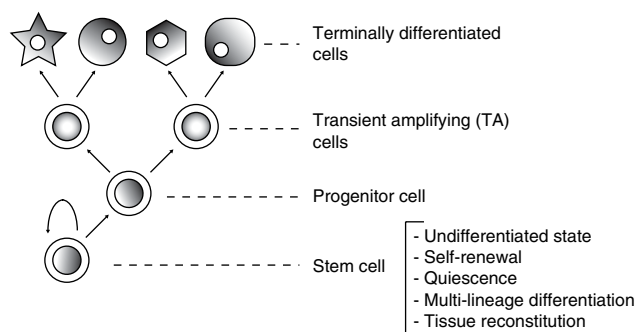

Figure 1 Adult stem cell division and differentiation. The adult stem cell resides in its niche and renews itself. This cell also gives rise to an immediate daughter/progenitor cell through asymmetric cell division. The progenitor cell further divides to generate transient amplifying (TA) population. The TA cells undergo rapid proliferation and expansion of cell numbers, ultimately leading to terminal differentiation into various functional cells with no further proliferative potentials. Adapted, with permission, from **Maruyama T** 2010 Stem/progenitor cells and the regeneration potentials in the human uterus. *Reproductive Medicine and Biology* **9** 9–16. © 2009 Japan Society for Reproductive Medicine.

Cells that possess these above-mentioned properties are, therefore, able to survive in a quiescent and undifferentiated state for a long span and give rise to multiple cell types through asymmetric cell division, eventually leading to regeneration and reconstitution of the corresponding tissue in vivo. Importantly, a specific 'niche' is required for each type of adult stem cell to elicit the stem cell activity (Martinez-Agosto et al. 2007). According to these criteria, a number of in vitro and in vivo assays have been widely used to examine the HSC and other adult stem cell functions including clonogenicity, quiescence, proliferative potential, self-renewal, differentiation, and in vivo tissue reconstitution (Gargett 2007). Notably, not all the criteria can be met for all classes and types of adult stem cells, partly because they may inevitably contain precursors/progenitors, and also a favourable stem cell niche is often difficult to be reproduced in the setting of in vitro and in vivo experiments.

Among those in vitro and in vivo assays, an in vivo reconstitution assay to generate the tissue that the putative stem cell population belongs to is considered as the gold standard to verify the adult stem cell property (Gargett 2007). Congenic strains of mice or immunocompromised mice have been widely used as hosts for the engraftment of putative stem cell populations (Gargett 2007). Transplantation sites of these cells are usually ectopic, for instance, the subrenal capsule or subcutaneous tissue (Gargett 2007), because these sites, in particular the subrenal capsule, not only furnish an abundant blood supply with rich vascularity but also confine transplanted cells to a desired position; however, they do not necessarily provide a 'niche' suitable for the survival and maintenance of stem cells (Gargett 2007).

Chan et al. (2004) provided the first evidence for the existence of endometrial stem/progenitor cells by identifying clonogenic human endometrial epithelial and stromal cells. Clonogenicity, which is defined as the ability of a single cell to give rise to a colony of cells when cultured at extremely low seeding densities, has been tested for characterization of various adult stem cells (van Os et al. 2004, Gargett 2007). Human endometrial stromal cells and epithelial cells are clonogenic, though the former have a more pronounced capacity in vitro (Chan et al. 2004). Interestingly, the highest clonogenicity of stromal cells is observed in the proliferative stage, whereas peak clonogenicity of epithelial cells is found in the secretory stage (Schwab et al. 2005).

These studies collectively suggest that two distinct types of stem/progenitor cells are present in the human endometrium. In particular, they are different in terms of their growth factor requirements. For instance, either epidermal growth factor (EGF), transforming growth factor-α (TGFA) or platelet-derived growth factor-BB (PDGF-BB) together with fibroblast feeder layers is required for clonogenic epithelial cells to achieve clonal

growth in serum-free medium (Chan *et al*. 2004, Schwab *et al*. 2005). Clonogenic stromal cells can clonally grow in serum-free medium containing fibroblast growth factor-2 (FGF2), in addition to either EGF, TGFA or PDGF-BB (Chan *et al*. 2004, Schwab *et al*. 2005). It is possible that they may reside within different types of niches in the endometrium. Epithelial and stromal stem cell-like precursors have high proliferative potential and undergo 30–32 population doublings before senescence or transformation, whereas endometrial non-stem epithelial and stromal cells undergo ∼12 population doublings (Gargett 2007, Gargett *et al*. 2009). Clonogenic cells derived from human endometrium are able to differentiate into mesenchymal lineages, including adipocytes, smooth muscle cells, chondrocytes and osteoblasts, *in vitro*, which are characteristics of the capacities of bone marrow (BM) and adipose tissue mesenchymal stem cells (Schwab & Gargett 2007).

Analysis of stem cell populations was facilitated by the finding that they were enriched in a fraction termed the 'side population' (SP). SP cells constitute a small fraction of cells possessing a unique ability to pump out intracellular DNA-binding dye Hoechst 33342 via the ATP-binding cassette transporter G2 (ABCG2; Goodell *et al*. 1996, Challen & Little 2006). After incubation with Hoechst 33342, they are determined as a Hoechst-understained and/or -unstained fraction by dual wavelength flow cytometric analysis. To date, SP cells have been isolated from various adult tissues and extensively characterized, implicating the SP phenotype as a common feature of adult stem cells (Challen & Little 2006).

We have previously demonstrated that singly dispersed human endometrial cells (SDECs) are capable to reconstitute endometrium-like tissues *in vivo* when xenotransplanted into severely immunocompromised mice (Masuda *et al*. 2007*b*), which prompted us to postulate that SDECs may contain stem/progenitor cells such as SP cells. Indeed, we successfully isolated endometrial SP (ESP) cells from SDECs (Masuda *et al*. 2005, 2008, 2010). Other laboratories have identified candidate endometrial stem/progenitor cells, including clonogenic endometrial cells (Chan *et al*. 2004), ESP cells (Kato *et al*. 2007, Tsuji *et al*. 2008), MCAM$^+$ PDGF-Rβ^+ stromal cells (Schwab & Gargett 2007), ITGB1$^+$NT5E$^+$THY1$^+$ stromal cells (Dimitrov *et al*. 2008) and some other populations (Meng *et al*. 2007, Musina *et al*. 2008). Only *in vitro* functional properties of these cells, however, have been investigated. To address the possible involvement of stem cells in regeneration of the endometrium, we tested whether ESP cells were able to recapitulate endometrial tissue possessing stromal and glandular structures when xenotransplanted into NOD/SCID/γ_c^{null} (NOG) mice (Masuda *et al*. 2005, 2008, 2010). NOG mice display multiple immunological deficiencies including no activity of T, B and natural killer (NK) cells together with dysfunction of macrophages and dendritic cells, and, therefore, they allow heterologous cells to engraft more efficiently than any other types of immunodeficient mice including nude mice and NOD/*scid* mice (Ito *et al*. 2002). Data revealed that ESP cells could indeed reconstitute various endometrial tissue components or even the entire endometrium when transplanted under the kidney capsule of NOG mice (Masuda *et al*. 2005, 2008, 2010).

Putative endometrial stem/progenitor cells are believed to reside in the basalis layer of the human endometrium. Since ESP cells express the characteristic marker ABCG2 (Zhou *et al*. 2001), we performed immunofluorescence staining of human cycling endometria to identify ABCG2$^+$ cells (Masuda *et al*. 2010). While we anticipated that ABCG2$^+$ cells would be predominantly located in the basalis layer, ABCG2$^+$ cells were in fact evenly distributed across the functionalis and basalis layers of the endometrium (Masuda *et al*. 2010; Fig. 2A). Furthermore, ABCG2$^+$ cells were localized to PECAM1$^+$ endothelium of both functionalis and basalis layers (Masuda *et al*. 2010; Fig. 2B), which is in agreement with a recent report (Tsuji *et al*. 2008). The distribution pattern of ABCG2$^+$ cells was unchanged throughout the menstrual cycle. The presence of ABCG2$^+$ cells in both endometrial layers suggests that not only basal layer but also functional layer may have potential to give rise to endometrial tissues.

Investigation of methylation patterns in individual endometrial glands is one of the retrospective approaches for studying the activity of endometrial stem/progenitor cells (Kim *et al*. 2005). A 'cellular history' encoded by epigenetic variations in individual glands reflects the methylation patterns arising from stem/progenitor cells, because such epigenetic changes are thought to be transmitted to daughter cells. Conversely, abandoned are epigenetic changes emerging from more mature descendant cells presumably present in the functionalis endometrium when these cells are lost during menstruation. Kim *et al*. (2005) analysed the methylation patterns found in individual glands derived from cycling and atrophic human endometrium mathematically, substantiating the idea that there exists a stem cell niche in an individual gland. Furthermore, an undetermined number of stem/progenitor cells with long life span, but not a single stem cell, may be present in each niche (Kim *et al*. 2005). The ageing endometrium still harbours such gland diversity. These collectively suggest that a pool of stem cells remains preserved even in the atrophic endometrium, consistent with data from clonogenicity studies (Schwab *et al*. 2005) and clinical data of regenerating endometrium in postmenopausal women on oestrogen replacement therapy (Ettinger *et al*. 1997).

In humans and rodents, BM is the most likely candidate as a potential source to produce endometrial epithelial and stromal stem/progenitor cells (Taylor 2004, Bratincsak *et al*. 2007). Women who underwent
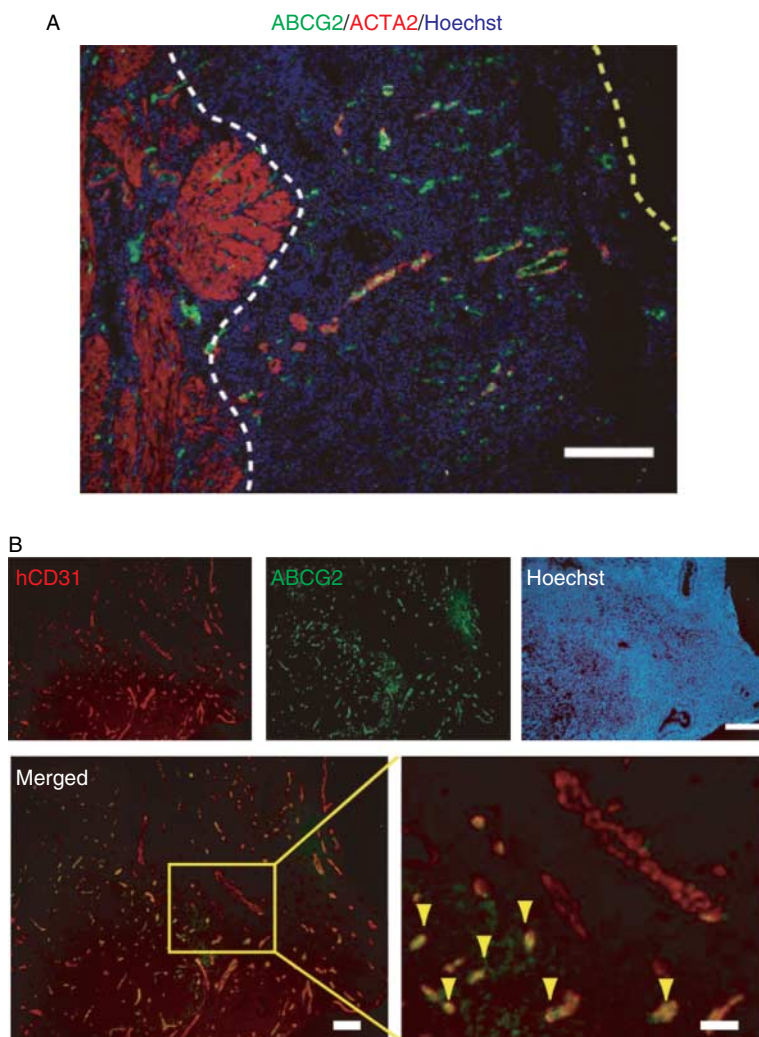
ABCG2/ACTA2/Hoechst

hCD31   ABCG2   Hoechst

Merged

Figure 2 Localization of ABCG2$^+$ cells in human endometrium. (A) A merged immunofluorescence image of human endometrium co-stained with Hoechst and antibodies against ABCG2 and α-smooth muscle actin (ACTA2, αSMA). The endometrium–myometrium junction and the luminal surface of the uterine epithelium are indicated by white and yellow dotted lines respectively. Bars, 200 μm. (B) Immunofluorescence images of human endometrium co-stained with Hoechst and antibodies against human PECAM1 and ABCG2. A small yellow box marks a region shown at a higher magnification in the adjacent panel as indicated. Yellow arrowheads indicate endothelial cells doubly positive for human PECAM1 and ABCG2. Bars, 200 μm (upper), 100 μm (lower left) and 50 μm (lower right). Adapted, with permission, from **Masuda H, Matsuzaki Y, Hiratsu E, Ono M, Nagashima T, Kajitani T, Arase T, Oda H, Uchida H, Asada H *et al.*** 2010 Stem cell-like properties of the endometrial side population: implication in endometrial regeneration. *PLoS ONE* **5** e10387.

single-antigen, HLA-mismatched, BM transplantation exhibit significant chimerism, ranging from 0.2 to 52%, in their endometrial glands and stroma (Taylor 2004). These collectively suggest that BM-derived stem cells participate in endometrial regeneration in the setting of cellular turnover and inflammatory stimuli (Taylor 2004, Bratincsak *et al.* 2007). Most gland profiles can be classified into either donor or host type; however, some chimerism is observed within individual glands. The incomplete monoclonality is in support with data on different methylation patterns across individual glands (Kim *et al.* 2005). Because cell trafficking can take place bidirectionally during pregnancy (Bianchi *et al.* 1996, Maloney *et al.* 1999), maternal-derived stem cells may be retained even in women who underwent transplantation in the childhood. Thus, precise origin of endometrial stem/progenitor cells remains elusive. It is also uncertain whether the endometrium regularly incorporates cells from the BM or foetus under normal physiological conditions, or which part of the endometrium these cells engraft, i.e. the basalis, functionalis region or both regions.

Regeneration of the human endometrium

Regeneration of the endometrium is achieved by endometrial epithelial regrowth, angiogenesis and proliferation of endometrial stromal cells. It was proposed many years ago that there exist endometrial stem/progenitor cells and that they mediate endometrial regeneration (Prianishnikov 1978, Padykula *et al.* 1989, Padykula 1991). This concept has been supported and substantiated by indirect evidence accumulated from proliferation studies, clinical observations and the demonstration of gland monoclonality (Tanaka *et al.* 2003, Gargett 2007). It is now generally accepted that the endometrial functionalis layer sheds at menstruation but subsequently is regenerated from the remaining endometrial basalis, suggesting that putative endometrial stem cells reside in the basalis (Prianishnikov 1978, Padykula *et al.* 1989, Padykula 1991). In this context, the remaining basal layer is believed to behave as a germinal compartment from which various types of endometrial cells proliferate and differentiate (Padykula *et al.* 1989). Figure 3 illustrates the zonation
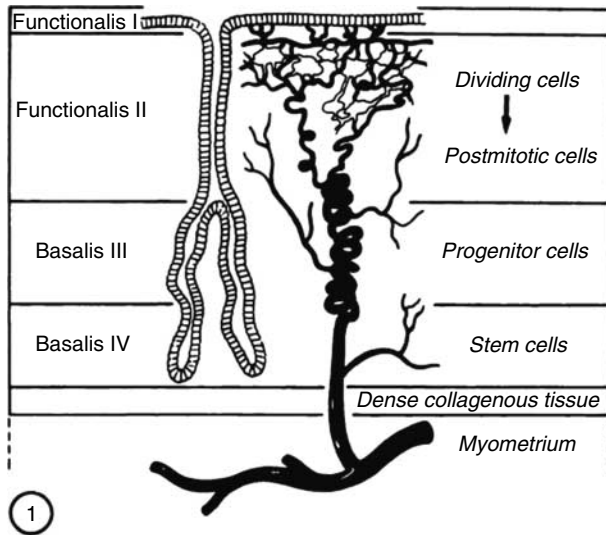
Figure 3 Zonation of the primate endometrial functionalis and basalis. Reprinted, with permission, from **Padykula HA, Coles LG, Okulicz WC, Rapaport SI, McCracken JA, King NW Jr, Longcope C & Kaiserman-Abramof IR** 1989 The basalis of the primate endometrium: a bifunctional germinal compartment. *Biology of Reproduction* **40** 681–690.

of primate (presumably human) endometrium and its corresponding cell types in relation to the mitotic activity (Padykula *et al*. 1989), which is consistent with the subsequent report that the proliferation kinetics differs between the functionalis and basalis layers (Brenner *et al*. 2003).

Following menstruation, regeneration of the human endometrium is oestrogen dependent. In the basal layer, epithelial cells receive mitogenic stimuli from TGFA, EGF and PDGF, with the first two factors acting through the EGF receptor (Chan *et al*. 2004). On the second day of menstruation, the epithelium initiates growth, most likely at the stumps of the glands. By the fourth day, two-third of the luminal surface is covered by epithelium which has grown out of the edges of cone-shaped glands. The epithelialization process is essentially done after 6 days (Jabbour *et al*. 2006). In those menstruating species characterized by spiral arterioles, regrowth of endometrial vessels is critical. Successful angiogenesis and remodelling of the vessel network are dependent upon an array of signalling molecules and receptors. For example, FGFs, angiopoietins, angiogenin, and the ephrins and their cognate receptors play important roles (Strauss & Lessey 2004). Also crucial is the vascular endothelial growth factor family of proteins. At this point, it is not clear how each of these factors makes specific contributions to the remodelling process.

Experimental model for endometrial regeneration and angiogenesis

Studies of the regenerative and angiogenic processes in the endometrium are complicated by major physiological differences between rodents (the most common subject of study) and humans. As is often the case with rodent models, information obtained may not be directly applicable to humans. *In vitro* approaches to molecular analyses have also proven difficult, as model systems have not accurately reflected physiological steps in tissue shedding and regeneration. As a consequence, progress has been slow in understanding normal uterine angiogenesis and pathologic states as endometriosis. In endometriosis, a relatively common gynaecological disease, functional endometrial-like tissue is found outside the uterine cavity. While it is known that the disorder is oestrogen dependent and that angiogenesis is involved in the establishment and development of endometriotic lesions (Donnez *et al*. 1998, Fujishita *et al*. 1999), the aetiology and pathophysiology are not at all understood (Giudice & Kao 2004, Bulun 2009).

To study the physiology of human endometrium and the pathophysiology of endometriosis, a variety of *in vivo* animal models have been developed in which autologous or heterologous endometrial cells/tissues or endometriotic tissues are transplanted (Grümmer 2006). However, these models fall short because they are not completely representative, and fail to meet the following all of the three criteria. First, in terms of more precise quantitative assessment, the transplanted human tissue should not be quantitatively and characteristically varied in each animal. Second, the model should recapitulate not only morphological but also functional changes as human eutopic and/or ectopic endometrium behaves *in vivo*. Finally, it should be possible to access the transplant in real time, for an extended period, without resorting to invasive techniques to obtain quantitative data.

To overcome these difficulties, we have recently established a novel mouse model that satisfies all of these requirements (Masuda *et al*. 2007*b*). This system utilizes severely immunodeficient NOG mice. When a small number of individual (dispersed) human endometrial cells (SDECs) containing epithelial, stromal, endothelial and immune cells are transplanted beneath the kidney capsule of NOG mice, we observed regeneration of functional endometrial tissue under treatment with oestrogen (Masuda *et al*. 2007*b*). In this system, the artificial endometrium mimics normal endometrium, showing hormone-dependent processes such as oestrogen-driven cellular proliferation, and progesterone-induced differentiation, as well as progesterone withdrawal-triggered tissue breakdown analogous to that observed in menstruation. In this model of endometrium, the mouse kidney parenchyma is invaded by human blood vessels. It is particularly interesting that the vasculature from the human tissue forms chimeric vessels with the host endothelium, producing a functional circulatory system. This observation suggests that human endothelial cells/progenitors derived from the endometrium can migrate, invade and

form vasculature in host tissue, even that of a different species (Masuda *et al*. 2007*b*). The summary of the *in vivo* mouse model for endometrial regeneration and angiogenesis is illustrated in Fig. 4. Given the unique angiogenic potential of endometrial endothelial cells, it is tempting to speculate that, in addition to the peritoneal environment (Gazvani & Templeton 2002), the properties of these cells *per se* may participate in the establishment and development of endometriotic lesions. Several groups have suggested that anti-angiogenic therapy has a potential as an alternative means to treat endometriosis (Dabrosin *et al*. 2002, Hull *et al*. 2003, Nap *et al*. 2004). Elucidation of mechanism(s) underlying endometrium- or endometriosis-specific angiogenesis will further rationalize and strengthen this therapeutic strategy.

Several experiments in which human endometrial tissues were transplanted into immunodeficient mice such as SCID and nude mice have shown that endometriotic lesions derive their blood supply from the surrounding vascular network (Nisolle *et al*. 2000, Grümmer *et al*. 2001, Bruner-Tran *et al*. 2002). Furthermore, native vessels derived from the human graft vanish gradually, whereas host-originated vessels instead migrate and invade accompanied by revascularization of endometrial transplants (Grümmer *et al*. 2001, Bruner-Tran *et al*. 2002, Hull *et al*. 2003, Eggermont *et al*. 2005). As functioning NK cells remain present in SCID and nude mice, and NK cells play a critical role in the interleukin-12-mediated inhibition of angiogenesis

(Yao *et al*. 1999), it is conceivable that the remaining NK cells may devastate various human immature vessel precursor cells that show low MHC expression level (Bix *et al*. 1991), resulting in disappearance of human endometrial graft-originated vessels in SCID and nude mice as observed in previous studies (Nisolle *et al*. 2000, Grümmer *et al*. 2001, Bruner-Tran *et al*. 2002, Hull *et al*. 2003, Eggermont *et al*. 2005). In contrast, we demonstrated that human-derived vessels are abundant in the endometrial reconstructs, and that they invade into the mouse kidney parenchyma and become connected with mouse vessels functioning as a circulation system (Masuda *et al*. 2007*b*). Because NK cells are functionally incompetent in the NOG mouse (Ito *et al*. 2002), the human-originated neovascularization is allowed to take place.

Recently, Alvarez Gonzalez *et al*. (2009) have demonstrated that human endometrial grafts retain their own vessels, which connect to the murine vasculature coming from the host tissue and become functional in SCID and nude mice. They suggest that the presence of NK cells may not inhibit the mixed origin of neovascularization. Intriguingly, chimeric vessels were never observed in fibrotic tissue around the grafts in their mouse model (Alvarez Gonzalez *et al*. 2009), whereas they were apparently present in the mouse kidney parenchyma adjacent to the endometrial constructs in our model (Masuda *et al*. 2007*b*), still suggesting a remaining possibility that the presence of host NK cells
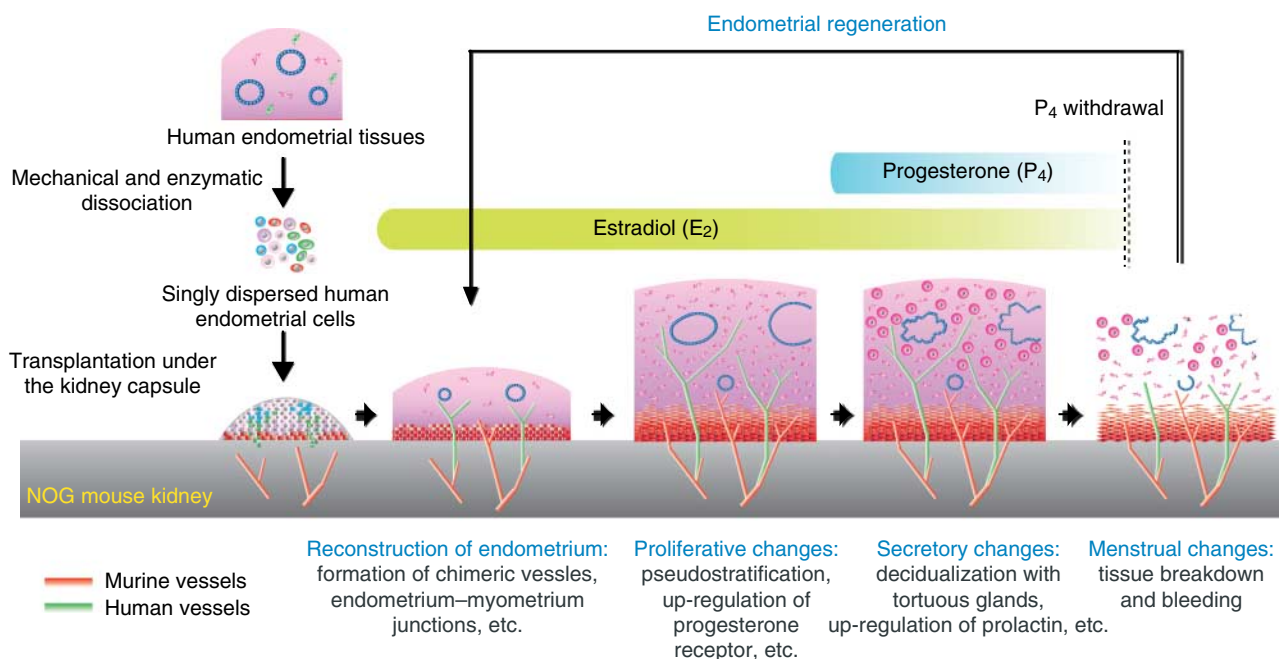

Figure 4 Summary of *in vivo* mouse model of human endometrium regeneration and angionensis. Reprinted, with permission, from **Masuda H, Maruyama T, Hiratsu E, Yamane J, Iwanami A, Nagashima T, Ono M, Miyoshi H, Okano HJ, Ito M *et al*.** 2007*b* Noninvasive and real-time assessment of reconstructed functional human endometrium in NOD/SCID/γ_c^{null} immunodeficient mice. *PNAS* **104** 1925–1930. © 2007 The National Academy of Sciences of the USA.

may be involved in the inhibition of donor-derived neovascularization in the host tissue. In addition to the type of the immunocompromised host animal, it is conceivable that preparations of endometrial transplants (tissue versus single cell suspension) and transplantation sites (kidney capsule versus skin versus peritoneum) may contribute to the differential behaviour of neo-vascularization among our and other mouse models. Elucidation of cellular and molecular mechanism underlying eutopic and ectopic endometrial angiogenesis awaits further studies.

A hypothetical model for eutopic and ectopic endometrial regeneration involving endometrial stem/progenitor cells

Human and primate endometria are thought to regenerate from the lower basalis layer, a germinal compartment that persists after menstruation to give rise to the new upper functionalis layer (Prianishnikov 1978, Padykula *et al*. 1989, Padykula 1991, Okulicz 2002). Notably, the growth of the surface epithelium occurs primarily from the upper ends of the remaining gland stumps through epithelial cell proliferation (Ferenczy 1976, Salamonsen 2003). These findings strongly support the idea that the basalis of the endometrium harbours stem/progenitor cells responsible for endometrial regeneration during menses as well as after parturition in both women and menstruating non-human primates (Okulicz 2002). It remains possible, however, that endometrial stem/progenitor cells also exist in the functionalis of the endometrium.

Several studies have questioned the current paradigm and/or provided alternative hypotheses for endometrial regeneration (Baggish *et al*. 1967, Horne & Blithe 2007, Garry *et al*. 2009). Using novel hysteroscopic plus histological and scanning electron microscopic approaches, Garry *et al*. (2009) demonstrated that endometrial surface epithelial regeneration takes place as a consequence of cellular differentiation from stromal cells, but not direct extension from the residual basal epithelial glands. Horne & Blithe (2007) have raised the possibility that the basalis layer may not serve as a source of stem cells for endometrial regeneration after normal menstruation.

As explained above, the precise mechanism of eutopic and ectopic regeneration of human endometrium is uncertain. Below, we present eight key findings from our own laboratory and those of others relevant to the issues of human endometrial stem/progenitor cells and endometrial regeneration.

1) The ABCG2$^+$ population presumably contains ESP cells and is localized exclusively in the endothelium of both the functional and basal layers of human endometrium (Fig. 3; Tsuji *et al*. 2008, Masuda *et al*. 2010).

2) ESP cells can produce endometrial epithelial and stromal cells *in vitro* and *in vivo* (Masuda *et al*. 2005, 2008, 2010, Kato *et al*. 2007).

3) BM-derived cells contribute to the formation of new blood vessels in the human and mouse endometria (Masuda *et al*. 2007*a*, Mints *et al*. 2008).

4) BM-derived cells give rise to uterine epithelial and stromal cells in humans (Taylor 2004) and mice (Bratincsak *et al*. 2007, Du & Taylor 2007).

5) A relatively small number of dispersed human endometrial cells (mainly derived from the functionalis layer of the endometrium) can generate endometrial tissue comprising glands, stroma, immune cells and vascular components when they are transplanted under the kidney capsule of severely immunodeficient mice (Masuda *et al*. 2007*b*).

6) A highly migratory population of human endometrial cells possesses angiogenic properties (Masuda *et al*. 2007*b*).

7) Mesenchymal stem-like cells expressing both MCAM and PDGF-Rβ are located perivascularly in the functionalis and basalis layers of the human endometrium (Schwab & Gargett 2007).

8) Endometrial surface epithelial regeneration occurs as a consequence of cellular differentiation from stromal cells, but not direct extension from the residual basal epithelial glands (Garry *et al*. 2009).

In view of these findings, we now propose a hypothetical model for eutopic and ectopic implantation of menstrual endometrial tissues/cells and subsequent endometrial regeneration and the establishment of endometriotic lesions as illustrated in Fig. 5. In this model, putative endometrial stem/progenitor cells containing ESP cells, perhaps derived from the BM, mainly reside in the vascular endothelial wall and/or perivascular region. Importantly, these stem/progenitor cells are present not only in the basalis but also in the functionalis endometrium. These cells, therefore, may be contained within the sloughed endometrium shed at menstruation. They may then implant onto the surface of ectopic sites such as the peritoneum through retrograde menstruation. Furthermore, some of these functional layer-derived endometrial stem/progenitor cells may remain in the uterine cavity even after menstruation and implant again (reimplant) onto the deconstructed eutopic endometrium. To our knowledge, no studies have ever demonstrated that shed menstrual endometrial tissues/cells are eliminated completely from the uterine cavity after menstruation. Indeed, each menstruated endometrium may contain heterogeneous areas, i.e. unshed, partially or completely shed late secretory phase endometrium, and partially or completely healed endometrium (Garry *et al*. 2009).

In both eutopic and ectopic implantation, endometrial stem/progenitor cells may generate endometrial tissues and vasculature, as well as endometriotic lesions.
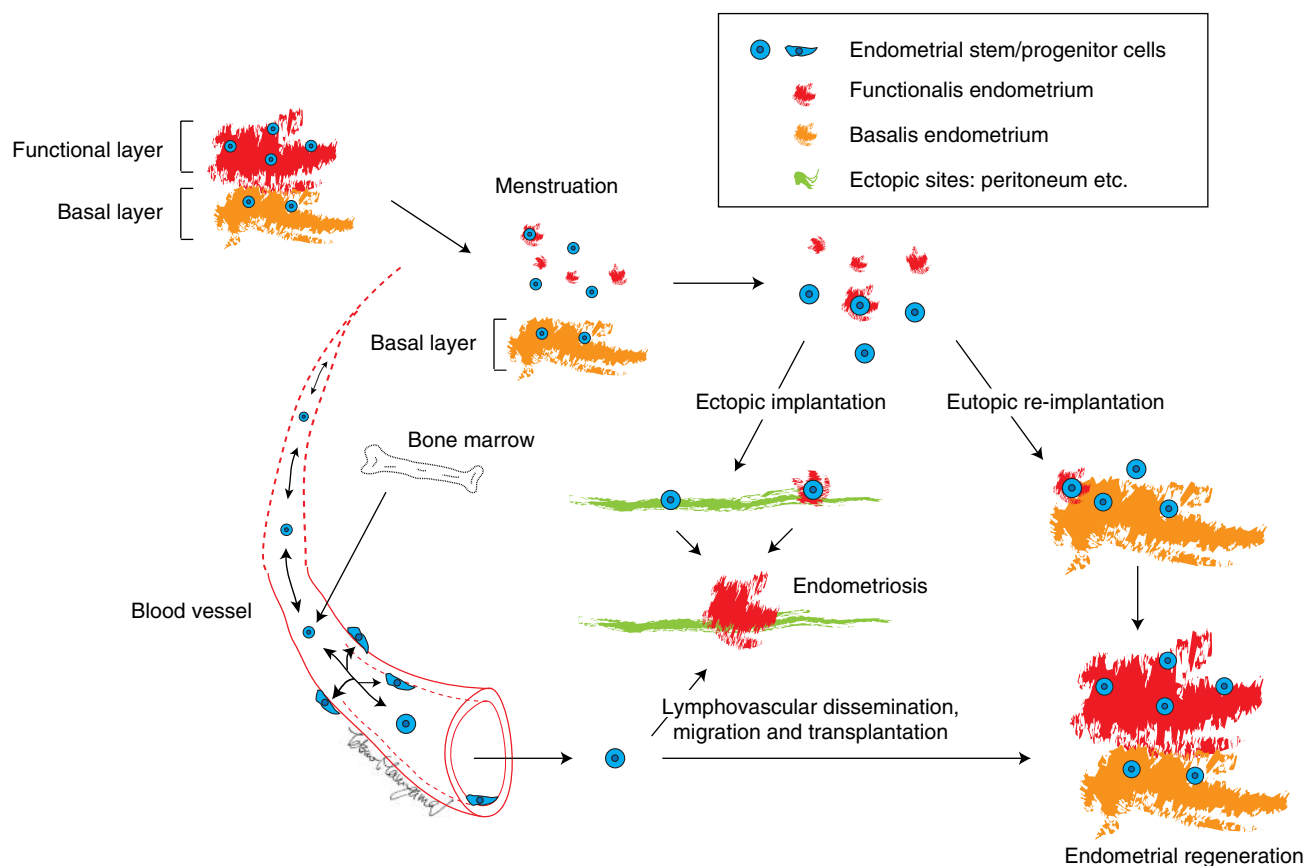
Figure 5 Proposed model for ectopic and eutopic implantation of menstrual endometrial tissues/cells and subsequent endometrial regeneration and establishment of the endometriotic lesion.

Our eutopic reimplantation hypothesis does not contradict the current theory but rather provides an additional mechanism for endometrial regeneration. It can be reconciled with the current paradigm that the human endometrium, in particular the functionalis layer, regenerates from the lower basalis layer that persists during menstruation.

In this model, putative endometrial stem/progenitor cells are derived from the BM, and they, at least some of them, reside in the endometrial vascular endothelium and/or perivascular regions. Taken together, it is conceivable that putative endometrial stem/progenitor cells may have endothelial progenitor cell (EPC)-like properties. EPCs are believed to be derived from the BM and to home to sites of neovascularization and neoendothelialization where they differentiate into endothelial cells (Urbich & Dimmeler 2004, Timmermans *et al.* 2008). Indeed, BM-derived EPCs have been shown to contribute to the formation of new blood vessels in the mouse endometrium (Masuda *et al.* 2007*a*). Furthermore, BM-derived cells have been shown to give rise to uterine epithelial and stromal cells in humans (Taylor 2004) and mice (Bratincsak *et al.* 2007, Du & Taylor 2007).

In addition to the recruitment of EPCs having endometrial stem/progenitor cell-like properties into the endometrium, the possible release of those EPCs from the uterus is also an interesting issue. Circulating endothelial cells are thought to appear in the bloodstream randomly after being shed from the vascular wall (Blann *et al.* 2005). Trauma induced by surgery or increased intravascular turbulence may also result in the introduction of endothelial cells into the peripheral circulation (Blann *et al.* 2005). Thus, it is conceivable that endometrial EPCs may be sloughed off from the endometrial vascular walls during tissue breakdown and shedding, and circulate via the blood and lymphatic vessels, eventually implanting and migrating at ectopic sites far from the uterus.

There are several theories regarding the pathogenesis of endometriosis (Sasson & Taylor 2008, Bulun 2009). The most widely accepted are retrograde menstruation and coelomic metaplasia, though they are not mutually exclusive. A third hypothesis claims transplantation of endometrial tissue secondary to surgery or via lymphovascular dissemination. Deep infiltrating endometriosis, characterized by the invasion of anatomical structures and organs by endometriotic foci, is occasionally

accompanied by the presence of endometriotic lesions and endometriotic-like cells in the pelvic sentinel lymph nodes (Mechsner *et al.* 2008). According to the embryonic rest theory, Müllerian-originated cells presumably present within the peritoneal cavity give rise to endometrial and/or endometriotic tissues upon exposure to as-yet-unidentified inducing stimuli. Most recent is the fascinating proposal that circulating cells originating from the BM differentiate into endometriotic tissue at various sites (Du & Taylor 2007, Sasson & Taylor 2008).

We suggest that our model reconciles all of these theories. Putative endometrial EPCs, shed from endometrial vascular walls during menstruation, may disseminate either artificially or spontaneously, via lymphatic and/or blood flow in addition to retrograde menstruation. EPCs mobilized at ectopic locations may then migrate into the parenchyma of the corresponding organ, differentiate into endometrial cell components and eventually give rise to endometriotic lesions at the ectopic implantation sites. In the coelomic metaplasia and embryonic rest theories, the cells capable of generating endometriotic lesions are thought to have been present in the mesothelium and other ectopic sites since embryonic development (Sasson & Taylor 2008, Bulun 2009). Thus, to prove our hypothesis, it is very important to characterize putative endometrial stem/progenitor cells, in particular ESP cells, and to examine whether they have endothelial cell- or EPC-like properties. Very recently, we have demonstrated that ESP cells display not only endometrial stem/progenitor cell-like properties but also endothelial cell-like characters (Masuda *et al.* 2010).

Stem/progenitor cells in human myometrium

Marked pregnancy-induced expansion of the human uterus (mainly composed of myometrial cells) can be repeated multiple times throughout the reproductive life. The dramatic enlargement of the pregnant uterus is attributable not only to myometrial hyperplasia (an increase in cell number) but also to hypertrophy (an increase in cell size) in both humans and rodents (Ramsey 1994). In the human, stretch-induced myometrial hypertrophy predominantly contributes to uterine enlargement during pregnancy. In the first weeks of pregnancy, however, significant myometrial hyperplasia also takes place, acting as the driving force for uterine growth (Ramsey 1994, Shynlova *et al.* 2006). This is similar with the pregnant rat uterus. Myometrial hyperplasia predominantly occurs during early gestation but decreases dramatically later, while myometrial hypertrophy is not evident at the beginning of pregnancy but becomes more considerably predominant with gestational age (Shynlova *et al.* 2006). Thus, we hypothesize that there may exist in the myometrium a population of putative stem/progenitor cells involved in the growth and remodelling of the pregnant uterus.

To support this hypothesis, we isolated SP cells from the myometrium (myoSP) from consenting patients undergoing hysterectomy (Ono *et al.* 2007). After the myometria were mechanically and enzymatically digested, the dissociated cells were subjected to flow cytometric sorting to isolate myoSP cells in a similar way to the ESP cells (Ono *et al.* 2007, Masuda *et al.* 2010). The Hoechst-stained cells contained a small fraction of
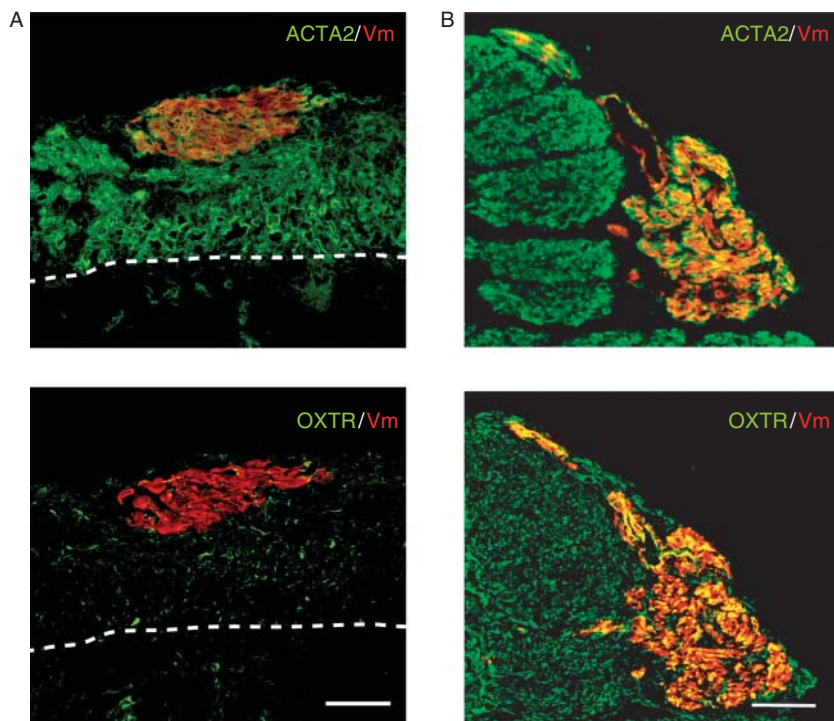

Figure 6 *In vivo* reconstitution of myometrium from myoSP in oestrogen-treated and pregnant uteri of NOG mice. (A) Ovariectomized NOG mice were xenotransplanted with myoSP into their uteri, subcutaneously implanted with an oestrogen pellet and hysterectomized 10 weeks after transplantation. The excised uteri were serially sectioned and subjected to immunofluorescence staining using antibodies against ACTA2, vimentin (Vm) and oxytocin receptor (OXTR), followed by confocal microscopic analysis. (B) Female NOG mice were mated to ICR males 2 weeks after transplantation of myoSP into the uterine horn. The pregnant uteri were excised at 7.5 days post coitum, serially sectioned and subjected to immunofluorescence staining as described in (A). Dotted lines indicate endometrium–myometrium junctions. Bars, 100 μm. Reproduced, with permission, from **Ono M, Maruyama T, Masuda H, Kajitani T, Nagashima T, Arase T, Ito M, Ohta K, Uchida H, Asada H *et al.*** 2007 Side population in human uterine myometrium displays phenotypic and functional characteristics of myometrial stem cells. *PNAS* **104** 18700–18705. © 2007 The National Academy of Sciences of the USA.

SP cells that were resting in G_0 phase. RT-PCR data revealed that only myoSP cells expressed *ABCG2* mRNA, a property of SP-type cells. In differentiation-inducing media, the cells differentiated into multiple lineages (Ono *et al*. 2007). Following transplantation to NOG mice, the cells regenerated myometrium-like tissues (Fig. 6A). Undifferentiated myoSP proliferated and differentiated into mature myometrial cells in mouse uteri (Fig. 6A), while non-SP cells (non-myoSP) lacked these capabilities (Ono *et al*. 2007). Furthermore, they could induce the expression of oxytocin receptor (Ono *et al*. 2007), a characteristic of 'activated' myometrium during late pregnancy and labour (Kimura *et al*. 1996), particularly in pregnant mouse uteri (Fig. 6B).

Collectively, our study revealed that the human myometrium harbours myoSP and that purified myoSP, but not non-myoSP, exhibits stem cell-like properties such as quiescent cell cycle status, *in vitro* potential for multi-lineage differentiation and *in vivo* capacities for reconstitution of the original tissue (Ono *et al*. 2007). Thus, myoSP satisfies the requirements of definition for adult stem cells as illustrated in Fig. 1.

Arango *et al*. (2005) reported that disruption of β-catenin in Müllerian duct mesenchyme results in a progressive turnover of the uterine myometrium to adipose tissue, suggesting the possible existence of myometrial stem cells harbouring a capacity for differentiation into adipocytes upon deletion of β-catenin. In this regard, it is tempting to speculate that they may have a potential to give rise to lipoleiomyomas. Subsequently, the same group isolated SP cells from murine Müllerian duct mesenchyme and demonstrated that they display several stem/progenitor cell-like properties (Szotek *et al*. 2007). This finding is consistent with our result that stem cells are enriched in myoSP in humans (Ono *et al*. 2007).

Concluding remarks

Many studies, including ours, have recently provided strong evidence for the existence of rare populations of adult stem cells in the human myometrium and endometrium. Stem cell biology in the female reproductive tract is still in its infancy, and, although surface markers of prospective isolation of human endometrial stromal colony-forming cells (putative endometrial stromal/progenitor cells) have recently identified (Gargett *et al*. 2008, Schwab *et al*. 2008), there remains a need for definitive markers of both myometrial and endometrial stem cells for more selective isolation and enrichment. Complete characterization of uterine stem/progenitor cells will improve our understanding of the mechanisms supporting physiological regeneration of the female reproductive tract. In addition, such studies will enhance our understanding of uterine cancer, hyperplasia, endometriosis, leiomyomas and adenomyosis. Indeed, our data and the published observations from other laboratories have enabled us to propose a novel hypothetical model for eutopic and ectopic endometrial regeneration. Confirmation of this hypothesis requires further studies. Finally, availability of these stem cells suggests new approaches to reconstruction of the human uterus and perhaps other organs as well.

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