Stem cells in human normal endometrium and endometrial cancer cells: Characterization of side population cells

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Abstract
Recently, adult stem cells have been identified in several mature tissues. The human endometrium is responsive to sex steroid hormone. It undergoes extraordinary growth in a cyclic manner and is shed and regenerated throughout a woman’s lifetime. It has been proposed that the human endometrium may contain a population of stem cells, which are responsible for its remarkable regenerative ability. It is also suggested that stem-like cells exist in cancer tissues. Stem-like cell subpopulations, referred to as “side population” (SP) cells, have been identified in several tissues and tumors based on their ability to efflux the fluorescent dye Hoechst 33342. Recently, we isolated and characterized the SP cells in normal human endometrium and in an endometrial cancer (EC) cell line. Endometrial SP cells can function as progenitor cells. EC SP cells show the following: (1) reductions in the expression levels of differentiation markers; (2) long-term repopulating properties; (3) self-renewal capacity; (4) enhancement of migration and podia formation; (5) enhancement of tumorigenicity; and (6) bipotent developmental potential (tumor cells and stroma-like cells), suggesting that these SP cells have cancer stem-like cell features. We review the articles that show the presence of stem cells in normal endometrium and EC cells and demonstrate the results of our studies.

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Endometrial stem cells

Recently, adult stem cells have been identified in several mature tissues, such as the adult intestine [1], skin [2], muscle [3], blood [4], and the nervous system [5–7]. A stem cell is an undifferentiated cell that is defined by its ability to both self-renew and to produce mature progeny cells [8].

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Stem cells are classified based on their developmental potential as being totipotent, pluripotent, oligopotent, and unipotent. Adult somatic stem cells were originally thought to be tissue specific and only able to give rise to progeny cells corresponding to their tissue of origin. Recent studies, however, have shown that adult mammalian stem cells are able to differentiate across tissue lineage boundaries [9, 10], although this “plasticity” of adult somatic stem cells remains controversial.

Stem cell subpopulations ("side population" (SP) cells) have been identified in many mammals, including humans, based on the ability of these cells to efflux the fluorescent dye Hoechst 33342 (Molecular Probes, Eugene, OR, USA) [11]. Recent evidence suggests that the SP phenotype is associated with a high expression level of the adenosine triphosphate (ATP)-binding cassette transporter protein ABCG2/Bcrp1 [12]. Most recently, established malignant cell lines, which have been maintained for many years in culture, have also been shown to contain SP cells as a minor subpopulation [13].

The human endometrium is a highly dynamic tissue undergoing cycles of growth, differentiation, shedding, and regeneration throughout the reproductive life of women. Endometrial adult stem/progenitor cells are likely to be responsible for endometrial regeneration [14]. Rare populations of human endometrial epithelial and stromal colony-forming cells [15] and SP cells have been identified by us and others [16–20]. Tsuji et al. [17] demonstrated that Breast cancer resistance protein (BRCP)/ABCG2, known as a marker of SP cells, was strongly expressed in the vascular endometrial. Several groups have shown that endometrial SP cells have multilineage developmental potential (Table 1) [18, 20].

Although coexpression of CD146 and platelet-derived growth factor β (PDGF β) isolates a population of mesenchymal stem-like cells from human endometrium [21], the specific stem cell markers of endometrium remain unclear. Recently, Gotte et al. [22] demonstrated that the adult stem cell marker, Musashi-1, was coexpressed with Notch-1 in a subpopulation of endometrial cells. Furthermore, they showed that telomerase- and Musashi-1-expressing cells were significantly increased in proliferative endometrium, endometriosis, and endometrial carcinoma tissue, compared with secretory endometrium, which suggested the concept of a stem cell origin of endometriosis and endometrial carcinoma.

Endometrial cancer stem-like cells

Recent evidence suggests that cancer stem-like cells exist in several malignant tumors, such as leukemia [23, 24], breast cancer [25], and brain tumors [26], and that these stem cells express surface markers similar to those expressed by normal stem cells in each tissue [23, 27].

Endometrial cancer (EC) is the most common gynecological malignancy in the industrialized world. Two different clinicopathological types can be distinguished: (1) estrogen-related ECs (Type I) developing in pre- and postmenopausal women, endometroid type and low cellular grade, frequently preceded by endometrial hyperplasia and with good prognosis. In Type I EC, estrogen receptor (ER, especially ERα) was expressed. (2) Non-estrogen-related ECs (Type II) occurring in postmenopausal women, nonendometroid type (mainly papillary serous or clear cell carcinomas), without associated hyperplasia. Type II ECs are negative for ER and progesterone receptor (PR), have high cellular grade, and have a poor prognosis. The most frequent genetic alteration in Type I EC is phosphatase and tensin homolog (PTEN) inactivation, followed by microsatellite instability and mutations of the K-ras and β-catenin. In Type II EC, p53 mutation is the most frequent genetic alteration, followed by amplification of human epidermal receptor 2 (HER2) (Table 2). Some of these pathways are important determinants of stem cell activity (Wnt-b-catenin and PTEN) [28–30]. These suggest that there is a stem cell contribution to EC development. Recently, Friel et al. [31] showed that SP cells were derived from two EC cell lines (AN3CA and Ishikawa). AN3CA had features of cancer stem-like cells, including low proliferative activity during 9 days of cultivation, chemoresistance, and enhanced tumorigenicity. Hubbard et al. [32] demonstrated that a small population of clonogenic cells from EC tissues showed self-renewing, differentiating, and tumorigenic properties. Gotte et al. [33] recently demonstrated that siRNA depletion of Musashi-1, an adult stem cell marker enriched in SP cells, in the endometrial carcinoma cell line Ishikawa leads to interference with the notch signaling pathway and p21 expression, resulting in an antiproliferative effect and induction of apoptosis.

### Table 1

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<th>The reports of endometrial SP cells</th>
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<td>Kato K et al. 2007 Hum Reprod</td>
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<td>Tsuji S et al. 2008 Paei Clin</td>
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<td>Masuda M et al. 2010 FLSOS One</td>
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### Table 2

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<td>Morphology</td>
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Characterization of normal endometrial SP cells

We have isolated SP cells from normal human endometrium and characterized their properties [16]. CD9 is an epithelial glandular marker and CD13 is a stromal marker in the normal human endometrium (Fig. 1). We analyzed 34 samples of the normal human endometrium. SP cells are present in the human endometrium (mean, 0.27%) (Fig. 2A). For the analysis of the surface marker of SP cells, endometrial cells were stained with Hoechst 33342, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated CD9 antibody and phycoerythrin (PE)-conjugated CD13 antibody. SP cells are enriched in negative fraction of both surface markers (Fig. 2B).

Morphologically, SP cells are small and round (Fig. 3). We followed a single SP cell for 48 hours. Daughter cells were generated from a single SP cell. We observed the long-term

Figure 1. CD9 and CD13 are surface markers in the normal human endometrium. The expressions of CD9 (A), CD13 (B), E-cadherin (C), and vimentin (D) in the human endometrium in the secretory phase were analyzed by immunohistochemistry using formalin-fixed histological sections. HE staining of normal human endometrium in the secretory phase. The brown color in the panel of CD9, CD13, E-cadherin, and vimentin shows the expression of each protein by immunohistochemistry using diaminobenzine tetrahydrochloride as the substrate (magnification: 200×, scale bar = 20 mm).

Figure 2. Side population (SP) cells were present in the human endometrium. (A) Endometrial cells were stained with Hoechst 33342 and analyzed by FACS. SP cells, which show a high level of dye efflux activity, were present in the human endometrium (mean = 0.27%, n = 34). Verapamil treatment increased the staining and made SP cells undetectable by FACS. (B) For analysis of the cell-surface markers of SP cells, cells stained with Hoechst 33342 dye were followed by incubation with FITC-conjugated anti-CD9 antibody and PE-conjugated anti-CD13 antibody. Most SP cells were present in the CD9−CD13− fraction. NSP = non–side population; SP = side population.
proliferative capacity of SP and non-SP (NSP) cells. NSP cells showed limited proliferation and finally became senescent within 3 months. In contrast, SP cells were successfully maintained for 9 months, finally differentiated into gland-like cells and constructed a gland-like structure (Fig. 3). These results demonstrate that isolated SP cells in the human endometrium have a long-proliferating potential and produce gland-like cells. They can function as progenitor cells.

After our publication, several groups have also reported the existence and characteristics of SP cells in endometrium. It has been shown that endometrial SP cells have a multilineage developmental potential [17–20].

Characterization of EC SP cells

Recent evidence suggests that cancer stem cells exist in several malignant tumors. We isolated and characterized SP cells from human EC cells [34].

We first analyzed primary EC cells freshly isolated from EC tissues after 7 days of cultivation (n = 7) and a human EC cell line, Hec1, by fluorescence-activated cell sorter (FACS). SP cells were present in both cells. Verapamil blocked the dye efflux (Fig. 4).

Next, both Hec1-SP and Hec1-NSP cells were cultured for 2 weeks, stained with Hoechst 33342, and then reanalyzed by FACS. Hec1-SP cell cultures generated both SP and NSP subpopulations. In contrast, NSP cell cultures produced NSP cells but never SP cells. We demonstrated that SP cells from human endometrium are negative for both CD9 and CD13. This was also the case in the EC cell lines. The expression levels of both CD9 and CD13 were lower in SP cells than those in NSP cells.

SP and NSP cells derived from Hec1 cells were cultured in mesenchymal stem cell maintenance medium (MF medium). Cell growth rate was analyzed for 2 months. Both cell types grew after 2 weeks of culture. SP cells continued to grow for 2 months and accumulated in colonies atop the confluent cell layer. In contrast, NSP cells stopped growing,
and the total cell number decreased. Finally, the cells became flat and enlarged after 2 months. These results show that SP cells, but not NSP cells, maintain a long-term proliferating capacity of the cell culture (Fig. 5A). Next, we analyzed self-renewal capacity by colony-forming potential in serial cloning.

When Hec1-SP cells were plated in 24-well collagen-coated dishes (50 cells/cm²), they proliferated and formed colonies. Hec1-NSP cells were more loosely arranged and did not form well-separated individual colonies. We dissociated the primary colonies into single cells and then cultured these cells. A single cell formed a secondary colony. The secondary colonies generate tertiary colonies. This indicates that the colony-forming cells isolated from existing colonies retain the same colony-forming potential. These results demonstrated that SP cells have a self-renewal capability. NSP cells also produced secondary clones, but there was much overlap. They did not form well-separated individual colonies (Fig. 5B).

Cellular activity was monitored with time-lapse video imaging studies. Interestingly, SP cells showed lamellipodia formation at the leading edge and uropodia formation at the trailing edge (Fig. 6). SP cells showed prominent migration. NSP cells did not show podia formation and migration (data not shown).

Hec1-SP or Hec1-NSP cells were inoculated into the subcutaneous tissues of nude mice. Tumors generated from the SP cells grew faster and larger than those from the NSP cells (Fig. 7A). The tumors were excised after 12 weeks of inoculation. Hematoxylin-eosin staining showed that the SP tumors were composed of tumor cells, surrounded by stroma-like tissues with a markedly enriched extracellular matrix (ECM). In contrast, most NSP tumors were composed of well-encapsulated tumor cells that were not accompanied by the ECM-enriched stroma.

The ECM is thought to be secreted by stromal cells. Therefore, we analyzed the expression of specific mesenchymal cellular markers, vimentin and α-smooth muscle actin (α-SMA), and collagen III as components of ECM. All vimentin, α-SMA, and collagen III stained more strongly in the SP tumors than in the NSP tumors (Fig. 7B).

The fact that the vimentin monoclonal antibody (V9) used in this study reacted with human and rat vimentin, but not with mouse vimentin, suggested the possibility that vimentin-positive, stromal-like tissues in the tumors originated from the inoculated SP cells of Hec1 cells.

To confirm the origin of the stroma-like cells in the Hec1-SP tumors, we also performed fluorescence in situ hybridization assay on these two areas of Hec1-SP tumor tissues using the spectrum orange-labeled CEP X (a satellite)
The DNA probe, which hybridizes to the centromere of human chromosome X, and the FITC-labeled DNA probe, which hybridizes to mouse pan-centromeric chromosome. We analyzed the signal numbers in three independent regions of tumor and stromal tissues. Only red human signals were detected in the area of tumor tissues without ECM. Both red human signals and green mouse signals were observed in the area of stroma-like cells with enriched ECM. The ratio of cells with red signals was significantly more than that of cells with green signals in stroma-like tissues. These results...

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**Figure 6.** Hec1-SP cells showed podia formation and prominent migration. In time-lapse video imaging studies, the frequency of cell division was higher in Hec1-SP cells (A) than that in Hec1-NSP cells (B). Hec1-SP cells showed podia formation (lamellipodia at the leading edge, shown by arrow, and uropodia at the trailing edge, shown by arrowhead). Hec1-NSP cells did not form podia. NSP = non–side population; SP = side population.

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**Figure 7.** Tumorigenicity was enhanced in Hec1-SP cells. (A) Side population (SP) cells or non–side population (NSP) cells were inoculated into the subcutaneous tissues of nude mice. (a, b) There was a trend toward an increased size of tumor from SP cells compared with that from NSP cells, although it was masked by large variation between experiments. The tumor size is shown as the mean ± standard error of the mean from three independent experiments. (b) y-axis: tumor size, length x width (cm²). (c) The tumor weight after 12 weeks of inoculation is shown as the mean ± standard error of the mean from three independent experiments—y-axis: tumor weight (g). SP: 1.72 ± 0.54 g and NSP: 0.18 ± 0.10 g (p = 0.049). (B) The expression levels of vimentin, α-smooth muscle actin, and collagen III were analyzed by immunohistochemistry. Both tumor cells and stroma-like cells in the SP tumor were stained with vimentin. Stroma-like cells in the SP tumor expressed α-SMA. Tumor cells in the NSP tumor also expressed vimentin, but the level of expression was less than that in the SP tumors. α-SMA and collagen III were not expressed in the NSP tumor (magnification: 20× and 50×, scale bar = 100 μm; 100×, scale bar = 50 μm). *p = 0.045, **p = 0.056, and ***p = 0.074. NSP = non–side population; α-SMA = α-smooth muscle actin; SP = side population.
Figure 8. Most of the stromal-like cells were derived from the inoculated Hec1-SP cells. Fluorescence in situ hybridization (FISH) assay was performed on Hec1-SP tumor tissues using the spectrum orange-labelled CEP X (a satellite) DNA probe, which hybridizes to the centromere of human chromosome X and the FITC-labeled DNA probe, which hybridizes to mouse pan-centromeric chromosome. In the area of tumor cells without ECM, only red signals (human chromosome) were detected. Both red signals and green signals (mouse chromosome) were detected in the area of stroma-like cells with enriched ECM. The ratio of cells with red signals was significantly more than that of cells with green signals (red: 76 ± 4%, green 24 ± 4% from three different regions of tumor cells or stroma-like cells with enriched ECM). The hematoxylin and eosin (HE) panel shows HE staining of tumor cells and stroma-like cells.

Figure 9. Side population cells differentiated into α-smooth muscle action (α-SMA)-expressing cells. 1 × 10^5 Hec1-SP or Hec1-NSP cells were cultured in Matrigel with a mesenchymal stem cell maintenance medium (MF-medium)(A, C), a standard growth medium (DMEM containing 10% FCS)(B, D). After 8 weeks, the level of α-SMA expression was analyzed in each Matrigel sample. SP cells cultured with either the standard growth medium (DMEM containing 10% FCS) expressed α-SMA. Hec1-NSP cells did not express α-SMA in any culture condition. NSP = non–side population; SP = side population.
clearly demonstrated that most of stroma-like cells were derived from the inoculated human Hec1-SP cells (Fig. 8).

To further demonstrate the mesenchymal developmental potential of SP cells in vitro, Hec1-SP or NSP cells were seeded into Matrigel (BD Bioscience, Bedford, MA, USA) and incubated with either a mesenchymal stem cell maintenance medium (MF medium) or Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS). After 8 weeks, the level of a-SMA expression was analyzed in each Matrigel sample. The SP cells were cultured with DMEM containing 10% FCS, which implied that SP cells differentiated to the smooth muscle cell lineage. The SP cells incubated with MF medium and the Hec1-NSP cells in MF medium, and DMEM failed to express a-SMA protein. These results suggest that SP cells have the potential to develop a-SMA-expressing cells in a differentiation medium (Fig. 9).

In this study, we demonstrated that an SP subpopulation is present in cultures of human EC cells. The biological characteristics of SP cells were distinct from those of NSP cells. SP cells showed the following: (1) a reduction in the expression levels of differentiation markers (CD9 and CD13); (2) long-term proliferating capacity of the cell culture; (3) self-renewal capacity in vitro; (4) enhancement of migration and lamellipodia and uropodia formation; (5) enhanced tumorigenicity; and (6) bipotent developmental potential (tumor and stroma-like cells). The association of epithelial-mesenchymal transition with the phenotype of migration enhancement and mesenchymal cell lineage differentiation in EC SP cells is currently under investigation.

In conclusion, recent evidence demonstrates the presence of stem-like cells in both normal endometrium and EC. Endometrial stem-like cells have a multilineage developmental potential. They may be applicable to the regeneration of not only injured endometrium but also of other tissues.

EC stem-like cells show enhanced tumorigenicity and migration. Development of target therapy to EC stem cells seems to contribute to the improvement of the prognosis for patients with EC.

Acknowledgments

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