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Characterization of side-population cells in human normal endometrium

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BACKGROUND: It has been proposed that the human endometrium may contain a population of adult stem cells that are responsible for its remarkable regenerative capability. Recently, a subset of stem cells or progenitor cells in adult tissue has been identified as side-population cells (SP cells) displaying low staining with Hoechst 33342 by fluorescence-activated cell sorter (FACS) analysis. In this study, we isolated SP cells from the human endometrium and analysed their properties. METHOD: Endometrial cells were obtained using enzymatic digestion from uterine hysterectomy for the treatment of uterine myoma and stained with Hoechst 33342 dye either alone or in combination with verapamil. The cells were then analysed using FACS. RESULTS: SP cells were present among normal human endometrial cells. Most SP cells were enriched in the $CD9^{-}CD13^{-}$ fraction. These SP cells showed long-term repopulating properties and produced gland ($CD9^{+}$)- and stroma ($CD13^{+}$)-like cells. $CD9^{-}CD13^{-}$ cells isolated from the endometrium also generated gland- or stroma-like cells. CONCLUSIONS: SP cells in the human endometrium can function as progenitor cells. This is the first report of the phenotype of SP cells from normal human endometrial cells.

Key words: CD9/CD13/endometrium/side-population cells/stem cells

Introduction

The human endometrium is responsive to sex steroid hormones, 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 that is responsible for its remarkable regeneration ability (Padykula, 1991; Chan *et al.*, 2004).

The human endometrium is composed mainly of two types of cells: endometrial glandular and stromal cells. CD9 is a 24–27 kD glycoprotein reported to be strongly expressed on the cell surface of the glandular epithelium throughout the menstrual cycle (Park *et al.*, 2000). On the other hand, CD13, known as aminopeptidase N, is expressed in endometrial stroma throughout the menstrual cycle and is significantly higher in the secretory phase compared with the proliferative phase (Seli *et al.*, 2001). These results suggest that CD9 and CD13 are used as surface markers in glandular and stromal cells, respectively, in the endometrium.

Recently, adult stem cells have been identified in many mature tissues, such as the adult intestine (Bjerknes and Cheng, 1999), skin (Alonso and Fuchs, 2003), muscle (Jankowski *et al.*, 2003), blood (Spangrude *et al.*, 1991) and the nervous system (Morrison *et al.*, 1999; Uchida *et al.*, 2000; Thomas *et al.*, 2001). Stem cells are undifferentiated cells defined by their ability at the single cell level to both self-renew and differentiate to produce mature progeny cells, including both non-renewing progenitors and terminally differentiated effector cells. Stem cells have been classified by their developmental potential as totipotent, pluripotent, oligopotent and unipotent. In adult soma, stem cells have generally been thought of as tissue-specific, able to give rise only to progeny cells corresponding to their tissue origin. Somatic stem cells represent a rare cell type capable of self-renewal and differentiation into one or more cell types in order to maintain and repair adult tissue (Reya *et al.*, 2001). Recent studies have shown that adult mammalian stem cells may be able to differentiate across tissue lineage boundaries (Goodell, 2003;

Herzog *et al.*, 2003), although studies proposing such 'plasticity' of adult somatic stem cells remain controversial.

Stem cell subpopulations have been identified in many mammals, including humans, using the fluorescent dye Hoechst 33342 [so-called 'side-population' (SP) cells] (Goodell *et al.*, 1996). An adult stem cell subpopulation has been identified that can rapidly efflux Hoechst dye to produce a characteristic SP profile on the basis of fluorescence-activated cell sorter (FACS) analysis. Recent evidence suggests that the SP phenotype is associated with the high-level expression of the ATP-binding cassette transporter protein ABCG2/Bcrp1 (Zhou *et al.*, 2001).

In this study, we isolated and analysed SP cells in the human endometrium that can function as progenitor cells. This is the first report of a phenotype in a subset of stem cells (SP cells) from normal endometrial cells.

Materials and methods

Human tissue

Endometrial tissues were obtained from uteri after hysterectomy for the treatment of uterine myomas (17 patients: age, 37–49 years, menstruation phase, 3 cases; proliferative phase, 9 cases; and secretory phase, 5 cases). This study was approved by the ethical committee of Kyushu University, Japan, and pre-operative informed consent was obtained from each patient.

Preparation of human endometrial epithelial and stromal cells

Cell suspensions of endometrial cells were obtained using enzymatic digestion and mechanical means as described elsewhere (Seli *et al.*, 2001; Chan *et al.*, 2004). The endometrium was scraped off the underlying myometrium, diced finely and dissociated in Hanks' balanced salt solution containing HEPES (25 mmol), penicillin (200 U ml^{-1}), streptomycin (200 $\mu g\ ml^{-1}$) and collagenase (1 mg ml^{-1}, 15 U mg^{-1}) (Sigma, St. Louis, USA) for 30 min at 37°C with agitation. The dispersed endometrial cells were separated by filtration through a wire sieve (32 μm diameter pores). Each endometrial tissue sample was separated into two samples (upper fraction and lower fraction) by filtration to obtain a total of 34 endometrial cell samples.

Cell culture

The endometrial glandular and stromal cells or immortalized human endometrial cells (Kyo *et al.*, 2003) were cultured with Dulbecco's modified Eagle's medium (DMEM) (Nissui Seika, Japan), supplemented with 20 $\mu g\ ml^{-1}$ Gly-His-Lys, 2 mM glutamine, 80 IU insulin (Sigma) and 10% fetal calf serum (FCS, Hyclone, Logan, USA), which was used as the medium for rat endometrial cells (Wiele *et al.*, 1990). Immortalized human endometrial cells were also incubated with the medium for mesenchymal stem cells (MF-medium, TOYOBO, Japan).

Immunohistochemistry

Formalin-fixed histological sections of endometrial tissue or cultured cells were used. Cultured cells were incubated on glass chamber slides (LAB-TEK; Nalge Nunc International Corp. Naperville, USA) and fixed by treatment with 10% formalin. Sections or cells were rinsed twice in phosphate-buffered saline (pH 7.4) for 5 min each. Samples were then incubated with 4% blocking horse serum (Vector Laboratories, Burlingame, USA) for 1 h at room temperature in a humidified chamber followed by incubation with the primary antibody (200 $\mu g\ ml^{-1}$, 1:100 diluted). The anti-CD9 polyclonal antibody (H-110), anti-CD13 polyclonal antibody (H-300), anti-E-cadherin polyclonal antibody (H-108) and anti-vimentin polyclonal antibody (S-20) were obtained from Santa Cruz Biotechnology Inc., USA. We also used non-immune rabbit immunoglobulin G as a control for the primary antibody. Staining with the primary or control antibody was performed overnight at 4°C. Bound antibodies were detected with a biotinylated anti-rabbit IgG secondary antibody (1.5 mg ml^{-1}) and an avidin–biotin complex linked to horse–radish peroxidase (HRP) (Vecstastain, Vector Laboratories), followed by incubation with diaminobenzidine tetrahydrochloride as the substrate.

Isolation of SP cells

To identify and isolate SP cells from endometrial cells, cells maintained for 24–48 h were removed from the culture dish with trypsin and EDTA, washed and suspended at 10^6 cells per millilitre in DMEM containing 2% FCS. The cells were then labelled in the same medium at 37°C for 90 min with 2.5 $\mu g\ ml^{-1}$ Hoechst 33342 dye (Molecular Probes Inc., Eugene, USA), either alone or in combination with 50 μM verapamil (Sigma). Finally, the cells were counterstained with 1 $\mu g\ ml^{-1}$ propidium iodide to label dead cells. The cells were then analysed in a Vantage FACS (Becton Dickinson, Bedford, USA) using dual wavelength analysis (blue, 424–444 nm; red, 675 nm) after excitation with 350-nm UV light. Propidium iodide-positive dead cells were excluded from the analysis. One analysis of SP cells was performed per sample. For analysis of the cell-surface markers of SP cells, cells stained with Hoechst 33342 dye were followed by incubation with 10 μl fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-CD9 antibody (200 $\mu g\ ml^{-1}$) and 10 μl phycoerythrin (PE)-conjugated anti-CD13 antibody (200 $\mu g\ ml^{-1}$) (Immunotech, Marseille, France) at 4°C for 15 min and analysed by FACS.

The SP cells or non-SP (NSP) cells were sorted and seeded in DMEM containing 100 ng ml^{-1} Interleukin-6 (IL-6), 100 ng ml^{-1} stem cell factor (SCF), 10 ng ml^{-1} thrombopoietin (TPO) (Calbiochem, La Jolla, USA) and 10% FCS on a collagen-coated 24 multiwell plate (2 cm^2) (300–500 cells cm^{-2})(Iwaki, Funabashi, Japan) for 2–4 weeks. The cells were then transferred onto feeder layers of aorta–gonad–mesonephros (AGM) stroma cells, derived from mouse embryos at 10.5 days post-coitum (Matsuoka *et al.*,2001) and treated with 10 $\mu g\ ml^{-1}$ mitomycine C (Calbiochem). As a medium for endometrial stem cells has not yet been reported, we used the medium for hematopoietic stem cells (Ueda *et al.*, 2000). The medium was replaced weekly. After 3 months, the SP cells were re-seeded into the matrigel-coated dishes (Becton Dickinson). Finally, a piece of matrigel was removed and stained with hematoxylin and eosin (HE).

SP cells and NSP cells from immortalized human endometrial cells were also isolated by the same method as decribed here. They were incubated on a collgen-coated plate or in the matrigel-coated dishes with MF medium or DMEM containing 10% FCS.

Magnetic cell sorting

To purify cells which expressed CD9 or CD13 or both, cultured endometrial cells were selected by positive or negative selection, using magnetic microbeads coated with anti-FITC or PE antibodies that recognize CD9- or CD13-expressing cells, respectively. Briefly, cells were incubated with the 10 μl FITC-conjugated monoclonal anti-CD9 antibody and 10 μl PE-conjugated monoclonal anti-CD13 antibody (200 $\mu g\ ml^{-1}$) followed by incubation with 10 μl anti-FITC or anti-PE antibody-coated magnetic microbeads (Miltenyi Biotec Auburn, USA). For example, $CD9^+$, and $CD13^-$ cells were purified by negative selection using anti-PE antibody-coated magnetic

microbeads followed by positive selection using anti-FITC antibody-coated magnetic microbeads. CD9- and CD13- cells were purified by negative selection using the co-incubation of anti-FITC antibody- and anti-PE antibody- coated magnetic microbeads.

Western blotting

To detect expression of each protein, subconfluent cells were lysed with ice-cold RIPA buffer (50mM Tris HCl, pH 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulphate (SDS), 0.5% sodium deoxycholate and 1% Nonidet P40) containing freshly added protease inhibitors (1 mM phenyl methyl sulphonylfluoride, 1 $\mu g\ ml^{-1}$ leupeptin and 10 $\mu g\ ml^{-1}$ aprotinin) (Sigma). After centrifugation at 13 000 *g* for 10 min to remove debris, 100 μg of the proteins were subjected to SDS–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane in a semi-dry transfer cell (Bio Rad Laboratories, Hercules, USA). The blots were incubated with diluted primary antibodies overnight at 4°C. Primary antibodies, including the anti-CD13 polyclonal antibody, anti-E-cadherin polyclonal antibody and anti-vimentin polyclonal antibody were obtained from Santa Cruz Biotechnology Inc. After incubation with each primary antibody (1:1000 diluted), the blots were incubated with HRP-linked anti-rabbit antibodies and analysed with an enhanced chemiluminescence system (Amersham Bioscience, Buckinghamshire, UK).

Data analysis

The SP cell proportion in each stage of the menstrual cycle was shown as the median, and the range of scatter. Data were also represented as the means ± SEM and analysed by Student's *t*-test.

A *P*-value of <0.05 was considered statistically significant.

Results

CD9 and CD13 are surface markers in the normal human endometrium

First, we investigated the expression of surface markers in the human endometrium. Previous studies demonstrated that CD9 and CD13 were expressed in glandular and stromal cells, respectively (Park *et al.*, 2000; Seli *et al.*, 2001). Thus, we analysed CD9 and CD13 expression levels in the human endometrium in the secretory phase by immunohistochemistry. CD9 was expressed in glandular cells and co-existed with E-cadherin, a well-known epithelial cell marker (Figure 1A). CD13 was expressed in both stromal and glandular cells (Figure 1A). This expression pattern was similar to vimentin. These results demonstrated that CD9 and E-cadherin were used as specific differential markers of glandular cells. In contrast, CD13 and vimentin were used as markers of whole endometrial cells (both glandular and stromal cells). Next, human endometrial cells in the proliferative phase were isolated by digestion with collagenase followed by filtration through a 32 μM filter. We analysed CD9 and CD13 expression levels using FACS. Cells from the upper fraction of the filter expressed much more CD9 than CD13, suggesting that most of these cells were glandular cells (Figure 1B). On the other hand, cells from the lower fraction of the filter expressed both CD13 and CD9, suggesting that these cells consisted of both stromal and glandular cells (Figure 1B). These results demonstrated that most of the endometrial glands (largely undispersed) were retained by the sieve, whereas the dispersed

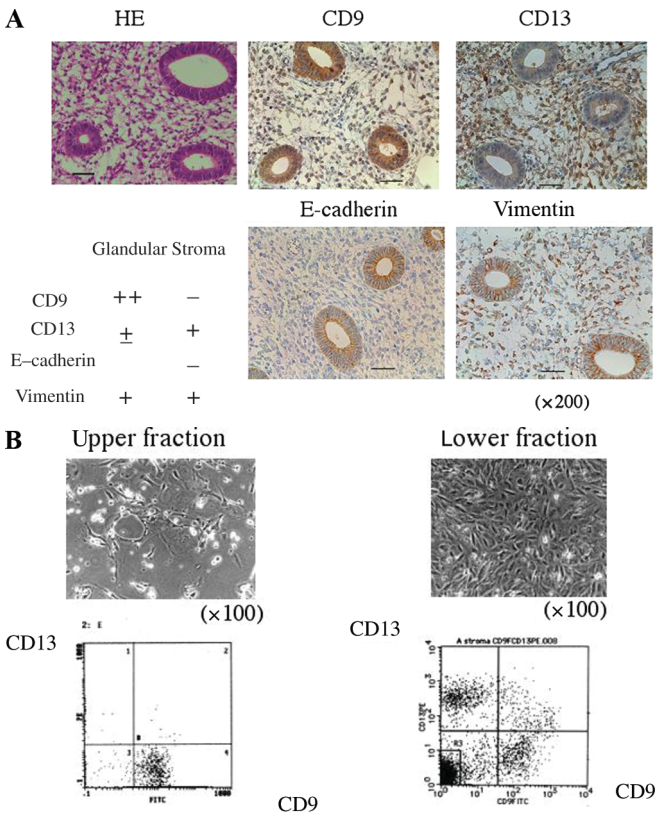
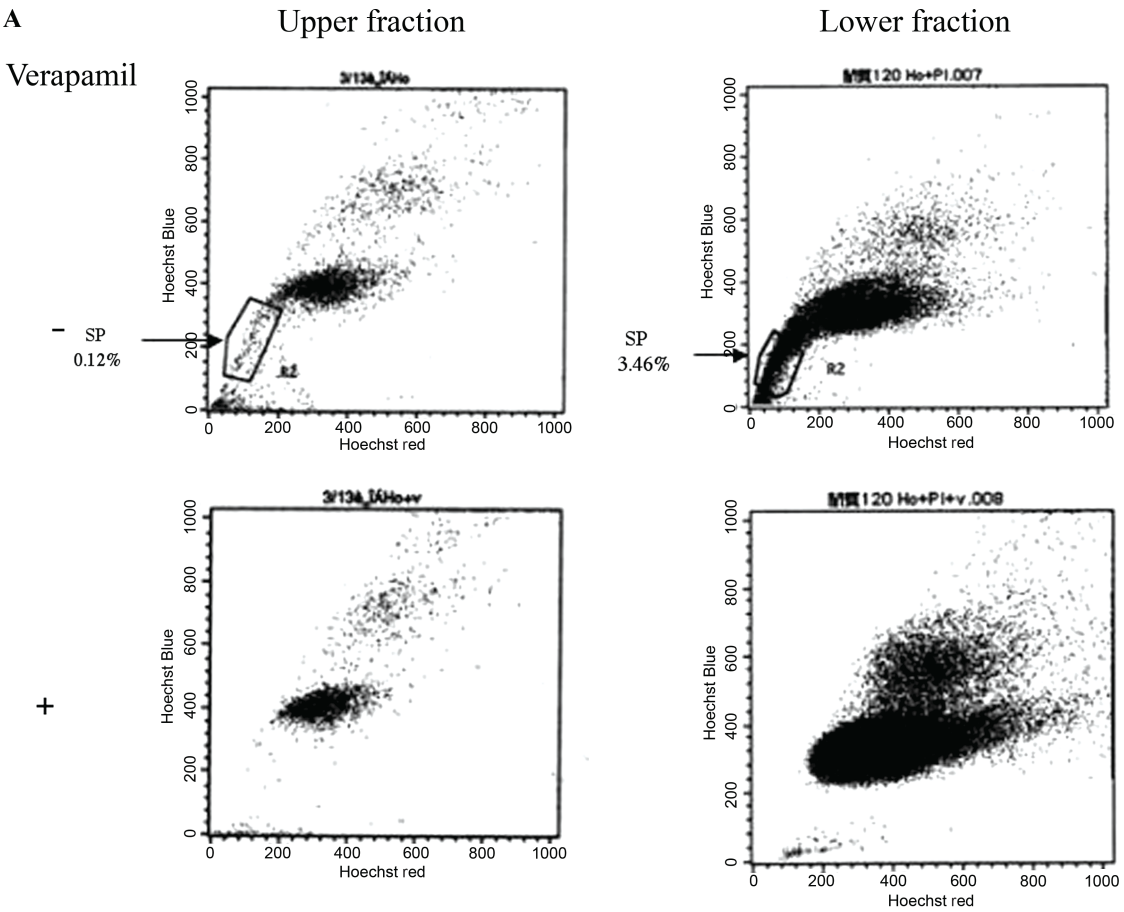


Figure 1. CD9 and CD13 are surface markers in the normal human endometrium. (**A**) The expression of CD9, CD13, E-cadherin and vimentin in the human endometrium in the secretory phase were analysed by immunohistochemistry using formalin-fixed histological sections. The HE panel shows hematoxylin–eosin (HE) stain of normal human endometrium in the secretory phase. The brown colour 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 μm). (**B**) Human endometrial cells in the proliferative phase (day 7) were isolated by digestion with collagenase followed by filtration. The levels of CD9 and CD13 expression were analysed by a fluorescence-activated cell sorter (FACS) using fluorescein isothiocyanate (FITC)-conjugated CD9 antibody or phycoerythrin (PE)-conjugated CD13 antibody.

stromal cells and a small number of glandular cells passed through the sieve into the filtrate.

SP cells were present in the human endometrium

Thirty-four samples of endometrial cells, prepared as described in the Materials and methods section, were stained with Hoechst 33342 and analysed by FACS. To determine the appropriate condition of incubation with Hoechst dye and to identify the SP population of human endometrial cells, we performed the experiments at various Hoechst concentrations for various incubation times (50, 100, 150 or 200 μM of Hoechst dye and 60, 90 or 120 min). Most SP cells were obtained at 50 μM of Hoechst dye with 90 min incubation. SP cells, which show a high level of dye efflux activity, were present in both the upper and lower fractions (Figure 2A) (representative results are shown). The sample was endometrial cells during menstruation; day 2. The SP cell proportion varied widely (0.00–5.11%) but was highest in endometrial cells



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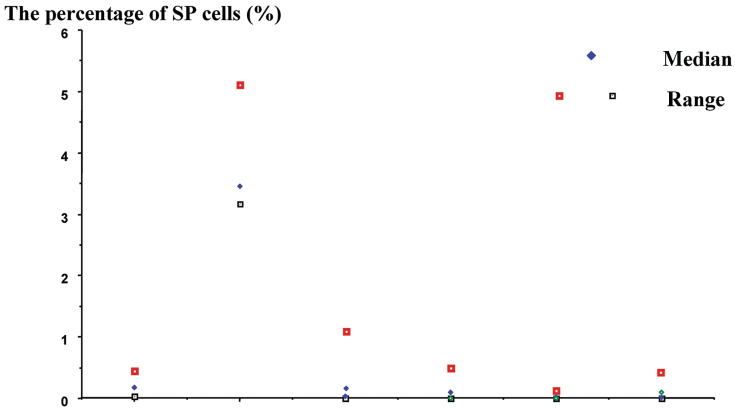

Phase	Upper	Lower	Upper	Lower	Upper	Lower	Total
	Menstruation		Proliferative		Secretory		
n	3	3	9	9	5	5	34
median (range)	0.18 (0.01–0.44)	3.46 (3.16–5.11)	0.14 (0–1.1)	0.10 (0–0.5)	0.01 (0–0.05)	0.05 (0–0.42)	0.21 (0–5.1)
mean ± SEM	0.21 ± 0.43	3.91 ± 1.0[a] (p<0.01)	0.15 ± 0.38	0.06 ± 0.16	0.02 ± 0.05	0.10 ± 0.18	0.27 ± 0.79

Figure 2. Side-population (SP) cells were present in the human endometrium. (**A**) Endometrial cells during menstruation (day 2) were stained with Hoechst 33342 and analysed by FACS. SP cells, which show a high level of dye efflux activity, were present in both the upper and lower fractions of the endometrium. Verapamil treatment increased the staining and made SP cells undetectable by FACS. (**B**) Summary of the SP cell percentage is shown. The graph shows the median and the range of scatter in each stage of the menstrual cycle. Data are also represented as the mean ± SEM. (a) The SP cell percentage was significantly higher in endometrial cells in the lower fraction of the menstrual phase than in all other samples ($P < 0.01$). (**C**) 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. (**a**) Most SP cells were present in the $CD9^{-}CD13^{-}$ fraction. (**b**) Cells in the $CD9^{-}CD13^{-}$ fraction were enriched with SP cells. R1: viable cells gated by propidium iodide; R3: $CD9^{-}CD13^{-}$ fraction. (**D**) (**a**) Morphologically, the SP cells were small and round in contrast to NSP cells. (**b**) Daughter cells were generated from a single SP cell (shown by arrows). (magnification: ×500).

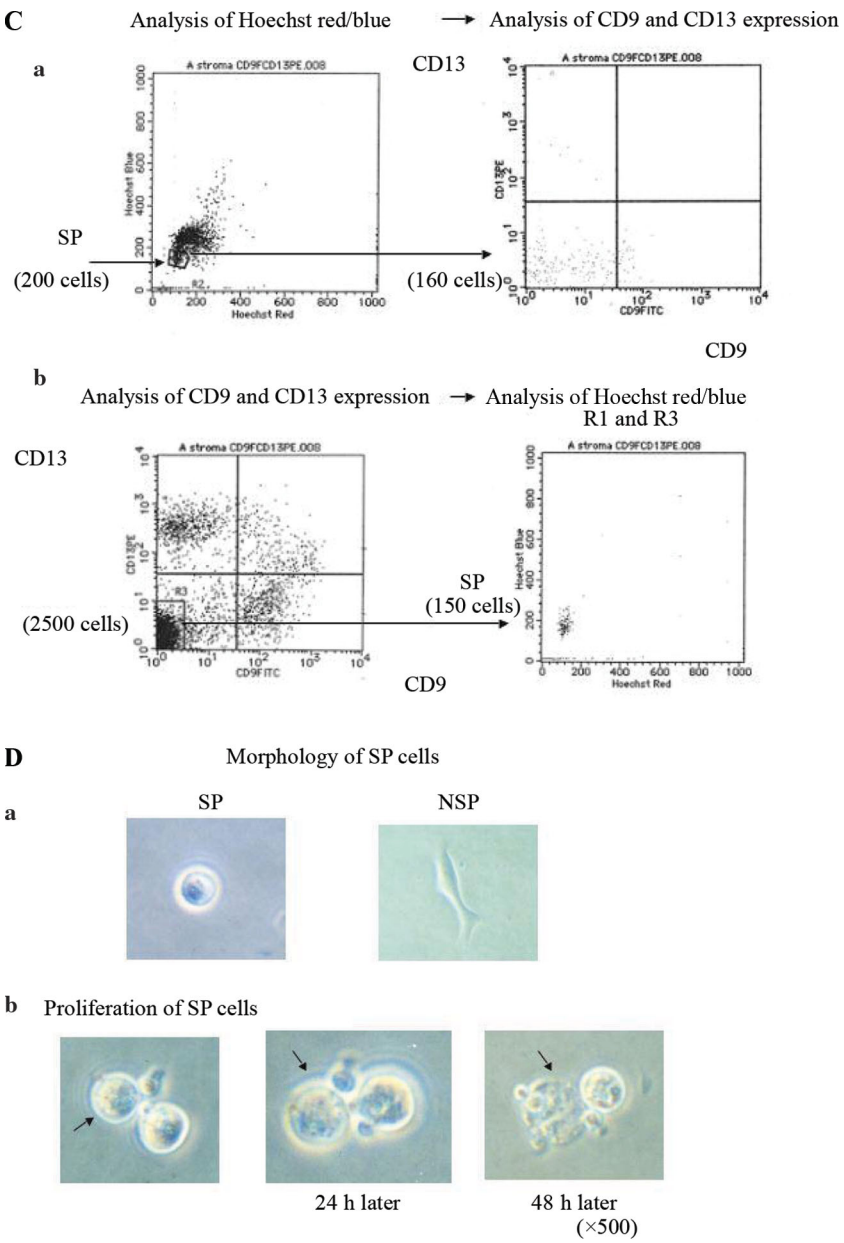

Figure 2. Continued.

derived from the lower fraction of endometria taken during the menstrual phase (Figure 2B). Verapamil treatment increased the staining and made SP cells undetectable by FACS.

For analysis of the cell-surface markers of SP cells, endometrial cells were stained with Hoechst 33342 followed by incubation with FITC-conjugated antibody against CD9 and PE-conjugated antibody against CD13. Finally, cells were stained with 1 μg ml^{-1} propidium iodide to label dead cells. The viable cells after the removal of dead cells by Hoechst staining were gated by propidium iodide-negative (R1 gate). CD9 and CD13 expression levels in SP cells isolated from the endometrium were analysed by FACS. Most of the SP cells were present in the $CD9^{-}CD13^{-}$ fraction (160 $^{CD9-,\ CD13\ -}$ cells/200 SP cells) (Figure 2Ca), although cells in the $CD9^{-}CD13^{-}$ fraction were enriched for SP cells (Figure 2Cb). In Figure 2Cb, we initially analysed cells by FITC and PE. Then cells in the $CD9^{-}$ and $CD13^{-}$ fraction (R3 gate) underwent dual wavelength analysis by UV light after the removal of dead cells (R1 gate). The cells in the $CD9^{-}$ and $CD13^{-}$ fraction (R3 gate) (∼2500 cells) were much more prevalent than SP cells (150 cells) in the right panel, because the former cells contained many dead cells and debris. We obtained the same results in three independent experiments from different samples. To exclude the possibility of contamination of blood cells, we also confirmed that SP cells were $CD34^{-}$ and $CD45^{-}$, and c-kit was expressed in both the SP cells and NSP cells (data not shown).

Morphologically, SP cells were small and round (Figure 2Da). We followed a single SP cell (shown by arrows) for 48 h. Daughter cells were generated from this single SP cell (Figure 2Db). We could obtain ∼500–1000 SP cells by sorting in one assay. We cultured these cells in a

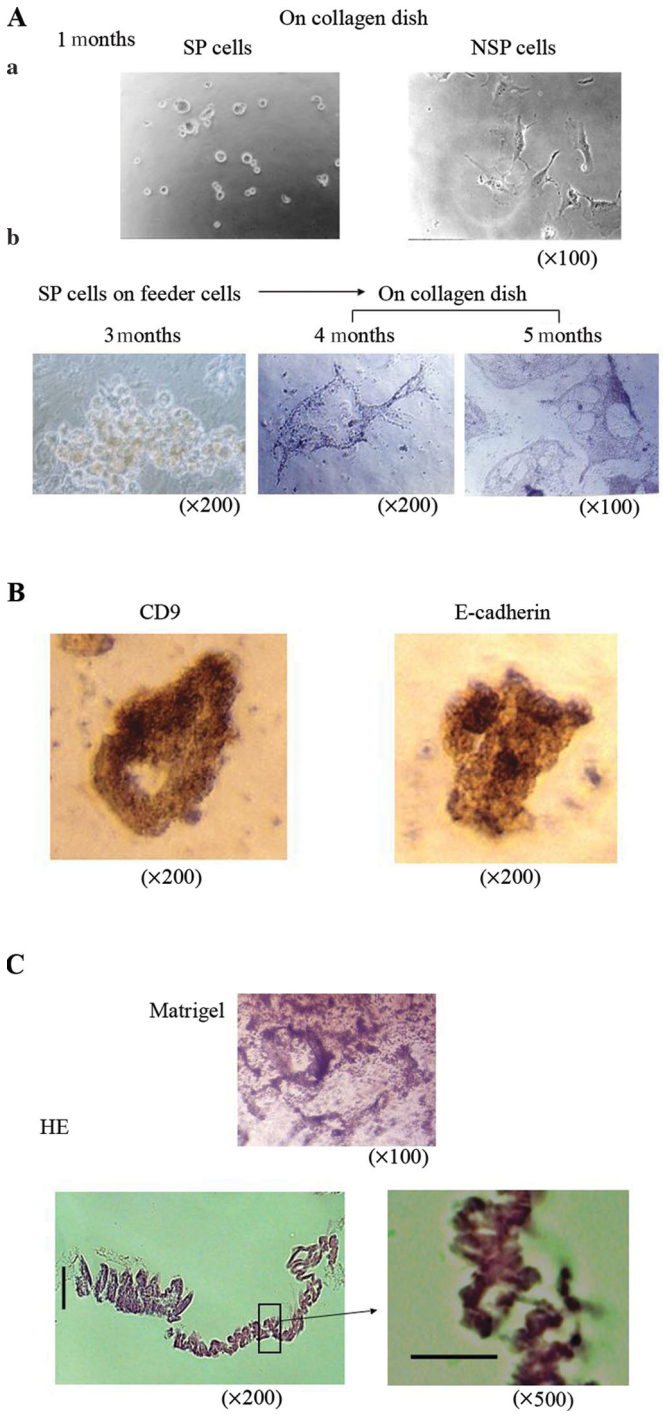

Figure 3. SP cells from the upper fraction differentiated into gland-like cells. (**A**) (**a**) Morphology of SP or NSP cells after 1 month of culture on a collagen-coated plate is shown (magnification: ×100). (**b**) SP cells from the upper fraction after 1 month of culture on a collagen plate were cultured on feeder cells (AGM cells), which were mitotically inactivated with 10 μg ml^{-1} mitomycin C (magnification: 3, 4 months ×200, 5 months ×100). (**B**) Cells forming a glandular-like structure were re-plated on a glass chamber slide. The expression of CD9 and E-cadherin was investigated by immunohistochemistry (magnification: ×200). (**C**) The cells on the collagen-coated plate were re-seeded in matrigel (upper panel). A piece of the matrigel was fixed with 4% paraformaldehyde, embedded in paraffin and stained with HE. An endometrial gland-like structure was observed (lower panel) (left; magnification: ×200, scale bar = 20 μm, right; magnification: ×500, scale bar = 10 μm).

collagen-coated 24-multi-well plate (Figure 3Aa) and all the cells were transferred onto a 35 mm feeder layer (AGM cells). SP cells from the endometrium were not maintained in DMEM containing 10% FCS on a plastic plate; however, they grew slowly in DMEM containing 100 ng ml^{-1} IL-6, 10 ng ml^{-1} TPO, 100 ng ml^{-1} SCF and 10% FCS on a collagen-coated plate or feeder cells (AGM cells) (19). The cells on the feeder cells started to proliferate slowly and formed colonies gradually (Figure 3Ab). The size of each colony was not significantly different. Representative data of alterations in morphology are shown, which were successfully maintained for >9 months; SP cells isolated from the upper fraction (case 1) and lower fraction (case 2). In both cases, NSP cells became flat and showed limited proliferation (Figure 3Aa) and finally became senescent within 3 months, suggesting that the long-term repopulating phenotype was specific to SP cells.

SP cells isolated from the human endometrium differentiated into gland- or stromal-like cells

SP cells in case 1 were cultured on feeder cells (AGM cells) (Matsuoka *et al.*, 2001), which were mitotically inactivated with 100 μg ml^{-1} mitomycin C. The medium was replaced weekly. They proliferated slowly and formed colonies on the feeder cells after 3 months in culture (Figure 3Ab). We split these colonies on a collagen-coated plate where they gradually aggregated after 4 months and finally formed a gland-like structure after 5 months (Figure 3Ab). After splitting these cells on collagen-coated plates, the expression of CD9 and E-cadherin were investigated by immunohistochemistry. Both proteins were expressed (Figure 3B). Neither protein was expressed in feeder cells, and feeder cells alone treated with mitomycin C died soon after splitting (data not shown), suggesting that cells forming a gland-like structure were generated from SP cells, but not feeder cells. We then re-seeded the matrigel with these cells, which continued to proliferate and formed a glandular structure in the matrigel for 2 months after culturing (Figure 3Ca). A piece of the matrigel was fixed with 4% paraformaldehyde, embedded in paraffin and stained with HE. Endometrial gland-like structures were observed (Figure 3Cb,c).

SP cells in case 2 slowly proliferated on the collagen-coated plate (Figure 4A). We investigated the expression of CD13, vimentin and E-cadherin by western blotting and CD9 by immunohistochemistry. The cells expressed CD13 and vimentin, but not E-cadherin (Figure 4B) and CD9 (data not shown). The pattern of marker expression in these cells was similar to that in stromal cells, as shown in Figure 1, and they were successfully maintained for >6 months. We re-seeded the matrigel with these cells, which formed clusters with several spindles, but not a gland-like structure (Figure 4C).

These results demonstrated that an SP cell isolated from the human endometrium differentiated into gland-like cells expressing CD9 or stromal cells expressing CD13. These results suggested that SP cells exhibited characteristics capable of self-renewal and differentiation into one cell type (glandular or stroma cells).

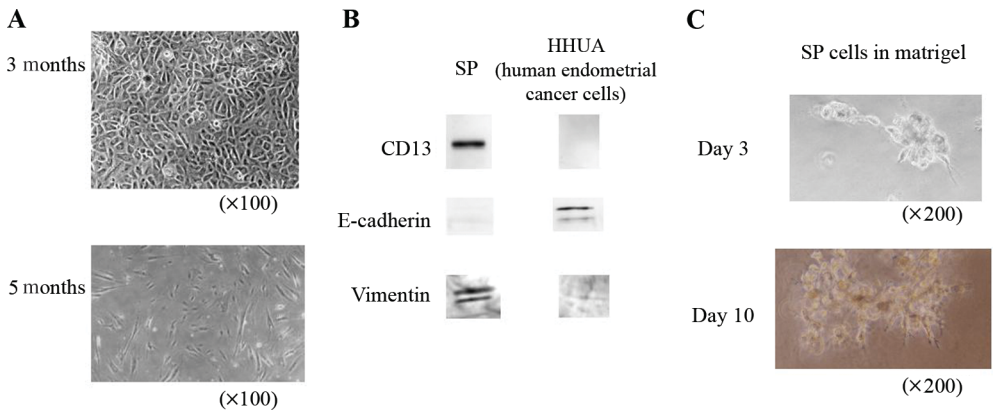

Figure 4. SP cells from the lower fraction differentiated into stroma-like cells. (**A**) SP cells from the lower fraction were cultured on a collagen-coated plate for 5 months (magnification: ×100). (**B**) We investigated the expression of CD13, vimentin and E-cadherin by western blotting. The lane of human endometrial cancer cells is shown as a control for endometrial epithelial cells. (**C**) These cells were re-seeded in matrigel (magnification: ×200)

CD9 and CD13 expression were induced during the process of differentiation

To eliminate contaminants during long culture, we isolated SP and NSP cells from immortalized human endometrial cells (Figure 5a) (Kyo *et al.*, 2003) and cultured them with different media: one an MF-medium to maintain mesenchymal stem cells and the other an ordinary growth medium (DMEM containing 10% FCS). We analysed the levels of CD9 and CD13 expression after 2 weeks of culturing (Figure 5B). The levels of both proteins were lower in SP cells than in NSP cells, when cells were incubated with the medium for mesenchymal stem cells. On the other hand, both proteins were expressed at similar levels between SP and NSP cells, when cells were incubated with ordinary growth medium. These results suggested that CD9 and CD13 expression were induced during the process of differentiation and were not an artifact effect of long culturing. Next, both SP and NSP cells were cultured in matrigel with an MF medium or DMEM containing 10% FCS for 2 months to show that SP cells have the ability to differentiate in morphology. Both SP and NSP cells cultured with MF medium grew slowly and individually (Figure 5Ca). In contrast, SP cells cultured with DMEM containing 10% FCS formed glandular- and stroma-like structures (SP cells Figure 5Cb) as did the NSP cells (data not shown). The levels of CD9, CD13, E-cadherin and vimentin expression were analysed by immunohistochemistry (Figure 5D). The levels of these proteins were lower in SP cells than in NSP cells, when cells were cultured with MF medium. In contrast, SP cells cultured with DMEM containing 10% FCS expressed these proteins (Figure 5D) as did the NSP cells (data not shown). These results demonstrated that SP cells induced these proteins during the process of morphological differentiation.

$CD9^-CD13^-$ cells exhibited a similar phenotype to SP cells

We showed that most SP cells were present in the $CD9^-CD13^-$ fraction using a FACS (Figure 2Ba). Next, we isolated $CD9^+CD13^-$ cells, $CD9^-CD13^+$ cells and $CD9^-CD13^-$ cells by magnetic cell sorting (MACS) from endometrial cells in the proliferative phase (day 7), digested with collagenase and cultured them on collagen-coated plates (Figure 6Aa,b). We isolated $CD9^-CD13^-$ cells by MACS, $\sim 0.5-1.0 \times 10^4$ cells from the upper fraction (mean 2.3%) and 1.0×10^4 cells from the lower fraction (mean 2.4%), which was more than SP cells (200–500 cells) sorted with FACS, and we cultured them on a collagen-coated plate with DMEM containing 10% FCS, IL-6, TPO and SCF. We compared the alteration of the phenotype in each cell fraction. $CD9^+CD13^-$ cells from the upper fraction aggregated after 1 month of culture and this phenotype was unchanged for 3 months. $CD9^-CD13^+$ cells from the lower fraction proliferated like stromal cells. Both $CD9^+CD13^-$ cells and $CD9^-CD13^+$ cells stopped growing after 2 months of culture and their morphology was not changed (Figure 6B). $CD9^-CD13^-$ cell viability from the upper fraction was not good. Viable $CD9^-CD13^-$ cells continued to proliferate slowly, remaining small and round for 2 months, aggregating gradually and finally forming a gland-like structure after 3 months of culture (Figure 6Ca), expressing both CD9 and E-cadherin (Figure 6Cb). In contrast, $CD9^-CD13^-$ cells from the lower fraction were differentiated into stroma-like cells, which expressed CD13 but not CD9 after 3 months of culture (Figure 6D). These results demonstrated that $CD9^-CD13^-$ cells isolated from the upper and lower fractions have a long-term repopulating phenotype and differentiate to gland or stroma-like cells, respectively. This phenotype was similar to that of SP cells.

The differentiated, gland-like structures from SP cells (Figure 3B) and from $CD9^-CD13^-$ cells (Figure 6Cb) seem quite small, particularly when compared with other figures, in which single undifferentiated cells (Figures 3A and 6C) appear larger than these structures at the same magnification, because the cells condense tremendously when they aggregate into such structures during the differentiation process (Figures 3Ab and 6Ca)

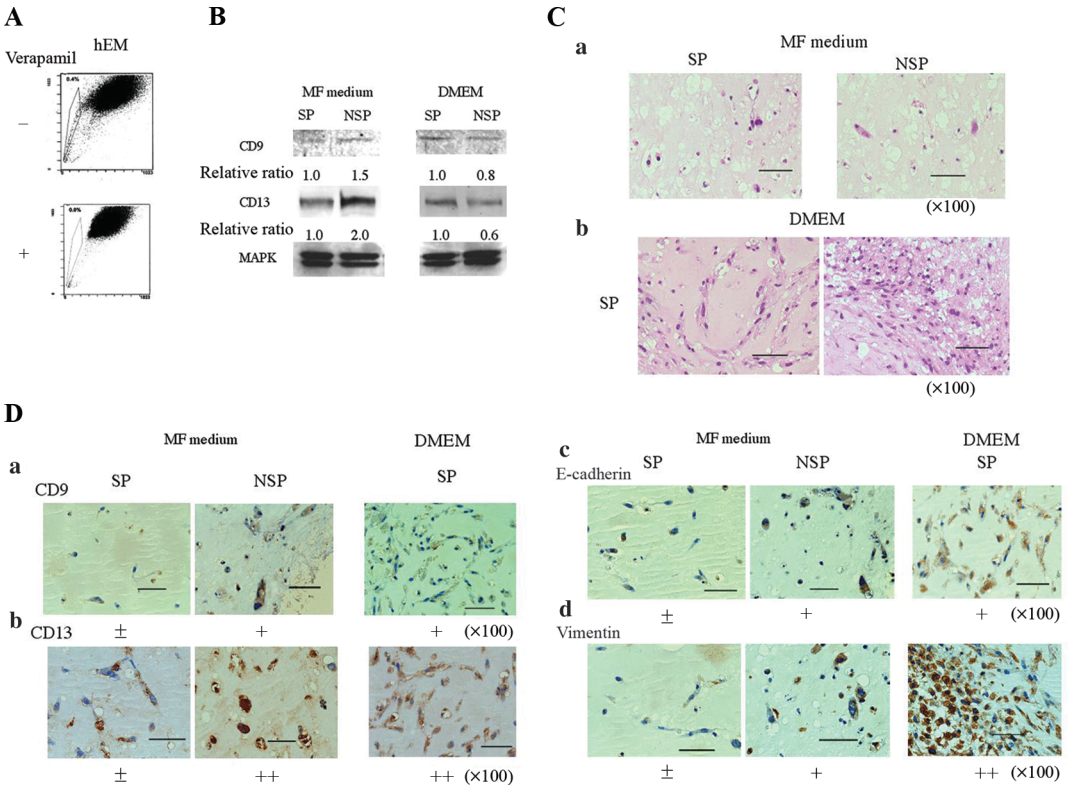

Figure 5. CD9 and CD13 expression were induced during the process of differentiation. (**A**) SP cells were present in immortalized human endometrial cells (hEM). (**B**) SP cells and NSP cells were isolated and cultured with the medium to maintain mesenchymal stem cells (MF medium) or Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) (growing medium) for 2 weeks. The levels of CD9 and CD13 expression were analysed by western blot. Mitogen-activated protein kinase (MAPK) was shown as an internal control. (**C**) Both SP and NSP cells were cultured in matrigel with MF medium (**a**) or DMEM containing 10% FCS for 2 months (**b**). Cell morphology stained with HE in each condition was shown (magnification: ×100, scale bar = 50 μm). (**D**) The levels of CD9, CD13, E-cadherin and vimentin expression were analysed by immunohistochemistry (magnification: ×100, scale bar = 50 μm). The proportion of positive, stained cells to negative cells was evaluated. ±:<50%, +: 50–100%, ++: 100% and strong stain.

Discussion

It has been proposed that the human endometrium may contain a population of stem cells that are responsible for its remarkable regenerative capability. The presence of rare clonogenic epithelial and stromal cells with high proliferative potential has been demonstrated, confirming the existence of putative endometrial epithelial and stromal stem cells (Chan *et al.*, 2004). Adult stem cell subpopulations have been defined using the fluorescent dye Hoechst 33342 (so-called SP cells) (Goodell *et al.*, 1996). In this study, we reported the presence of SP cells in the normal human endometrium for the first time.

Hoechst 33342 staining for SPs is a simple technique. Compared with the method using stem cell markers for isolating cells, it is unconventional and has many confounding technical issues. For example, Hoechst 33342 is a DNA intercalating dye and is potentially toxic to the cells in which it accumulates. As every tissue has a different profile, the concentration and staining period must be optimized for the tissue of interest. In this study, we found that incubation of the endometrial cells with 2.5 μg ml^{-1} dye for 90 min produced optimal results.

Since Goodell *et al.* reported this method in 1996, the presence of SP cells has been shown in many adult tissues and the SP phenotype might represent a common molecular feature for a wide variety of stem cells. The detection of SP cells might be useful to isolate stem cells in adult tissue, where stem cell markers remain unidentified, like human endometrial cells. However, although the heterogeneity of SP cells has been shown (Uzumi *et al.*, 2006), it is critical to identify stem cell markers for the purification of true stem cells.

Recently, several reports have described SP cells in adult, solid tissue (Challen and Little, 2006). For example, the proportion of SP cells was reported as 0.2–0.5% in the human mammary gland (Smalley and Clarke, 2006). We demonstrated in this study that the proportion of SP cells in whole live endometrial cells was 0.01–3%. Chan *et al.* (2004) have reported that 0.22–0.52% of human endometrial epithelial cells and 1.25% of stromal cells possessed clonogenic activity. Although it is unknown whether SP cells are identical to cells possessing clonogenic activity, the proportion was similar between these cells.

Regeneration begins during menstruation. The surface of the endometrium is rapidly covered by epithelial cells, which is complete within 48 h after shedding. The growth of the new endometrial functionalis begins in the proliferative phase in response to estrogen. Progesterone blocks epithelial mitosis during the secretory phase and cells begin to differentiate (Chan *et al.*, 2004). In our analysis, the population of SP cells was highest in the stroma-enriched fraction of

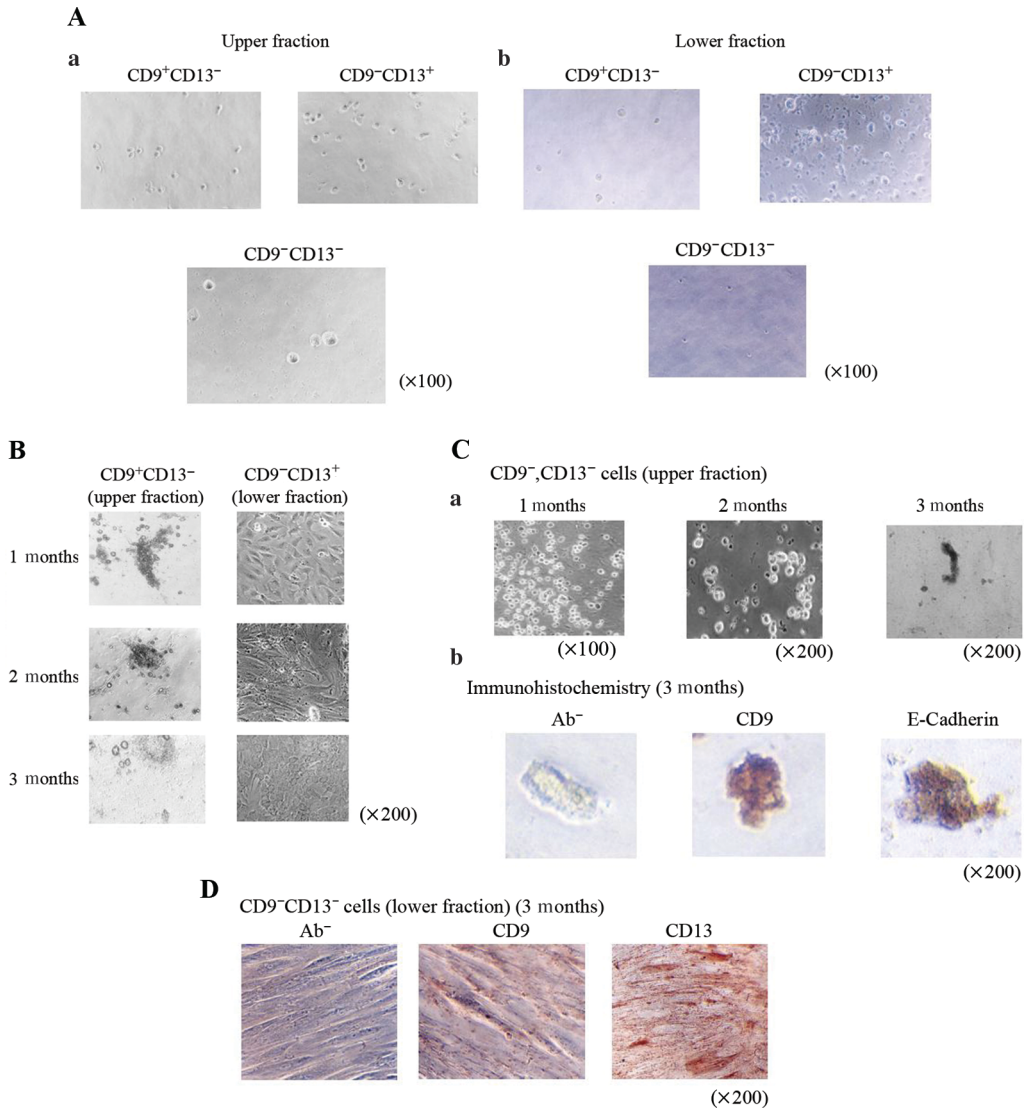

Figure 6. $CD9^-CD13^-$ cells exhibited a similar phenotype to SP cells. (**A**) $CD9^+CD13^-$ cells, $CD9^-CD13^+$ cells and $CD9^-CD13^-$ cells were isolated by magnetic cell sorting from endometrial cells in the proliferative phase (day 7) digested with collagenase and were cultured on collagen-coated plates. (**a**) Cells from the upper fraction of the filter (cell density: $CD9^+CD13^-$ cells, 2500 cells cm^{-2}, $CD9^-CD13^+$ cells, 2500 cells cm^{-2}, $CD9^-CD13^-$ cells, 250 cells cm^{-2}) (magnification: ×100). (**b**) Cells from the lower fraction of the filter (cell density: $CD9^+CD13^-$ cells, 500 cells cm^{-2}, $CD9^-CD13^+$ cells, 2500 cells cm^{-2}, $CD9^-CD13^-$ cells, 250 cells cm^{-2}) (magnification: ×100) (**B**) Morphology of $CD9^+CD13^-$ cells and $CD9^-CD13^+$ cells after 1, 2 and 3 months of culture. Both $CD9^+CD13^-$ cells and $CD9^-CD13^+$ cells stopped proliferating after 2 months of culture (magnification: ×100). (**C**). (**a**) Morphology of $CD9^-CD13^-$ cells from the upper fraction after 1, 2, and 3 months of culture (magnification: 1 months ×100, 2, 3 months ×200). (**b**) The expression of CD9 and E-cadherin in cells generated from $CD9^-CD13^-$ cells from the upper fraction after 3 months of culture was investigated by immunohistochemistry (magnification: ×200). (**D**) Morphology and expression of CD9 and CD13 by immunohistochemistry was shown in $CD9^-CD13^-$ cells from the lower fraction after 3 months of culture (magnification: ×200). Ab^- represents no added antibody.

endometrial cells just after menstruation (mean 3.91%). It is difficult to explain why the population of SP cells was highest at the end of menstruation. One explanation is that the population of SP cells in the endometrium was enhanced in response to estrogen or growth factors, which increase during or just after menstruation. Alternatively, SP cells increase proportionally in this phase because the total number of cells is lower at the end of menstruation and during the proliferative phase than in the secretory phase.

To identify stem cells, it is essential to provide evidence that a single cell differentiates into multiple lineages. First of all, we showed that a single SP cell generated daughter cells (Figure 2Ca). In addition, these daughter cells formed colonies and finally either glandular- or stromal-like structures. The results implicated the possibility that these SP cells showed the characteristics of progenitor cells, which are more mature than stem cells, and have the potential to differentiate to one cell type, not multiple lineages. We showed evidence of the progenitor cell potential of SP cells, which showed long-term repopulation properties (>24 weeks), and cultured SP cells produced gland ($CD9^+$)- and stroma ($CD13^+$)-like cells. In contrast, NSP cells became senescent within 1–3 months. It is unclear whether common stem cells of glandular or stroma cells are present in the human endometrium.

SP cells were present in the $CD9^{-}CD13^{-}$ fraction using FACS analysis. $CD9^{-}CD13^{-}$ cells isolated by MACS generated gland- or stroma-like cells after long culturing. $CD9^{+}CD13^{-}$ cells generated gland-like cells and $CD9^{-}CD13^{+}$ cells generated stroma-like cells. These results suggested that stem cells in the endometrium were involved at least in the $CD9^{-}CD13^{-}$ fraction and that they differentiated into $CD9^{+}CD13^{-}$ (glandular) cells or $CD9^{-}CD13^{+}$ (stroma) cells. CD9 and CD13 could be used as negative selection markers for immature endometrial cells (progenitor cells). In order to purify stem cells from SP cells, the identification of undifferentiated markers of endometrial cells is currently under investigation.

An adequately developed, receptive endometrium is important for normal pregnancy. Curettage or infection causes intrauterine damage with destruction of the endometrium, which becomes a cause of infertility. If a technique of regenerating the endometrium using stem cells is established, it will be clinically significant for the treatment of uterine disorders.

A great deal of attention has been paid to stem cells as the targets for genetically or environmentally induced neoplasia. Recent evidence suggests that cancer stem cells exist in several malignant tumours, leukaemia (Lapidot *et al.*, 1994; Bonnet and Dick, 1997), breast cancer (AI-Haji *et al.*, 2003) and brain tumours (Singh *et al.*, 2003), and that these stem cells express surface markers similar to normal stem cells in each tissue (Bhatia *et al.*, 1997; Bonnet *et al.*, 1997). Most recently, it has been shown that established malignant cell lines that have been maintained for many years in culture contained a minor subpopulation of stem cells enriched in SP cells (Kondo *et al.*, 2004). We also identified SP cells from both a human endometrial cancer cell line and freshly dispersed human endometrial cancer cells from endometrial cancer tissue (K. Kato, unpublished data). The characteristics of SP cells as cancer stem cells are under investigation. This will lead to an understanding of the aetiology of endometrial cancer to clarify the similarities and differences in biology between normal and cancer stem cells in endometrial tissue.

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