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Research Article

Gene-expression profile and postpartum transition of bovine endometrial side population cells†

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Abstract

The mechanism of bovine endometrial regeneration after parturition remains unclear. Here, we hypothesized that bovine endometrial stem/progenitor cells participate in the postpartum regeneration of the endometrium. Flow cytometry analysis identified the presence of side population (SP) cells among endometrial stromal cells. Endometrial SP cells were shown to differentiate into osteoblasts and adipocytes. RNA-seq data showed that the gene expression pattern was different between bovine endometrial SP cells and main population cells. Gene Set Enrichment Analysis identified the enrichment of stemness genes in SP cells. Significantly (false discovery rate < 0.01) upregulated genes in SP cells contained several stem cell marker genes. Gene ontology (GO) analysis of the upregulated genes in SP cells showed enrichment of terms related to RNA metabolic process and transcription. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of upregulated genes in SP cells revealed enrichment of signaling pathways associated with maintenance and differentiation of stem/progenitor cells. The terms involved in TCA cycles were enriched in GO and KEGG pathway analysis of downregulated genes in SP cells. These results support the assumption that bovine endometrial SP cells exhibit characteristics of somatic stem/progenitor cells. The ratio of SP cells to endometrial cells was lowest on days 9–11 after parturition, which gradually increased thereafter. SP cells were shown to differentiate into epithelial cells. Collectively, these results suggest that bovine endometrial SP cells were temporarily reduced immediately after calving possibly due to their differentiation to provide new endometrial cells.

Summary sentence: The ratio of bovine endometrial side population cells, showing characteristics similar to those of somatic stem/progenitor cells, was the lowest on days 9–11 after parturition and then increased gradually.

Key words: side population cell, endometrium, postpartum period, cow, RNA-sequencing.

Introduction

A low reproductive efficiency due to reduced conception rates after embryo transfer (ET) in cattle results in diminished profitability for livestock farmers. Previous findings have demonstrated that early embryonic death before the pregnancy recognition period is a major cause of infertility after ET in cattle [1]. Histotroph secreted by the endometrium is essential for embryo development and survival [2]. The uterine fluid of cows with abnormal embryos has been reported to exhibit a different protein composition from those cows with normal embryos [3]. Based on these facts, it is presumed that deterioration of uterine function is a cause of reproductive failure in cattle.

The uterus is an organ with high regenerative potency. It is well known that the functional layer in the endometrium is sloughed off and then regenerated, and in humans, this occurs repeatedly every menstrual cycle [4]. Several lines of evidence have shown that somatic stem cells contribute to the regeneration of the endometrium. Masuda et al. [5] demonstrated that human endometrial stem cells could differentiate in vitro into various cells of the endometrium. Moreover, human endometrial stem cells were shown to construct endometrial tissue containing glandular structures and blood vessels when transplanted into the kidney capsule of immunodeficient mice [5]. Endometrial stem cells are a type of the mesenchymal stem cells, which can be isolated by stem cell markers such as melanoma cell adhesion molecule (MCAM) and platelet derived growth factor receptor-beta (PDGFRβ) or sushi domain containing 2 (SUSD2), expressed on the cell membrane in humans [6, 7]. However, specific stem cell markers differ between organs and species, and the stem cell markers in cattle have not been identified. Side population (SP) analysis is another method used to identify mesenchymal stem cells. SP cells were identified by staining with Hoechst 33342 and Vybrant DyeCycle Violet [8, 9]; stem cells highly express ATP-binding cassette subfamily G member 2 (ABCG2), which effluxes the dye, and these cells therefore show low fluorescence by flow cytometry analysis. It has been observed that the SP cells exist in the endometrium and show stem cell potency in mice [10], rats [11], pigs [12], and humans [13, 14].

Bovine endometrial cells have been demonstrated to differentiate into osteoblasts, adipocytes, and chondrocytes [15–17]. This finding indicates that the stem cells are present in bovine endometrium. It has previously been reported that regeneration of murine liver after injury is enhanced to by activation of proliferative somatic stem cells [18]. Because menstruation is not an innate system in cattle, there are no drastic endometrial replacements during the estrus cycle, although cell proliferation and apoptosis occur periodically [19]. At parturition, the endometrium is heavily damaged through shedding of the placenta, but it is subsequently regenerated. Thus, there may be a possibility that the endometrial stem cells are involved in endometrial regeneration after parturition in cattle. To determine the validity of this hypothesis, we first examined whether SP cells in the bovine endometrium had stem cell-like characteristics by clarifying their gene expression profiles. We also subsequently investigated the postpartum transition of bovine endometrial SP cells.

Materials and methods

Experiment 1: differentiation assay and SP analysis of bovine endometrial cells

Isolation of bovine endometrial cells. Healthy bovine uteri were collected from local slaughterhouses, and healthy uteri were used for isolation of endometrial cells. The stages of the estrus cycle of the tissues were estimated based on ovarian morphology, as described previously [20, 21]. Each uterine horn was washed with 50 mL saline and then filled with 30–50 mL of 0.3% (w/v) trypsin (Gibco, Grand Island, NY, USA)-0.02% (w/v) EDTA (DOJINDO, Kumamoto, Japan)-0.01 M phosphate buffered saline (PBS, pH 7.4; Sigma–Aldrich, St Louis, MO, USA). Luminal epithelial cells were isolated after incubation at 37°C for 30 min. The uterine horn was then cut vertically, and the remaining luminal epithelial cells were removed by scraping with the scoopula. After washing with PBS, the intracaruncular endometrium was minced and incubated for 1 h at 37°C with gentle shaking with 0.2% (w/v) collagenase (FUJIFILM WAKO Chemical, Tokyo, Japan)-Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 4 mM $NaHCO_3$ (FUJIFILM WAKO Chemical), 100 U/mL penicillin (Sigma–Aldrich), and 100 µg/mL streptomycin (Nacalai Tesque, Kyoto, Japan) to isolate endometrial stromal cells. Each cell suspension was filtered through a 70 µm cell strainer (Falcon, Corning, NY, USA) and centrifuged at 100 g for 10 min. The collected cell pellet was washed twice with DMEM supplemented with 4 mM $NaHCO_3$, 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin.

Sorting of SP cells from bovine endometrial cells. Luminal epithelial cells and stromal cells were separately resuspended at 1×10^6 cells/mL in DMEM supplemented with 4 mM $NaHCO_3$, 2% (v/v) FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 mM HEPES buffer (Gibco). Cells were stained with 10 µg/mL Hoechst 33342 (Sigma–Aldrich) for 90 min at 37°C either alone or in combination with 50 µM verapamil (Nacalai Tesque), an inhibitor of ABCG2. After centrifugation (100 g, 5 min, 4°C), the cell pellet was washed twice and resuspended in cold Hanks' balanced salt solution (HBSS, 14025; Thermo Fisher Scientific, Waltham, MA, USA) containing 2% FBS and 10 mM HEPES. Propidium iodide (2 µg/mL, Sigma–Aldrich) was simultaneously added to Hoechst-stained cells in order to exclude dead cells prior to flow cytometry analysis and sorting. Flow cytometry analysis and cell sorting were performed using a FACS SORP Aria2 (Becton Dickinson, Franklin Lakes, NJ, USA). Hoechst 33342 was excited at 355 nm, and fluorescence emission was detected using 450/50 and 670/LP nm band-pass filters for Hoechst blue and Hoechst red, respectively. After collecting 5×10^4 events, the SP and main population (MP) cells were defined by the ability to pump out Hoechst 33342. SP and MP cells were sorted and stored at −80°C for subsequent RNA-seq and quantitative RT-PCR (qRT-PCR) analysis. Some sorted SP cells were cultured for the differentiation assay and immunocytochemistry.

Differentiation assay for adipocytes and osteocytes from bovine endometrial cells. Luminal epithelial cells, stromal cells, and SP cells were cultured in adipogenic and osteogenic differentiation media. Briefly, cells were maintained in a humidified CO_2 incubator at 37°C in 5% CO_2: 95% air. Luminal epithelial cells, stromal cells, and SP cells were treated with the osteogenic induction medium (LONZA, Basel, Switzerland) and the adipogenic induction medium (LONZA) for 3 weeks in accordance with the manufacturer's instructions. After differentiation, the cells were fixed using 10% formalin (FUJIFILM WAKO Chemical) and stained with 2% alizarin red S solution (pH 5.5; FUJIFILM WAKO Chemical) or 0.18% Oil red O (Sigma–Aldrich)-60% (v/v) 2-propanol (Sigma–Aldrich) solution to detect osteogenic and adipogenic differentiation, respectively.

Differentiation assay for epithelial cells from bovine endometrial cells. Bovine endometrial stromal cells and endometrial SP cells were seeded onto a collagen type IV coated 24-well plate. After attachment, the cells were replaced with medium contained 0 or 10 μM All-trans retinoic acid (FUJIFILM WAKO Chemical) according to the procedure described previously [22]. The treated day was defined as day 0. After 1 week of culture, these cells were used for immunocytochemistry to detect cytokeratin.

Immunocytochemistry of SP cells for cytokeratin. Cultured bovine endometrial stromal cells and endometrial SP cells were fixed using 10% formalin (FUJIFILM WAKO Chemical). Fixed cells were blocked with 1.5% Blocking reagent (Roche Diagnostics) in 0.3% (v/v) Triton X-100 (Sigma–Aldrich) in 0.05 M phosphate buffered saline (PBST). The primary antibodies used were anticytokeratin pan (1:200 dilution, 1017390, Boehringer Mannheim, Baden-Wurttemberg, Germany) at 4°C for 2 night. After washing with PBST, fixed cells were incubated with Alexa Fluor 488-conjugated antimouse IgG secondary antibodies (ab150117, 1:400, Abcam, Cambridge, UK). Nuclei were stained with DAPI (Invitrogen, Carlsbad, CA, USA).

RNA-seq analysis of SP and MP cells. Since SP cells were extremely rare, SP and MP cells of 2–5 uteri at the same estrus stage were combined to obtain enough RNA. Total RNA was extracted from each of the three replicates of SP and MP cells using a QIAGEN RNeasy Plus micro kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA-sequencing was performed by Genebay Inc. (Kanagawa, Japan). TruSeq Stranded mRNA Library Prep (Illumina, San Diego, CA, USA) and MGI Easy Universal Library Conversion Kit (App-A, MGI Tech, Shenzhen, China) were used to prepare sequencing libraries from extracted total RNA, following the manufacturer's recommendations. These libraries were sequenced as 100 bp paired-end reads on a DNBSEQ-G400 (MGISEQ-2000RS, MGI Tech). Sequenced raw reads were processed using Cutadapt [23] ver 2.5 to remove adapter and trim low-quality reads. Clean reads were aligned to the *Bos taurus* reference genome (ARS-UCD1.2) using HISAT [24] v2.1.0. Fragments per kilobase of transcript per million mapped reads (FPKM) were calculated from the mapping results and were used to detect differentially expressed genes (DEGs) between SP cells and MP cells. DEGs were identified by Cutdiff [25] v2.2.1, with a false discovery rate (FDR) < 0.01. Gene Set Enrichment Analysis (GSEA) was performed using the gene sets of RAMALHO_STEMNESS_UP and RAMALHO_STEMNESS_DN collected common DEGs in mouse embryonic, neural, and hematopoietic stem cells compared to differentiated cells [26]. With 1000 permutations, FDR < 0.05 was used as the significance level. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs were carried out using the functional annotation tool in DAVID (http://david.abcc.ncifcrf.gov/). Upregulated and downregulated DEGs in SP cells were analyzed, and GOTERM_BP_5 was used in GO enrichment analysis for biological processes. The statistical significance of GO and KEGG pathway enrichment analysis was set at $P < 0.05$.

Quantitative RT-PCR. Total RNA was extracted from SP ($n = 7$) and MP cells ($n = 7$) using a QIAGEN RNeasy Plus micro kit. Single-stranded cDNA was synthesized from total RNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan) in accordance with the manufacturer's instructions. qRT-PCR analysis was performed using the AriaMx real-time PCR system (Agilent, Santa Clara, CA, USA). The primer sequences for *MCAM,* endoglin *(ENG) ABCG2, CD34,* platelet and endothelial cell adhesion molecule 1 (*PECAM1)* and 18S rRNA (*RN18S1*) are listed in Supplementary Table S1. Each reaction mixture consisted of cDNA, forward and reverse primers, THUNDERBIRD® SYBR qPCR Mix (TOYOBO), and nuclease-free water at a total reaction volume of 20 μL. Thermocycling conditions were a denaturation step of 95°C for 1 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 45 s. SYBR Green fluorescence was measured at the end of each extension step. Serial dilutions of plasmids containing the PCR products of each gene were used as standards. For each gene, expression was normalized to *RN18S1*expression to adjust for any variation in the qRT-PCR.

Immunohistochemistry for ABCG2. The bovine uteri collected from a local slaughterhouse was fixed using 10% formalin (FUJIFILM WAKO Chemical) overnight. Then, the fixed samples were replaced in 30% sucrose (FUJIFILM WAKO Chemical) −0.1 M phosphate buffer (pH 7.4; Sigma–Aldrich) solution. Subsequently, 6-μm-frozen sections were produced and attached to MAS-coated slide glass. Antigen activation was performed in 0.1 M pH 9.0 Tris–HCl (Nacalai Tesque) at 90°C for 5 min, and then the samples were cooled to room temperature. After washing with PBST, sections were incubated in 3% H_2O_2 in methanol to remove endogenous peroxidase activity. Sections were blocked in PBST contained 3% normal goat serum-1%bovine serum albumin (Sigma–Aldrich) and incubated with primary antibody anti-ABCG2 (1:500 dilution, BXP-53, Santa Cruz Biotechnology, Dallas, TX, USA) for 2 night at 4°C. After washing with PBST, sections were incubated for 2 h at room temperature with biotinylated antirat IgG secondary antibody (1:200 dilution, BA-9400, Vector Laboratories, Peterborough, UK), and then incubated with avidin biotin peroxidase complex (ABC standard kit; Vector Laboratories). Staining was detected with liquid diamino-benzidine (Dako North America, Inc., Carpinteria, CA, USA). Nuclei were stained with hematoxylin (Merck, Darmstadt, Germany).

Experiment 2: quantification of the ratio of SP cells in bovine endometrial cells during the postpartum period and estrus cycle

Animals. Female Japanese Black cows were used ($n = 8$). Information on age, body weight, body condition score, and parity of experimental animals is shown in Supplementary Table S2. All animals were housed under natural conditions at the Institute of Livestock and Grassland Science, National Agriculture, and Food Research

Organization (NARO) and provided with hay and concentrate twice per day and water ad libitum. All procedures involved in the animal experiments were approved by the Committee of the Care and Use of Experimental Animals of NARO (No. 1711D034).

SP cell analysis of endometrial cells during the postpartum period. In this experiment, the SP and MP cells were detected using the DyeCycle Violet reagent with fumitremorgin C, as DyeCycle Violet appeared to be effluxed by the same molecular mechanisms as Hoechst 33342 and DyeCycle Violet SP cells could be blocked with the ABCG2 inhibitor fumitremorgin C [9]. Eight female Japanese Black cows were used in this experiment. The day of parturition was defined as day 0. Several endometrial tissues, including both the intercaruncular and caruncular areas, were obtained from the uterine horn of each cow using uterine biopsy forceps (Amco Inc., Tokyo, Japan) on days 9–11, 29–32, 49–52, and 99–101. Endometrial cells were immediately isolated from the biopsy sections using the procedure mentioned earlier with collagenase. Endometrial cells were resuspended at 1×10^6 cells/mL in DMEM supplemented with 4 mM $NaHCO_3$, 2% (v/v) FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 mM HEPES (Gibco). Cells were stained with 5 mM Vybrant DyeCycle Violet stain (Thermo Fisher Scientific) for 90 min at 37°C, either alone or in combination with 5 mM fumitremorgin C (Sigma–Aldrich). Stained cells were washed with cold HBSS supplemented with 2% FBS and 10 mM HEPES, and then stained with 2 µg/mL propidium iodide before flow cytometry. Flow cytometry analysis was performed using an Attune® acoustic focusing cytometer (Applied Biosystems, Life Technologies, Carlsbad, CA) with Attune® Cytometric Software. Vybrant DyeCycle Violet stain was excited by a 405 nm violet laser and 440/50 and 603/48 nm band-pass filters were used to detect the fluorescence emission. SP cells were distinguished using the same procedure as in Experiment 1, and 5.0×10^4 events were recorded. The ratio of SP cells in the gated live singlet cells was calculated.

SP cell analysis of endometrial cells during the estrus cycle. Four animals were tested on each sampling day, and a cumulative total of eight female Japanese Black cows were used in this experiment. All animals were treated with a controlled intravaginal drug release (CIDR) device (Eazi-Breed CIDR, Zoetis Inc., Parsippany-Troy Hills, NJ, USA), estradiol benzoate (estradiol injection KS, Kyoritsu Seiyaku, Tokyo, Japan), and prostaglandin $F_{2\alpha}$ analog (Zenoadin C, Nippon Zenyaku Kogyo, Fukushima, Japan) for estrus synchronization, as described in a previous study [27]. The day of standing estrus was defined as day 0. Several endometrial tissues were biopsied from the ipsilateral uterine horn to ovulation on days 1–2, 7–8, 12–13, and 17–18 in each cow. Samplings were performed once per estrus cycle. Next samplings were performed after two cycles of the first sampling. Biopsy, isolation of endometrial cells, and flow cytometry analysis for SP cells were performed as described above.

Statistical analyses

Statistical analyses were conducted using JMP 14.0 software (SAS Institute Inc. Cary, NC, USA). In experiment 1, significant differences ($P < 0.05$) in mRNA expression between SP and MP cells were analyzed using the Student's *t*-test. In experiment 2, statistical differences ($P < 0.05$) in the ratios of SP cells during the postpartum period (days 9–11, 29–32, 49–52, 99–101) and estrus cycle (days 1–2, 7–8, 12–13, and 17–18 after standing estrus) were analyzed using one-way repeated ANOVA followed by the contrast test.

Results

Flow cytometric analysis of bovine endometrial cells

Low fluorescent cells appeared in the flow cytometric results of endometrial stromal cells (Figure 1A), and these cells disappeared after verapamil treatment (Figure 1B). The mean ratio of SP cells in live stromal cells ($n = 13$) was $3.34 \pm 0.53\%$. Luminal epithelial cells did not show any low fluorescent cells with or without verapamil treatment (Figure 1C and D).

Differentiation assay for adipocytes and osteocytes from bovine endometrial cells

After culturing in the differentiation media, endometrial stromal cells differentiated into osteoblasts (Figure 2B) and adipocytes (Figure 2J), whereas luminal epithelial cells did not show any differentiation (Figure 2A and I). Bovine endometrial SP cells differentiated into adipocytes and osteocytes in the differentiation media (Figure 2C and K).

Differentiation of bovine endometrial cells into epithelial cells

Bovine endometrial stromal cells were differentiated into epithelial cells by all-trans retinoic acid treatment (10 µM) (Figure 3C). Stromal cells did not differentiate in the control (0 µM, Figure 3B), whereas endometrial SP cells differentiated regardless of the all-trans retinoic acid treatment (0 and 10 µM) (Figure 3E and F).

RNA-sequencing, GSEA, GO analysis, and KEGG pathway analysis

Sequencing of the RNA-seq libraries generated 43.2–107.0 million total reads, which were mapped at 94.58–96.14% for each sample to the *Bos taurus* reference genome (ARS-UCD1.2). GSEA using the calculated FPKM clarified RAMALHO_STEMNESS_UP and RAMALHO_STEMNESS_DN were significantly enriched as upregulated and downregulated gene sets in SP cells, respectively (Figure 4A and B).

Cutdiff identified 3,601 DEGs (FDR < *0.01*) between SP and MP cells from the calculated FPKM. Among the 3,601 DEGs, 1,971 (Supplementary Table S3) and 1625 genes (Supplementary Table S4) were identified as upregulated and downregulated genes in SP cells, respectively. GO analysis of the upregulated genes identified 684 enriched biological processes ($P < 0.05$; Supplementary Table S5). The top 10 terms included intracellular signal transduction, regulation of gene expression, RNA metabolic process, regulation of RNA metabolic process, regulation of macromolecule biosynthetic process, transcription, DNA-templated, regulation of cellular macromolecule biosynthetic process, RNA biosynthetic process, regulation of RNA biosynthetic process, and regulation of transcription, DNA-templated (Figure 4C). GO analysis of the 1625 downregulated genes identified involvement in 355 biological processes ($P < 0.05$; Supplementary Table S6). The top 10 terms included cell surface receptor signaling pathway, osteoblast differentiation, regulation of cell motility, cell migration, ATP synthesis coupled electron

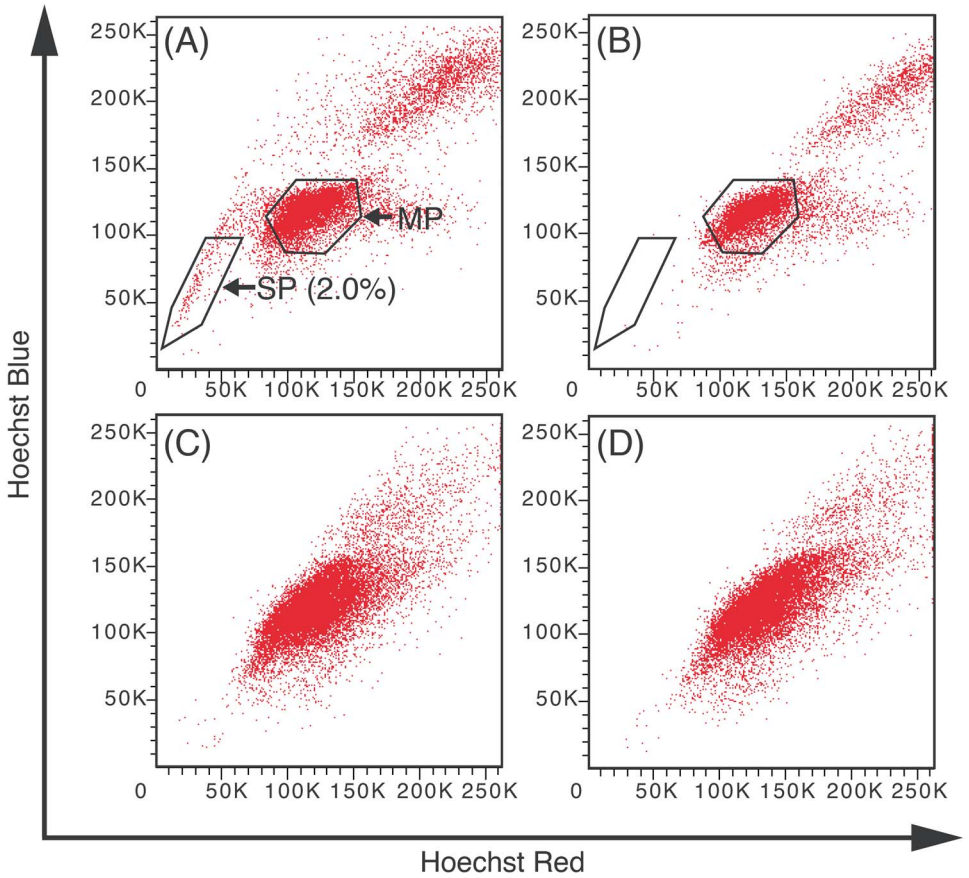

Figure 1. Representative flowcytometric distribution of the endometrial side population (SP) and main population (MP) of Hoechst 33342 dye-stained living cells in bovine endometrial stromal (A) and luminal epithelial cells (C). Verapamil, an inhibitor of Hoechst 33342 dye transport, was combined with Hoechst 33342 in bovine endometrial stromal (B) and luminal epithelial cells (D).

transport, regulation of cell migration, negative regulation of cell projection organization, collagen fibril organization, cartilage development, and respiratory electron transport chain (Figure 4D).

KEGG pathway analysis of 1971 genes upregulated in SP cells revealed 90 enriched pathways ($P < 0.05$; Supplementary Table S7), and the top 10 pathways included TNF signaling pathway, influenza A, herpes and simplex infection, pathways in cancer, hepatitis C, osteoclast differentiation, FoxO signaling pathway, measles, Rap1 signaling pathway, and Toll-like receptor signaling pathway (Figure 4E). Among the 1625 downregulated genes in SP cells, 36 KEGG pathways were enriched, including oxidative phosphorylation, Alzheimer's disease, Parkinson's disease, ECM-receptor interaction, metabolism of xenobiotics by cytochrome P450, nonalcoholic fatty liver disease, metabolic pathways, glutathione metabolism, focal adhesion, and chemical carcinogenesis as the top 10 pathways ($P < 0.05$; Figure 4F and Supplementary Table S8).

MCAM, ENG, ABCG2, CD34, and PECAM1 mRNA expression in SP and MP cells

qRT-PCR analysis showed that the expression of *MCAM, ENG, ABCG2, CD34*, and *PECAM1* mRNAs was significantly higher in bovine endometrial SP cells than in MP cells ($P < 0.05$, Figure 5).

Distribution of SP cells in bovine endometrium

Immunohistochemistry for ABCG2 was stained several cells in endometrial stroma layer. ABCG2 was found to be distributed in fibroblasts (Figure 6A) and vascular endothelial cells (Figure 6B).

The ratio of SP cells in bovine endometrial cells during the postpartum period and estrus cycles

The ratio of endometrial SP cells in the live singlet cells was lowest on days 9–11 after parturition and gradually increased as postpartum days passed (Figure 7A). The ratio of endometrial SP cells on days 9–11 after parturition was 0.85%, which was significantly lower than the values on days 29–31, 49–51, and 99–101 after parturition. The ratio of endometrial SP cells on days 29–31 was also significantly lower than that on days 99–101 after parturition. On the other hand, the ratios of endometrial SP cells did not change throughout the estrus cycle (Figure 7B), remaining at 2.63–3.32%.

Discussion

This study revealed that SP cells were present in the endometrial stromal cells, but not in the luminal epithelial cells. It has been shown that the expression of ABCG2 is stronger in the SP cell population than in the non-SP cell population [28], and similar results have been obtained from RNA-seq and qRT-PCR analysis in this experiment. Our immunohistochemical results clarified that

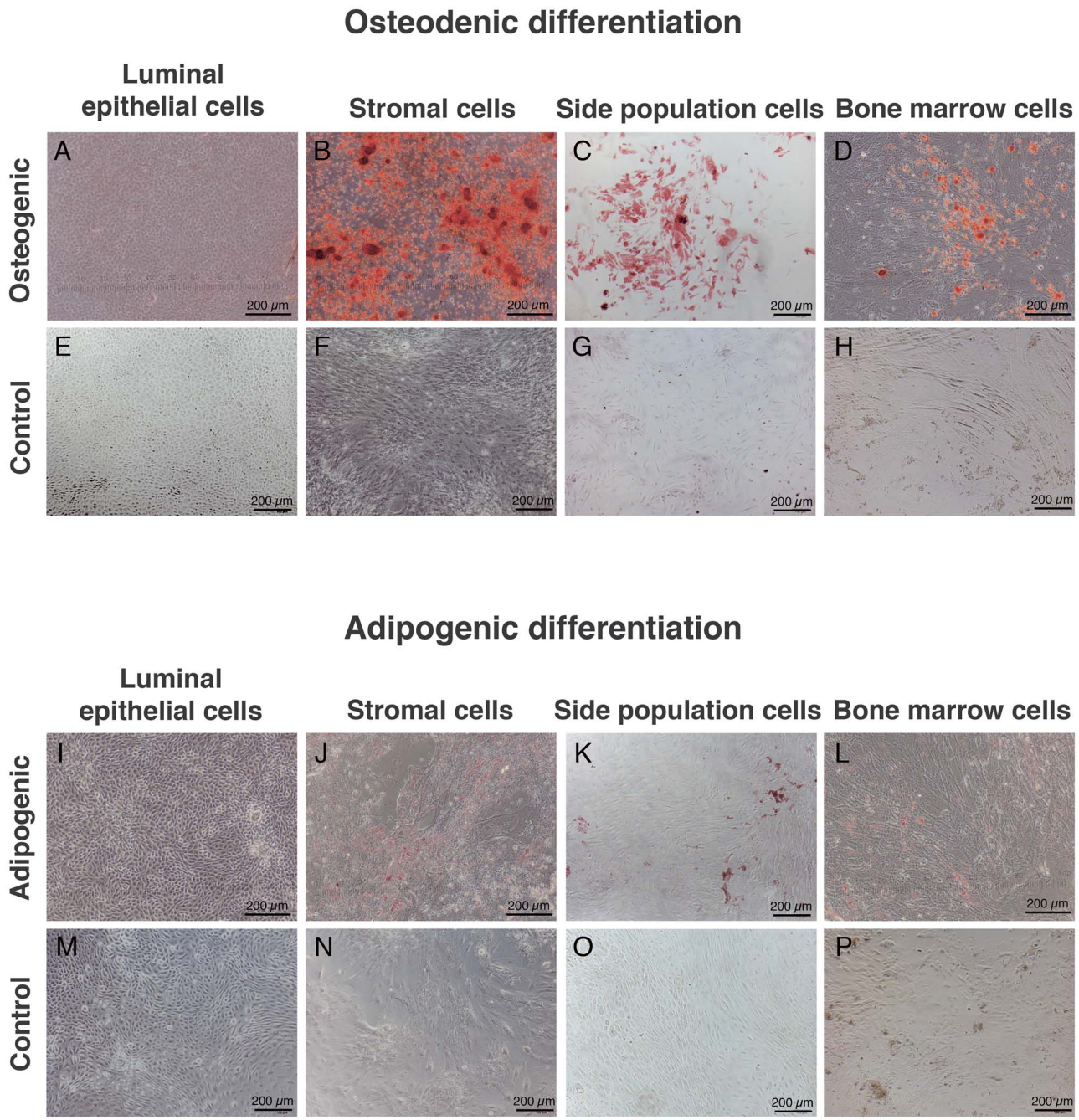

Figure 2. Representative photomicrographs of calcium deposition staining of bovine endometrial epithelial cells (A), stromal cells (B), side population (SP) cells (C), and bone marrow cells (D) cultured in osteoblast differentiation medium. Representative photomicrographs of Oil Red O staining of bovine endometrial epithelial cells (I), stromal cells (J), SP cells (K) and bone marrow cells (L) cultured in adipocyte differentiation medium. Negative controls were cultured without differentiation medium (E–H, M–P). Scale bars are 200 μm.

ABCG2-positive cells are localized in fibroblast cells and vascular endothelial cells. Additionally, in this study, SP cells were shown to differentiate into epithelial cells. Bovine endometrial SP cells can also differentiate into adipocytes and osteocytes. In humans, SP cells in the endometrium have been shown to exhibit pluripotency [29, 30]. Collectively, these findings indicate that the bovine SP cells in the endometrium may be pluripotency.

Bovine endometrial SP cells showed 1971 upregulated genes when compared to MP cells. These upregulated genes included *MCAM*, *ENG* (CD105) and *CD200* (Supplementary Table S3), which have been known to be mesenchymal stem cell markers in humans [31]. Genes with upregulated expression in bovine endometrial SP cells also included the genes of RNA-binding proteins such as ZFP36 ring finger protein like 2 (*ZFP36L2*), zinc finger protein 131 (*ZNF131*), and zinc finger protein 326 (*ZNF326*) (Supplementary Table S3). The mRNA of RNA-binding proteins, which are related to RNA transcriptional regulation, have been shown to be expressed at higher levels in human hematopoietic

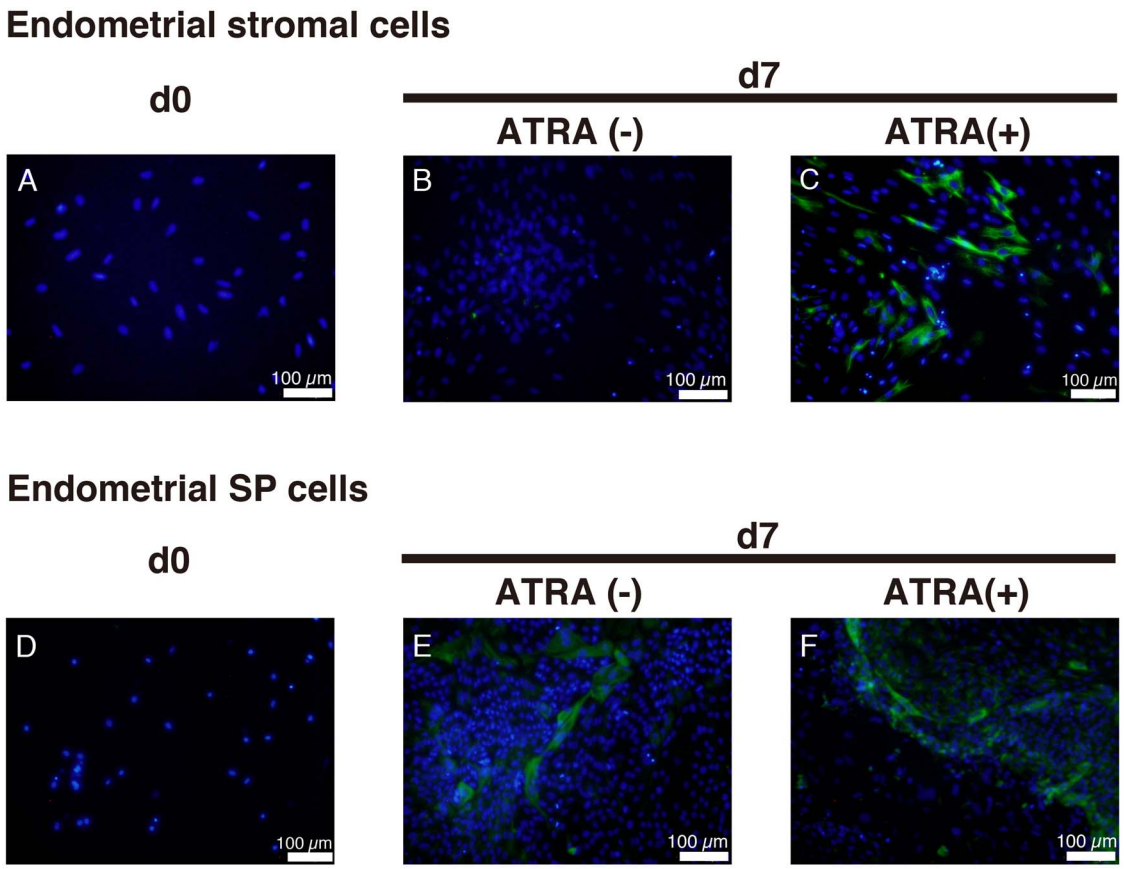

Figure 3. Representative photomicrographs showing immunohistochemistry for decting cytokeratin (green) in cultured endometrial stromal cells (A) and side population (SP) cells (D) at day 0. Representative photomicrographs showing immunohistochemistry for cytokeratin (green) in cultured endometrial stromal cells with 0 μM (B), and 10 μM (C) of all-trans retinoic acid treatment at day 7. Representative photomicrographs showing immunohistochemistry for cytokeratin (green) in cultured endometrial SP cells with 0 μM (E) and 10 μM (F) of all-trans retinoic acid treatment at day 7. Scale bars are 100 μm.

stem cells, myeloid progenitors, and granulocyte progenitors when compared with differentiated monocytes [32]. In this study, functional annotation using GSEA identified the differences in gene expression between SP cells and MP cells that were significantly enriched in the gene sets derived from mouse embryonic stem cells, neuronal stem cells and hematopoietic stem cells (Figure 4A and B). These results indicated that the mRNA expression pattern of bovine endometrial SP cells was similar to that of stem cells. Moreover, GO analysis of the 1971 upregulated genes in bovine endometrial SP cells showed that the enrichment of terms related to RNA metabolic process and transcription were present in the top 10 enriched pathways (Figure 4C). KEGG pathway analysis of 1971 upregulated genes also revealed enrichment of Wnt (Supplementary Figure S1), Notch (Supplementary Figure S2), Jak-STAT (Supplementary Figure S3), PI3K-Akt (Supplementary Figure S4), MAPK (Supplementary Figure S5), FoxO (Supplementry Figure S6), and TGF-β (Supplementary Figure S7 and Supplementary Table S7) signaling pathways. The Wnt signal from the niche maintains quiescence in hematopoietic stem cells in mice [33]. Notch signaling maintains the undifferentiated status in murine hematopoietic stem cells [34] and intestinal epithelial stem/progenitor cells [35]. Jak-STAT and PI3K-Akt signaling maintain multipotency and self-renewal in embryonic stem cells [36, 37], whereas the FoxO signaling suppresses DNA damage in murine hematopoietic stem and progenitor cells [38]. As mentioned above, these pathways are associated with the maintenance of somatic stem/progenitor cells. In contrast, MAPK and TGF-β signaling involved in both the maintenance and differentiation of somatic stem/progenitor cells. MAPK signaling has been shown to promote the differentiation of murine embryonic stem cells [39]. TGF-β signaling regulates the differentiation of mesenchymal stem cells from bone marrow into osteoblasts, adipocytes, chondrocytes, myocytes, and tenocytes [40]. In addition, stem cells are known to decrease ATP synthesis in the TCA cycle by maintaining quiescent status [41]. In the present study, the terms involved in TCA cycles, such as the fatty acid metabolic process, cellular respiration, and respiratory electron transport chain were enriched in the GO analysis (Supplementary Table S6) and KEGG pathway analysis (Supplementary Table S8) of 1625 downregulated genes in endometrial SP cells. These results support the notion that bovine endometrial SP cells have the characteristic of somatic stem/progenitor cells.

Because the bovine endometrial SP cells in this study contained vascular endothelial cells in addition to fibroblast cells, the SP cells showed significantly higher expression of the *PECAM1*, *CD34*, *MCAM*, *ENG*, von Willebrand factor (*VWF*), kinase insert domain receptor (*KDR*), and fms related receptor tyrosine kinase 1 (*FLT1*) genes (Figure 5 and Supplementary Table S3), which are endothelial marker genes in humans [42, 43]. GO analysis of the 1971 upregulated genes in SP cells identified the terms involved in vascular development (Supplementary Table S5). It is considered that in humans

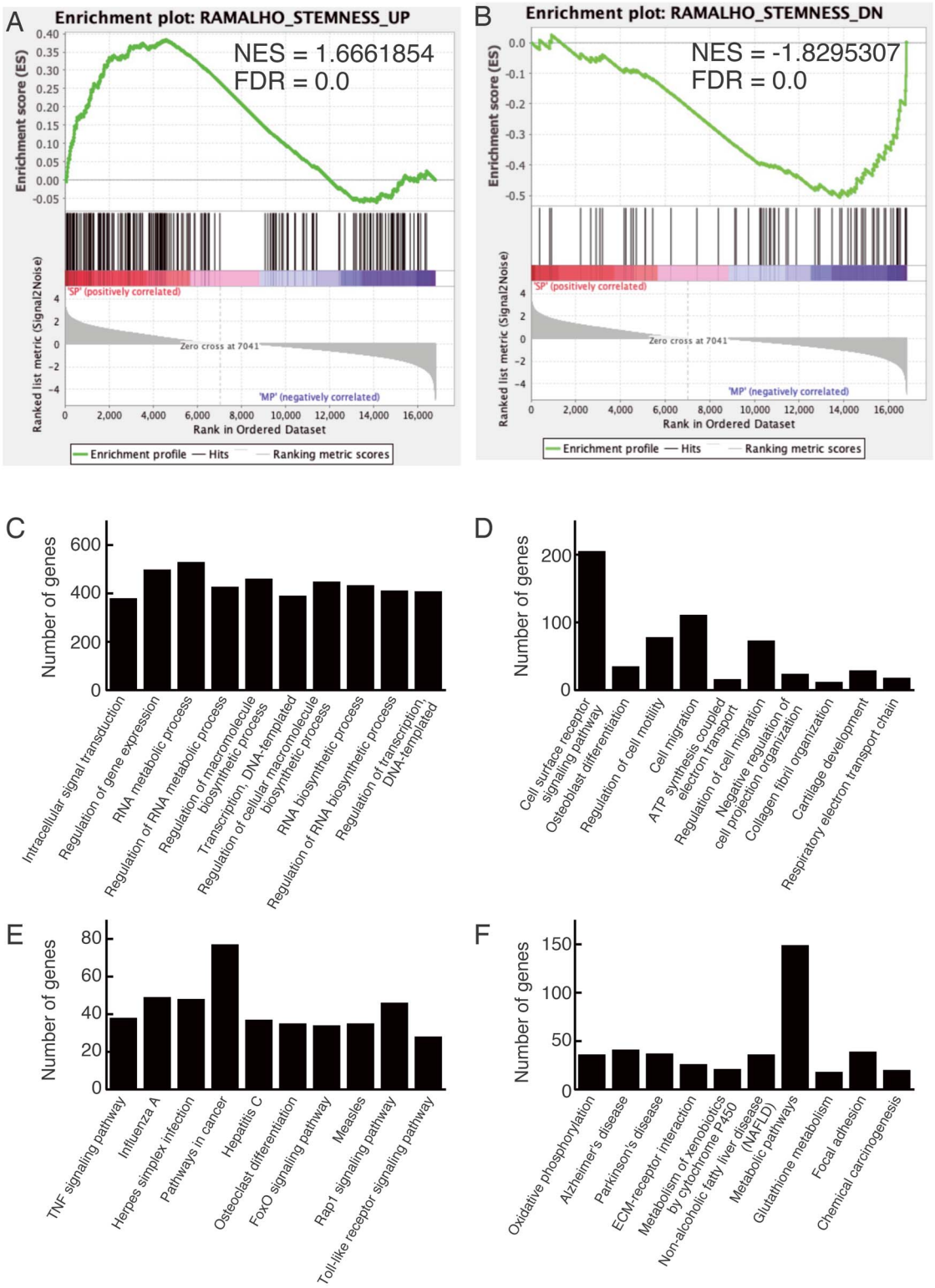

Figure 4. Gene set enrichment analysis of mRNA expression between bovine endometrial side population (SP) cells and main population (MP) cells using the gene sets of RAMALHO_STEMNESS_UP (A) and RAMALHO_STEMNESS_DN (B). NES, normalized enrichment score; FDR, false discovery rate. Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for differentially expressed genes (DEGs) between SP cells and MP cells. Enriched functional biological process of upregulated (C) and downregulated (E) DEGs in SP cells. Enriched KEGG pathways of upregulated (D) and downregulated (F) DEGs in SP cells.

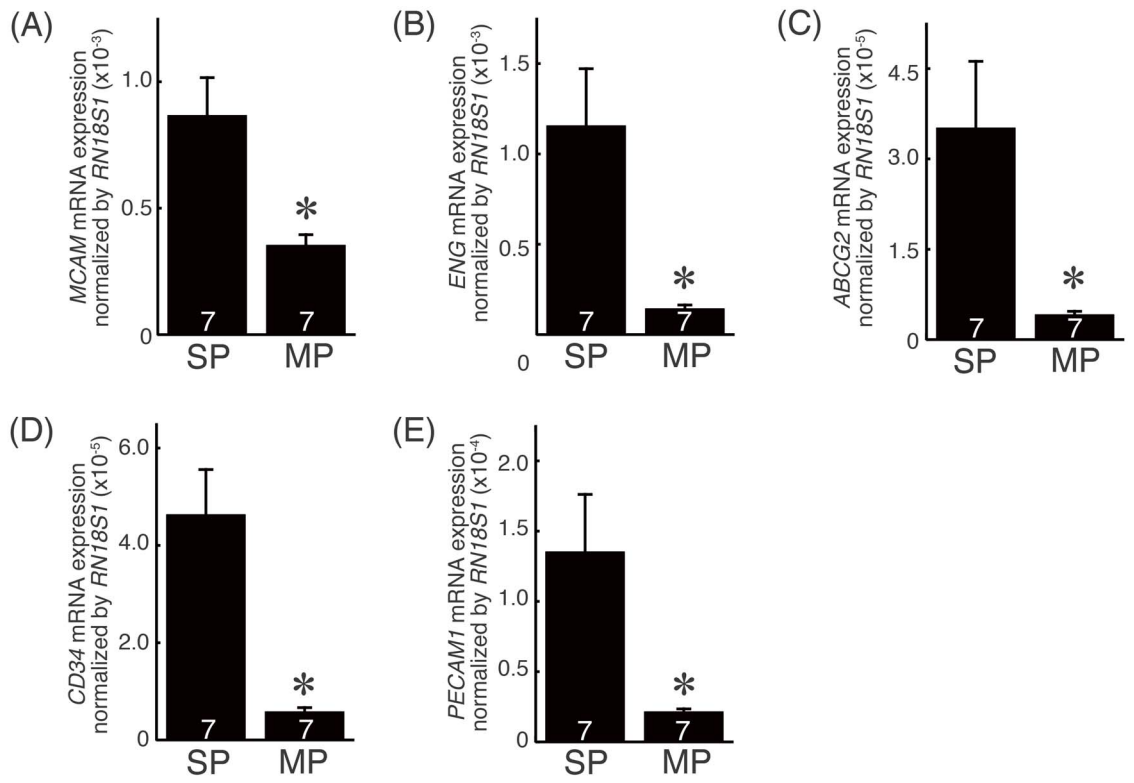

Figure 5. Comparison of relative mRNA expression of *MCAM* (A), *ENG* (B), *ABCG2* (C), *CD34* (D), *PECAM1* (E) determined using qRT-PCR analysis of bovine endometrial side population (SP) cells and main population (MP) cells. Values are expressed as means ± SEM. Data were analyzed using the Student's *t*-tests. ∗Significant differences as compared to MP cells ($P < 0.05$).

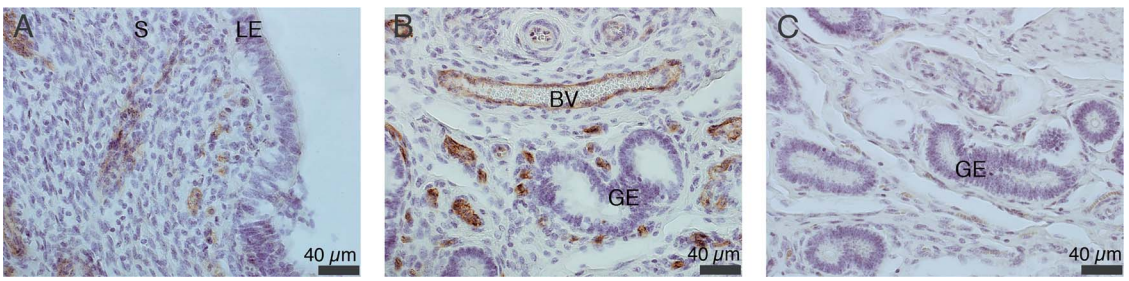

Figure 6. Photomicrographs showing the distributions of ABCG2 immunoreactivity in bovine endometrium. Immunoreactivities were detected in the stromal layer (A, B) but not in the luminal epithelial layer (A). Immunoreactivities were not detected without primary antibody (C). S, stroma; LE, luminal epithelium; BV, blood vessel; GE, glandular epithelium. Scale bars are 40 μm.

at least some SP cell populations contain cells derived from bone marrow because endometrial SP cells have properties similar to those of vascular endothelial progenitor cells [5, 44]. Therefore, the bovine endometrial SP cells identified in this experiment might be derived from the bone marrow.

The ratio of bovine SP cells to endometrial cells was the lowest at days 9–11 after parturition and then gradually increased in this study. In mice, it has been demonstrated that a significant portion of putative endometrial stem/progenitor cells transiently proliferated on postpartum day 1 and that this proliferation of putative endometrial stem/progenitor cells resulted in a significant decline in the proportion of cells in the uterus during the period from 7 days after calving until 21 days after delivery [45] In addition, the subpopulation of stromal cells, which are putative stromal stem/progenitor cells, have been shown to differentiate into both luminal and glandular epithelial cells after parturition in mice [46]. We have shown that bovine endometrial SP cells are more likely to differentiate into the epithelial cells, although both endometrial SP cells and stromal cells have the ability to differentiate into epithelial cells. Accordingly, the temporarily reduction in SP cells in the bovine endometrium immediately after calving may be due to the differentiation of SP cells to provide the new endometrial cells. Further studies are required to prove that the transplanted SP cells reconstitute the endometrium in vivo in order to prove that SP cells contribute to endometrial regeneration.

The mean ratios of bovine endometrial SP cells at postpartum days 49–52 and 99–101 were 3.78% and 4.91%, respectively, which were higher than those during estrus cycles. In mice, the ratio of SP cells in the endometrium was increased by postpartum day 18 and then decreased thereafter [47], although uterine contraction

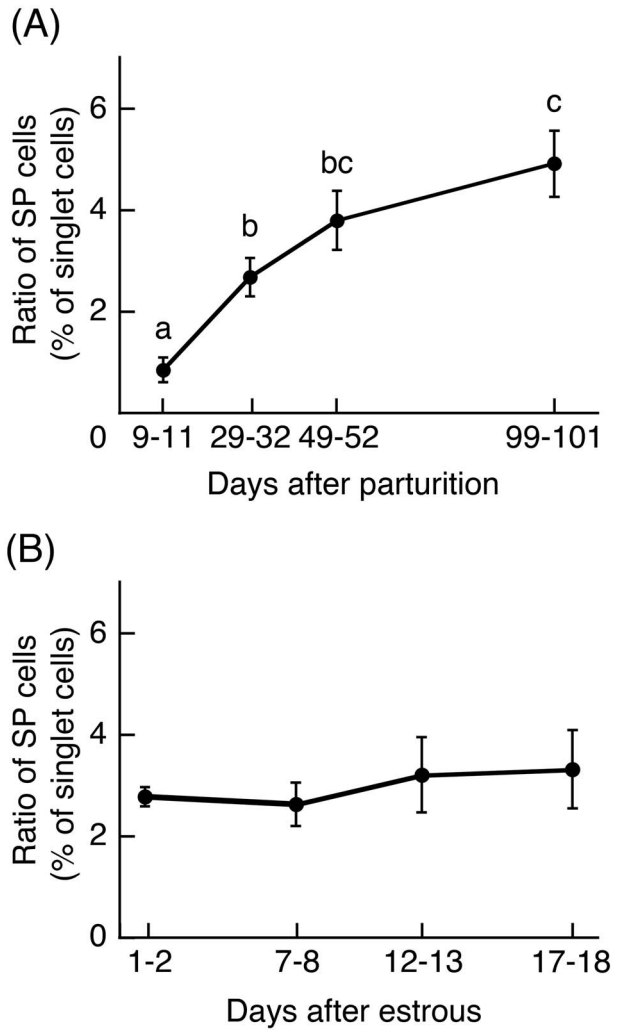

Figure 7. The ratios of endometrial side population (SP) cells in live singlet cells during postpartum periods (A) and estrus cycles (B). The days of parturition and estrus were designated as day 0. Values are expressed as mean ± SEM. Values with different letters indicate a significant difference ($P < 0.05$, one-way repeated ANOVA followed by the contrast test).

was completed on postpartum day 10 [48]. In cows, it is generally considered that the endometrium is completely recovered around 40 days after parturition [49, 50]. Therefore, as in the case of mice, the ratio of SP cells might continue to be high for a period of time after uterine recovery, and then gradually decrease, although this could not be confirmed in the current experiment. The increase in the ratio of endometrial SP cells after parturition in mice may be attributed to the effect of estrogen [47]. The ratio of human endometrial SP cells has been shown to change during the estrus cycle, reaching a peak in the early proliferative phase [5]. Human endometrial SP cells express estrogen receptor 2 (*ESR2)* [5], suggesting the possibility that the change in the ratio of human endometrial SP cells during menstrual cycles also depends on estrogen. On the other hand, the ratio of bovine SP cells in the endometrium was maintained constant throughout the estrus cycle. This result reflects the fact that the drastic reconstruction of the endometrium, like in menstruation, does not occur during the estrus cycle in cattle. Although the effects of estrogen on bovine endometrial SP cells have not yet been fully understood, the mechanism of SP cell proliferation may differ between species, and further research is needed to clarify this mechanism.

In summary, the present study revealed the existence of SP cells in bovine endometrial cells, except for luminal epithelial cells. Bovine endometrial SP cells showed characteristics similar to those of somatic stem/progenitor cells. The changes in the ratio of endometrial SP cells after parturition, which was at its lowest level at 9–11 days after parturition and then gradually increased, suggested that SP cells contribute to endometrial regeneration in cattle.

Authors' contributions

R.T. participated in Experiment 1 and drafted the manuscript. S. N. and S. Minabe participated in Experiment 2. T. F. participated in Experiment 2 and designed this study. R.A. and M. K. participated in Experiment 1. Y. M. and S. O. contributed to the discussion and helped to draft the manuscript. K. K. conceptualized and designed this study. S. Matsuyama conceptualized and designed this study and drafted the manuscript.

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