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CD34 and CD49f Double-Positive and Lineage Marker-Negative Cells Isolated from Human Myometrium Exhibit Stem Cell-Like Properties Involved in Pregnancy-Induced Uterine Remodeling[1]

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ABSTRACT

Repeated and dramatic pregnancy-induced uterine enlargement and remodeling throughout reproductive life suggests the existence of uterine smooth muscle stem/progenitor cells. The aim of this study was to isolate and characterize stem/progenitor-like cells from human myometrium through identification of specific surface markers. We here identify CD49f and CD34 as markers to permit selection of the stem/progenitor cell-like population from human myometrium and show that human CD45$^-$ CD31$^-$ glycophorin A$^-$ and CD49f$^+$ CD34$^+$ myometrial cells exhibit stem cell-like properties. These include side population phenotypes, an undifferentiated status, high colony-forming ability, multilineage differentiation into smooth muscle cells, osteoblasts, adipocytes, and chondrocytes, and in vivo myometrial tissue reconstitution following xenotransplantation. Furthermore, CD45$^-$ CD31$^-$ glycophorin A$^-$ and CD49f$^+$ CD34$^+$ myometrial cells proliferate under hypoxic conditions in vitro and, compared with the untreated nonpregnant myometrium, show greater expansion in the estrogen-treated nonpregnant myometrium and further in the pregnant myometrium in mice upon xenotransplantation. These results suggest that the newly identified myometrial stem/progenitor-like cells influenced by hypoxia and sex steroids may participate in pregnancy-induced uterine enlargement and remodeling, providing novel insights into human myometrial physiology.

CD34, CD49f, myometrium, pregnancy, stem cells, uterus

INTRODUCTION

The human myometrium undergoes profound physiological tissue remodeling in volume up to 1000-fold and increases its weight more than 20-fold during pregnancy. Both in humans and rodents, myometrial hyperplasia (cell number increase) and hypertrophy (cell size increase) contribute to the dramatic expansion of the uterus [1–3]. New myometrial cells must be generated when the myometrium increases its size. These pregnancy-driven processes can reportedly occur more than 20 times throughout a woman's reproductive life [4–5]. However, precisely how the myometrium regenerates itself is poorly understood. New smooth muscle cells might develop from differentiated cells, but they are more likely to be generated by primitive, tissue-specific stem cells.

After completion of embryonic development, tissue-specific stem cells (also termed somatic or adult stem cells) remain throughout the body and play critical roles both in replacing dying cells and in regenerating injured tissues. In this fashion, they contribute to the structural and functional maintenance of their tissue of origin [6]. Candidate tissue-specific stem cells have been identified in many tissues based on their side population (SP) phenotype [7]. This characteristic is due to the unique ability of primitive cells to reduce the intracellular concentration of Hoechst 33342, a DNA-binding dye, via the ATP-binding cassette, subfamily G, member 2 (ABCG2) [7]. In our original search for primitive myometrial cells, we initially employed the SP technique because no myometrium-specific surface makers were known. In this way, we demonstrated that SP cells, but not main population (MP) cells, isolated from human myometrium exhibited stem/progenitor-like cell properties [8]. However, SP sorting methodology is very expensive, a flow cytometric cell sorter equipped with an ultraviolet laser is required, and extreme care and skill is needed for success [7]. In addition, the method is not necessarily benign for sorted cells as they are exposed to toxic Hoechst dye and ultraviolet light [7]. Therefore, to overcome these obstacles and to facilitate possible future clinical use, we sought to identify surface markers to permit selection of the myometrial stem/progenitor-like cell population.

We analyzed cell surface antigens of myometrial SP (myoSP) and myometrial MP (myoMP) fractions and found that CD49f and CD34 were preferentially expressed on myoSP cells. The myoSP cells, in turn, preferentially existed in the fraction of CD49f$^+$ and CD34$^+$ fraction. CD49f is known as

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very late activation antigen 6 (VLA-6) and integrin, α6 (ITGA6). Integrins constitute a family of integral cell surface heterodimeric receptors consisting of two distinct subunits called α and β and mediate binding and responding to the extracellular matrix. Some types of integrins such as CD49a/integrin α1/ITGA1 and CD49b/integrin α2/ITGA2 have recently emerged as potential markers for hematopoietic and uterine leiomyoma stem/progenitor cells [9–11]. Because SP cells exhibit tissue-specific stem cell phenotypes in a variety of tissues and also in myometrium [8, 12–13], we further analyzed $CD49f^+$ and $CD34^+$ fraction as a candidate of myometrial stem/progenitor-like cells.

MATERIALS AND METHODS

Preparation of Human Myometrial Cells

Normal myometrial tissues were obtained from women (age range: 30- to 52-yr-old) undergoing hysterectomy. Additional basic endocrine information was also obtained, that is, day of menstrual cycle, age of menarche, and oral contraception status. Written informed consent was obtained from each patient, and the use of these human specimens was approved by the Keio University Ethics Committee. None of these cases had any previous history of uterine cancer, and all samples were confirmed to be free of malignancy by histopathological examination. Single dispersed myometrial cells were obtained from the collected tissues as previously described [8, 14]. In brief, the myometrial tissue was immediately cut manually into small pieces of less than 1 mm^3. They were then incubated in Dulbecco-modified Eagle medium (Sigma-Aldrich) containing 0.2% (w/v) collagenase (Wako), 0.05% DNase I (Life Technologies), 1% (v/v) antibiotic-antimycotic mixture (Life Technologies), 10% (v/v) fetal bovine serum (FBS) and 10 mM HEPES buffer solution (Life Technologies) at 37°C on a shaker. The digested tissue was filtered through a sterile 400 μm polyethylene mesh filter to remove particulates and then filtered through a 40-μm cell strainer (BD Falcon). The pooled cells were depleted of red blood cells by Ficoll-Paque PLUS (GE Healthcare Bio-Sciences).

Cell Sorting and Flow Cytometric Analysis

Myometrial cells were sorted on a FACS Vantage SE flow cytometer (BD Biosciences) and analyzed with Cell-Quest (BD Biosciences) and FlowJo software (Tree Star). The FACS Vantage SE flow cytometer is equipped with an argon laser (488 nm excitation), a He/Ne laser (633 nm excitation), and a multiline ultraviolet laser (334–364 nm excitation). Establishment of the gates was based on the staining profiles of the negative controls. To eliminate lineage-committed cells from living cells singly dissociated from human myometrium, CD31, CD45, and glycophorin (GlyA) were used as lineage markers, and the myometrial cells positive for these markers were separated and designated as Lin^+ cells (Fig. 1A, left). Lineage negative (Lin^-) cells were subjected to double staining for CD34 and CD49f (Fig. 1A, right) and then divided into four fractions (I–IV): I) $CD49f^+/CD34^+/Lin^-$ (double-positive/Lin^- cells, DP/Lin^-), II) $CD49f^+/CD34^-/Lin^-$, III) $CD49f^-/CD34^-/Lin^-$, and IV) $CD49f^-/CD34^+/Lin^-$ (Fig. 1B, fractions I–IV). Each fraction was then sorted and subjected to Hoechst dye staining as previously described [8]. The nozzle size of the FACS Vantage was 70 μm. Sort speed was <1000 cells/sec. Residual erythrocytes, debris, doublets, and dead cells were excluded by forward scatter, side scatter, and propidium iodide gating. Viability of the sorted cells exceeded 90% as assessed by trypan blue exclusion. Antibodies used for cell sorting and flow cytometric analysis are listed in Table 1.

Reverse-Transcription PCR

The primers used for PCR amplification are listed in Table 2. Total RNA was extracted using TRIzol reagent (Life Technologies) and reverse transcribed with Superscript III reverse transcriptase (Life Technologies) and random hexamers, according to the manufacturer's instructions. Complementary DNA was synthesized from 100 000 DP/Lin^-, non-DP/Lin^-, or Lin^+ cells. Estrogen receptor 1 (*ESR1*), estrogen receptor 2 (*ESR2*), progesterone receptor (*PGR*), calponin 1, basic, smooth muscle (*CNN1*), and smoothelin (*SMTN*) were used as markers for uterine myometrial cells. *ABCG2* was used as a marker for SP cells. Freshly sorted cells were used to analyze the expression of myometrial markers. Bone gamma-carboxyglutamate (Gla) protein (*BGLAP*), runt-related transcription factor 2 (*RUNX2*), alkaline phosphatase, liver/bone/kidney (*ALPL*), collagen, type I, alpha 2 (*COL1A2*), integrin-binding sialoprotein (*IBSP*), and parathyroid hormone 1 receptor (*PTH1R*) were used as osteoblast markers. Lipoprotein lipase (*LPL*) and peroxisome proliferator-activated receptor gamma (*PPARG*) were used as adipocyte markers. Aggrecan (*ACAN*) and collagen, type II, alpha 1 (*COL2A1*) were used as chondrocyte markers. Cultured cells were used to analyze the cell differentiation. An aliquot was then assayed for the relative amount of *GAPDH* signal. The data were then used to calculate a dilution factor for each sample so that each contained the same concentration of *GAPDH* cDNA.

Cell Culture

The following were plated and cultured under normoxic or hypoxic conditions: I) DP/Lin^-, II) $CD49f^+/CD34^-/Lin^-$, III) $CD49f^-/CD34^-/Lin^-$, and IV) $CD49f^-/CD34^+/Lin^-$ and Lin^+ fractions. These sorted cells from human myometria were cultured in Mesenchymal Stem Cell Growth Medium (MSCGM) (Lonza) under normoxic, that is, 20% (v/v), O_2 or hypoxic, that is, 2% (v/v) O_2, conditions for 2–3 wk. Cell proliferation activities were measured using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. For induction of osteogenic and adipogenic differentiation, DP/Lin^-, non-DP/Lin^-, and Lin+ fractions were plated at a density of approximately 5×10^3 cells/well in each 96-well plate with MSCGM and grown in an hypoxic environment until the cells reached confluence (14–21 days). For osteogenic induction, the cultures were then exported to a normoxic environment and fed with osteocyte differentiation media (Cambrex Bio Science) every 3–4 days for 2–3 wk. The cells were then harvested for RNA extraction or subjected to alkaline phosphatase staining using the Histofine New Fuchsin Substrate kit (Nichirei). For adipogenic induction, confluently cultured cells were exported to a normoxic environment and exposed to three cycles of incubation with adipogenic induction/maintenance media (Cambrex Bio Science). Each cycle consisted of incubation with the supplemented adipogenesis induction media for 3 days, followed by 1–3 day incubation with adipogenic maintenance media. After three cycles of incubation, cells were cultured for at most another week in the maintenance media and then harvested for RNA extraction or subjected to Oil Red O staining. For induction of chondrogenic differentiation, hypoxic cell culture was performed in 60 mm cell culture dishes. Subsequently, the cultures were moved to a normoxic environment, and fed with chondrogenic induction medium (Lonza) in a 15 ml centrifuge tube. MSCGM were used as noninduction media for controls.

As the positive control, human mesenchymal stem cells (hMSCs) purchased from Lonza were subjected to in vitro differentiation assays according to the manufacturer's instruction. In brief, hMSCs were cultured in MSCGM under a normoxic condition for 2 wk, then plated at a density of approximately 4×10^4 cells/well in 24-well plates with MSCGM and grown until the cells reached confluence (3–7 days). For induction of osteogenic differentiation, the confluent hMSC cultures were fed with osteocyte differentiation media every 3–4 days for 2–3 wk. For induction of adipogenic differentiation, the confluent hMSC cultures were exposed to three cycles of incubation with adipogenic induction/maintenance media for 3 wk. For induction of chondrogenic differentiation, the confluent hMSC cultures were transferred into a 15-ml centrifuge tube and fed with chondrogenic induction medium for 4 wk. MSCGM were used as noninduction media for controls. Human MSCs with or without differentiation were subjected to both RNA extraction followed by RT-PCR analysis and each corresponding dye or immunohistochemical staining.

In Vitro Colony Assay

Freshly isolated I) DP/Lin^-, II+III) $CD34^-/Lin^-$, IV) $CD49f^-/CD34^+/Lin^-$ and Unfractionated fractions were plated on 35-mm culture dishes at a density of 200 cells/cm^2 and cultured in MSCGM under hypoxic conditions. Scratches in the culture dish serve to identify the field. The number of colonies was counted after 14 days of culture. The colony was defined as an aggregate containing over 15 cells. The data were obtained from five independent experiments.

Transplantation Analysis

All the experiments using severely immunodeficient NOD/SCID/γc null (NOG) mice that exhibit multiple immunological deficiencies (Central Institute for Experimental Animals, Kanagawa, Japan) were conducted in accord with the Guide for the Care and Use of Laboratory Animals of the Keio University School of Medicine. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee

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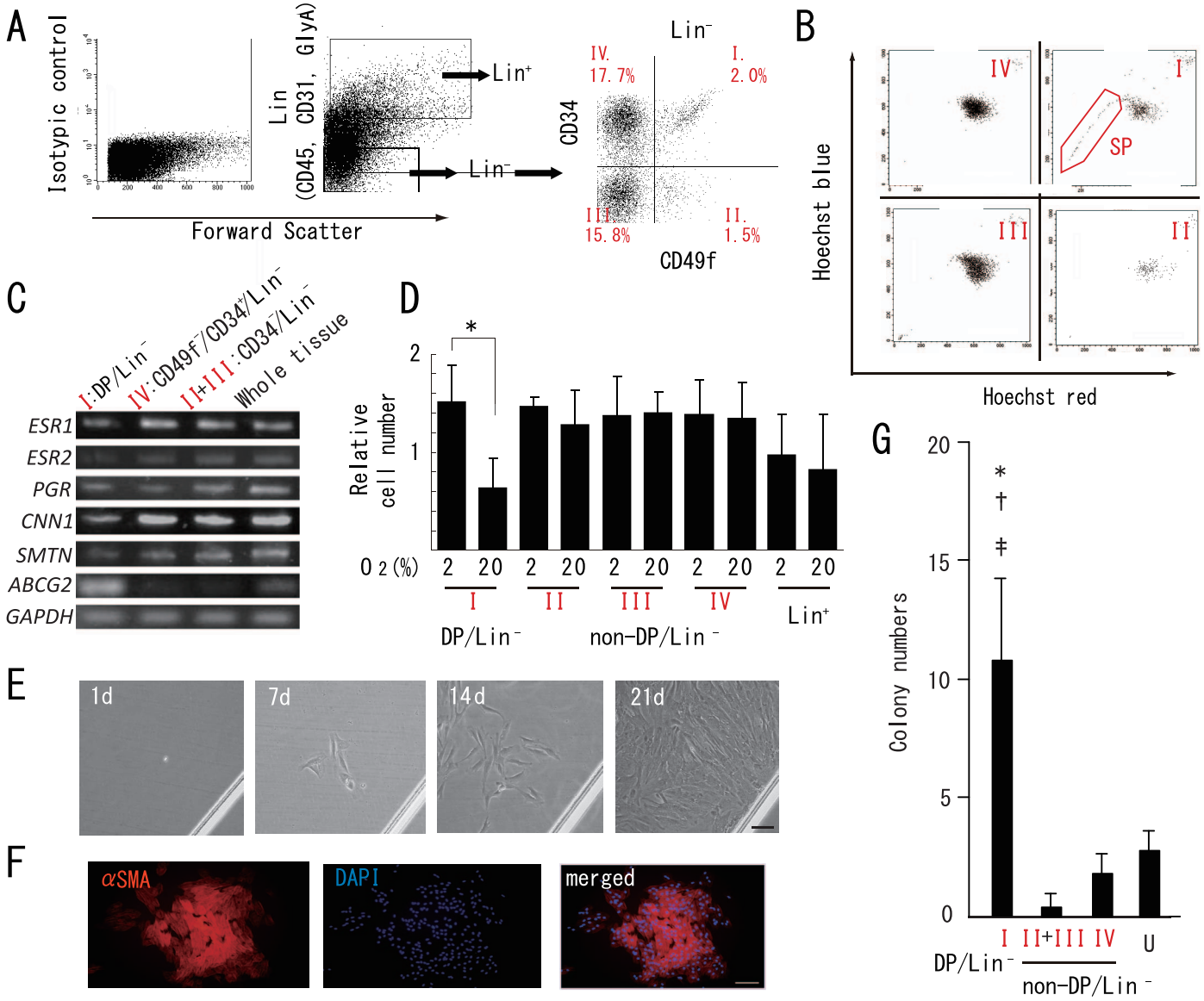

FIG. 1. Isolation and characterization of the human myometrial DP/Lin$^-$ fraction. **A**) Flow cytometric profiles of Lin$^+$ and Lin$^-$ fractions. Lin$^-$ cells were divided into four fractions (I–IV) based on the expression pattern of CD34 and CD49f. The proportion of each fraction as a percentage of total living cells is shown. **B**) Hoechst-staining profiles of Lin$^-$ populations subsequently sorted by their CD34 and CD49f staining intensities. The percentage of SP cells is 2.73% in fraction I, 0% in fraction II, 0.07% in fraction III, and 0.06% in fraction IV. **C**) Messenger RNA expression of genes in DP/Lin$^-$, CD49f$^-$/CD34$^+$/Lin$^-$, CD34$^-$/Lin$^-$, and whole myometrial tissues as determined by RT-PCR. Representative of five independent experiments. **D**) Cell proliferation activities of Lin$^+$ and fractions I–IV of the Lin$^-$ population under normoxic and hypoxic conditions as determined by the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay. Each bar indicates the mean + SEM of the absorbance at 490 nm obtained from three independent experiments using three individual samples. *$P < 0.05$. **E**) Colony formation by a single sorted DP/Lin$^-$ cell after the indicated days of hypoxic culture. After seeding 200 cells/cm^2, single sorted DP/Lin$^-$ cells attached to the culture dish 1 day (one d) after seeding. The cells then formed colonies after the indicated days of culture. Scratches in the culture dish serve to identify the field. Bar = 100 μm. **F**) Immunofluorescence of a colony generated from a single sorted DP/Lin$^-$ cell using 4′,6-diamidino-2-phenylindole dihydrochloride and an antibody against αSMA. Representative of five independent experiments. Bar = 100 μm. **G**) Colony formation potential of DP/Lin$^-$ under hypoxic conditions. Each bar indicates mean + SEM of the average colony number in 30 dishes for unfractionated myometrial cells and each indicated cell subpopulation obtained from five independent experiments. U, unfractionated myometrial cells. *$P < 0.005$ versus unfractionated myometrial cells; †$P < 0.005$ versus CD49f$^-$/CD34$^+$/Lin$^-$; ‡$P < 0.005$ versus CD34$^-$/Lin$^+$.

on the Ethics of Animal Experiments of Keio University School of Medicine. All the surgery was performed under anesthesia, and all efforts were made to minimize suffering. Freshly isolated DP/Lin$^-$ cells (5×10^4) were injected into each uterine horn of nine NOG mice using a 29 gauge needle. Each uterine horn was exteriorized through a dorsal-horizontal incision. DP/Lin$^-$ cells (5×10^4) were injected into each uterine horn of NOG mice using a 29 gauge needle. The 29 gauge needle was introduced into the uterine horn at a site apart from the transplanted region. The uterine horn was penetrated, and the tip of the needle was held just beneath the serous membrane. Subsequently, the single-cell suspension of DP/Lin$^-$ cells was injected into the myometrium. Non-DP/Lin$^-$ and Lin$^+$ cells were similarly transplanted into each of NOG mice. Freshly isolated non-DP/Lin$^-$ and Lin$^+$ cells were similarly transplanted into each of nine NOG mice. Four to five weeks after transplantation, three NOG mice that had been injected with DP/Lin$^-$ cells were subcutaneously implanted with two E_2 pellets (1.5 mg E_2 per pellet; Innovative Research of America). Three NOG mice also underwent sham operations. Finally, three NOG mice were mated to ICR males. To minimize the adverse effects of transplantation operation on pregnancy, we started mating or implanted E2 pellets 4–5 wk after transplantation. To standardize the experimental conditions between the three groups, we did not perform ovariectomy prior to implantation. All the uteri were excised when the pregnant NOG mice were at 18.5 days postcoitum.

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TABLE 1. List of antibodies used in this study.[a]

Antigen	Clone	Fluorochrome and isotype (usage)	Supplier
CD31 (FACS)	WM59	PE-conjugated mouse IgG1 (20 μl/1 × 10^6 cells)	BD Biosciences (San Jose, CA)
CD45 (FACS)	HI30	PE-conjugated mouse IgG1 (5 μl/1 × 10^6 cells)	BD Biosciences
Glycophorin (FACS)	GAR-2	PE-conjugated mouse IgG2b (20 μl/1 × 10^6 cells)	BD Biosciences
CD49f (FACS)	GoH3	FITC-conjugated rat IgG2a (20 μl/1 × 10^6 cells)	BD Biosciences
CD34 (FACS)	581	APC-conjugated mouse IgG1 (20 μl/1 × 10^6 cells)	BD Biosciences
Collagen type II	Polyclonal	Rabbit (1:50)	Quartett (Berlin, Germany)
α-Smooth muscle actin	1A4	Mouse IgG2a (1:100)	DAKO Cytomation (Glostrup, Denmark)
Human vimentin	V9	Cy3-conjugated mouse IgG1 (1:200)	SIGMA Chemical (St. Louis, MO)
Oxytocin receptor	2F8	Mouse IgM (1:1000)	ROHTO Pharmaceutical (Osaka, Japan)

[a] FACS, fluorescence-activated cell sorting; PE, phycoerythrin; IgG, immunoglobulin G; FITC, fluorescein isothiocyanate; APC, allophycocyanin; IgM, immunoglobulin M.

Immunofluorescence and Confocal Microscopy

Immunofluorescence analyses were performed on glass coverslips placed in the culture dish for in vitro colony assay or cryosections derived from the right uterine horns transplanted with DP/Lin^-, non-DP/Lin^-, or Lin^+ fractions. Glass slides onto which the sections were mounted were fixed with 4% paraformaldehyde for 20 min and washed with PBS, followed by permeabilization with 0.2% Triton X-100 in PBS for 10 min. After blocking with 10% FBS for 60 min, slides were successively stained with various antibodies as listed in Table 1, followed by incubation with secondary antibodies conjugated with Alexa Fluor 488, 555, or 647 (Life Technologies) to visualize the primary antibodies. Nuclei were stained using 4′,6-diamidino-2-phenylindole dihydrochloride (Vector) or TOTO3 (Life Technologies). Anti-human vimentin (Vm) antibody (V9) recognizes only human Vm [8, 15, 16]. Images were collected using an inverted Leica DMIRE2 fluorescent microscope (Leica Microsystems) equipped with a charge-coupled device camera (VB-700; Keyence) and a Leica TCS SP2 confocal microscopy system (Leica Microsystems).

Quantification of Human Cells in Chimeric NOG Mice by Real-Time PCR

This analysis was performed on the left uterine horns transplanted with DP/Lin^-, non-DP/Lin^-, or Lin^+ fractions. The left uterine horn was immediately cut manually into small pieces of less than one mm^3, which were then incubated for 4 h in Dulbecco-modified Eagle medium containing 0.2% (w/v) collagenase (Wako), 0.05% DNase I (Life Technologies), 1% (v/v) antibiotic-antimycotic mixture (Life technologies), 10% (v/v) FBS and 10 mM HEPES buffer solution (Life Technologies) at 37°C on a shaker. Then, the digested tissue was filtered through a sterile 400 μm polyethylene mesh filter to remove particulates and then through a 40 μm cell strainer. Genomic DNA was harvested from 5 × 10^5 cells. The assay is based on separate amplification of the human specific endogenous retroviral sequence ERV-3 [17]. LightCycler PCR (Roche) was performed in a final reaction volume of 20 μl including 2 μl LightCycler-Fast Start DNA Master Hybridization Probes mix (Roche).

TABLE 2. List of primer sets used in this study.

Gene name (symbol)	Primer sets	Accession no.
Estrogen receptor 1 (ER alpha) (*ESR1*)	5′-ACAAGCGCCAGAGAGATGAT-3′ 5′-CAGATTCATCATGCGGAACC-3′	NM_000125
Estrogen receptor 2 (ER beta) (*ESR2*)	5′-GCCTTAATTCTCCTTCCTCC-3′ 5′-TACATCCTTCACACGACCAG-3′	NM_001214902
Progesterone receptor (*PGR*)	5′-GACTGAGCTGAAGGCAAAGG-3′ 5′-TCCAAGACACTGTCCAGCAG-3′	NM_000926
Calponin 1, basic, smooth muscle (*CNN1*)	5′-AAGTATGACCACCAGCGGGAGCAG-3′ 5′-TAGGCGGAATTGTAGTAGTTGTGT-3′	NM_001299
Smoothelin (*SMTN*)	5′-GCTGAGGAGCTGATGACTAT-3′ 5′-TTGAGAAGCTGGAGAAGGAG-3′	NM_134270
ATP-binding cassette, sub-family G (WHITE), member 2 (*ABCG2*)	5′-GGCCTCAGGAAGACTTATGT-3′ 5′-AAGGAGGTGGTGTAGCTGAT-3′	NM_004827
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	5′-TCACCATCTTCCAGGAGCG-3′ 5′-CTGCTTCACCACCTTCTTGA-3′	NM_002046
Bone gamma-carboxyglutamate (gla) protein (*BGLAP*)	5′-ATGAGAGCCCTCACACTCCTC-3′ 5′-GCCGTAGAAGCGCCGATAGGC-3′	NM_199173
Runt-related transcription factor 2 (*RUNX2*)	5′-GTGGACGAGGCAAGAGTTTCA-3′ 5′-TGGCAGGTAGGTGTGGTAGTG-3′	NM_004348
Alkaline phosphatase, liver/bone/kidney (*ALPL*)	5′-TGGAGCTTCAGAAGCTCAACACCA-3′ 5′-ATCTCGTTGTCTGAGTACCAGTCC-3′	NM_000478
Collagen type I, alpha 2 (*COL1A2*)	5′-GGACACAATGGATTGCAAGG-3′ 5′-TAACCACTGCTCCACTCTGG-3′	NM_000089
Integrin-binding sialoprotein (*IBSP*)	5′-AATGAAAACGAAGAAAGCGAAG-3′ 5′-ATCATAGCCATCGTAGCCTTGT-3′	NM_004967
Parathyroid hormone 1 receptor (*PTH1R*)	5′-AGGAACAGATCTTCCTGCTGCA-3′ 5′-TGCATGTGGATGTAGTTGCGCGT-3′	NM_000316
Lipoprotein lipase (*LPL*)	5′-GAGATTTCTCTGTATGGCACC-3′ 5′-CTGCAAATGAGACACTTTCTC-3′	NM_000237
Peroxisome proliferator-activated receptor gamma (*PPARG*)	5′-GCTGTTATGGGTGAAACTCTG-3′ 5′-ATAAGGTGGAGATGCAGGCTC-3′	NM_005037
Aggrecan (*ACAN*)	5′-TGAGGAGGGCTGGAACAAGTACC-3′ 5′-GGAGGTGGTAATTGCAGGGAACA-3′	NM_001135
Collagen, type II, alpha 1 (*COL2A1*)	5′-CAGGTCAAGATGGTC-3′ 5′-TGCAGCACCTGTCTCACCA-3′	NM_001844
Endogenous retroviral sequence 3 (*ERV3*)	5′-CATGGGAAGCAAGGGAACTAATG-3′ 5′-CCCAGCGAGCAATACAGAATTT-3′	NM_001007253

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Cycling conditions were 10 min at 95°C followed by 45 cycles of 8 sec at 95°C, 8 sec at 51°C, and 8 sec at 72°C.

Statistical Analysis

Each experiment was repeated using cells from at least three subjects followed by statistical analysis. All of the data were expressed as the mean ± SD. Analyses were performed using the Student *t*-test and a Kruskal-Wallis test followed by the Sheffe *F* test. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Isolation and Characterization of the Human Myometrial Doubly Positive for CD49f and CD34 (DP/Lin$^-$) Fraction

We first analyzed cell surface antigens of myoSP and myoMP fractions and found that CD49f and CD34 were preferentially expressed on myoSP cells (Supplemental Fig. S1; Supplemental Data are available online at www.biolreprod.org). Because stem/progenitor cells are immature and undifferentiated cells, we eliminated mature and/or differentiated cells such as leukocytes, endothelial cells, and red blood cells from human dissociated myometrial cells using antibodies against CD31, CD45, and GlyA, respectively, as lineage markers (Fig. 1A, left). Lin$^-$ (CD31$^-$/CD45$^-$/GlyA$^-$) cells were then subjected to double staining for CD34 and CD49f (Fig. 1A, right) and divided into four fractions for flow cytometric analysis (Fig. 1A, fractions I–IV). Each fraction was then sorted and subjected to Hoechst dye staining. Separation of the myoSP cells was blocked by the addition of 50 μM reserpine, an ABCG2 blocker. SP cells were exclusively enriched in DP/Lin$^-$ cells that constituted 2.22 ± 0.99% (mean ± SD) of total viable cells (Fig. 1B). Consistent with our previous results on myoSP and myoMP cells, DP/Lin$^-$ cells preferentially expressed SP-associated marker *ABCG2*, whereas they hardly expressed ovarian steroid hormone receptors (*ESR1* and *PGR*) and smooth muscle cell differentiation markers, including *SMTN* and *CNN1* (Fig. 1C). We did not find differences in the expression of ESR2. Thus, DP/Lin$^-$ cells exhibited a less differentiated phenotype characteristic of tissue-specific stem cells. We confirmed that isolated DP/Lin$^-$ cells proliferated poorly under normoxia but markedly better under hypoxic condition (Fig. 1D), which is consistent with our previous study showing that myoSP cells grow efficiently under hypoxia [8]. Stem cells are able to clonally expand from a single cell to form a colony [18]. Colony assays revealed that a single cell derived from the DP/Lin$^-$ fraction grew into a large colony 14 days after it adhered to the dish (Fig. 1E). Clonally expanded cells were positive for the smooth muscle cell differentiation marker, α-smooth muscle actin (ACTA2) (Fig. 1F). DP/Lin$^-$ cells gave rise to significantly more colonies than either non-DP/Lin$^-$ cells or unfractionated myometrial cells (Fig. 1G). In summary, DP/Lin$^-$ cells prefer a hypoxic environment for proliferation, they possess a high clonogenic activity, and they spontaneously differentiate into smooth muscle cells. Thus, they have the properties expected for human myometrial-specific stem cells.

Multipotential Differentiation of the DP/Lin$^-$ Population

We examined the potential of DP/Lin$^-$ cells to undergo multilineage differentiation. We found that DP/Lin$^-$ cells (but neither non-DP/Lin$^-$ nor Lin$^+$ cells), differentiated into osteoblasts (Fig. 2A), adipocytes (Fig. 2B), and chondrocytes (Fig. 2C) under appropriate inducing conditions, as judged by morphology, specific staining patterns, and expression of the corresponding terminal differentiation marker genes. DP/Lin$^-$ cells also differentiated into myometrial smooth muscle cells (Fig. 3, B, C, and E). The plasticity and multidifferentiation abilities are characteristics of tissue-specific stem cells. As the positive control, we subjected hMSCs to similar in vitro differentiation assays and found that they successfully differentiated into adipocytes, osteoblasts, and chondrocytes (Supplemental Fig. S2). The multilineage differentiation potential of DP/Lin- cells was comparable to that of hMSCs.

In Vivo Reconstitution of Human Myometrial Tissues from DP/Lin$^-$ Cells in Pregnant and Nonpregnant Uteri of NOG Mice

To investigate the in vivo myometrial reconstitution potential of the DP/Lin$^-$ population, we transplanted DP/Lin$^-$, non-DP/Lin$^-$, or Lin$^+$ cells into the uteri of NOG mice. Four to five weeks after transplantation, the NOG mice were divided into three groups: no further intervention (Cx), implantation of 17β-estradiol pellet(s) (E_2), or mating with male mice to conceive (Preg) (Fig. 3A). Uterine horns were excised from Cx and E_2 mice 18.5 days later or 18.5 days after confirmation of the plug (Fig. 3A). They were then subjected to immunofluorescence staining and confocal microscopy. Human vimentin (Vm)-positive cells were present in all of the uteri obtained from the Cx (Fig. 3B), E_2 (Fig. 3C), and Preg (Fig. 3E) groups transplanted with DP/Lin$^-$. In contrast, no or very few Vm$^+$ cells were found in E_2 (Fig. 3D) group transplanted with non-DP/Lin$^-$ cells. Mature human myometrial cells that coexpressed Vm and αSMA (yellow-colored cells) were abundant in the E_2 and Preg groups as compared to the Cx group transplanted with DP/Lin$^-$ (Fig. 2, B, C, and E). Upregulation of oxytocin receptors (OTR) is associated with activated myometrium during late pregnancy and labor in humans and mice [19]. Human myometrial cells doubly positive for αSMA and Vm in the pregnant uteri contained a large number of OTR-positive cells (Fig. 3E) as compared to the nonpregnant uteri (Fig. 3, B and C). To quantify the amount of human-derived reconstituted tissues, we employed real-time PCR to assess human-specific endogenous retroviral sequence ERV3 [17] using genomic DNA extracted from xenotransplanted mouse uteri. The human specific ERV-3 PCR product was significantly more abundant in pregnant uteri transplanted with DP/Lin$^-$ than in the nonpregnant uteri (Fig. 4A). Furthermore, the amount of ERV3 PCR products was E_2 dose-dependent in the uteri of ovariectomized NOG mice transplanted with DP/Lin$^-$, but not in non-DP/Lin$^-$ or Lin$^+$ fractions (Fig. 4B).

DISCUSSION

In this study we identified cell surface markers, CD49f and CD34, to permit selection of the stem/progenitor cell-like population from human myometrium. CD49f has been demonstrated as a useful surface marker to isolate single human hematopoietic stem cells capable of long-term multilineage engraftment [10, 20]. CD49f has also been suggested as a potential marker of cancer stem cells, including human glioblastoma and liposarcoma [21, 22]. Also, increasing bodies of evidence indicate that CD34 is a common marker for diverse stem/progenitor cells not only in hematopoietic cells but also in several other nonhematopoietic cell types, including vascular endothelial progenitors, embryonic fibroblasts, MSCs, interstitial dendritic cells, and epithelial progenitors [23]. Very recently, uterine leiomyoma stem cells have been identified as cells positive for both CD34 and CD49b, which is integrin α2/ITGA2, similar to but distinct from CD49f/integrin α6/

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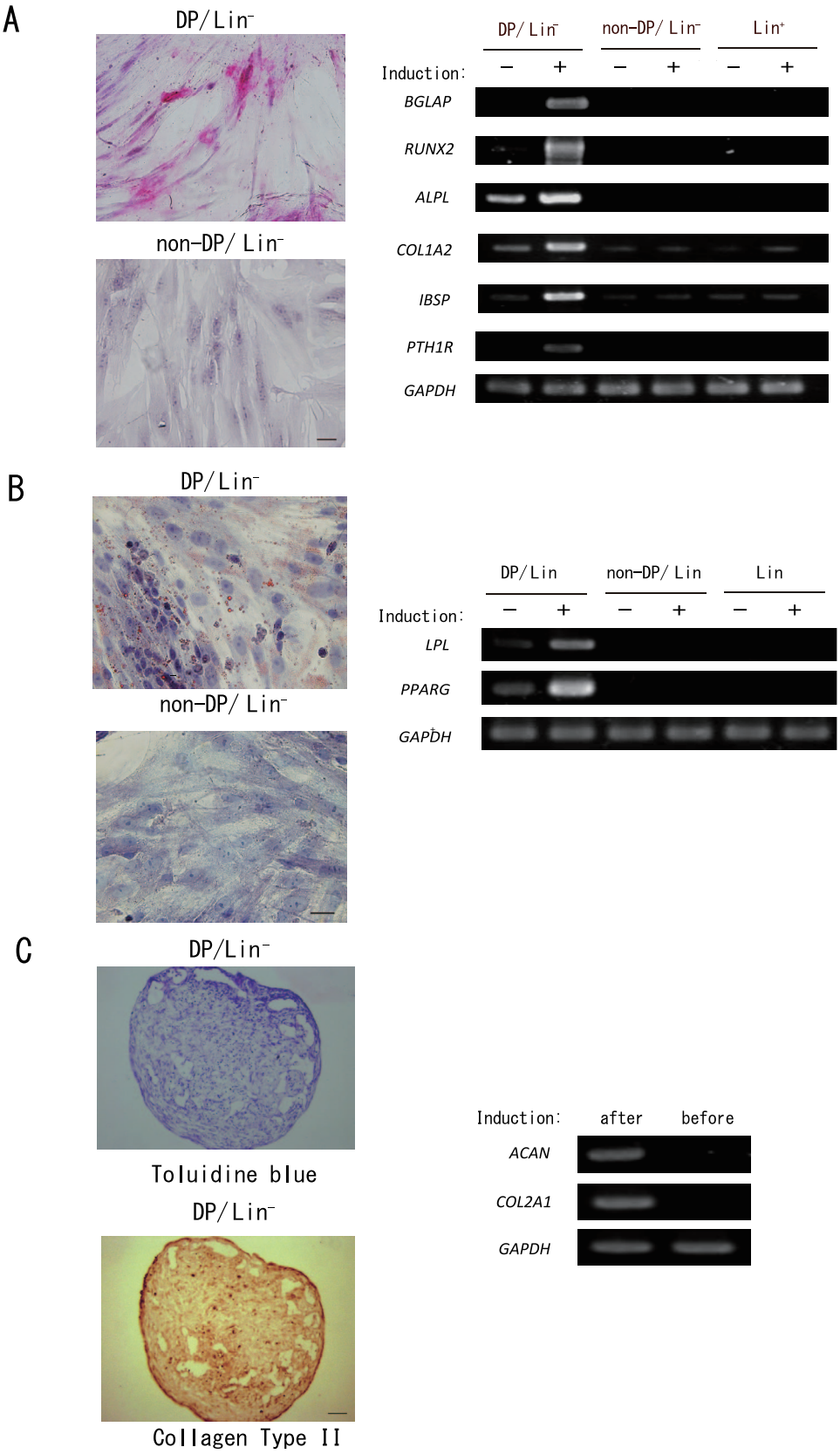

FIG. 2. Multipotential differentiation of the DP/Lin$^-$ population. **A**) Osteoblast-differentiation capacity of the DP/Lin$^-$ population. Neither non-DP/Lin$^-$ nor Lin$^+$ treated with (+) or without (–) osteoblast-inducing medium differentiated to the osteoblast lineage as determined by alkaline phosphatase staining and by RT-PCR for the expression of osteoblast lineage-specific genes. *BGLAP*, bone gamma-carboxyglutamate protein; *RUNX2*, runt-related transcription factor 2; *ALPL*, alkaline phosphatase, liver/bone/kidney; *COL1A2*, collagen type I, alpha 2; *IBSP*, integrin-binding sialoprotein; *PTH1R*, parathyroid hormone 1 receptor. Representative of three independent experiments. Bar = 50 μm. **B**) Adipocyte-differentiation capacity of DP/Lin$^-$ cells. Neither non-DP/Lin$^-$ nor Lin$^+$ treated with (+) or without (–) adipocyte-inducing medium differentiated to the adipocyte lineage, as determined by Oil red-O staining and by RT-PCR for the expression of adipocyte lineage-specific genes. *LPL*, lipoprotein lipase; *PPARG*, peroxisome proliferator-activated

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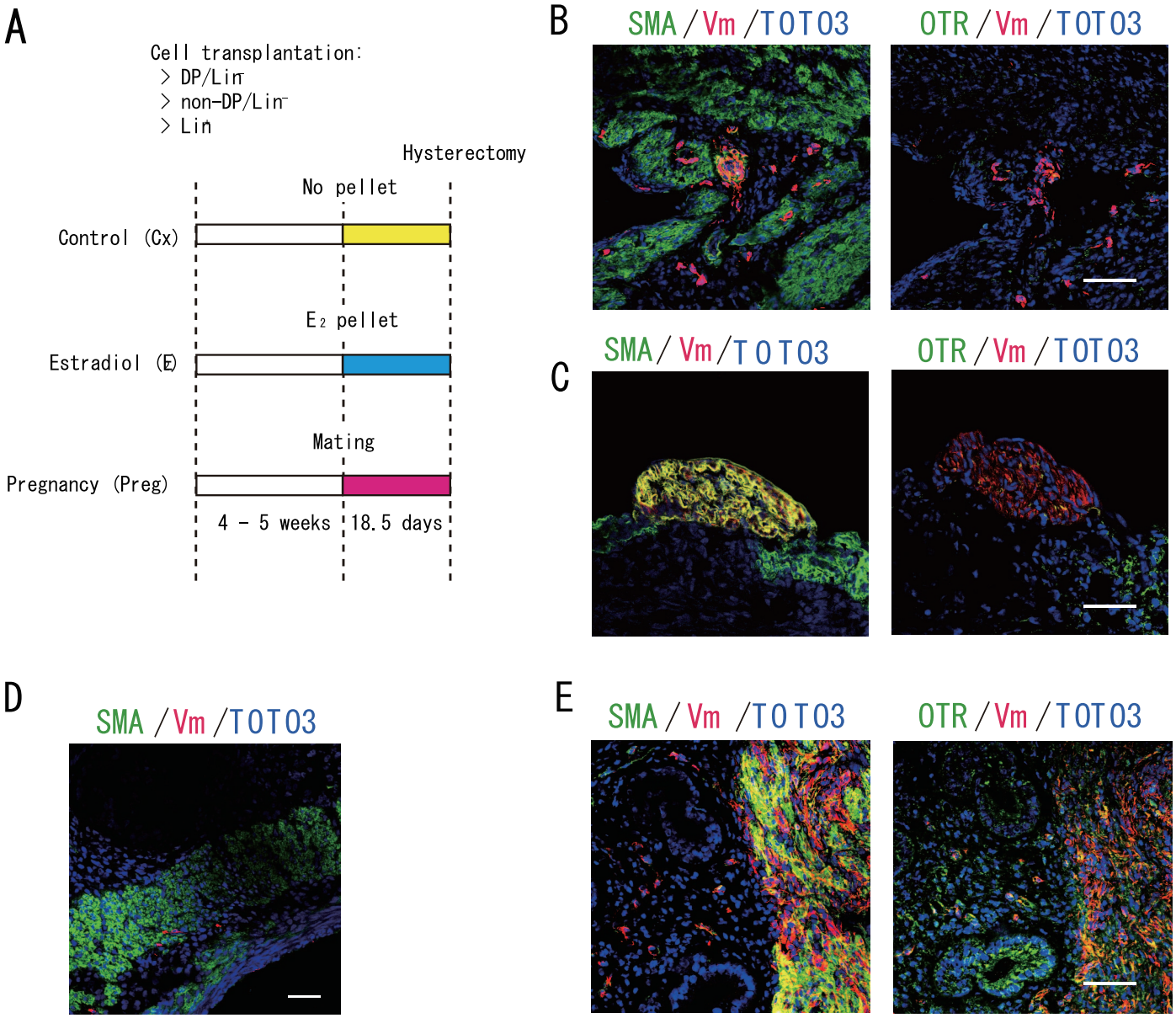

FIG. 3. In vivo reconstitution of human myometrial tissues from DP/Lin$^-$ cells in pregnant and nonpregnant uteri of NOG mice. **A**) Experimental design for in vivo transplantation analysis. **B**–**E**) Immunofluorescence and confocal microscopy of serial sections obtained from NOG mouse uteri transplanted with DP/Lin$^-$ (**B**, **C**, **E**) or non-DP/Lin$^-$ (**D**) that then received no further treatment (**B**), treatment with E_2 (**C**, **D**), or mating (**E**). These sections were immunostained for αSMA, Vm, OTR, or with TOTO3. Representative of three independent experiments. Bars = 100 μm.

ITGA [11]. Thus, these facts collectively substantiate the validity of CD49f and CD34 as potential cell surface markers for stem/progenitor-like cells in myometrium, although the biological significance and relevance of differential expression of CD49b and CD49f between myometrial and leiomyoma stem/progenitor cells remain to be elucidated.

Recently, telocytes have been described as a new cell type in the interstitial space of organs, including myometrium [24–28]. Cretoiu et al. reported that telocytes are found to be positive for CD34 and platelet-derived growth factor receptor, alpha polypeptide (PDGFRA) in myometrium [25, 29]. A growing bodies of evidence showed that telocytes could be located around stem-cell niches and related to stem cells [30]. Telocytes have been also proposed to act as progenitor cells for the development of myocardial cells in normal and/or injured heart [31]. Because telocytes and our DP/Lin$^-$ cells share CD34 positivity, it is tempting to speculate that these two myometrial cell types may have some interaction and share some common characteristics in myometrium.

A further novel finding is that DP/Lin$^-$ cells proliferate under hypoxic conditions and, more significantly, participate in estrogen-dependent and pregnancy-induced uterine enlargement and remodeling under possible hypoxic conditions. Hypoxic conditions promote the growth of many types of

receptor gamma. Representative of three independent experiments. Bar = 30 μm. **C**) Chondrocyte-differentiation capacity of DP/Lin- cells. Induction of chondrocyte differentiation of DP/Lin$^-$, as determined by staining with toluidine blue or collagen type II and by RT-PCR for the expression of chondrocyte lineage-specific genes as indicated. *ACAN*, aggrecan; *COL2A1*, collagen, type II, alpha 1. Representative of three independent experiments. Bar = 50 μm.

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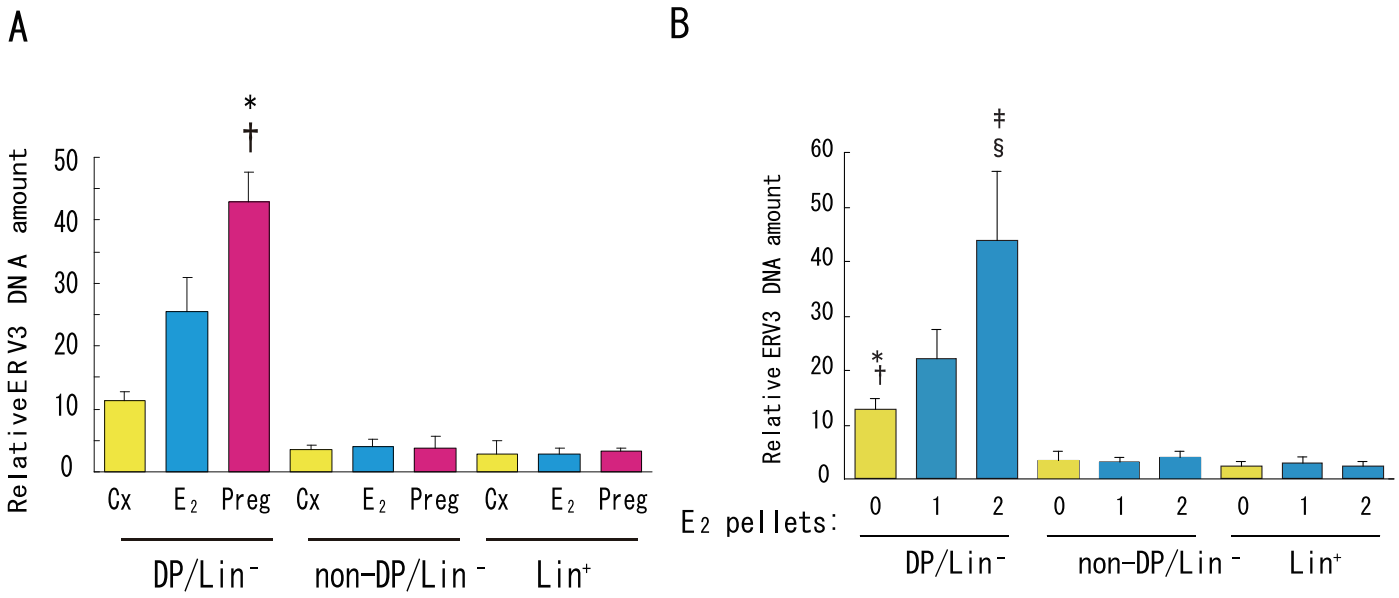

FIG. 4. Quantification of human-derived cells in NOG mouse uteri. **A**) Quantification of human-derived cells in nonpregnant, E_2-treated, or pregnant NOG mouse uteri xenotransplanted with DP/Lin$^-$, non-DP/Lin$^-$, or Lin$^+$ using real-time PCR amplification for human *ERV3* retroviruses. Each bar indicates the mean + SEM of the relative amount of ERV3 PCR products obtained from three independent experiments. *$P < 0.05$ versus DP/Lin$^-$ (Cx); †$P < 0.05$ versus DP/Lin$^-$ (E_2). **B**) The uteri of ovariectomized NOG mice were transplanted with each indicated fraction, treated with or without one or two E_2 pellets, excised 7 wk later, and subjected to real-time PCR amplification for human ERV3 retroviruses. Each bar indicates the mean + SEM of the relative amount of ERV3 PCR products obtained from three independent experiments. *$P < 0.05$ versus non-DP/Lin$^-$(0); †$P < 0.05$ versus Lin$^+$ (0); ‡$P < 0.05$ versus DP/Lin$^-$ (0); §$P < 0.05$ versus DP/Lin$^-$ (1).

stem cells, including embryonic stem cells and MSCs [32]. Indeed, myoSP cells that exhibit myometrial stem cell-like properties can only proliferate efficiently in vitro under hypoxia [8]. Consistent with those observations, like myoSP, isolated DP/Lin$^-$ cells proliferated poorly under normoxia but markedly better under hypoxia (Fig. 1D). Shynlova et al. demonstrated that mechanical stretch of the uterine wall induces hypoxia in the rat myometrium during pregnancy [3]. Thus, it is conceivable that hypoxia resulting from pregnancy-induced mechanical stretching may promote the proliferation of DP/Lin$^-$ cells, leading to the contribution of DP/Lin$^-$ cells to pregnancy-induced uterine enlargement and remodeling.

In addition to hypoxia, E_2 and progesterone (P_4) may be important to upregulate the proliferation of DP/Lin$^-$ cells in the pregnant myometrium. Because DP/Lin$^-$ cells underexpressed *ESR1* and *PGR* (Fig. 1C), it is possible that more differentiated myometrial cells may produce as yet unidentified factors in response to E_2 and/or P_4, which, in turn, may promote DP/Lin$^-$ cell proliferation in a paracrine manner. In support of this idea, an indirect paracrine effect of steroid hormones on stem cells via the mature neighboring cells have been suggested in the pathogenesis of leiomyoma [33–35]. Intriguingly, hypoxia as well as E_2 and/or P_4 are involved in the pathogenesis of leiomyomas [36]. Recently, it has been reported that SP cells are also present in leiomyoma [33, 37, 38] and that leiomyoma SP cells may have the potential to behave as leiomyoma-initiating cells [33, 38]. Because DP/Lin$^-$ cells exclusively contain SP cells (Fig. 1B), it is conceivable that DP/Lin$^-$ cells might be more primitive leiomyoma-initiating cells.

In conclusion, we identified CD49f and CD34 as markers to permit selection of the stem/progenitor cell-like population from human myometrium. The newly identified myometrial stem/progenitor-like cells proliferated preferentially by hypoxia in vitro and sex steroids in vivo and further showed greater expansion in the mouse pregnant myometrium upon xeno-tranplantation, suggesting the involvement of myometrial stem/progenitor cells in sex steroid- and pregnancy-induced uterine enlargement and remodeling. Further studies, however, are needed to investigate the function of these cells in myometrium as well as in pathologic conditions.

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