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Human Placenta-Derived Cells Have Mesenchymal Stem/Progenitor Cell Potential

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ABSTRACT

Mesenchymal stem/progenitor cells (MSCs) are widely distributed in a variety of tissues in the adult human body (e.g., bone marrow [BM], kidney, lung, and liver). These cells are also present in the fetal environment (e.g., blood, liver, BM, and kidney). However, MSCs are a rare population in these tissues. Here we tried to identify cells with MSC-like potency in human placenta. We isolated adherent cells from trypsin-digested term placentas and established two clones by limiting dilution. We examined these cells for morphology, surface markers, gene expression patterns, and differentiation potential and found that they expressed several stem cell markers, hematopoietic/endothelial cell–related genes, and organ- specific genes, as determined by reverse transcription– polymerase chain reaction and fluorescence-activated cell sorter analysis. They also showed osteogenic and adipogenic differentiation potentials under appropriate conditions. We suggest that placenta-derived cells have multilineage differentiation potential similar to MSCs in terms of morphology, cell-surface antigen expression, and gene expression patterns. The placenta may prove to be a useful source of MSCs. *Stem Cells 2004;22:649–658*

INTRODUCTION

Multipotential mesenchymal stem/progenitor cells (MSCs) can be induced to differentiate into bone, adipose, cartilage, muscle, and endothelium if these cells are cultured under specific permissive conditions [1, 2]. In rodents, a specific type of MSC (termed multipotent adult progenitor cell) can be isolated from bone marrow (BM) and contributes to most somatic cell types when injected into early blastocysts at the single-cell level [3]. Because MSCs have unique immunologic characteristics that suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo [4], persistence in a xenogeneic environment is favored [1]. With such multiple differentiation capacities and unique immunoregulatory features plus self-renew potential [5], MSCs show promise as a possible therapeutic agent. Data from preclinical transplantation studies suggested that MSC infusions not only prevent the occurrence of graft failure but also have immunomodulatory effects [6].

MSCs are a rare population (approximately 0.001%–0.01%) of adult human BM [7]. Moreover, numbers of BM MSCs significantly decrease with age [8]. MSCs are also relatively few in adult peripheral blood [9] and in term cord

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blood [10]. A recent study showed that the population of MSC-like cells exists within the umbilical vein endothelial/subendothelial layer [11]. Furthermore, MSCs are present in fetal organs, such as liver, BM, and kidney, and circulate in the blood of preterm fetuses [10, 12, 13]. However, fetal samples can be difficult to procure, and term cord blood compared with preterm is a poor source of MSCs [10–12]. Such being the case, searching for appropriate sources, avoiding ethical issues, and establishing suitable culture systems are a challenge.

In this study, we evaluated the possibility that MSCs or cells with MSC-like potency are present in the human term placenta, and we obtained evidence that cells with the phenotype of MSCs exist in this tissue.

Materials and Methods

Isolation and Culture of Placenta-Derived Cells

Term placentas (*n* = 57; clinically normal pregnancies, caesarean section) were collected after obtaining written informed consent from donors to the Tokyo Cord Blood Bank.

The internal area (approximately 1 cm^3) of central placenta lobules was minced, hemolyzed, trypsinized (37°C for 5 minutes), and finally prepared in both single-cell suspensions and small digested residues. These samples were cultured with α-minimum essential medium (MEM; Sigma-Aldrich Co., St. Louis, http://www.sigmaaldrich.com) and supplemented with 15% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, http://www.hyclone.com), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Paisley, U.K., http://www.invitrogen.com). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. Three to 5 days after initiating incubation, the small digested residues were removed and the culture was continued. Approximately 3 to 4 weeks later, there were some colonies that contained 50 or more fibroblast-like cells that were more than 50% confluent; they were then trypsinized using 0.05% trypsin (Invitrogen) and replated at a 1:4 dilution. Under the same conditions, placenta-derived cells were continued to culture.

Fluorescence In Situ Hybridization Analysis

Human X/Y chromosomes of placenta-derived cells (male, *n* = 3; female, *n* = 3; passages two and three) were cultured on silica-coating slides and examined using CEP X/Y DNA probe kits (Vysis, Inc., Downers Grove, IL, http://www.vysis.com) according to the manufacturer's instructions. The slides were scanned under a fluorescence microscope using a rhodamine/fluorescein isothiocyanate (FITC) filter for X/Y chromosomes and a UV filter for 4',6-diamidine-2'-phenylindole dihydrochloride–stained cell nuclei.

Fluorescence-Activated Cell Sorter Analysis

Frozen and thawed placenta-derived cells (*n* = 3, passages 9–12) were trypsinized and incubated with medium containing 15% FBS-2 mM EDTA (pH 8.0) for 3 hours. Next the cells were stained with anti-human specific antibodies CD45-phycoerythrin (PE), CD31-PE, CD54-PE, CD29-FITC or CD29-PE, CD44-FITC or CD44-PE (BD Pharmingen, San Diego, http://www.bdbiosciences.com), AC133/1-PE (Miltenyi Biotec GmbH, Germany, http://www.miltenyibiotec.com), or PE- or FITC-conjugated isotype control (BD Biosciences, San Jose, CA, http://www.bd.com). After staining, cells were analyzed using fluorescence-activated cell sorter (FACS) Calibur flow cytometry (Becton, Dickinson, Mountain View, CA).

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction

Total RNA from 10^5–10^6 placenta-derived cells (*n* = 15, passages 2–18, including frozen and thawed samples) was isolated using ISOGEN (Nippon Gene, Tokyo). RNA extracts were treated with deoxyribonuclease I (Amplification Grade, Invitrogen) for digesting contaminated genomic DNA.

Reverse transcription (RT) reactions were carried out on 1 μg of total RNA using the ThermoScript™ RT–polymerase chain reaction (PCR) system (Invitrogen), and 40 cycles of PCR were run using the Platinum PCR SuperMix (Invitrogen) according to the manufacturer's instructions. Evaluation of all PCRs was estimated using appropriate human tissue RNA (Clontech Laboratories, Inc., Palo Alto, CA, http://www.clontech.com), human BM-derived MSCs (Bio Whittaker, Inc., Walkersville, MD), and human cell lines [14, 15]. cDNA synthesis and genomic DNA contamination were examined using HOXB4 primers, which give products of 268 bp and 1.1 kb when amplifying cDNA and genomic DNA, respectively. Human-specific primers used were as follows: Oct-4 (866 bp), CCGCCGTATGAGTTCTGTGG/AGAGTGGTGACAGAGACAGG; Rex-1 (449 bp), ATGGCTATGTGTGCTATGAGC/CCTCAACTTCTAGTGCATCC; HOXB4 (268 bp), CTACCCCTGGATGCGCAAAG/CGAGCGGATCTTGGTGTTGG; CBFβ (300 bp), TCGTGCCCGACCAGAGAAGC/TCAGAATCATGGGAGCCTTC; β2-microglobulin (341 bp), GAGTGCTGTCTCCATGTTTG/TAACCACAACCATGCCTTAC; GATA-2 and Tie-2 [16]; TAL-1 [17]; CD34, AC133, flk-1, myogenin, nestin, and α-1-fetoprotein [18]; flt-1 [19]; Nkx2.5 and GATA-4 [20]; renin and albumin [21]; GFAP [22]; and amylase and insulin [23].

Differentiation Studies

Passage 2 through 11 placenta-derived cells, including frozen and thawed samples ($n = 8$), were cultured either in an osteogenic (0.1 μM dexamethasone, 10 mM β-glycerol phosphate, 50 μM ascorbate) or adipogenic (1 μM dexamethasone, 5 μg/ml insulin, 0.5 mM isobutylmethylxanthine, 60 μM indomethacin) medium (all chemicals from Sigma) [10] on two-well Permanox slides (Nalge Nunc International, Naperville, IL). After 2 weeks, osteogenic differentiation was evaluated after 1% Alizarin Red S (Sigma) staining, and adipogenic differentiation was assessed using Oil Red O (Sigma) staining [2].

Subcloning and Characterization of Placenta-Derived Clones

The MSCV-IRES-GFP retroviral plasmid was transfected in PLAT-A packaging cells. Retroviral supernatants were collected and infected in No. 40 placenta-derived cells (passage five). The green fluorescent protein (GFP)–positive cells (passage seven) were sorted by FACS Vantage flow cytometry (Becton, Dickinson) and then subcultured at 5 or 10 cells per well (passage nine). After subcloning, we selected single retroviral-inserted subclones by Southern blot analysis using a GFP cDNA probe. Two clones were obtained, and then we carried out a FACS and RT-PCR analysis and differentiation studies for characterization of these clones.

Results

Characterization of Placenta-Derived Cells

Searching for alternative sources of MSCs, we attempted to prepare human term placentas and isolated fibroblast-like cells from every placenta isolation ($n = 57$; Fig. 1). In a single-cell suspension culture of the isolated placenta, cells firstly formed colony-forming unit fibroblast (CFU-F)–like colonies (Figs. 1A, b). On the other hand, in the culture of small trypsin-digested residues of placenta, cells began to migrate and proliferate (data not shown). After the first passage, cells from both samples expanded in the same monolayer manner (Fig. 1A, a–c). Cord blood (CB) is a rich source of hematopoietic stem cells and MSCs, and the term placenta contains much CB, primarily adherent cells derived from freshly isolated CB mononuclear cells ($n = 77$). However, CBs were obtained (after receiving the informed consent from Kiyosenomori Hospital, Tokyo) but did not survive in α-MEM containing 15% FBS. To determine whether these cells were from maternal or fetal parts of the placenta, we did a fluorescence in situ hybridization analysis using X- and Y-probes. These cells were positive for X- and Y-signals, indicating that they were from a fetal part of the placenta (Fig. 1B). The placenta-derived cells were classified into two groups according to growth characteristics; one could proliferate more than 20 passages (Fig. 1C, Nos. 40 and 29), and the other went into replicative senescence between 10 and 20 passages (Fig. 1C, Nos. 41 and 44). The former type had a small and homogeneous morphology, but the latter type was of a bigger shape than the former. We also examined the surface marker profile of the above three representative placenta-derived cell lines using FACS, and these three lines had a similar phenotype, as follows: $CD45^{low}CD31^{-}AC133^{-}CD54^{+}CD29^{+}CD44^{+}$ (Fig. 1D), which closely resembles the phenotypes of BM-derived and CB-derived MSCs [2, 7, 10, 24].

Gene Expression Patterns of Placenta-Derived Cells

For a closer study of placenta-derived cells, we did a RT-PCR analysis for various genes, including stem cell markers, hematopoietic/endothelial cell–related genes, and organ-specific genes. The placenta-derived cells expressed many of the genes derived from mesoderm, ectoderm, and endoderm (Fig. 2). Additionally, expression patterns of stem cell markers and hematopoietic/endothelial cell–related genes in placenta-derived cells were similar to those of human BM (hBM)–derived MSCs (Fig. 2, lane 2).

Differentiation Potential of Placenta-Derived Cells

To estimate the potential to differentiate into osteoblasts and adipocytes, the placenta-derived cells were cultured in osteogenic or adipogenic medium. At the end of the induction periods, most of the cells were Alizarin Red S–positive (Figs. 3B, 3C) or Oil Red O–positive (Figs. 3E, 3F), indicating differentiation to osteoblasts or adipocytes, respectively. In contrast, cells cultured with regular medium were not significantly stained (Figs. 3A, 3D). Such data indicate that the placenta-derived cells had bidirectional differentiation potency.

Subcloning of Placenta-Derived Cells

The placenta-derived cells used in the above experiments are obviously heterogeneous and may be a mixture of progenitors that can differentiate into specific lineages. To

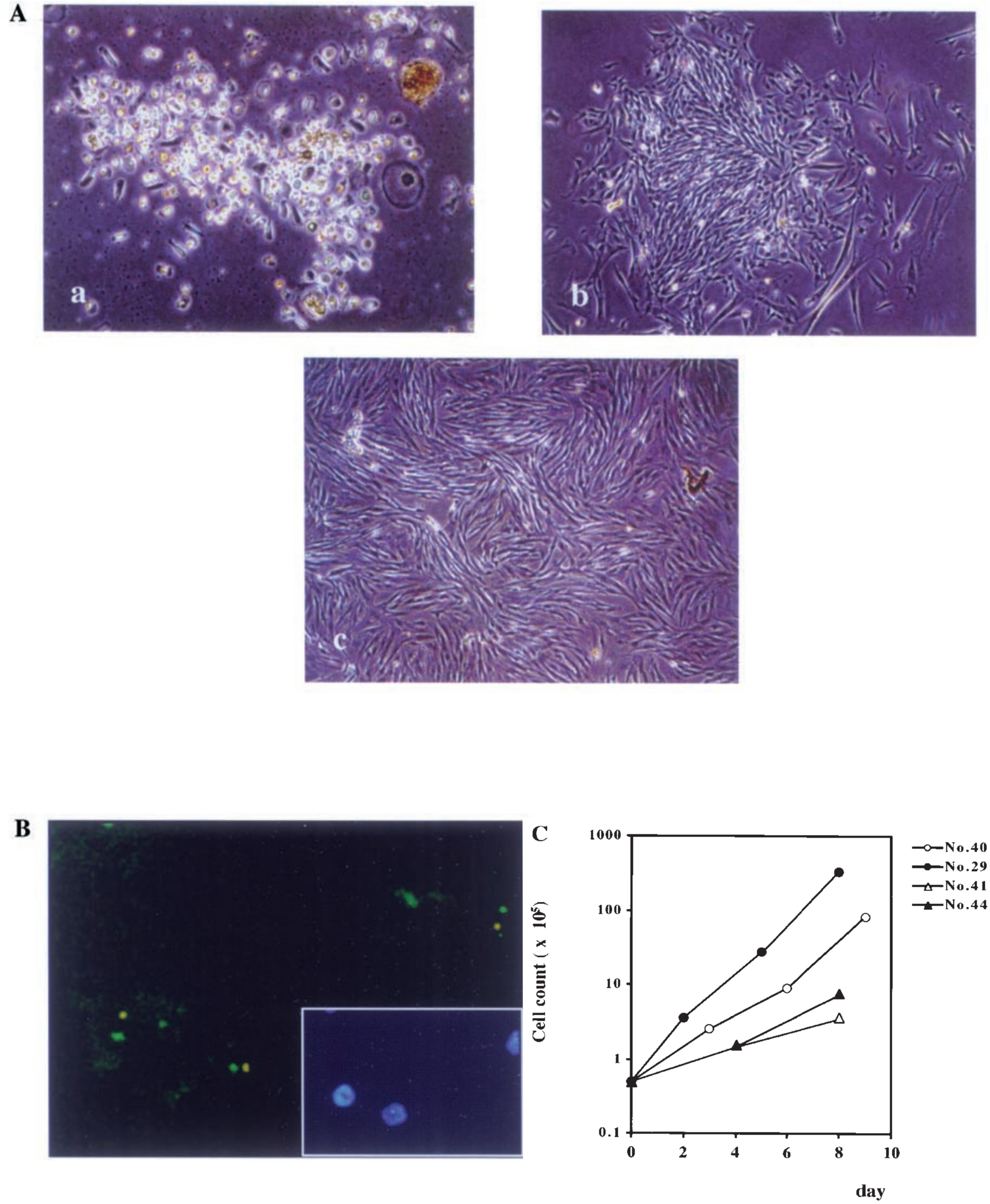

Figure 1. Isolation and characterization of placenta-derived cells. **(A):** Morphology of placenta-derived cells. Cells from a single-cell suspension easily expanded through the formation of colony-forming unit fibroblast–like colonies. a: 10 days after isolation (× 100 magnification); b: 3 weeks after isolation (× 40 magnification); c: 6 weeks after isolation (passage 3; × 40 magnification). **(B):** Fluorescence in situ hybridization analysis for human X/Y chromosomes. Cells from male placenta have Y-positive (green) and X-positive (orange) signals. **(C):** Growth curve of placenta-derived cells. Frozen and thawed cells ($n = 4$, started at passage six or seven) were seeded at 0.5×10^5 cells per well and cultured until 90% confluence was reached. These cells were resuspended, enumerated, and reseeded at the same density for 10 days. **(D):** Immunophenotype of placenta-derived cells. Cells were stained with phycoerythrin-conjugated or fluorescein isothiocyanate–conjugated antibodies against CD45, CD31, AC133, CD54, CD29, CD44, or immunoglobulin isotype control antibodies. Cells were analyzed using fluorescence-activated cell sorter Calibur. Individual placenta-derived cells were given serial numbers of placenta isolation. Representative samples were used for these figures. **(Figure 1 D continued on next page.)**

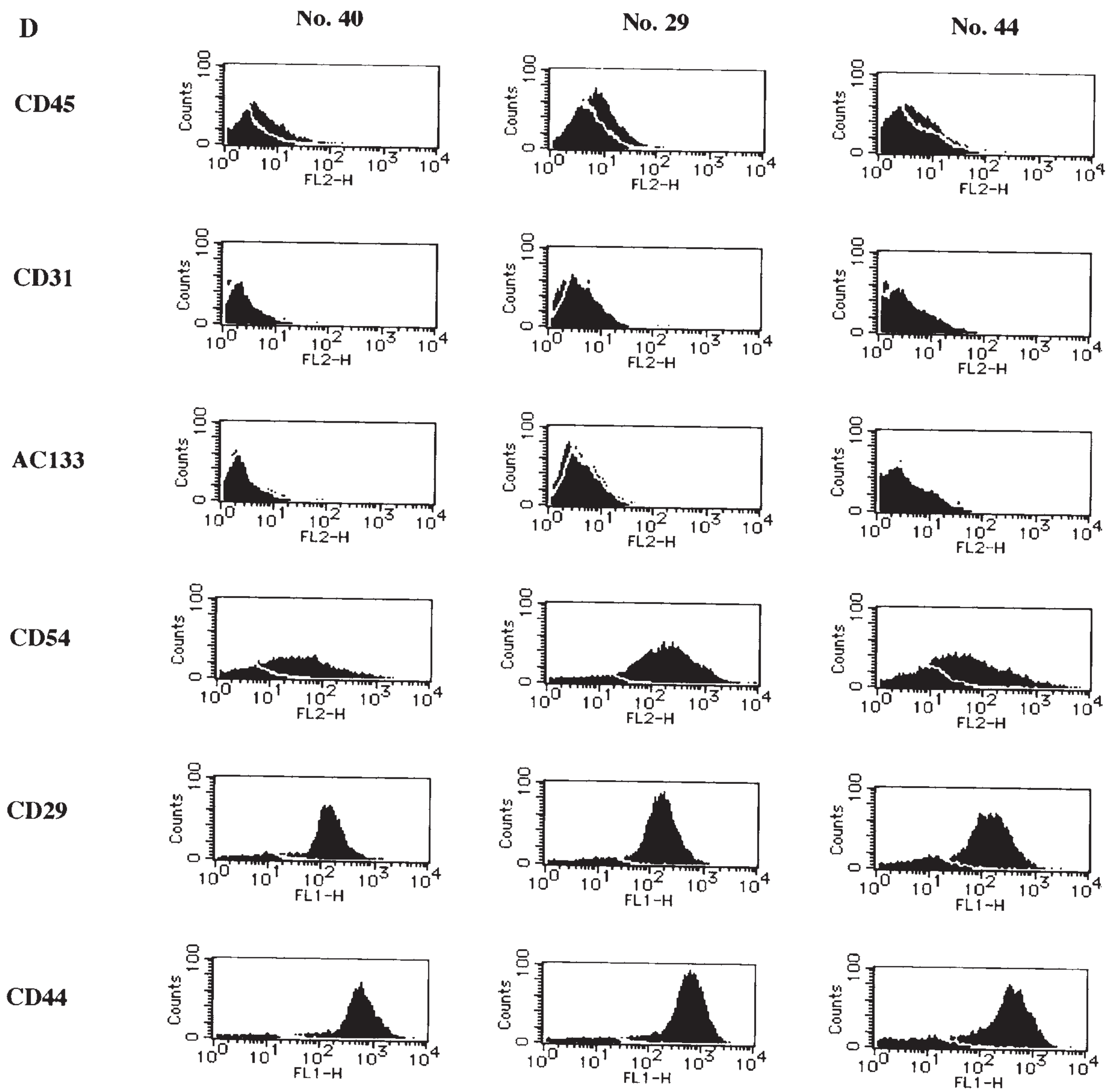

Figure 1 (continued).

exclude this possibility, we attempted to subclone No. 40 placenta–derived cells showing the human MSC (hMSC)–like gene expression pattern using RT-PCR (Fig. 2, lanes 2 and 7). We established two clones, B2 and F4 (Fig. 4A, lanes 1 and 9), which retained almost all of the phenotypes of their parental cells; surface marker expression ($CD45^{low}CD31^{-}AC133^{-}CD54^{+}CD29^{+}CD44^{+}$), gene expression patterns, and differentiation potential (Figs. 4B–4D versus Figs. 1–3). Moreover, these phenotypes were similar to those of other placenta-derived cell lines. Such data suggest that although the placenta-derived cells are considered to be polyclonal, most of the clones are similar in gene-expression profiles and retain the differentiation capacity to osteoblasts and adipocytes.

Discussion

In this study, we successfully isolated placenta-derived cells from human term placentas (n = 57) and then characterized morphology, cell-surface antigens, gene expression patterns, and differentiation capacity of these cells. Results of RT-PCR analysis of 15 individual placenta-derived cells showed that the expression patterns of seven genes (HOXB4, CD34, AC133, flk-1, Tie-2, GATA-4, and myogenin) varied but expressions of 14 other genes were quite similar (Fig. 2

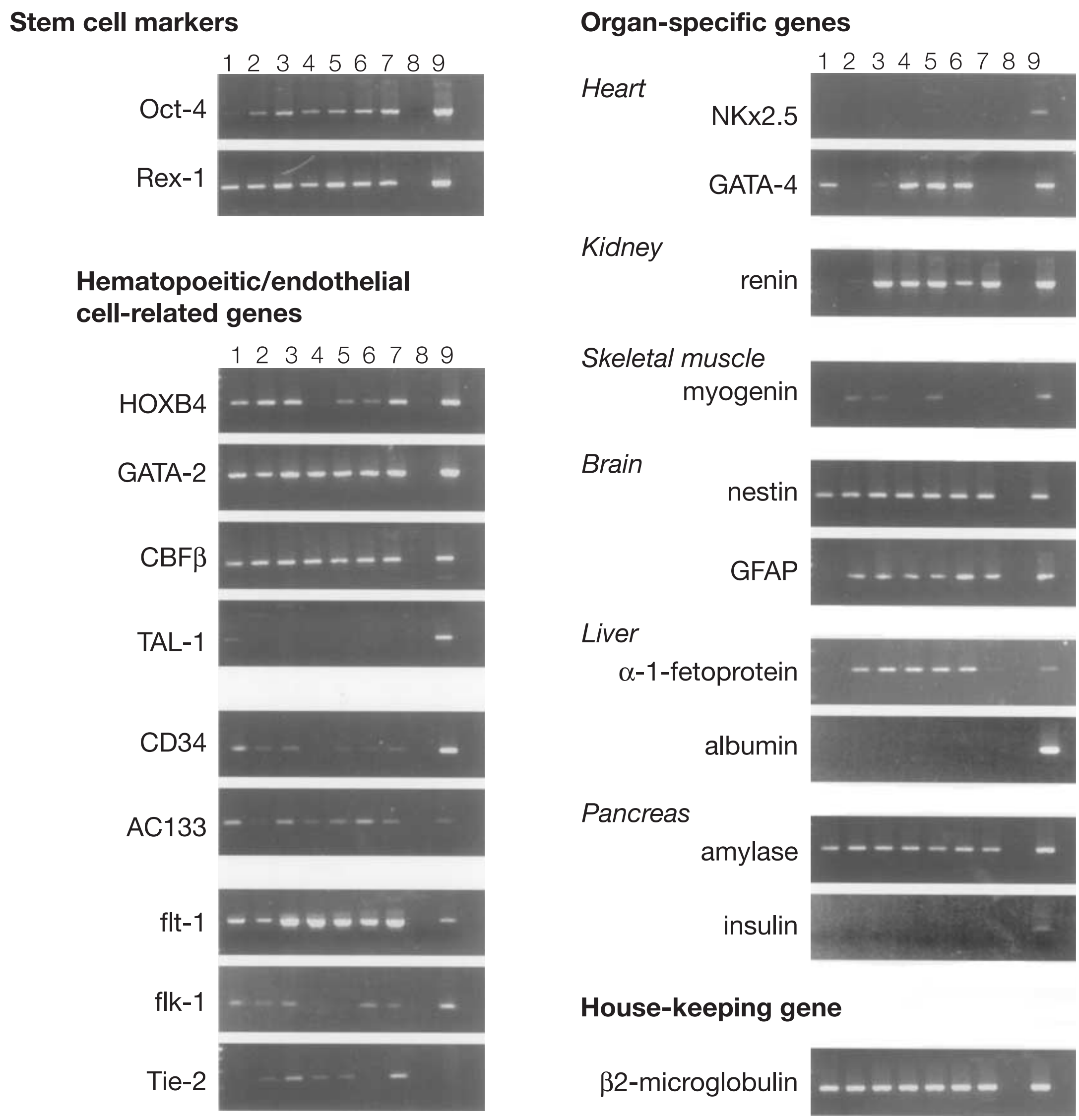

Figure 2. Gene expression patterns of placenta-derived cells. Placenta-derived cells were characterized using reverse transcription–polymerase chain reaction. Samples are as follows: lane 1, noncultured placenta (trypsin-digested residue); lane 2, human bone marrow–derived mesenchymal stem/progenitor cells; lanes 3–7, placenta (Nos. 29, 41, 42, 44, and 40)-derived cells; lane 8, reagent control; lane 9, positive control (i.e., Oct-4, Rex-1, HOXB4, and β2-microglobulin were used for EoL-3. GATA-2, CBFβ, TAL-1, CD34, AC133, flk-1, and flt-1 were used for TF-1. NKx2.5 and GATA-4 were used for human heart RNA. Renin and myogenin were used for human kidney RNA and skeletal muscle RNA, respectively. Nestin and GFAP were used for human brain RNA. α-1-fetoprotein and albumin were used for human liver RNA. Amylase and insulin were used for human pancreas RNA). In this figure, we took up the data from the five representative placenta-derived cells, and each of placenta-derived cells was shown by serial numbers of placenta isolation.

shows evidence of six placenta-derived cells; nine are not shown). These expression patterns resemble those of hBM-derived MSCs, except for renin and flt-1 (Fig. 2, lane 2). Comparison of the two types of placenta-derived cells with distinct growth characteristics (one that propagates more than 20 passages [Fig. 1C, Nos. 40 and 29] and the other with growth limitation [Nos. 41, 42, and 44]) showed that the expressions of HOXB4, CD34, Tie-2, and GATA-4 were different among these groups. The former more resembled the hBM-derived MSCs for gene expression patterns (Fig. 2). Collectively, these results indicate that the placenta-derived cells have MSC-like gene expression patterns. In addition, they showed a differentiation capacity toward both osteoblasts and adipocytes (Fig. 3), suggesting that these cells have MSC-like differentiation potential.

Because the original culture of 57 placenta-derived cell lines should be a mixture of a variety of cell types, we attempted to subclone these cells to do a detailed analysis.

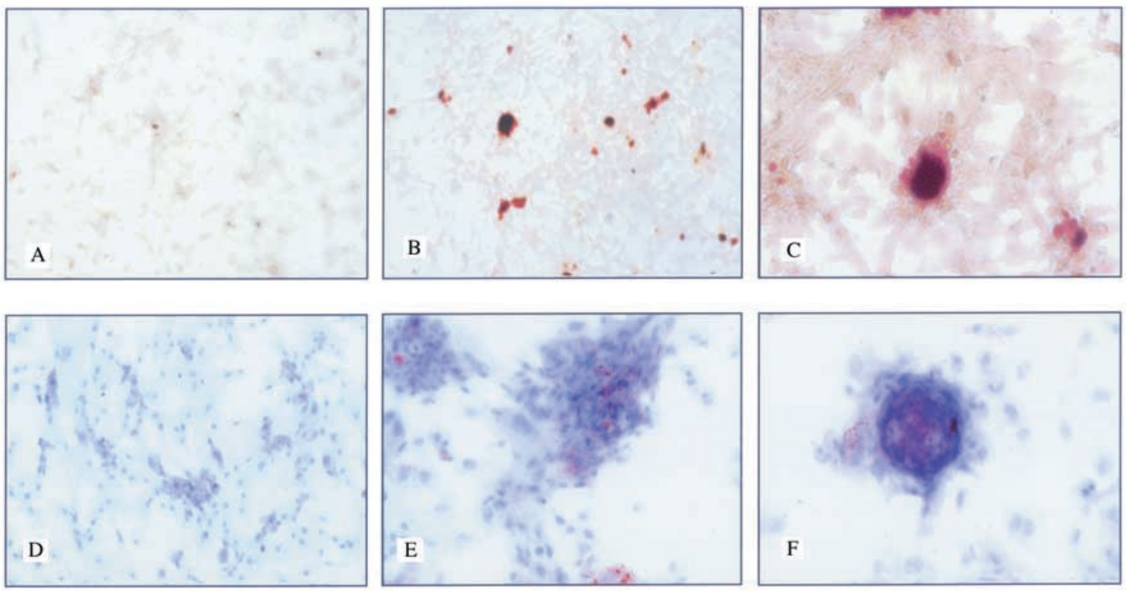

Figure 3. Differentiation potential of placenta-derived cells. After a 2-week culture in osteogenic **(B, C)** or adipogenic **(E, F)** medium or regular medium **(A, D),** each of the placenta-derived cells was evaluated for osteogenic or adipogenic differentiation using specific staining and hematoxylin counterstaining. Magnification: A, B, D, E, ×40; C, F, ×100. A representative sample was used for this figure.

Two established clones retained almost all of the phenotypes of parental No. 40 placenta-derived cells, including morphology, cell-surface populations, gene expression patterns, and differentiation capacity. However, these clones also had some differences in mRNA expression, such as CD34 and α-1-fetoprotein. These genes were upregulated compared with the parent mixture cells (Figs. 2, 4C). In some reports, small proportions of hMSCs expressed low levels of CD34 [6, 25]. Further experiments are required to determine the meaning of expressions of these genes.

Rex-1 is known to be important for maintaining undifferentiated embryonic stem cells [26, 27]. However, the role of this gene in MSCs is not clear. The result of RT-PCR analysis showed that Rex-1 is expressed in both BM-derived MSCs and placenta-derived cells (Fig. 2) but not in the two clones (Fig. 4C). Analysis of parental placenta-derived cells at various time points during passages (passages 3, 5, 9, and 18 for original cells; passages 13 and 26 for GFP-labeled mixture cells) using RT-PCR showed that only Rex-1 expression switched from positive (before passage 5, Fig. 2) to negative (after passage 9; data not shown). Additional analysis is required to know the role of Rex-1 in placenta-derived cells.

Interestingly, cell-surface markers analyzed using FACS revealed that the placenta-derived mixture cells and clones had the $CD45^{low}CD31^{-}AC133^{-}CD54^{+}CD29^{+}CD44^{+}$ phenotype (Figs. 1D, 4B), and the expression of CD45 and AC133 antigens differed from MSCs derived from other sources [2, 7, 10, 24]. As for the expression of AC133, the results were negative with FACS yet positive with RT-PCR analysis. This contradictory finding may be due to a damaged AC133 epitope by trypsin treatment of the cells. As for the expression of CD45, some reports showed that unprocessed or fresh MSCs were $CD45^{med,low}$, whereas cultured MSCs and more mature cells were $CD45^{-}$ [24, 28]. However, as our results showed, the expression of CD45 was low during passages. The $CD45^{low}$ phenotype might be one of the specific characteristics of the placenta-derived cells.

This study showed that the placenta-derived MSC-like cells could be easily isolated and expanded without morphological and characteristic changes in medium supplemented only with FBS. Therefore, the placenta may prove to be an attractive and rich source of MSCs. Further studies are required to better understand the precise nature of placenta-derived cells and to explore their potential clinical applications.

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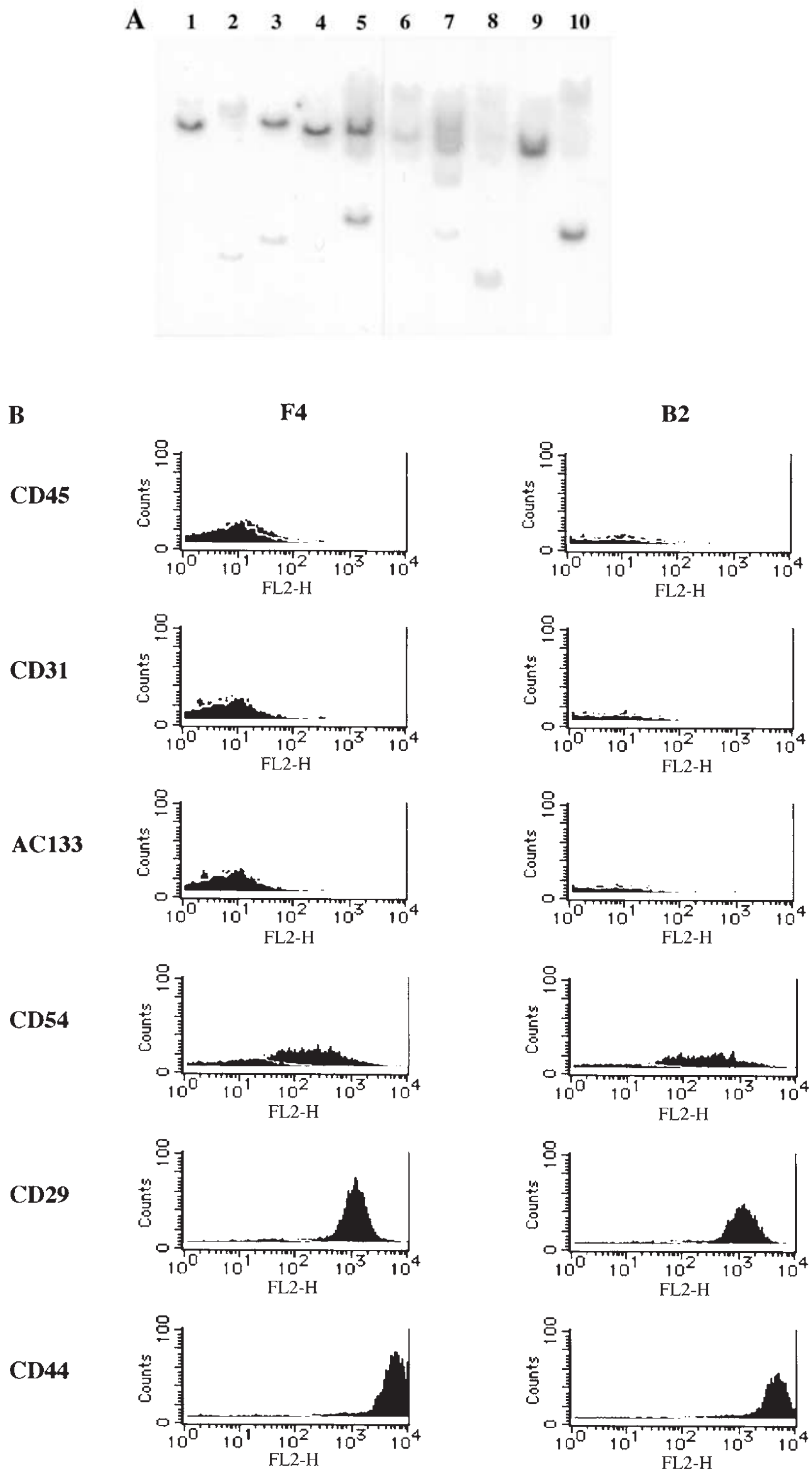

Figure 4. Establishment and characterization of two clones from No. 40 placenta-derived cells. **(A):** Establishment of placenta-derived clones. No. 40 placenta-derived cells were transduced with MSCV-IRES-GFP retrovirus, and green fluorescent protein (GFP)-positive population was sorted by fluorescence-activated cell sorting, then replated onto a 96-well dish at 5 or 10 cells per well and expanded. DNAs from these GFP-positive No. 40 placenta-derived subclones were digested overnight with BamHI (cut only once in the MSCV-IRES-GFP plasmid), and fragments were separated by electrophoresis and probed with a 32P-labeled GFP cDNA probe. Samples are as follows for lanes 1-6, 8, and 9: subclones B2, B4, D2, D3, E4, G3, F1, and F4, respectively. These subclones were obtained from subcloning of five cells per ell. Lanes 7 and 10, subclones E4 and G4. (Figure 4C and D continued on next page.) **(Figure 4 C and D continued on next page.)**

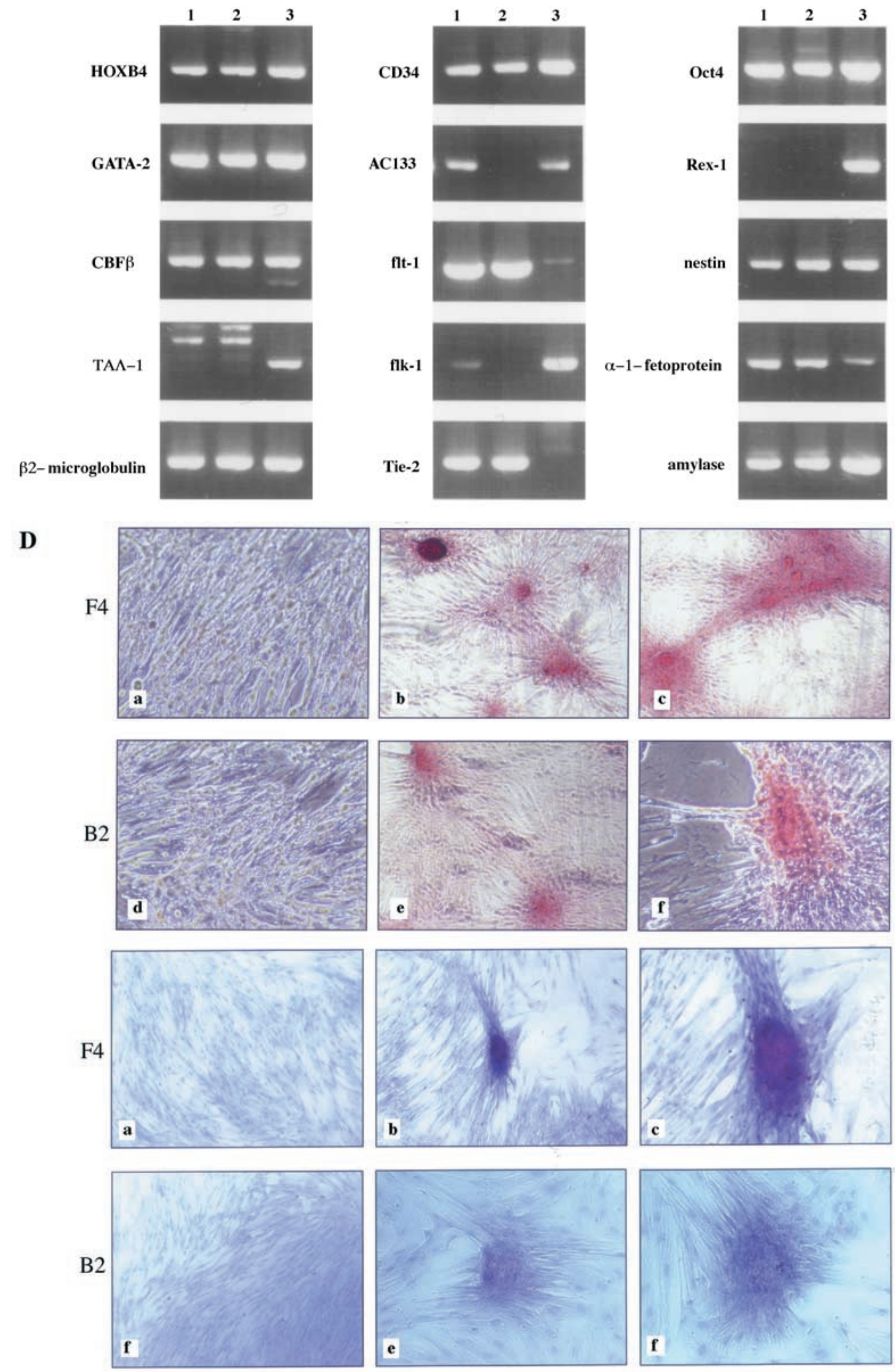

Figure 4 (continued). These were obtained from subcloning of 10 cells per well. Subclones B2 (lane 1) and F4 (lane 9) had a single retroviral insert. **(B):** Immunophenotype of clones. Clones F4 and B2 were stained with phycoerythrin-conjugated antibodies against CD45, CD31, AC133, CD54, CD29, and CD44 or immunoglobulin isotype control antibodies then analyzed by fluorescence-activated cell sorter Calibur. **(C):** Gene expression patterns of clones. Clones F4 and B2 were characterized by reverse transcription–polymerase chain reaction analysis. Samples are as follows: lane 1, clone F4; lane 2, clone B2; lane 3, positive control (same positive controls were used in Fig. 2). **(D):** Differentiation potential of clones. Clones F4 and B2 were cultured in osteogenic (b, c, e, f), adipogenic (h, i, k, l), or regular medium (a, d, g, j) for 2 weeks. After the culture periods, each of the clones was evaluated for osteogenic or adipogenic differentiation using specific staining and hematoxylin counterstaining. Magnification: a, b, d, e, g, h, j, k, ×40; c, f, i, l, ×100.

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