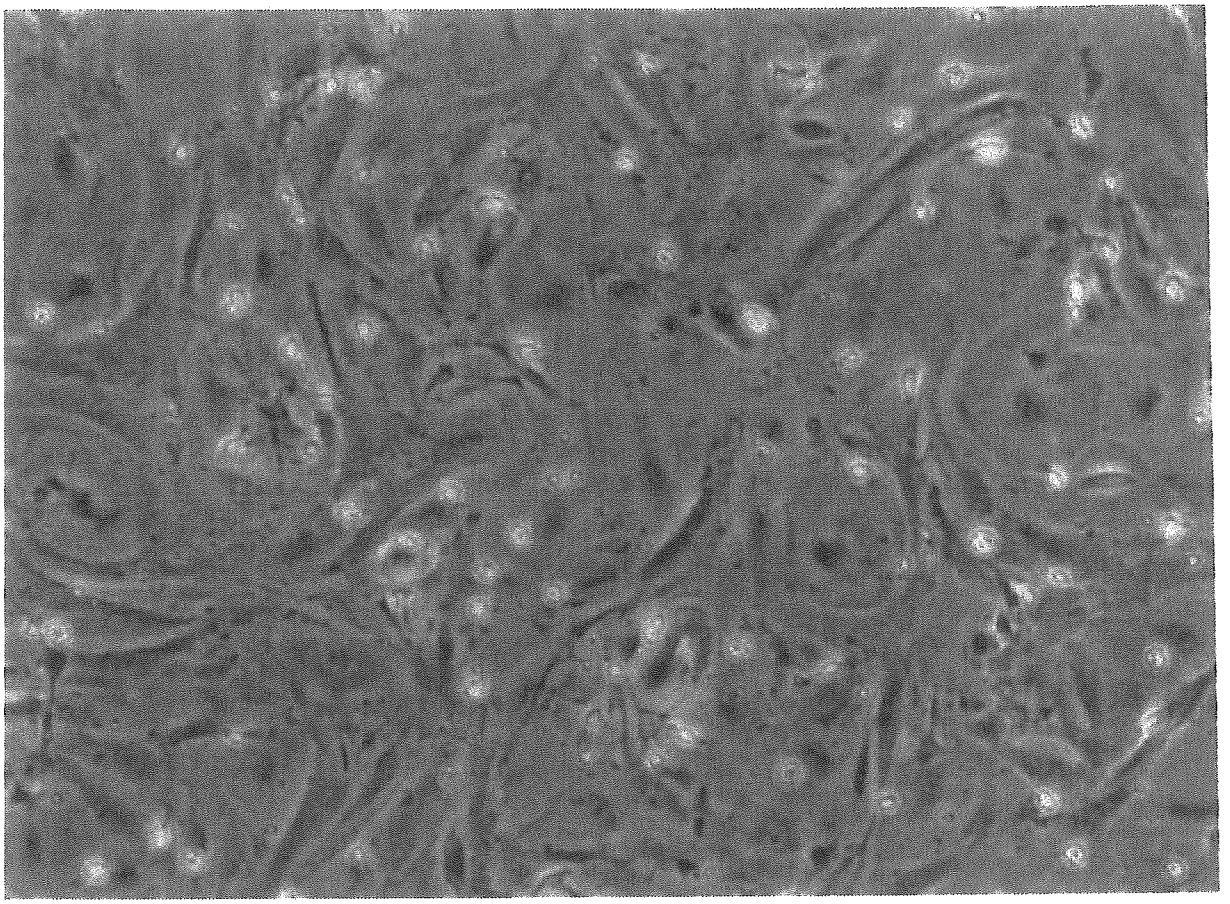
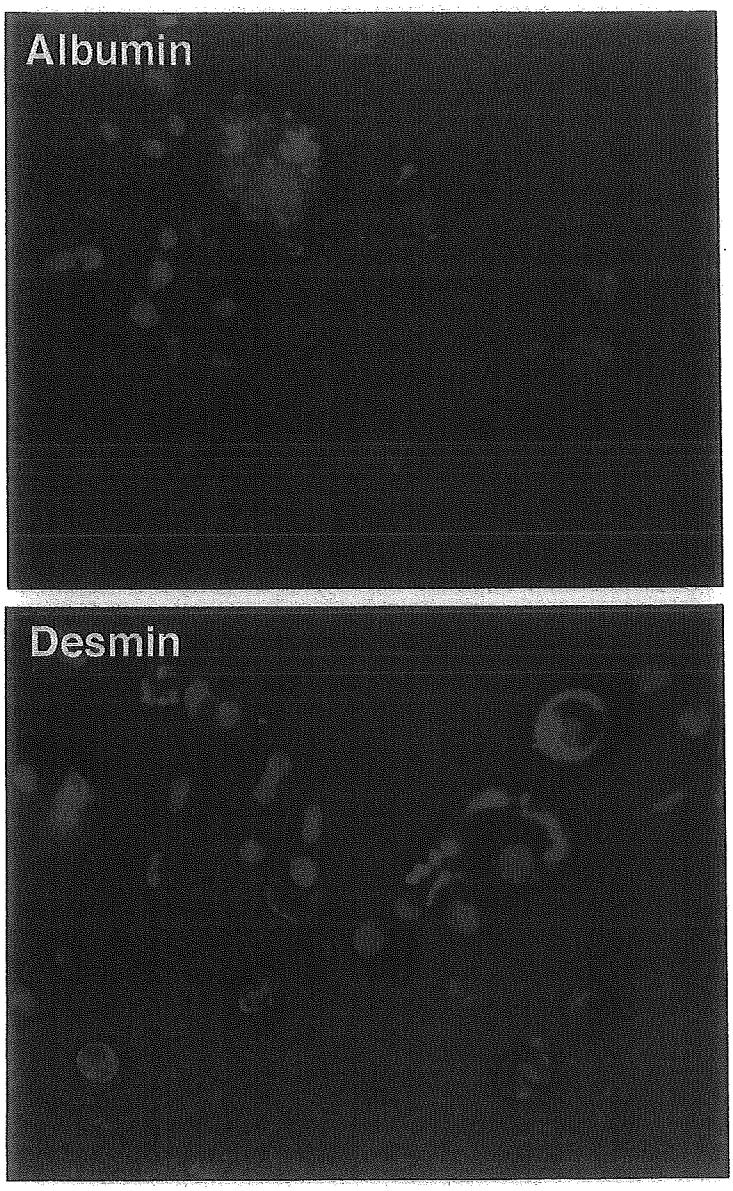
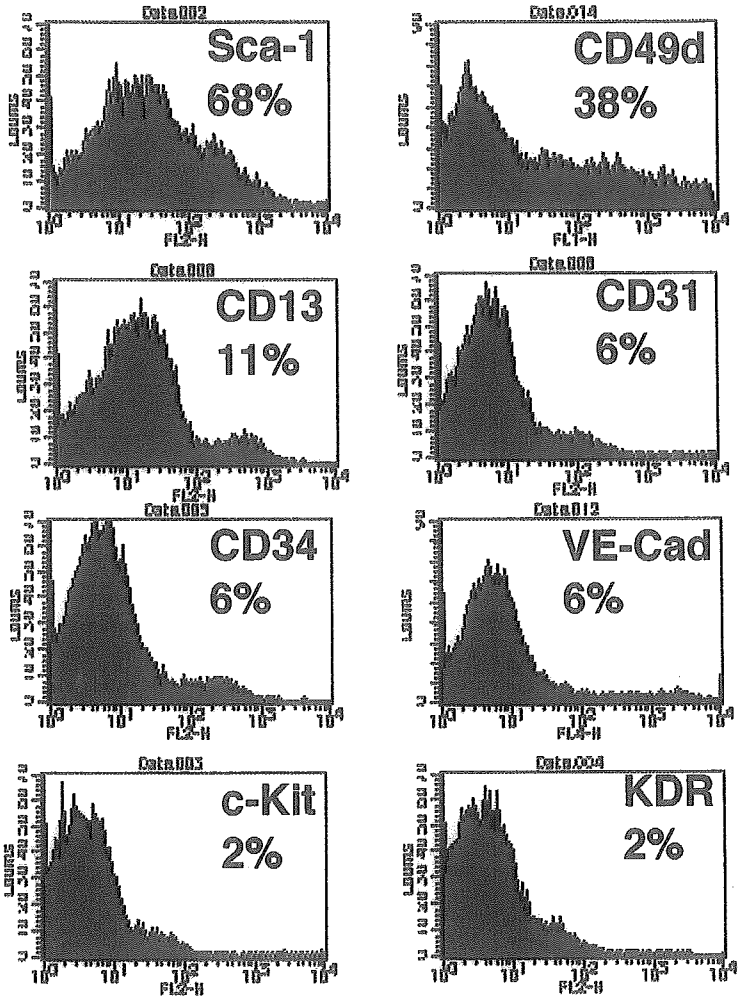
（資料２）マウス胎仔肝由来ストローマ細胞



（資料 3）マウス胎仔肝由来ストローマ細胞における Albumin および Desmin の発現

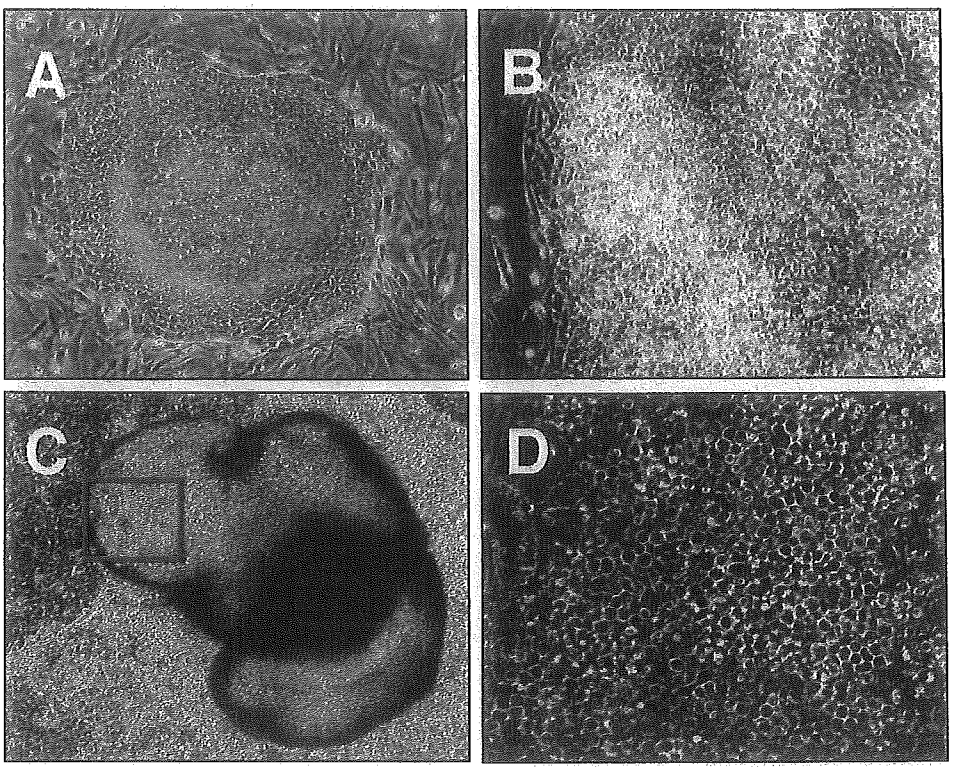


(資料 4)マウス胎仔肝由来ストローマ細胞のフローサイトメトリーによる解析



（資料5）マウス胎仔肝由来ストローマ細胞と共培養されたヒトES細胞

A. 培養3日目のヒトES細胞
B. 培養5日目のヒトES細胞
C. 培養12日目のヒトES細胞
D. 培養12日目の観察されたcobblestone area（Cの赤線で囲まれた四角形の部分）を強拡大したもの



（資料 6）ヒト ES 細胞とマウス胎仔肝由来ストローマ細胞の共培養における RT-PCR による解析

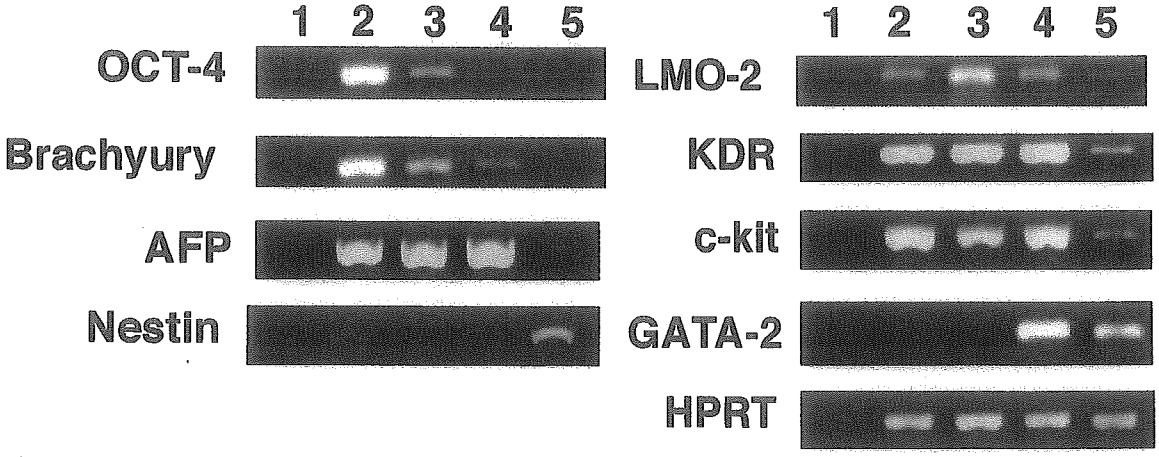
Lane 1: no transcripts

2: MEF で維持された未分化なヒト ES 細胞

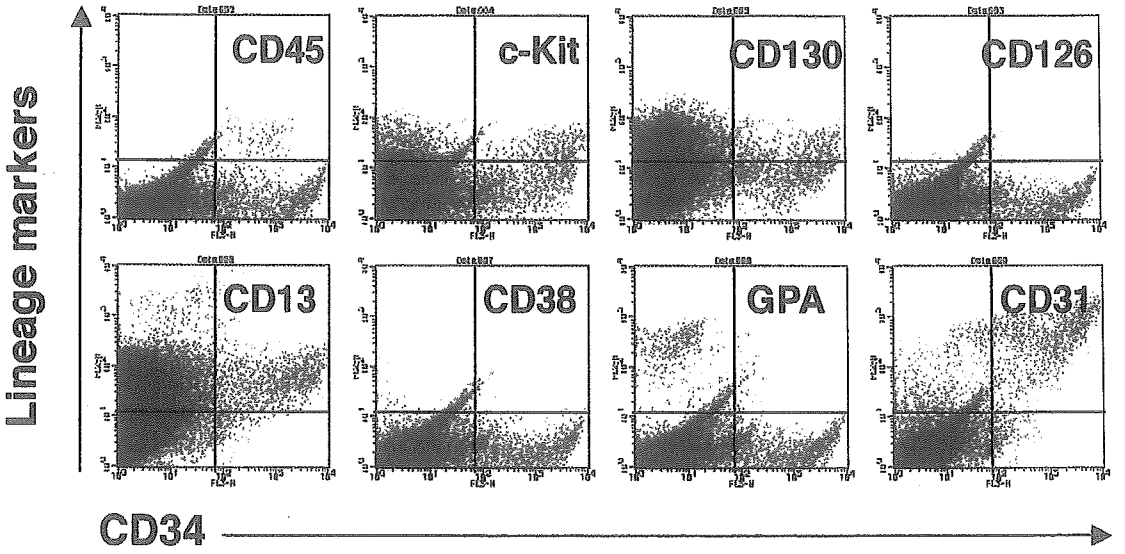
3: マウス胎仔肝由来ストローマ細胞で 5 日間共培養されたヒト ES 細胞

4: マウス胎仔肝由来ストローマ細胞で 9 日間共培養されたヒト ES 細胞

5: マウス胎仔肝由来ストローマ細胞で 13 日間共培養されたヒト ES 細胞



（資料7）ヒトES細胞とマウス胎仔肝由来ストローマ細胞の共培養中の培養細胞のフローサイトメトリーによる検討（培養14日目）



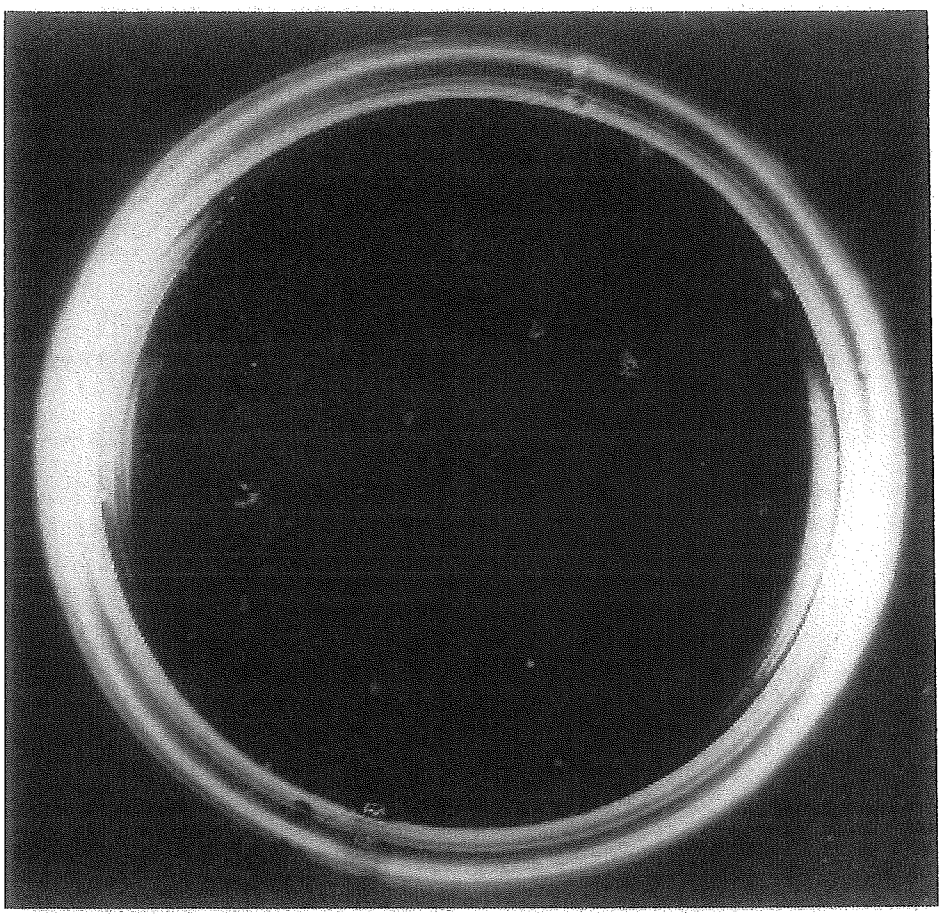
（資料 8）マウス胎仔肝由来ストローマ細胞と共培養されたヒト ES 細胞による血液細胞コロニー形成の解析

Days in culture	Colonies					
	G	M	GM	E	Mix	Total
mFLSC alone						
	0	0	0	0	0	0
Co-culture with mFLSC						
Day 0	0	0	0	0	0	0
Day 8	0	1.2±0.4	0	0	0	1.2±0.4
Day 15	5.4±1.7	60.6±4.1	32.5±7.5	6.6±3.0	9.4±2.5	114.5±7.0

Each value shows the mean ± SD of colony numbers formed from 2 x 10^5 cells.

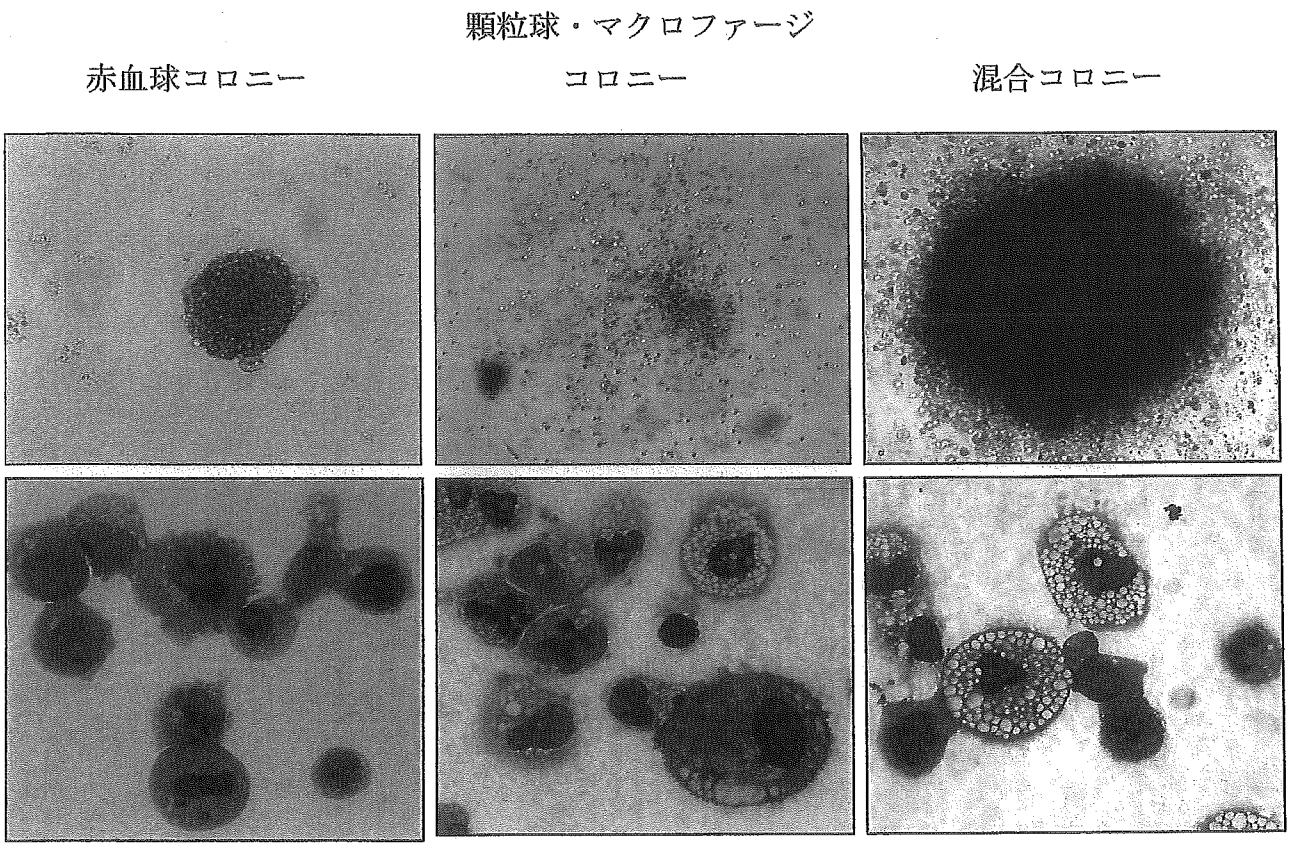
Abbreviations: G, granulocyte; M, macrophage, GM, granulocyte-macrophage; E, erythroid; and Mix, mixed lineage colonies.

(資料9)マウス胎仔肝由来ストローマ細胞と共培養されたヒトES細胞による血液細胞コロニー形成

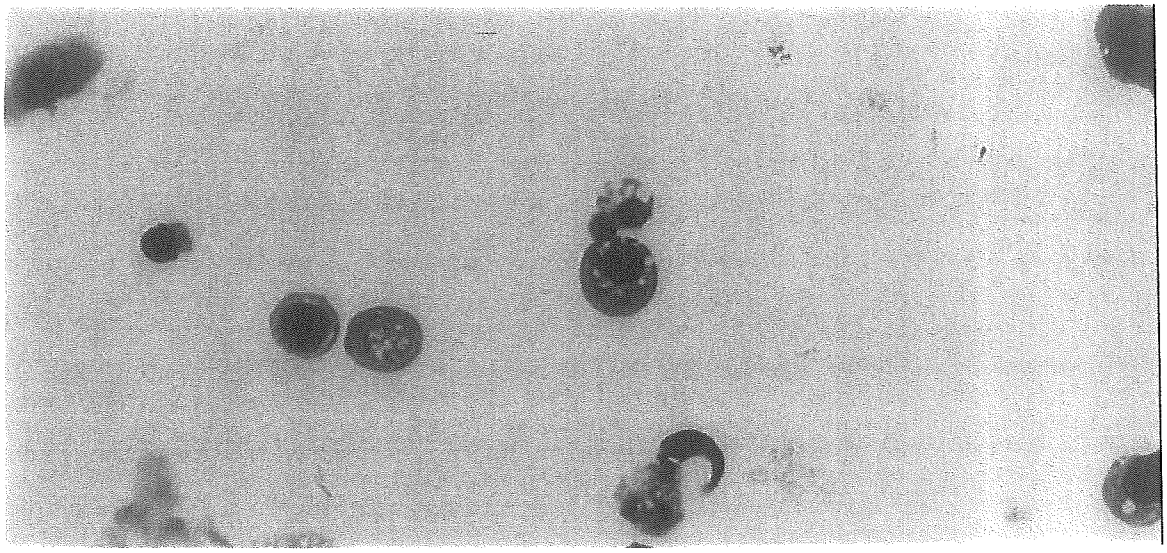


(資料 10) マウス胎仔肝由来ストローマ細胞と共培養されたヒト ES 細胞による
血液細胞コロニー形成

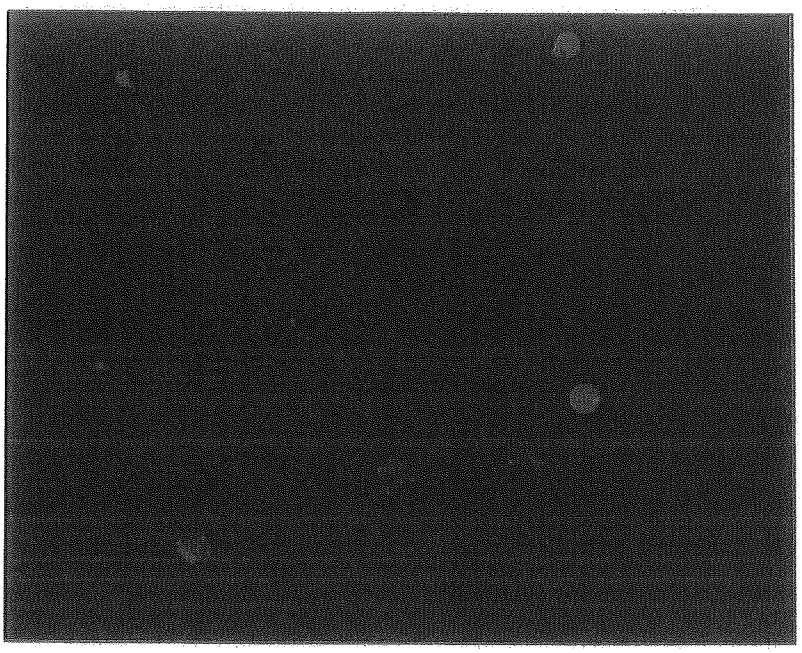
(上段) 倒立顕微鏡下での観察
(下段) サイトスピン標本での観察



（資料 11）ヒト ES 細胞由来赤血球



（資料 12）ヒト ES 細胞由来混合コロニー中に含まれる赤血球における β グロビンの発現



Ⅱ. 研究成果の刊行に関する一覧表

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Sugiyama D, Tsuji K	Definitive hematopoiesis from endothelial cells in mouse embryo: A simple guidance.	Trends Cardiovas Med	In press		
Ma F, Wang D, Hanada S, Ebihara Y, Kawasaki H, Zaike Y, Heike T, Nakahata T, Tsuji K	Novel method for efficient production of multipotential hematopoietic progenitors from human embryonic stem cells.	Blood	In press		
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辻浩一郎	Embryonic stem cell の造血幹細胞移植への応用	今日の移植	印刷中		
発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
辻浩一郎	ヒト ES 細胞からの血液細胞の産生	臨床血液	印刷中		
辻浩一郎	末梢血幹細胞	血液・腫瘍科	印刷中		
辻浩一郎	がんの細胞療法	日本アフェレシス学会雑誌	印刷中		

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

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Key Words. Human placenta • Placenta-derived cells • Mesenchymal stem/progenitor cells • Cell culture

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), CCGCCGTATGAGTTCT GTGG/AGAGTGGTGACAGAGACAGG; Rex-1 (449 bp), ATGGCTATGTGTGCTATGAGC/CCTCAACTTCTAGT GCATCC; HOXB4 (268 bp), CTACCCCTGGATGCG CAAAG/CGAGCGGATCTTGGTGTTGG; CBFβ (300 bp), TCGTGCCCGACCAGAGAAGC/TCAGAATCATGGGA GCCTTC; β2-microglobulin (341 bp), GAGTGCTGTCTC CATGTTTG/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