introduced in the near future. RFID may have advantages over bar code-based technology, i.e. more user-friendly, and facilitating the recording of more information. Technology-based solutions designed to prevent mistransfusion have recently been developed, and the effectiveness of the different systems in detecting errors has been reported [24]. The economic aspects and availability of products remain issues regarding implementing these technologies. To reduce human error and the risk of mistransfusion, we have to address the issue at the hospital level, employing a system-based approach.

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RAPID COMMUNICATION

Is parainfluenza virus a threatening virus for human cancer cell lines?

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Abstract

Immortalized cell lines, such as human cancer cell lines, are an indispensable experimental resource for many types of biological and medical research. However, unless the cell line has been authenticated prior to use, interpretation of experimental results may be problematic. The potential problems this may cause are illustrated by studies in which authentication of cell lines has not been carried out. For example, immortalized cell lines may unknowingly be infected with viruses that alter their characteristics. In fact, parainfluenza virus type 5 (PIV5) poses a threat to the use of immortalized cell lines in biological and medical research; PIV5 infection significantly alters cellular physiology associated with the response to interferon. If PIV5 infection is widespread in immortalized cell lines, then a very large number of published studies might have to be re-evaluated. Fortunately, analyses of a large number of immortalized cell lines indicate that PIV5 infection is not widespread.

Key words: authentication, cell bank, cell line, parainfluenza virus, quality check.

INTRODUCTION

Immortalized cell lines, such as human cancer cell lines, are very useful and indispensable experimental materials in current biology and medical science. However, without the authentication of such cell materials prior to use, experimental results using cell materials don't make sense.

Parainfluenza virus type 5 (PIV5), a paramyxovirus that harbors a non-segmented negative-stranded RNA genome, was originally isolated from simian kidney cell cultures and was called simian virus 5 (SV5). Subsequently, the virus has also been isolated from various mammalian species such as dogs, pigs, and humans. In dogs, PIV5 is responsible for a respiratory disease.[1]

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The following report by Young *et al.* was surprising: "AGS and other tissue culture cells can unknowingly be persistently infected with PIV5; a virus that blocks interferon signaling by degrading STAT1".[2] When screening various cell lines for their ability to respond to interferon (IFN), the authors noted that in comparison to other tissue culture cells AGS tumor cells, which are widely used in biomedical research, had very low levels of STAT1. The reason for this was that AGS cells were persistently infected with PIV5, a virus that blocks the IFN response by targeting STAT1 for proteasome-mediated degradation.

Thus, the AGS cell line infected with PIV5 is not appropriate material to analyze the mechanism by which gastric cancer acquires resistance to IFN. The authors cautioned that some of the conclusions drawn from previous studies that used AGS cells may have to be reevaluated, especially as not only does the V protein of PIV5 target STAT1 for degradation, but it also binds to, and inhibits the activity of, MDA-5, a cellular protein that recognizes dsRNA and activates the IFN induction cascade.[3,4]

In addition, PIV5 appears to infect a wide variety of cells including an oligodendrocyte cell line.[2] Thus, we evaluated the possibility of PIV5 infection for the human cancer cell lines deposited in our cell bank so as to exclude the erroneous interpretation due to the use of cells infected by PIV5.

METHODS

Cells

The AGS cell line was obtained from the American Type Culture Collection (ATCC, http://www.atcc.org/; Monassas, VA, USA). Other cell lines were obtained from the Cell Engineering Division of RIKEN Bio-Resource Center (RIKEN BRC, http://www.brc.riken.jp/lab/cell/english/; Tsukuba, Ibaraki, Japan). The human cancer cell lines analyzed in the present study are listed in Table 1.

Western blot analysis

Total cell extracts were generated by lysing cells (1×10^7) in CellLytic-M (Sigma-Aldrich, St. Louis, MO, USA). After removing cell debris, protein concentration was quantified with Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Ten micrograms of protein samples were electrophoresed through sodium dodecyl

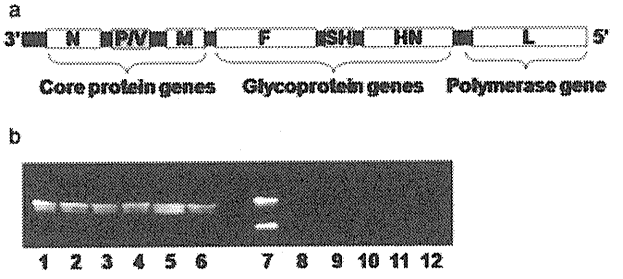

Figure 1 Genomic structure of parainfluenza virus type 5 (PIV5) and an example of a screen for PIV5 infection. (A) Genomic structure of PIV5. PIV5 possesses eight genes and produces nine different proteins. P and V proteins are produced from the same coding region by RNA editing. Thus, P and V proteins commonly possess the same N-terminal amino acid sequences. (B) An example of a western blot analysis. β-actin was used as the internal control for the amount of protein (lanes 1–6). A monoclonal antibody against both P and V proteins was used to detect PIV5 infection (lanes 7–12). The AGS cell line was used as the positive control. Lanes 1 and 7, protein sample prepared from AGS cell line; lanes 2 and 8, Lu-138 cell line; lanes 3 and 9, HLC-1 cell line; lanes 4 and 10, MIA Paca2 cell line; lanes 5 and 11, S1 cell line; lanes 6 and 12, MR10-1 cell line. The protein sample prepared from AGS cells clearly showed two bands for both P and V proteins (lane 7), whereas the other cells did not.

Table 1 List of cell lines used for screening

Screening	Cell lines
Esophageal cancer	TE-1, TE-4, TE-6, TE-8, TE-10, TE-11, TE-14, TE-15, EC-GI-10
Gastric cancer	HGC-27, NUGC-4, MKN1, MKN7, MKN45, MKN74, Kato III, HuG1-PI, HuG1-N, GCIY, SF8657, ECC10, ECC12, LMSU, TGBC11TKB, KE-39, KE-97, H-111-TC, SH-10-TC, AZ521, NCC-StC-K140, GSS, GSU
Colon cancer	CW-2, CACO-2, COLO-320, LoVo, JHCA-ov, COLO205, TT1TKB, PMF-ko 14, JHCOLOYI, JHSK-rec
Hepatic cancer	HuH-7, Hep G2, Li-7
Bile duct cancer	TGBC24TKB, HuH-28, HuCCT1, RBE, SSP-25, TKKK
Pancreatic cancer	PK-1, PK-45H, PK-45P, PK-59, MIA Paca2, PANC-1, NOR-P1
Breast cancer	ZR-75-1
Oral cavity cancer	T3M-1 Cl-10, CJM, HSQ-89, Sa3, T3M-1 Clone2, HSC-2, HSC-3, Ho-1-u-1
Cervical cancer	OMC-1, TCS, HeLa, BU25TK-, TC-YIK, HOKUG, SKG-II-SF, QG-U, TCO-2, TOM-2, JHUS-nk1, JHUCS-3
Sub-lines of HeLa cell line:	MR10-1, HEp-2, HeLa TG, HeLa.S3, HeLa.S3(SC), MR1-3, MR6, HeLa.S3(Mer∧(-)), HeLa P3
Urinary bladder cancer	5637
Lung cancer	RERF-LC-AI, RERF-LC-KJ, Sq-1, EBC-1, LK-2, LC-1/sq-SF, LC-1F, LC-1/sq, MS-1, Lu-134-A, Lu-134-B, Lu135, Lu-138, Lu-139, Lu-140, Lu-141, Lu-143, Lu-165, T3M-12, S1, 87-5, WA-hT, S2, IA-LM, 86-2, IA-5, A549, II-18, LU65, 633, Lu-24, HLC-1, LC-2/ad, PC-14

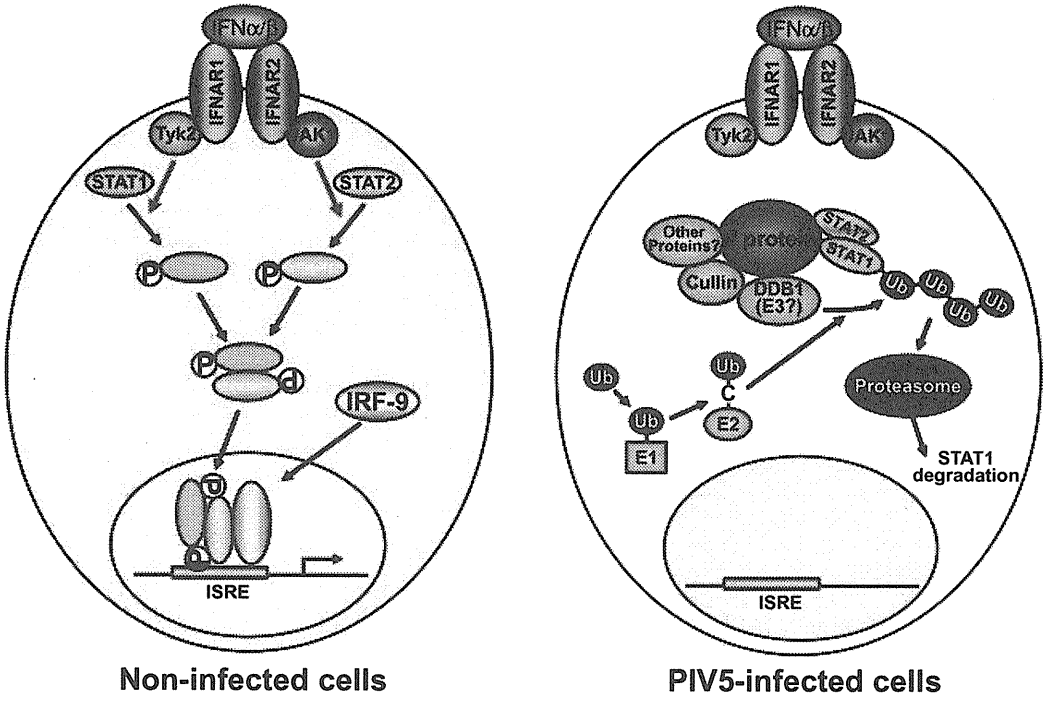

Figure 2 Mechanism of STAT1 degradation by the V protein of parainfluenza virus type 5 (PIV5).

sulfate (SDS)-15% polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated with either a monoclonal antibody (MoAb) against P/V protein produced by PIV5 (AbD Serotec, Oxford, UK) or a MoAb against β-actin (an internal control; Abcam, Cambridge, UK), and bound antibody was revealed by horseradish peroxidase-conjugated goat anti-mouse IgG (GE Healthcare, Fairfield, CT, USA) by using enhanced chemiluminescence (Pierce, Rockford, IL, USA).

RESULTS AND DISCUSSION

Parainfluenza virus type 5 can infect cells derived from the stomach, such as AGS cells and oligodendrocytes.[2] In addition, respiratory tract epithelial cells and bone marrow cells can be infected by PIV5.[1] At the moment, we cannot rule out the possibility of PIV5 infection of any cell type. Thus, we evaluated the PIV5 infection status of many cancer cell lines derived from various tissues (Table 1).

The genomic structure of PIV5 is shown in Figure 1A. To detect PIV5 infection, we carried out western blot analyses, using a monoclonal antibody against the P/V proteins of PIV5, on cell lysates prepared from each cell line. We used the AGS cell line as a positive control in these analyses; Young *et al.* reported previously that this cell is infected with PIV5.[2] We analyzed 123 of the approximately 600 human cell lines deposited in our cell bank (see Methods, Table 1). Fortunately, PIV5 infection was not detected in any of the cell lines analyzed in this study. One example of a western blot analysis is shown in Figure 1B; the results from the other such analyses are not shown.

Of note, the authentication of immortalized cell lines has not necessarily been accomplished. For example, misidentification of cell lines has frequently been detected.[5–8] BBC radio also recently reported this fact with a 40-min broadcast titled "Cancer studies wasted millions" (20 November 2007). Short tandem repeat (STR) polymorphism analysis to exclude misidentification among human cell lines is a very powerful method[9,10] and is routinely carried out at present in cell

banks around the world. Thus, if researchers would use only the cell lines that have been tested by STR polymorphism analysis in cell banks, misidentification of human cell lines will be eradicated in the future.

On the other hand, virus infection is another critical issue in the use of immortalized cell lines. First, biohazard of cell lines should be excluded. Thus, in relation to the cell lines derived from liver cells, we routinely evaluate the infection of hepatitis B and C viruses (HBV and HCV). In relation to the cell lines derived from hematopoietic cells, we routinely evaluate the infection of human immunodeficiency virus type 1 (HIV1) and human T cell leukemia virus type 1 (HTLV-1). Second, the effects of virus infection on the characteristics of the infected cell lines must be taken into account, since it is clear that alteration of cell characteristics by virus infection leads to erroneous interpretation of the experimental results.

The AGS cell line infected with PIV5 is a sub-clone that has been deposited in a cell bank; the parental AGS cell line was not infected with PIV5.[2] Thus, the PIV5 infection of the AGS sub-line appears to have occurred during the culture prior to the deposition of the sub-line in the cell bank. Since PIV5 can infect the cells of various animal species,[1] PIV5 may be present in some of the materials typically used for cell culture, such as serum. Although the infection of AGS cells appears to be a secondary event during culture, it is still possible that primary cancer cells may be infected with PIV5 and that this infection produces resistance to IFN in the clinic.

Although the cell lines deposited in our cell bank and tested in this study were free of PIV5 infection, we want to emphasize the importance of testing PIV5 infection for immortalized cell lines, especially when they are used to analyze the mechanism of signal transduction of IFN (Fig. 2). In addition, it is important for the scientific community to be aware that cells may be persistently infected with viruses that significantly alter cellular physiology. Since many viruses able to infect cell materials are present and the effects of such infection on cellular physiology are largely unknown, the authentication of immortalized cell lines regarding virus infection is necessary and should be continued enthusiastically in cell banks around the world as one of the most pivotal missions.

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RESEARCH ARTICLE

Induced *in vitro* differentiation of pancreatic-like cells from human amnion-derived fibroblast-like cells

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Abstract

There is growing evidence that the human amnion contains various types of stem cells. As amniotic tissue is readily available, it has the potential to be an important source of material for regenerative medicine. In the present study, we evaluated the potential of human amnion-derived fibroblast-like (HADFIL) cells to differentiate into pancreatic islet cells. Two HADFIL cell populations, derived from two different neonates, were analyzed. The expression of pancreatic cell-specific genes was examined before and after *in vitro* induction of cellular differentiation. We found that *Pdx-1*, *Isl-1*, *Pax-4*, and *Pax-6* showed significantly increased expression following the induction of differentiation. In addition, immunostaining demonstrated that insulin, glucagon, and somatostatin were present in HADFIL cells following the induction of differentiation. These results indicate that HADFIL cell populations have the potential to differentiate into pancreatic islet cells. Although further studies are necessary to determine whether such *in vitro*-differentiated cells can function *in vivo* as pancreatic islet cells, these amniotic cell populations might be of value in therapeutic applications that require human pancreatic islet cells.

Key words: amnion, diabetes mellitus, insulin, pancreas, regenerative medicine.

INTRODUCTION

The pancreas has important roles in the digestive and endocrine systems of vertebrates and functions in both an exocrine and endocrine manner. As an exocrine gland, the pancreas secretes various digestive enzymes that are passed to the small intestine where they aid digestion of food. The endocrine function in the pancreas is carried out by cells in the islets of Langerhans that secrete several important hormones, including insulin, glucagon, and somatostatin.

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Diabetes mellitus is a disease caused by either the loss of insulin production from pancreatic β-cells in the islets of Langerhans (type I) or an insufficiency in insulin production or resistance to insulin (type II). Diabetes mellitus is a global disease and affects the lives of millions of people every year. Islet-based transplantation and regimens such as the Edmonton protocol can restore the insulin producing islet cells and are considered promising approaches for the treatment of diabetes mellitus, in particular for that of type I diabetes.[1] However, numerous obstacles remain to transplantation-based therapy, such as limitations in the availability of donor cells and the difficulty of islet isolation. The procedure is also costly and its success is unpredictable; factors that have largely restricted its use in the treatment of diabetic patients to date.[2]

The induction of specific functional cells from embryonic stem (ES) cells or induced pluripotent stem (iPS)

cells is currently being intensively studied. However, the risks, such as tumorigenicity, associated with the use of cells derived from ES or iPS cells in clinical applications will not be so easily resolved. Therefore, for the moment at least, specific functional cells derived from somatic tissues appear to be more useful. One source of somatic tissues is the placenta, which is comprised of three layers, the amnion, the chorion, and the deciduas, that are derived from different origins. The amnion and the chorion are formed by the neonate, whereas the decidua is maternally derived. In culture, epithelial and fibroblast-like cells can be obtained from human amniotic cells.[3] The fibroblast-like cells produced in culture are usually derived from mesenchymal cells.[4] We previously reported that human amnion-derived fibroblast-like (HADFIL) cell populations could differentiate into various types of cell, for example, mesoderm-derived osteoblasts,[5] chondrocytes,[6] ectoderm-derived neural cells,[7] and endoderm-derived hepatocytes.[3] In addition, it has also been shown that amniotic epithelial cells can differentiate into ectoderm-, mesoderm-, and endoderm-derived cells.[8]

A number of studies have recently reported that pancreatic cells, including insulin-producing cells, can be induced from human amniotic epithelial cells,[9,10] human placenta-derived multipotent stem cells,[11] human adipose tissue-derived mesenchymal stem cells,[12] human umbilical cord blood-derived stem cells,[13] and human bone marrow-derived mesenchymal stem cells.[14] Of note, human amniotic epithelial cells, but not amniotic mesenchymal cells, can differentiate into insulin-producing cells.[9] These results prompted us to investigate whether HADFIL cells (amniotic mesenchymal cells) can differentiate into pancreatic cells.

MATERIALS AND METHODS

Cell culture

The use of human amniotic tissue was approved by the institutional ethical committee of the RIKEN Tsukuba Institute before the study was initiated. The HADFIL cell populations were prepared by first rinsing amniotic membranes three times with phosphate buffered saline (PBS; Sigma, St. Louis, MO, USA). The tissue was then minced thoroughly with scissors, and incubated in 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Invitrogen-Gibco, Carlsbad, CA, USA) at 37 °C for 45 min. Cells were collected by centrifugation and incubated in α-MEM (Sigma) supplemented with 10% fetal bovine serum (FBS; Tissue Culture Biologicals, Los Alamitos, CA, USA), 1.0 mg/mL collagenase (Wako, Osaka, Japan), 0.1% dispase (Invitrogen-Gibco) and 0.1% papain (Wako) at 37 °C for 60 min. Finally, the cells were filtered through a 100-μm cell strainer (BD Falcon, Franklin Lakes, NJ, USA), collected by centrifugation, and cultured at 37 °C in 5% CO_2 under humidified conditions in the growth medium: α-MEM (Sigma) supplemented with 10% FBS, 10 ng/mL human epithelial growth factor (hEGF; Sigma), and 10 ng/mL human leukemia inhibitory factor (hLIF; Sigma). The medium was changed twice a week. When the cells reached 90% confluence, they were treated with 0.25% trypsin-EDTA (Invitrogen-Gibco), and one-third to one-half of the cells was re-plated on a new dish. Cells were used for experimental analyses after three to five passages.

Differentiation of HADFIL cells into pancreatic cells

To evaluate the potential of HADFIL cells to differentiate into pancreatic-like cells, 1×10^5 HADFIL cells were seeded onto a collagen I-coated 60 mm plastic dish (BD Falcon) and cultured in the growth medium described above. When the cells reached 100% confluence, we used a "three-step" protocol to induce pancreatic cell differentiation. In the first step, the medium was replaced with high glucose Dulbecco's Modified Eagle Medium (DMEM) (Sigma) supplemented with 10% FBS (Tissue Culture Biologicals) and 10^{-6} M retinoic acid (Sigma) and cultured for 2 days; the medium was then replaced with high glucose DMEM (Sigma) supplemented with 10% FBS (Tissue Culture Biologicals) alone and cultured for 2 days. In the second step, the cells were dissociated by PBS (Sigma) supplemented with 0.25% EDTA (Sigma), plated in a Matrigel matrix six-well plate (BD Falcon), and cultured for 7 days in low glucose DMEM (Sigma) supplemented with 10% FBS (Tissue Culture Biologicals), 10 mM nicotinamide (Sigma), 20 ng/mL hEGF, and 1xN_2 supplement (Invitrogen-Gibco). In the third step, the medium was replaced with low glucose DMEM (Sigma) supplemented with 10 nM exendin-4 (Sigma) and the cells were cultured for 7 days.

Semi-quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from cells using Isogen reagent (Wako, Osaka, Japan). Reverse-transcription (RT) was carried out using 5 μg total RNA and the Ready-To-Go

Table 1 Information regarding polymerase chain reaction

Primer	Sequence (5′→3′)	Product size (bp)	Annealing temperature (°C)	Cycle no.
Insulin	5′-ggaacgaggcttcttctacac-3′ 5′-acaatgccacgcttctgc-3′	144	58	38
Glucagon	5′-gaataccaagaggaacaggaataa-3′ 5′-ctttcaccagccaagcaatg-3′	140	58	38
Somatostatin	5′-cgctgtccatcgtcctg-3′ 5′-gggcatcattctccgtctg-3′	168	58	38
CK19	5′-atggccgagcagaaccggaa-3′ 5′-ccatgagccgctggtactcc-3′	317	60	36
HNF-1 α	5′-aggacctgagcctgccgagcaac-3′ 5′-agggctctccataggcccaggct-3′	281	56	36
HNF-3 β	5′-aatggacctcaaggcctacg-3′ 5′-cctgcaacaacagcaatgg-3′	441	56	36
HNF-6	5′-tatcaccaccgagctcaagc-3′ 5′-acaccatacaccttcgtggc-3′	889	59	38
Isl-1	5′-attccctatgtgttggttgcg-3′ 5′-cgttcttgctgaagccgatg-3′	229	58	40
GLUT-2	5′-cctgtttatgcaaccattgg-3′ 5′-gcagcaggacgtggtccttg-3′	292	57	36
GCK	5′-aagaaggtgatgagacggatgc-3′ 5′-catctggtgtttggtcttcacg-3′	230	56	35
GATA-4	5′-ccaagcaggactcttggaac-3′ 5′-cagcgtgtaaaggcatctga-3′	263	57	36
Sox-17	5′-gacgaccagagccagacc-3′ 5′-cgcctcgcccttcacc-3′	114	59	36
Pdx-1	5′-ttagaccgaaggggaaaacc-3′ 5′-ttagggagccttccaatgtg-3′	267	58	38
Pax-4	5′-tttgtgctgaagggctttgc-3′ 5′-gggagaagatagtccgattccg-3′	216	58	40
Pax-6	5′-agattcagatgaggctcaaa-3′ 5′-aattggttggtagacactgg-3′	313	56	40
Nkx2.2	5′-gaagaacccccttctacgaca-3′ 5′-tcgccgctttcgcttcttg-3′	216	58	40
Nkx6.1	5′-acacgagacccactttttccg-3′ 5′-tgctggacttgtgcttcttcaac-3′	336	56	40
Neuro D	5′-cagaaccaggacatgccc-3′ 5′-atcaaaggaagggctggtg-3′	216	60	37
Ngn 3	5′-ggtagaaaggatgacgcctc-3′ 5′-ccgagttgaggtcgtgcat-3′	313	55	36
Nestin	5′-agaggggaattcctggag-3′ 5′-ctgaggaccaggactctcta-3′	490	56	36
β-actin	5′-gagaaaatcttgcaccacac-3′ 5′-ctcggtgaggatcttcat-3′	340	56	30

CK19, cytokeratin 19; GCK, glucokinase; GLUT-2, glucose transporter-2; HNF-1α, hepatocyte nuclear factor-1α; HNF-3β, hepatocyte nuclear factor-3β; HNF-6, hepatocyte nuclear factor-6; Isl-1, islet-1; Ngn 3, neurogenin 3; Nkx-2.2, NK2 transcription factor-related locus 2; Nkx-6.1, NK6 transcription factor-related locus 1; Pax-4, paired box gene-4; Pax-6, paired box gene-6; Pdx-1, pancreatic and duodenal homeobox gene-1; Sox-17, SRY-related HMG box-17.

T-primed First-Strand kit (Amersham Biosciences, Piscataway, NJ, USA) in a 33-μL reaction volume. After RT, 167 μL of TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) was added to the reaction mixture, and 5 μL (1/40) of this was used in each polymerase chain reaction (PCR). PCR was carried out using a recombinant Taq polymerase kit (TaKaRa Bio, Otsu, Shiga, Japan) with the following cycling parameters: denaturation at 94 °C for 1 min, annealing at 56–60 °C (see Table 1) for 1 min, and extension at 72 °C for 1 min. Annealing temperatures

and cycle numbers for each PCR are shown in Table 1. PCR products were separated on 1.2% agarose gels and visualized by ethidium bromide staining. β-actin amplification was used as an internal control.

Immunostaining

Cells adhering to a dish were washed three times with PBS (Sigma), fixed with 4% paraformaldehyde (Wako) in PBS at room temperature for 10 min, re-fixed with methanol at −20 °C for 10 min, and washed three times at room temperature with PBS supplemented with 0.05% Tween-20 (Wako). Nonspecific binding of antibodies was blocked by washing in PBS with 0.5% casein (Sigma) and 0.05% sodium azide (NaN_3; Wako) for 60 min at room temperature. The cells were then incubated with monoclonal antibodies against insulin (H-86; Santa Cruz Biotechnology, Santa Cruz, CA, USA), glucagon (FL-180; Santa Cruz Biotechnology), or somatostatin (H-106; Santa Cruz Biotechnology) for 60 min at room temperature. The cells were washed three times with PBS supplemented with 0.05% Tween 20, and then incubated at room temperature for 60 min with Alexa Fluor-488 Fluoro Nanogold-antirabbit IgG antibody (Nanoprobes, Yaphank, NY, USA) to detect insulin, glucagon, or somatostatin. The cells were analyzed using a fluorescence microscope.

RESULTS

Morphological changes in HADFIL cells following the induction of differentiation into pancreatic cells

In the present study we tested two HADFIL cell populations, HADFIL-52 and HADFIL-57, that both have fibroblast-like morphology (Fig. 1A), and express vimentin, a marker of mesenchymal cells, but not epithelial antigen (data not shown).[7]

Morphological changes in the HADFIL cell populations were not observed after the first step of the differentiation protocol, but were observed at the second step. During the second step, the HADFIL cells changed from a fibroblast-like morphology to becoming rounder epithelial-like cells (Fig. 1B). At the end of the second step, many islet-like clusters were observed (Fig. 1C) and these islet-like clusters grew larger during the third step (Fig. 1D).

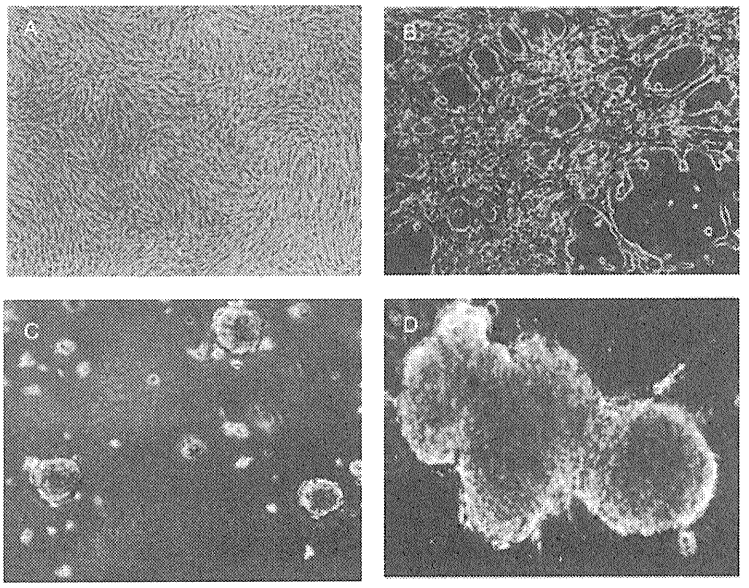

Figure 1 Morphology of human amnion-derived fibroblast-like (HADFIL)-57 cells before (A) and after (B–D) induction of pancreatic cell differentiation. (A) Before differentiation, HADFIL cells have a fibroblast-like appearance. Magnification, ×40. (B) During the second step of differentiation induction, the morphology of HADFIL cells changed from fibroblast-like to rounder, epithelial-like cells. (C) At the end of the second step of differentiation induction, islet-like cell clusters were formed. (D) At the end of the third step of differentiation induction, the islet-like cell clusters grew larger. (B–D) Magnification, ×100. Results shown are representative of three independent experiments. The same experiments with HADFIL-52 showed similar results.

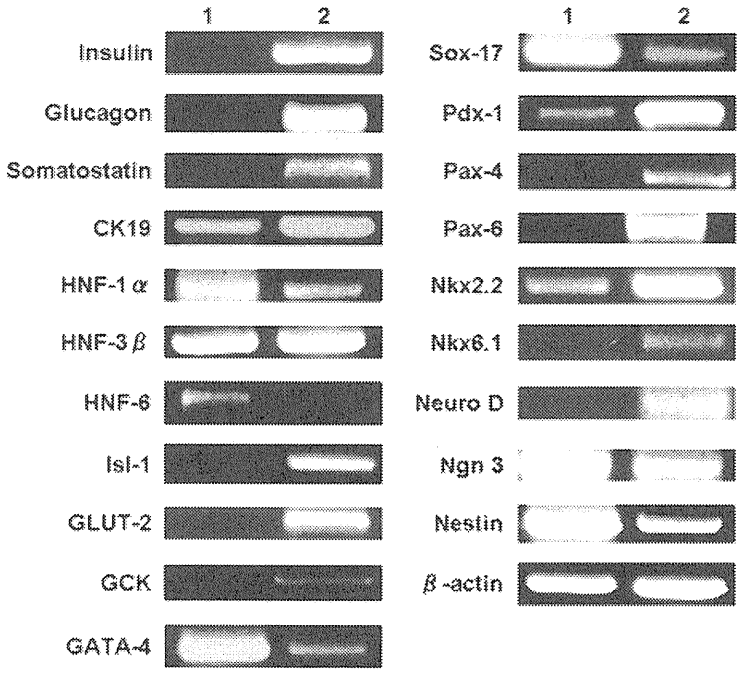

Figure 2 Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of human amnion-derived fibroblast-like (HADFIL)-57 cells for genes expressed specifically in pancreatic cells. CK19, cytokeratin 19; GCK, glucokinase; GLUT-2, glucose transporter-2; HNF-1α, hepatocyte nuclear factor-1α; HNF-3β, hepatocyte nuclear factor-3β; HNF-6, hepatocyte nuclear factor-6; Isl-1, islet-1; Ngn 3, neurogenin 3; Nkx-2.2, NK2 transcription factor-related locus 2; Nkx-6.1, NK6 transcription factor-related locus 1; Pax-4, paired box gene-4; Pax-6, paired box gene-6; Pdx-1, pancreatic and duodenal homeobox gene-1; Sox-17, SRY-related HMG box-17. Lane 1, untreated cells. Lane 2, the cells after induction of pancreatic cell differentiation. Results shown are representative of three independent experiments. The same experiments with HADFIL-52 showed similar results.

Expression of pancreas-specific genes in HADFIL cells following induction of pancreatic cell differentiation

We analyzed the expression of pancreas-specific genes in the HADFIL cell populations before and after induction of pancreatic cell differentiation. The following pancreas-specific genes were screened: insulin, glucagon, somatostatin, cytokeratin 19 (*CK19*), hepatocyte nuclear factor-1α (*HNF-1α*), *HNF-3β*, *HNF-6*, Islet-1 (*Isl-1*), glucose transporter-2 (*GLUT-2*), glucokinase (*GCK*), *GATA-4*, SRY-related HMG box-17 (*SOX-17*), pancreatic and duodenal homeobox gene-1 (*Pdx-1*), paired box gene-4 (*PAX-4*), *PAX-6*, NK2 transcription factor-related locus 2 (*Nkx-2.2*), NK6 transcription factor-related locus 1 (*Nkx-6.1*), NeuroD, neurogenin3 (*Ngn3*), and nestin.

Expression of insulin, glucagon, somatostatin, *Isl-1*, *GLUT-2*, *GCK*, *PAX-4*, *PAX-6*, *Nkx-6.1*, and NeuroD was not detected in untreated HADFIL cells but were found after induction of differentiation (Fig. 2). Although expression of *CK19*, *Pdx-1*, and *Nkx2.2* was detected in untreated HADFIL cells, their levels of expression increased after induction of differentiation (Fig. 2). In contrast, expression of *HNF-1α*, *HNF-6*, *GATA-4*, *Sox-17*, *Ngn 3*, and nestin was detected in untreated HADFIL cells but their levels of expression decreased after induction of differentiation (Fig. 2). *HNF-3β* was expressed in untreated HADFIL cells and after induction of differentiation (Fig. 2).

Immunostaining of pancreatic islet cell-specific proteins

Human amnion-derived fibroblast-like cells were subjected to immunostaining before and after induction of pancreatic cell differentiation. Insulin, glucagon, and somatostatin were not detected in untreated HADFIL cells (Fig. 3A,C,E), but were found after induction of differentiation (Fig. 3B,D,F).

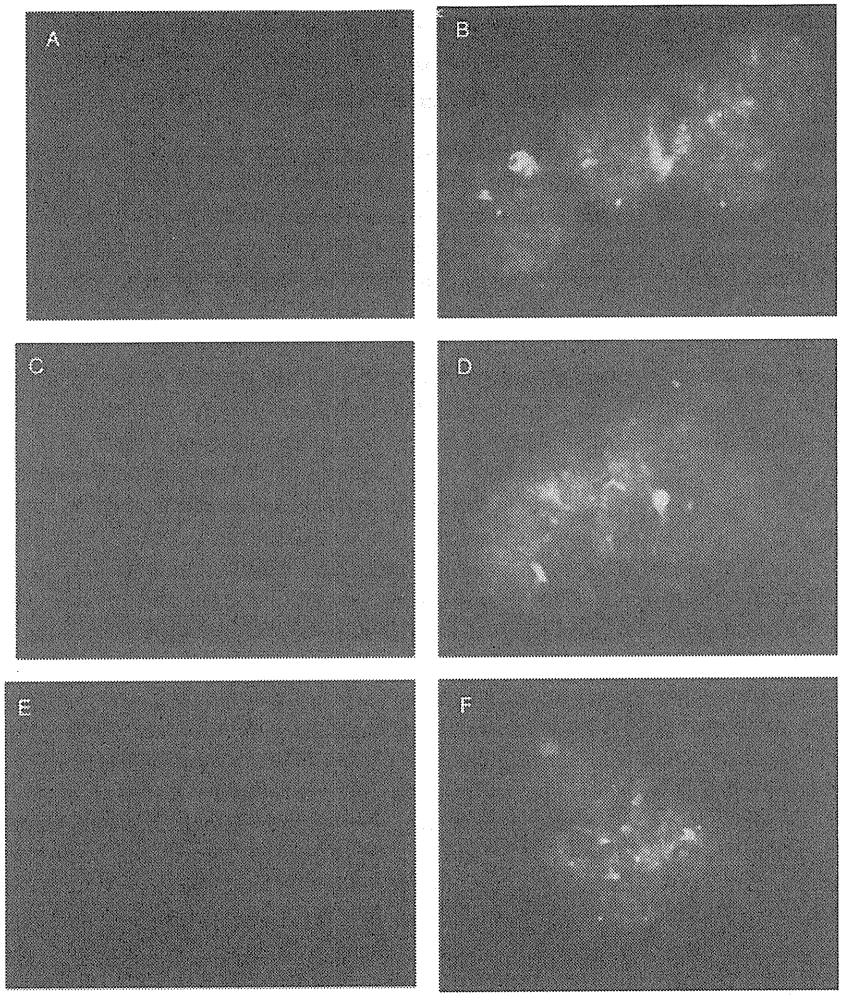

Figure 3 Immunostaining of human amnion-derived fibroblast-like (HADFIL)-57 cells for insulin (A, B), glucagon (C, D), and somatostatin (E, F) before (A, C, E) and after (B, D, F) induction of pancreatic cell differentiation. No staining is present in untreated cells (A, C, E), but clear staining is present after induction of pancreatic cell differentiation (B, D, F). Magnification, ×100. Results shown are representative of three independent experiments. The same experiments with HADFIL-52 showed similar results.

DISCUSSION

Refinement of the method to induce pancreatic cell differentiation

We developed the three-step protocol as a means of efficiently inducing the differentiation of pancreatic cells in HADFIL cell cultures. In the first step, we used a medium that included a high dose of glucose and retinoic acid. It was previously shown that a high-glucose culture is a critical factor for adult stem cell *trans*-differentiation into insulin-producing cells[15,16] and that retinoic acid induces *Pdx-1* positive endoderm.[17]

In the second step, we used Matrigel coated-dishes. Matrigel, a type of extracellular matrix, supports the migration of pancreatic progenitor cells, formation of 3-D cystic structures, and protrusion of islet buds.[18] When ES cells are cultured on Matrigel, they can form insulin positive, islet-like clusters.[19] We found that HADFIL cells cultured without Matrigel did not form islet-like cell clusters (data not shown). It seems that there is a close relationship between the formation of 3-D structures and pancreatic endocrine cell maturation. The probable reason for this is that cell clusters enable increased contact and interaction between adjacent cells and thereby accelerate the differentiation process and promote maturation of induced cells. Matrigel contains collagen and laminin, and appears to provide a suitable matrix for the formation of 3-D structures.

In the second step, we also used nicotinamide and EGF. Nicotinamide is a poly adenosine diphosphate (ADP)-ribose synthetase inhibitor that can induce islet formation from pancreatic progenitor cells, *trans-*

differentiation, and maturation of liver stem cells into insulin-producing cells.[20,21] Nicotinamide can also induce differentiation and maturation of human fetal pancreatic islet cells.[22] Human duct tissues treated with nicotinamide differentiated into glucose-responsive islet tissues *in vitro*.[23] EGF can increase the number of undifferentiated endocrine precursor cells, and the removal of EGF induces β cell formation.[24]

In the third step, we used exendin-4; a potent *GLP-1* agonist that has been shown to stimulate both β cell replication and neogenesis from ductal progenitor cells.

Overall, the method we developed for use in the present study appears to provide an efficient means for inducing pancreatic islet-like cells from HADFIL cells.

Characteristics of HADFIL cells

The transcription factor *HNF-3β* is critical for endoderm lineage cell development[25] and regulates the expression of *Pdx-1*, an islet cell-specific transcription factor.[26] HADFIL cells expressed *HNF-3β* even before the induction of differentiation (Fig. 2). Previously, we showed that HADFIL cells could differentiate into hepatocyte-like cells;[3] HADFIL cells therefore appear to possess the characteristics of endoderm cells.

The transcription factor *HNF-6* is expressed in early pancreatic epithelial cells that are precursors of the exocrine and endocrine cells.[27] *HNF-6* controls expression of *HNF-3β* and *Ngn3* and regulates pancreatic endocrine cell differentiation.[27] *HNF-6* regulates the expression of *HNF-3β* and *HNF-3β* controls the expression of *Pdx-1*. Untreated HADFIL cells expressed *HNF-6* but expression was suppressed after induction of differentiation (Fig. 2). This suggests that untreated HADFIL cells are the precursors of the exocrine and endocrine cells and are committed to forming endocrine cells following the induction of differentiation. Therefore, untreated HADFIL cells developed matured pancreatic like cells.

Nestin-positive cells are present in rodent pancreatic ducts and islets.[28,29] It was reported that nestin-positive cells might be the progenitors of insulin-producing cells.[30] In our study, untreated HADFIL cells showed a high level of expression of nestin, which was suppressed after induction of differentiation (Fig. 2). Therefore, untreated HADFIL cells appeared to contain some progenitors of insulin-producing cells and, following differentiation into more mature insulin-producing cells (Fig. 3), they express lower levels of nestin (Fig. 2).

Roles of transcription factors in the differentiation of HADFIL cells into pancreatic islet cells

In mouse embryos lacking a functional *Pdx-1* gene, pancreatic growth and development is arrested shortly after the emergence of the pre-pancreatic buds.[31] *Pdx-1* also regulates insulin gene expression and is involved in islet cell-specific expression of various genes.[32] Moreover, bone marrow-derived stem cells can be induced to differentiate into functional insulin-producing cells by the introduction of *Pdx-1*.[33] In the present study, HADFIL cells expressed *Pdx-1* after induction of differentiation (Fig. 2) and also expressed insulin (Fig. 3B).

The transcription factor *Isl-1* is essential for the development of pancreatic islet cells.[34] Expression of *Isl-1* was not detected in untreated HADFIL cells but was detected after the induction of differentiation (Fig. 2).

The transcription factors *Pax-4* and *Pax-6* are expressed in the developing gut and in the adult pancreas, and are essential factors in the generation of various cell types. Pax-4 is a key factor in the differentiation of insulin-producing β cells and somatostatin-producing δ cells.[35] On the other hand, *Pax-6* is required for the generation of glucagon-secreting α cells.[36] Neither *Pax-4* nor *Pax-6* was detected in untreated HADFIL cells but both were detected in the cells after induction of differentiation (Fig. 2). Consistent with the patterns of expression of these genes, insulin, glucagon, and somatostatin were detected in the cells after the induction of differentiation (Figs 2,3).

Application of HADFIL cells

Although further studies will be necessary to determine whether these *in vitro* differentiated cells can function *in vivo* as pancreatic islet cells, HADFIL cell populations clearly have the potential to be useful for various therapeutic applications that require human pancreatic cells.

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Research Article

Human Hematopoietic Stem Cells Can Survive In Vitro for Several Months

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We previously reported that long-lasting in vitro hematopoiesis could be achieved using the cells differentiated from primate embryonic stem (ES) cells. Thus, we speculated that hematopoietic stem cells differentiated from ES cells could sustain long-lasting in vitro hematopoiesis. To test this hypothesis, we investigated whether human hematopoietic stem cells could similarly sustain long-lasting in vitro hematopoiesis in the same culture system. Although the results varied between experiments, presumably due to differences in the quality of each hematopoietic stem cell sample, long-lasting in vitro hematopoiesis was observed to last up to nine months. Furthermore, an in vivo analysis in which cultured cells were transplanted into immunodeficient mice indicated that even after several months of culture, hematopoietic stem cells were still present in the cultured cells. To the best of our knowledge, this is the first report to show that human hematopoietic stem cells can survive in vitro for several months.

1. Introduction

Identification of in vitro culture protocols that enable somatic stem cells to survive and proliferate will be of value not only for basic research but also clinical applications that require somatic stem cells. The development of an efficient method for in vitro proliferation of mesenchymal stem cells, for example, has allowed cultured mesenchymal stem cells to be used in clinical applications [1].

Although hematopoietic stem cells have been extensively analyzed and characterized [2], in vitro proliferation of these cells remains problematic using established culture methods [3, 4]. In addition, the length of time that hematopoietic stem cells can survive in an in vitro culture system remains uncertain. CD34-positive ($CD34^+$) cells have been identified in long-term in vitro cultures of hematopoietic stem cells [5–9]. However, as none of these previous studies performed an in vivo assay of the cultured cells, such as transplantation into mice, it is uncertain whether hematopoietic stem cells with the capacity to reconstitute long-term in vivo hematopoiesis were present in these prolonged in vitro cultures.

We previously described a culture method that produced long-lasting in vitro hematopoiesis using non-human primate embryonic stem (ES) cells [10]. We speculated that hematopoietic stem cells derived from ES cells could sustain long-lasting in vitro hematopoiesis. To test this hypothesis, we initiated long-term in vitro cultures of human hematopoietic stem cells using the same culture method as previously [10]. In addition, we evaluated the in vivo function of cells cultured in vitro for several months by transplanting them into immunodeficient mice.

2. Materials and Methods

2.1. Cell Culture. We purchased human umbilical cord blood samples from the Cell Engineering Division of RIKEN BioResource Center (Tsukuba, Ibaraki, Japan). The ethical committee of the RIKEN Tsukuba Institute approved the use of human umbilical cord blood before the study was initiated. $CD34^+$ hematopoietic stem/progenitor cells were collected from human umbilical cord blood using a magnetic cell sorting system, MACS CD34 Isolation kit (Miltenyi

Biotec Inc., Sunnyvale, Calif, USA), according to the manufacturer's instructions.

Mouse-derived cell lines (OP9 and C3H10T1/2) were purchased from the Cell Engineering Division of RIKEN BioResource Center (Tsukuba, Ibaraki, Japan) and were cultured as follows: OP9 in Minimum Essential Medium-α (MEM-α; Invitrogen, Carlsbad, Calif, USA) containing 20% fetal bovine serum (FBS; Invitrogen, Calif, USA); C3H10T1/2 in Basal Medium Eagle (BME; Invitrogen) containing 10% FBS (BioWest, Miami, Fla, USA). The cell lines were γ-irradiated (50 Gy) before use as feeder cells.

$CD34^+$ cells were cultured on feeder cells in a 100 mm dish in Iscove's modified Dulbecco's medium (IMDM; SIGMA, St Louis, Mo, USA) containing 10% FBS (BioWest), 10 μg/mL bovine insulin, 5.5 μg/mL human transferrin, 5 ng/mL sodium selenite (ITS liquid MEDIA supplement; SIGMA-Aldrich, Mass, USA), 100 unit/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine (PSQ; Invitrogen), 50 ng/mL stem cell factor (SCF; R&D Systems, Minneapolis, Minn, USA), 50 ng/mL Flt-3 ligand (Flt-3L; R&D Systems), and 50 ng/mL thrombopoietin (TPO; R&D Systems). The initial number of $CD34^+$ cells placed in culture varied between experiments: 5×10^3 cells in Exp-OP9-A, Exp-10T1/2-A, and Exp-OP9-B; 4×10^4 cells in Exp-OP9-F and Exp-10T1/2-F; 5×10^4 cells in Exp-OP9-C, Exp-OP9-E, Exp-10T1/2-E, Exp-OP9-H, and Exp-10T1/2-H; 8×10^4 cells in Exp-OP9-G and Exp-10T1/2-G; 1×10^5 cells in Exp-10T1/2-I; 2×10^5 cells in Exp-OP9-D and Exp-10T1/2-D. The letters "A" to "I" after Exp-OP9 or Exp-10T1/2 indicate 9 different umbilical cord blood samples derived from 9 different neonates. Samples A, B, C, F, G, H, and I were frozen after collection, while samples D and E were used immediately as fresh samples.

Twenty-four hours after initiation of culture, the medium together with any detached cells was removed and fresh medium was added to the culture. Thereafter, the medium was changed every 3-4 days (twice a week). The number of cells attached to the feeder cells increased gradually. Approximately four weeks after initiation of culture, attached cells were dissociated using a 0.25% trypsin EDTA solution (SIGMA-Aldrich) and cultured again on new feeder cells. Thereafter, similar passages of attached cells were performed every 3-4 weeks.

The number of viable cells was assessed using an automated cell counter and an assay based on the trypan blue dye exclusion method, ViCell (BECKMAN COULTER, Fullerton, Calif, USA). The morphology of the cells was analyzed by microscopic examination after Wright staining (Muto Pure Chemicals, Tokyo, Japan).

2.2. Flow Cytometry. Cells were stained with monoclonal antibodies (MoAbs) and analyzed using a FACS Calibur (BD Biosciences, San Jose, Calif, USA). The following MoAbs were purchased from BD Biosciences: fluorescein isothiocyanate- (FITC-) conjugated MoAb against human CD14 (FITC-CD14), FITC-CD34, FITC-CD41a, and FITC-CD45; phycoerythrin-conjugated MoAb against human CD4(PE-CD4), PE-CD11b, PE-CD13, PE-CD34, PE-CD45, PE-CD56, and PE-CD235a (Glycophorin A); allophycocyanin-conjugated MoAb against human CD3 (APC-CD3), APC-CD8, APC-CD19, APC-CD33, and APC-CD45. PE-CD33 was purchased from eBiosciences (San Diego, Calif, USA). FITC-mouse IgG1, PE-mouse IgG1, APC-mouse IgG1, FITC-mouse IgG2a, and PE-mouse IgG2b were also purchased from BD Biosciences and were used as isotype controls. Cell viability was monitored by staining with propidium iodide (SIGMA-Aldrich). Flow cytometry data were analyzed using CellQuest (BD Biosciences) analysis software.

2.3. Colony Formation Assay. Cells (1×10^4) were cultured in a 35 mm dish with 1 mL of Methocult (H4435; Stem cell technology, Vancouver, British Columbia, Canada) for 10–14 days, and the number of separate colonies was determined by macroscopic morphology. Representative colonies were picked up and cell morphology was analyzed by microscopic examination after Wright staining (Muto Pure Chemicals).

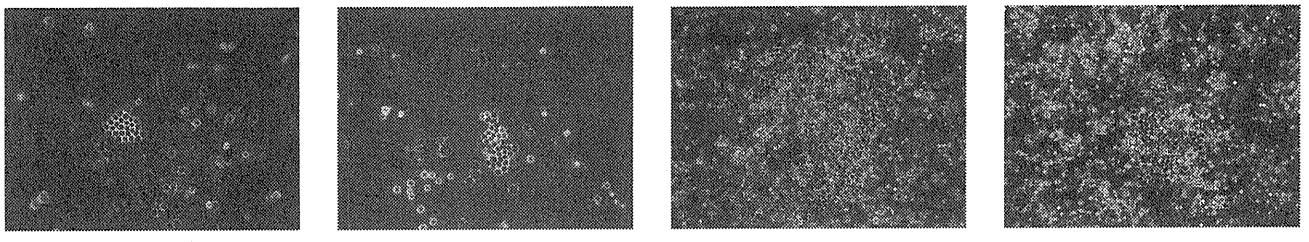
2.4. Cell Transplantation into Mice. Eight-week-old male NOD/Shi-*scid* IL-2Rγ^{null} (NOG) mice were purchased from the Central Institute for Experimental Animals (Kawasaki, Kanagawa, Japan) and used within two weeks of delivery in all experiments. Prior to cell transplantation, the mice were given a sublethal dose of γ-rays (3.0 Gy). A 200 μL cell suspension in phosphate-buffered saline (PBS; SIGMA) containing 5% FBS (BioWest) was injected intravenously into the tail vein of each mouse. All experimental procedures on the mice were approved by the Institutional Animal Care and Use Committee of the RIKEN Tsukuba Institute.

3. Results and Discussion

3.1. Long-Lasting In Vitro Hematopoiesis from Human Hematopoietic Stem Cells. Human $CD34^+$ cells were cultured on feeder cells in the presence of SCF, Flt-3L, and TPO. We used the mouse-derived cell lines, OP9 and C3H10T1/2, as feeder cells. Both OP9 [10–15] and C3H10T1/2 [10, 16–18] have been used in many studies to maintain in vitro hematopoiesis. As we show below, both OP9 and C3H10T1/2 cells supported long-lasting in vitro hematopoiesis from human hematopoietic stem cells.

About one week after initiation of culture, cobblestone areas were observed on the feeder cells (Figures 1(a) and 1(b)), indicating that human hematopoietic cells were proliferating. The numbers of cells attached to the feeder cells increased gradually (Figures 1(c) and 1(d)) and the numbers of detached cells, derived from the attached cells, also increased gradually. When the medium was changed, the numbers of detached cells in the medium were counted and the cells were subjected to analyses such as flow cytometric analysis. Detached cells were removed during the medium changes and were not cultured further in any of the experiments.

Detached cells were continuously produced for several months in experiments Exp-OP9-A, Exp-OP9-F, Exp-OP9-H, Exp-10T1/2-A, and Exp-10T1/2-H (Figure 2). As we



(a) (b) (c) (d)

FIGURE 1: Appearance of the cells attached to feeder cells. Representative examples of CD34(+) human hematopoietic stem/progenitor cells cultured on either OP9 feeder cells ((a), (c)) or C3H10T1/2 feeder cells ((b), (d)) for 7 days ((a), (b)) or 22 days ((c), (d)).

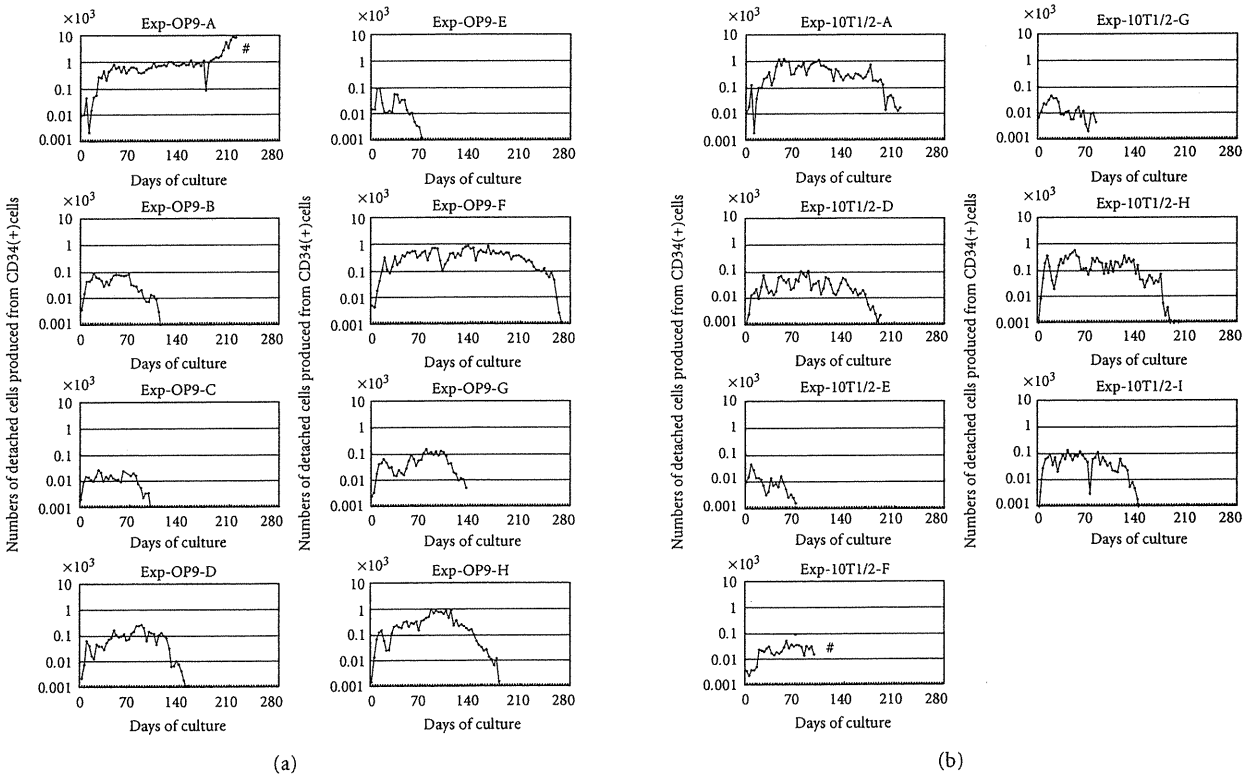


FIGURE 2: Production of hematopoietic cells in long-term cultures of human hematopoietic stem cells. (a) Eight independent experiments were performed using eight different umbilical cord blood samples and OP9 cells as feeder cells. (b) Seven independent experiments were performed using seven different umbilical cord blood samples and C3H10T1/2 cells as feeder cells. ((a), (b)) The number of detached cells in the overlying medium was counted at each medium change (approximately half weekly). The data are shown as the mean number of detached cells produced from a single CD34(+) cell, that is, the total number of detached cells divided by the number of CD34(+) cells used to initiate the culture. Exp: experiment. A to I after Exp-OP9 and Exp-10T1/2 indicate 9 different umbilical cord blood samples derived from 9 different neonates. #: Cultures Exp-OP9-A and Exp-10T1/2-F were terminated because of fungal infection.

detail below, flow cytometric analysis and a transplantation assay demonstrated that the detached cells produced in this culture method included both mature and immature hematopoietic cells, such as colony-forming cells and hematopoietic stem cells. We found that production of detached cells eventually ceased in all experiments except for Exp-OP9-A. Unfortunately, we were forced to halt Exp-OP9-A because of fungal infection although the cells in this culture proliferated efficiently and robustly before fungal contamination (Figure 2).

The numbers of detached cells varied among the experiments (Figure 2) and, notably, showed no correlation with the initial number of $CD34^+$ cells used in each experiment. Thus, 5×10^3 $CD34^+$ cells were used in Exp-OP9-A and Exp-10T1/2-A and both cultures produced substantial numbers of detached cells over a prolonged period (Figure 2). In contrast, a larger number of $CD34^+$ cells (2×10^5) was used to initiate the Exp-OP9-D and Exp-10T1/2-D cultures, but they produced considerably fewer detached cells (Figure 2). These results indicate that the rate of production of detached

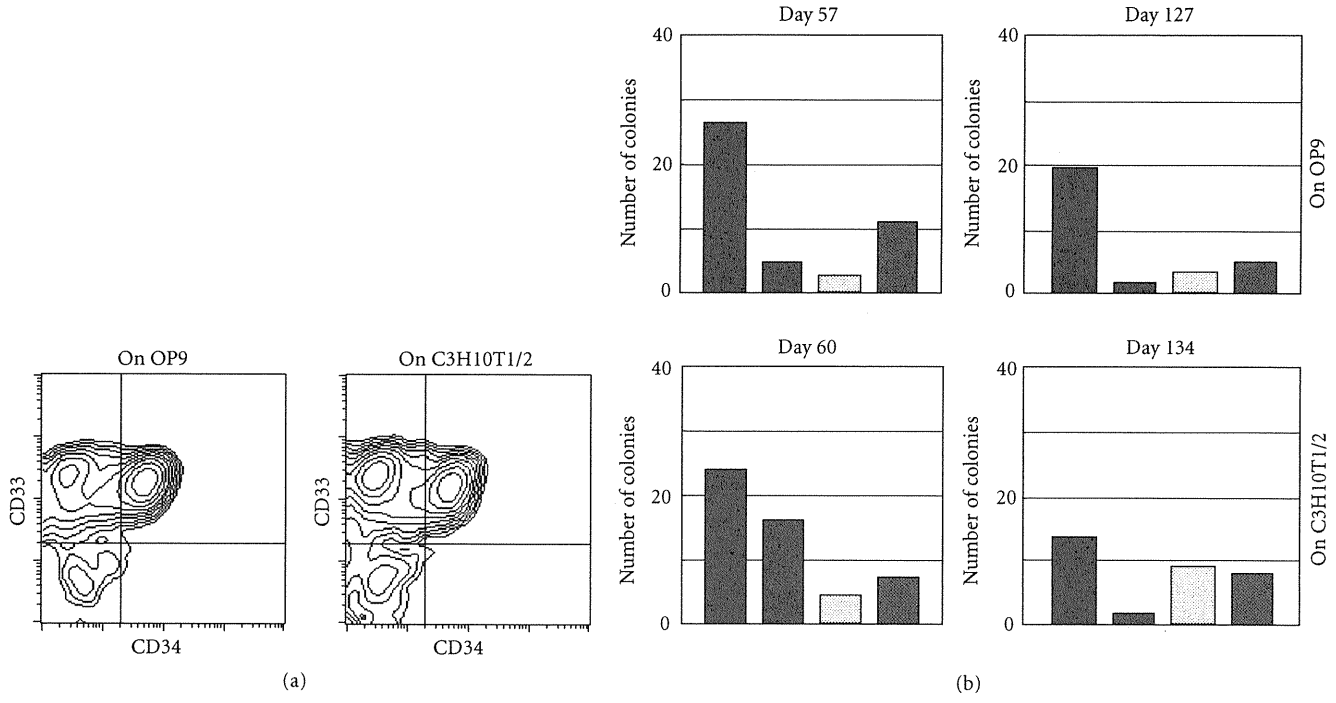


FIGURE 3: Characterization of cultured cells. (a) Flow cytometric analysis of detached cells produced in cultures on OP9 (Exp-OP9-A) and C3H10T1/2 (Exp-10T1/2-A) feeder cells and collected on Day 218 of culture. The detached cells were stained for CD33, a marker specific for granulocyte/macrophage lineage cells, and CD34, a marker specific for hematopoietic stem/progenitor cells. Flow cytometric analyses of detached cells from other experiments showed similar results. (b) Colony-formation assays. Detached cells produced in culture on OP9 feeder cells (Exp-OP9-A) were collected on Days 57 and 127 of culture. Similarly, detached cells produced in culture on C3H10T1/2 feeder cells (Exp-10T1/2-D) were collected on Days 60 and 134 of culture. The cell samples were used in a standard colony-formation assay. Black bars: colony-forming unit of monocyte/macrophage lineage cells, CFU-M. Blue bars: colony-forming unit of granulocyte lineage cells, CFU-G. Yellow bars: colony-forming unit of granulocyte and monocyte/macrophage lineage cells, CFU-GM. Red bars: burst-forming unit of erythroid cells, BFU-E. Similar results were obtained in colony-formation assays using detached cells from other cultures.

cells depended on the quality rather than the number of $CD34^+$ cells used in each experiment. In other words, when the quality of $CD34^+$ cells was high, 5×10^3 $CD34^+$ cells were sufficient to generate efficient in vitro hematopoiesis as shown in Exp-OP9-A and Exp-10T1/2-A (Figure 2).

The majority of detached cells had the morphological characteristics of granulocyte/macrophage lineage cells (Supplementary Figure S1, available at doi 10.1155/2009/936761) although some blast-like cells were also present. Consistent with their morphological phenotype, the majority of the detached cells expressed CD33, a marker of granulocyte/macrophage lineage cells (Figure 3(a)). Of note, $CD34^+CD33^+$ cells, which are less mature than $CD34^-CD33^+$ cells, were abundant among the detached cells even at 7 months after initiation of culture (Figure 3(a)).

A colony-formation assay demonstrated that granulocyte, macrophage, and erythrocyte progenitor cells were present among the detached cells (Figure 3(b)). As mentioned above, when the culture medium was changed, detached cells were removed and were not cultured further. Instead, they were either used in experimental analyses or discarded. As is shown in Figure 2, detached cells were continuously produced, and they included abundant colony-forming cells even at Day 127 and Day 134. As one example, the calculated total number of colony-forming cells present in detached cells at Day 127 (upper right, Figure 3(b)) was 13 311, which corresponded to a greater than ten-fold increase in the numbers of colony-forming cells compared to the starting material of this culture, that is, 983 colony-forming cells in 5×10^3 $CD34^+$ cells. Thus, the culture method we describe here could continuously produce abundant colony-forming cells for several months.

Although a mixed colony (a colony derived from very immature hematopoietic cells) was not observed in the colony formation assay (Figure 3(b)), it nevertheless remained possible that hematopoietic stem cells were present at a very low frequency among the detached cells. Therefore, we performed a transplantation assay in which detached cells were injected into an immunodeficient NOD/Shi-*scid* IL-$2R\gamma^{null}$ (NOG) mouse (mentioned hereafter).

3.2. Hematopoietic Stem Cells Cultured In Vitro for Several Months Give Rise to Long-Lasting In Vivo Hematopoiesis after Transplantation into Mice. Detached cells were collected from Exp-OP9-A on Day 169 after initiation of culture and transplanted into an NOG mouse (3.9×10^6 cells) (Figure 4(a)). Peripheral blood samples from the mouse were subjected to flow cytometric analysis on Days 56 and

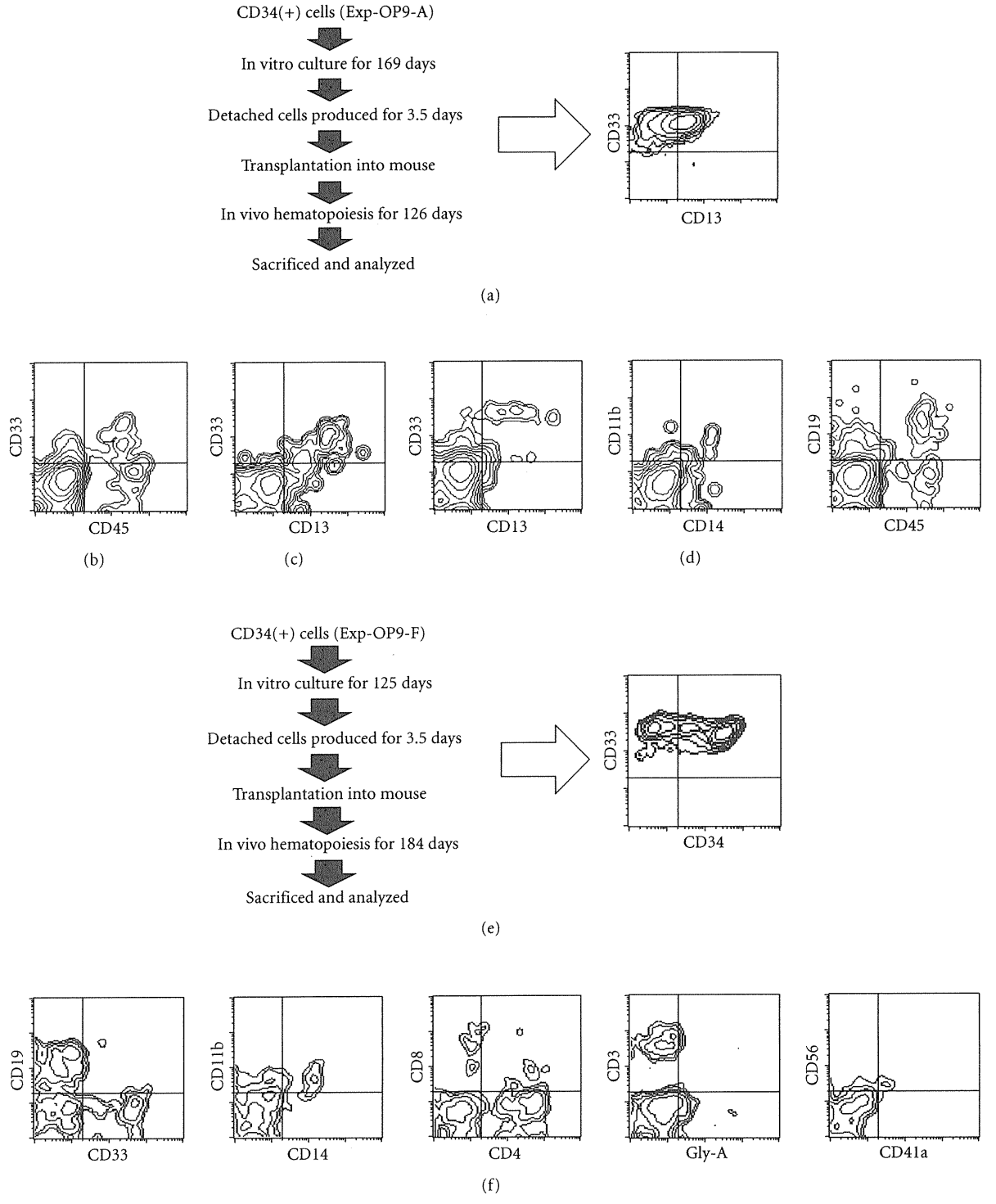


FIGURE 4: Flow cytometric analysis of hematopoietic cells of the mouse that had been transplanted with human hematopoietic cells produced by in vitro culture. ((a), (e)) Schema of the experimental procedure and flow cytometric analysis of transplanted cells. ((a)–(d)) Detached cells (3.9×10^6 cells) produced on OP9 feeder cells (Exp-OP9-A) were collected on Day 169 of culture and transplanted into an immunodeficient NOG mouse. Peripheral blood was collected on Days 56 (b) and 112 (c) after transplantation, and bone marrow cells were collected on Day 126 after transplantation (d). ((e), (f)) Detached cells (2.4×10^6 cells) produced on OP9 feeder cells (Exp-OP9-F) were collected on day 125 of culture and transplanted into an immunodeficient NOG mouse. The bone marrow cells were collected on Day 184 after transplantation and were analyzed. ((a)–(f)) The cells were stained using monoclonal antibodies against CD45, a leukocyte common antigen, CD34, a marker specific for hematopoietic stem/progenitor cells, CD33 and CD13, markers of granulocyte and monocyte/macrophage lineage cells, CD11b and CD14, markers of monocyte/macrophage lineage cells, CD19, a marker of B lymphocyte lineage cells, CD3, CD4, and CD8, markers of T lymphocyte lineage cells, Gly-A (Glycophorin A), a marker of erythroid cells, CD56, a marker of large granular lymphocytes and natural killer cells, and CD41a, a marker of megakaryocyte/platelet lineage cells.

112 after transplantation. Human hematopoietic cells were clearly present in the peripheral bloods (Figures 4(b), 4(c)). The mouse was sacrificed on Day 126 after transplantation, and bone marrow and spleen cells were subjected to flow cytometric analysis. Human hematopoietic cells were present in the bone marrow (Figure 4(d)) but were present at a very low rate in spleen (data not shown). The estimated rate of chimerism of human $CD45^+$ cells in bone marrow was 2.6% when compared to the number of mouse $CD45^+$ cells. The human hematopoietic cells detected in the bone marrow included cells of the myeloid lineage ($CD13^+CD33^+$: 9.7% of the human $CD45^+$ cells), the monocyte/macrophage lineage ($CD11b^+CD14^+$: 3.8% of the human $CD45^+$ cells), the B cell lineage ($CD19^+$: 80.5% of the human $CD45^+$ cells) (Figure 4(d)), and other lineages at very low levels (data not shown).

Detached cells were collected from Exp-OP9-F on day 125 after initiation of culture and transplanted into an NOG mouse (2.4×10^6 cells) (Figure 4(e)). $CD45^+$ human hematopoietic cells were present in peripheral blood from the mouse one month after transplantation (data not shown). The mouse was sacrificed on Day 184 after transplantation, and the bone marrow cells were subjected to flow cytometric analysis. The rate of chimerism of human $CD45^+$ cells in the bone marrow was 0.2% when compared to the number of mouse $CD45^+$ cells. The bone marrow contained human hematopoietic cells, which included cells of the myeloid lineage ($CD33^+$: 23.5% of the human $CD45^+$ cells), the monocyte/macrophage lineage ($CD11b^+$ $CD14^+$: 6.7% of the human $CD45^+$ cells), as well as the B ($CD19^+$: 53.7% of the human $CD45^+$ cells) and T cell lineages ($CD3^+$, $CD4^+$, $CD8^+$, and $CD4^+CD8^+$: 10.7% of the human $CD45^+$ cells) (Figure 4(f)). Erythroid (Glycophorin A^+) and megakaryocyte ($CD41a^+$) lineage cells were present at very low rates (Figure 4(f)).

In the transplantation assay described above, human hematopoietic cells of various lineages were present in mice up to six months after cell transplantation. In general, it is impossible that in vivo hematopoiesis derived from transplanted cells is maintained for several months solely by committed progenitor cells. Therefore, the transplanted cells appeared to include hematopoietic stem cells, that is, even after in vitro culture for several months, hematopoietic stem cells were still present in cultures Exp-OP9-A and Exp-OP9-F. In light of the numbers of cells produced in culture and of the duration of this production, the hematopoietic stem cells also appeared to have survived for several months in Exp-OP9-H, Exp-10T1/2-A, and Exp-10T1/2-H.

The in vitro expansion of human hematopoietic stem cells is known to be very difficult [3]. In agreement with this, a mixed colony (a colony derived from very immature hematopoietic cells) was not observed in the colony formation assay in this study (Figure 3(b)), indicating that hematopoietic stem cells did not expand to any great extent in our culture method. However, since it was highly unlikely that in vitro hematopoiesis could be maintained for several months solely by committed progenitor cells present in the starting materials, the long-lasting in vitro hematopoiesis was likely maintained by hematopoietic stem cells.

On the basis of a previous estimation of the numbers of hematopoietic stem cells capable of repopulating in NOD/SCID mice [19], the starting materials we used in Exp-OP9-A and Exp-OP9-F should have included a very low number of NOD/SCID-repopulating cells. However, the transplantation assay demonstrated that NOD/SCID-repopulating cells were present among the detached cells that were continuously produced in our culture method (Figure 4), strongly suggesting that our culture method continuously produced small numbers of new NOD/SCID-repopulating cells throughout the long-term culture period. Hence, the total number of NOD/SCID-repopulating cells that were produced as detached cells throughout the whole long-term in vitro culture was likely greater than the number of such cells that were present in the starting materials.

Taken together, the hematopoietic stem cells capable of repopulating in NOD/SCID mice in our culture system appeared to be maintained by asymmetric cell division, that is, one of the daughter cells retained the characteristics of hematopoietic stem cells and another did not.

Leukemic stem cells might also survive and/or proliferate in our culture method for a prolonged period, enabling basic research or screening for effective anticancer drugs to be performed on these cultured cells. In addition, basic research on specific diseases, such as aplastic anemia or paroxysmal nocturnal hemoglobinuria, might benefit from a long-term culture system for hematopoietic stem cells derived from patients.

4. Conclusions

To the best of our knowledge, this is the first report to show that human hematopoietic stem cells can survive in vitro for several months. Since the duration of in vitro hematopoiesis appeared to depend on the quality of hematopoietic stem cells present in each sample, our culture method may be of value for assessing the quality of hematopoietic stem cells prior to their use in the clinic. In particular, our method could be used for the evaluation of umbilical cord bloods since these samples are routinely used in the clinic following preservation for several months. For example, in this study the quality of hematopoietic stem cells derived from samples A, F, and H appeared to be higher compared to other samples.

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