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Original Article

Human Umbilical Cord Blood as a Source of Transplantable Hepatic Progenitor Cells

SEI KAKINUMA,[a,b] YUJIRO TANAKA,[a] RYOKO CHINZEI,[a,b] MAMORU WATANABE,[a] KEIKO SHIMIZU-SAITO,[b] YUZURU HARA,[a,b] KENICHI TERAMOTO,[a] SHIGEKI ARII,[a] CHIFUMI SATO,[a] KOZO TAKASE,[a] TAKEHIKO YASUMIZU,[c] HIROBUMI TERAOKA[b]

[a]Graduate School, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan; [b]Medical Research Institute, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo, Japan; [c]Department of Obstetrics and Gynecology, Kanto Medical Center NTT EC, Shinagawa-ku, Tokyo, Japan

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ABSTRACT

Human umbilical cord blood (UCB) cells have many advantages as grafts for cell transplantation because of the immaturity of newborn cells compared with adult cells. In contrast to their hematopoietic and mesenchymal potential, it remains unclear whether UCB cells have endodermal competence. Here, with a view to utilize UCB cells for cell transplantation into injured liver, we investigated the hepatic potential of UCB cells both in vitro and in vivo. We determined the most efficient conditions leading UCB cells to produce albumin (ALB). In a novel primary culture system supplemented with a combination of growth/differentiation factors, about 50% of UCB cells in 21-day cultures expressed ALB, and the ALB$^+$ cells coexpressed hepatocyte lineage markers. The ALB-expressing cells were able to proliferate in the culture system. Moreover, in the cell-transplantation model into liver-injured severe combined immunodeficient mice, inoculated UCB cells developed into functional hepatocytes in the liver, which released human ALB into the sera of the recipient mice. In conclusion, this study demonstrates that human UCB is a source of transplantable hepatic progenitor cells. Our findings may have relevance to clinical application of UCB-derived cell transplantation as a novel therapeutic option for liver failure. *Stem Cells 2003;21:217-227*

INTRODUCTION

Umbilical cord blood (UCB) contains circulating stem/progenitor cells, and the cellular contents of UCB are known to be quite distinct from those of bone marrow (BM) and adult peripheral blood. Over the past two decades, the presence and characteristics of hematopoietic stem cells in UCB have been clarified [1-3]. The frequency of UCB hematopoietic stem/progenitor cells equals or exceeds that of BM and greatly surpasses that of adult peripheral blood [4]. Compared with adult cells, UCB hematopoietic stem cells produce larger hematopoietic colonies in vitro, have different growth factor requirements, are able to expand in long-term culture in vitro, and have longer telomeres [5-7]. UCB transplantation for various hematopoietic diseases has resulted in successful hematopoietic reconstitution and a lower incidence of graft-versus-host disease than expected

Correspondence: Hirobumi Teraoka, Ph.D., Department of Pathological Biochemistry, Medical Research Institute, Tokyo Medical and Dental University, 2-3-10 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062, Japan. Telephone: 81-3-5280-8074; Fax: 81-3-5280-8075; e-mail: hteraoka.pbc@mri.tmd.ac.jp *Received September 9, 2002; accepted for publication October 11, 2002.*

with conventional therapies [8, 9]. Recently, it has been reported that UCB contains mesenchymal progenitor cells capable of differentiating into marrow stroma, bone, cartilage, muscle, and connective tissues [10]. Furthermore, UCB provides no ethical problems for basic studies and clinical applications. UCB cells can be collected without any harm to the newborn infant, and UCB hematopoietic stem cell grafts can be cryopreserved and transplanted to a host after thawing without losing their repopulating ability [11]. For these reasons, UCB could be a prominent source of cells for transplantation in various diseases. It remains obscure, however, whether UCB contains stem/progenitor cells leading to endodermal cells, including hepatocytes.

In the present study, we investigated, in vitro and in vivo, whether UCB is a potential source of cells for transplantation for the support of liver injury. We found that human UCB cells generated hepatocyte lineage cells in our original primary culture system supplemented with a combination of growth/differentiation factors. In the cell-transplantation model for the support of liver injury, human UCB cells displayed the characteristics of functionally differentiated hepatocytes and released human albumin (ALB) into the recipient's peripheral blood after engraftment in liver-injured severe combined immunodeficient (SCID) mice, indicating that UCB-derived hepatocytes may play a role in the support for liver injury. Therefore, human UCB is a suitable source of transplantable cells for liver injury.

Materials and Methods

Materials

UCB samples from full-term deliveries were collected after informed consent had been obtained in writing. The study protocol was approved by the ethics committees of the Medical Research Institute, Tokyo Medical and Dental University, and Kanto Medical Center NTT EC. Dulbecco's modified Eagle's medium (DMEM) and monothioglycerol were purchased from Sigma (St. Louis, MO; http://www.sigmaaldrich.com). Fetal bovine serum ([FBS] JRH Biosciences; Lenexa, KS; http://www.jrhbio.com), HEPES buffer solution, recombinant human fibroblast growth factor (FGF)-1, recombinant human FGF-2, penicillin/streptomycin/L-glutamine (100×), and amphotericin B (GIBCO/BRL; Grand Island, NY; http://www.invitrogen.com), recombinant human leukemia inhibitory factor (LIF), recombinant human stem cell factor (SCF), and recombinant human oncostatin M ([OSM] R&D Systems; Minneapolis, MN; http://www.rndsystems.com) were used. Recombinant human hepatocyte growth factor (HGF) was kindly provided by Mitsubishi Chemical Co. (Tokyo, Japan; http://www.m-kagaku.co.jp) to *S.A*. Hydroxyethyl starch was from Nipro Co. (Osaka, Japan; http://www.nipro.co.jp). Normal human liver sections were obtained from the region containing no tumor at surgical resection of hepatocellular carcinoma. Human serum was obtained from healthy volunteers.

Cell Culture

Equal volumes of UCB and 6% hydroxyethyl starch were mixed in sterile centrifuge tubes and left to stand for 90 minutes. The red cells were allowed to settle by gravity. Nucleated cells were obtained from the supernatant. After washing once with sterilized phosphate-buffered saline (PBS), isolated UCB cells were primarily cultured in DMEM supplemented with 15% FBS, 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 300 μM monothioglycerol, and a combination of several growth/differentiation factors including FGF-1 (20 ng/ml), FGF-2 (10 ng/ml), LIF (10 ng/ml), SCF (10 ng/ml), HGF (10 ng/ml), and OSM (10 ng/ml). UCB cells were plated onto 0.1% gelatin-coated tissue culture dishes and maintained at 37ºC in a 5% CO_2 atmosphere. Culture media were replaced every 7 days.

mRNA Detection by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total mRNA was extracted from freshly isolated UCB cells and cultured cells with an mRNA isolation kit for blood/bone marrow (Roche Diagnostics; Basel, Switzerland; http://www.roche-applied-science.com). First-strand cDNA was synthesized using Superscript II RNase H RT (GIBCO/BRL) according to the manufacturer's instructions. The resulting cDNA was amplified by use of a Light Cycler (Roche) under the following conditions: human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5′-GTC TTCTCCACCATGGAGAAGGCT, 5′-CATGCCAGTGAG CTTCCCGTTCA) at 95ºC for 120 seconds followed by 30 cycles at 94ºC for 0 seconds, 58ºC for 5 seconds, and 72ºC for 16 seconds; mouse GAPDH (5′-TGAAGGTCGGTGTG AACGGATTTGGC, 5′-TGTTGGGGGCCGAGTTGGG ATA) at 95ºC for 120 seconds followed by 30 cycles at 94ºC for 0 seconds, 55ºC for 5 seconds, and 72ºC for 50 seconds; human ALB (5′-TTGGAAAAATCCCACTGCAT, 5′-CTCC AAGCTGCTCAAAAAGC) at 95ºC for 120 seconds followed by 35 cycles at 94ºC for 0 seconds, 58ºC for 5 seconds, and 72ºC for 20 seconds; human glutamine synthetase (GS) (5′-GTCAAGATTGCGGGGACTAA, 5′-TACGATTGGCT ACACCACCA) at 95ºC for 120 seconds followed by 35 cycles at 94ºC for 0 seconds, 58ºC for 5 seconds, and 72ºC for 20 seconds; human cytokeratin (CK)-18 (5′-GAGATCGAG GCTCTCAAGGA, 5′-CAAGCTGGCCTTCAGATTTC) at 95ºC for 120 seconds followed by 40 cycles at 94ºC for 0 seconds, 58ºC for 5 seconds, and 72ºC for 20 seconds; and

human alpha-fetoprotein (AFP) (5′-TGCCAACTCAGTGAG GACAA, 5′-TCCAACAGGCCTGAGAAATC) at 95ºC for 120 seconds followed by 40 cycles at 94°C for 0 seconds, 58ºC for 5 seconds, and 72ºC for 20 seconds. The amplified products were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

Immunofluorescent Staining Analysis

Cultured UCB cells were fixed with 80% acetone for 20 minutes at -20ºC. Samples were washed three times with PBS then incubated for 1 hour with diluted primary antibodies at room temperature. Rabbit anti-human ALB antibody (DAKO; Kyoto, Japan; http://www.dako.dk), anti-human CK-18 monoclonal antibody (mAb) (clone DC10; DAKO), anti-human CK-19 mAb (clone IF15, Oncogene Research Products; Cambridge, MA; http://www.apoptosis.com), and anti-proliferating cell nuclear antigen (PCNA) mAb (clone NA03; Oncogene Research Products) were used. Samples were washed three times with PBS then incubated for 30 minutes at room temperature with anti-rabbit IgG-fluorescein isothiocyanate ([FITC] Sigma) or anti-mouse IgG-tetramethyl rhodamine isothiocyanate ([TRITC] Santa Cruz Biotechnology; Santa Cruz, CA; http://www.scbt.com). For each analysis, negative control was performed either by addition of an appropriate nonimmune serum or by removal of the primary antibody from the protocol. To evaluate the number of immunostained cells, photographs of 100 random fields were taken, and more than 1,000 immunostained cells were counted.

Cell Transplantation

C.B-17/Icr SCID mice were purchased from Clea Japan, Inc. (Tokyo, Japan; http://www.clea-japan.co.jp). Mice were injected with 0.4 mg/body 2-acetylaminofluorene (2-AAF), then 7 days after injection, they were subjected to one-third partial hepatectomy and infusion of a total of 1×10^7 isolated UCB cells in 0.1 ml of PBS into the liver via the portal vein under anesthesia. Control mice were subjected only to 2-AAF injection and partial hepatectomy. These mice were sacrificed from 4-55 weeks after cell transplantation. The animals were managed according to Tokyo Medical and Dental University guidelines.

Fluorescence In Situ Hybridization (FISH) and Immunohistology

In FISH for human X chromosome, slides containing 5-μm frozen liver sections were digested at 37ºC with 100 μg/ml RNase A for 30 minutes then with 0.005% pepsin solution (pH 2.0) for 5 minutes. Human X chromosome DNA probe labeled with FITC (CEP X Spectrum Green, Vysis; Downers Grove, IL; http://www.vysis.com) was treated according to the manufacturer's instructions. Slides were heated for 5 minutes at 73ºC then hybridized with the denatured probe overnight at 37ºC. After washing, slides were mounted in 4',6-diamidino-2-phenylindole (DAPI) antifade. Multicolored immunofluorescent staining was analyzed by use of a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss; Oberkochen, Germany; http://www.zeiss.com). For subsequent immunohistochemistry of human ALB, slides containing FISH-positive signals were treated with 3% hydrogen peroxide at room temperature then immunostained with rabbit-polyclonal antibody against human ALB (DAKO), which cross-reacts very slightly with mouse ALB. The primary antibody was detected using horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody. The complex was visualized with 3,3-diaminobenzidine tetrahydrochloride (DAB).

For immunohistological analysis, formalin-fixed, paraffin-embedded liver sections were obtained from recipient mice livers, and 5-μm liver sections were deparaffinized. Slides were heated in 10 mM Na-citrate buffer (pH 6.0) at 95ºC for 40 minutes then treated with 3% hydrogen peroxide at room temperature. Immunodetection was performed with an MOM immunodetection kit (Vector; Burlingame, CA; http://www.vectorlabs.com) according to the manufacturer's instructions. Anti-human hepatocyte mAb (clone OCH1E5; DAKO) was used as the primary antibody, which reacts with only human hepatocytes and human bile duct cells, but not with mouse liver cells [12]. The primary antibody was detected using biotin-conjugated anti-mouse IgG antibody then incubated with streptoavidin-HRP. The complex was visualized with DAB and counterstained with hematoxylin. In the case of double-fluorescent staining, streptoavidin-FITC was used instead of streptoavidin-HRP. After washing, slides were incubated for 1 hour with diluted rabbit polyclonal anti-human ALB antibody (DAKO) at room temperature. Samples were washed three times with PBS then incubated for 30 minutes at room temperature with anti-rabbit IgG-TRITC (Sigma). After washing, slides were mounted in DAPI antifade.

Immunoblotting

Mouse sera (1 μl of 20-fold dilution with distilled water) were subjected to 0.1% SDS-7.5% PAGE, and the separated proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech; Uppsala, Sweden; http://www.apbiotech.com). The human ALB protein was detected on immunoblots using an mAb against human ALB (clone 4761; Institute of Immunology; Tokyo, Japan) diluted with 0.05% Triton X-100 in Tris-buffered saline containing 1% gelatin, followed by HRP-conjugated anti-mouse IgG (Bio-Rad; Hercules, CA; http://www.

bio-rad.com). Immunoreactive bands were developed by ECL (Amersham) and blots were exposed to Fuji medical x-ray film (Fuji Film; Tokyo, Japan; http://www.fuji film.com).

Results

A Novel Primary Culture System of UCB Cells Produces ALB$^+$ Cells

In order to investigate the hepatic competence of UCB cells in vitro, we established a novel primary culture system. Nucleated cells isolated from UCB were cultured in the basic medium supplemented with various combinations of growth/differentiation factors (FGF-1, FGF-2, LIF, SCF, HGF, and OSM), each of which contributes in a different manner to proliferation and differentiation of hepatic progenitor cells in the rodent and human liver [13-18]. Cultured cells were examined for the expression profile of ALB mRNA, which is expressed abundantly in hepatocytes (Fig. 1A). In the 21-day culture without exogenous growth/differentiation factors, UCB cells scarcely attached on the culture dishes, and ALB mRNA was not detected in any samples examined. Similarly, in the medium with a single supplement of HGF, FGF-1, FGF-2, LIF, SCF, or OSM, attached cells were rarely observed, and ALB mRNA was not detected. In contrast, ALB mRNA was detected in the medium supplemented with the combination of FGF-1, FGF-2, LIF, SCF, and HGF.

To determine which of these five factors is necessary for the induction of ALB mRNA, we tested combinations lacking either LIF, SCF, or HGF with UCB cells obtained from an identical donor (Figs. 1B and 1C). With the combination lacking HGF, mainly spindle-shaped cells attached to the dish, and the expression of ALB mRNA was only weakly detectable at 21 days of culture. With the combinations lacking SCF or LIF, fewer round cells attached to the dish than with the combination of all five factors, while the expression of ALB mRNA was comparable with that for the five factors when normalized to GAPDH mRNA. These findings indicate that HGF is essential for UCB cells to generate ALB-producing cells in this culture system. In the same way, we compared the effects of 34 different combinations or sequential additions of FGF-1, FGF-2, LIF, SCF, HGF, and OSM. Human ALB mRNA was detected at the highest level in the cultured cells with the combination of FGF-1, FGF-2, LIF, SCF, and HGF and was detectable in 21 of 28 samples from different donors (75%). On the basis of

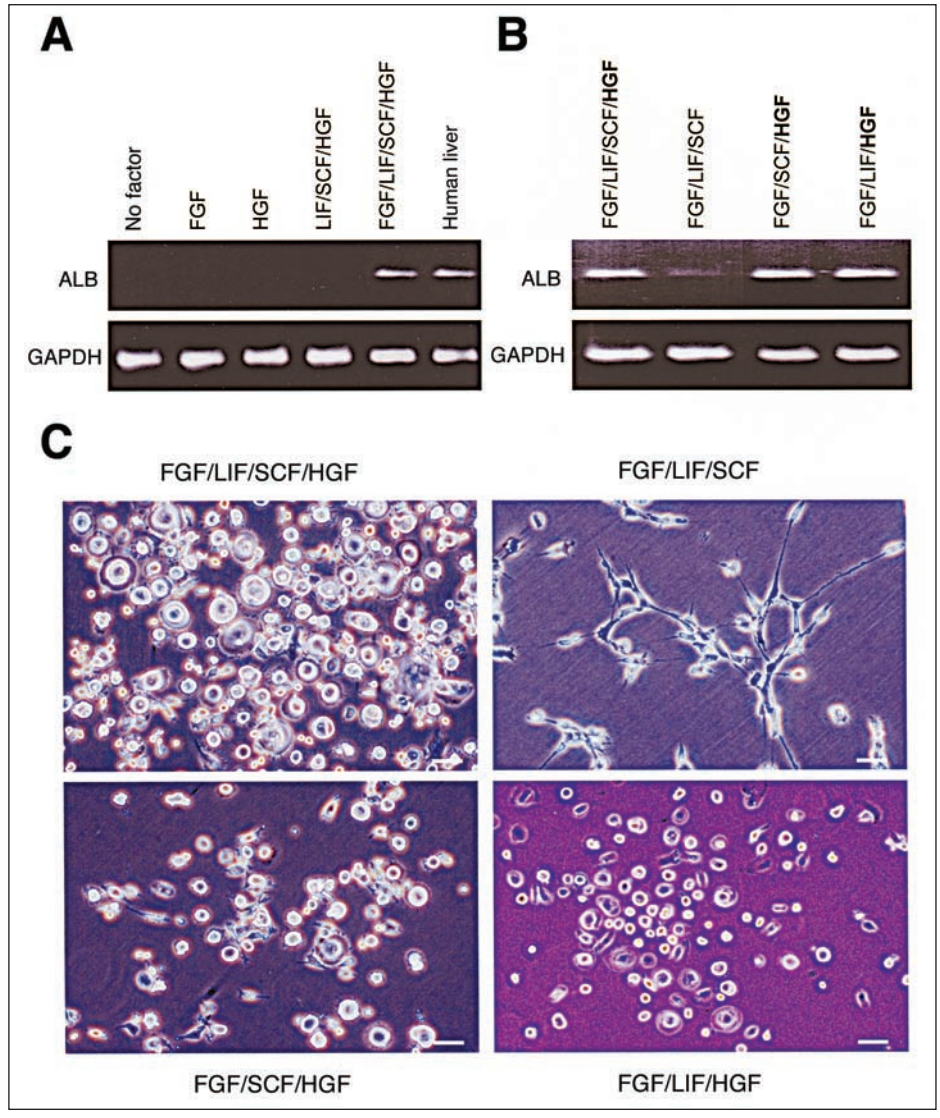

Figure 1. ALB-mRNA expression and morphology of 21-day cultured UCB cells. *UCB cells were cultured for 21 days with or without growth/differentiation factors. A, B) RT-PCR products of ALB (350 bp) and GAPDH (395 bp) are shown. Human liver was employed as a positive control. C) Phase contrast view of UCB cells at 21 days with the growth/differentiation factors indicated. Different combinations of growth/differentiation factors were added to the culture media. The combinations were: FGF/LIF/SCF/HGF (upper left), FGF/LIF/SCF (upper right), FGF/SCF/HGF (lower left), and FGF/ LIF/HGF (lower right), respectively. Scale bars = 10 µm.*

this examination, we determined that this combination was the most efficient in leading UCB cells to express ALB mRNA.

We next examined the time dependency of the ALB mRNA expression and the production of ALB in the UCB cells obtained from the same donors. RT-PCR analysis revealed the expression of ALB mRNA in the 7-day culture of UCB cells, and greater expression was observed at 14 and 21 days, whereas no transcript of ALB was detected at day 0 (Fig. 2A). Quantitative RT-PCR analysis by use of the Light Cycler showed that the ALB mRNA level of the 21-day cultured cells was approximately 40-fold higher than that of the 7-day cultured cells (data not shown). This finding indicates that freshly isolated UCB cells of these donors did not appreciably contain hepatic progenitor cells expressing ALB mRNA at detectable levels and that ALB-expressing cells appeared with subsequent increases in the number of cultured cells. From a morphological viewpoint, small round cells appeared at 7 days, and both round and spindle-shaped cells were observed at 14 and 21 days (Fig. 2B, left panels). Immunofluorescent staining analysis demonstrated that the round cells, but not the spindle-shaped cells, expressed ALB, and the number of ALB-producing round cells increased with culture time (Fig. 2B, right panels). ALB^+ cells accounted for about 50% of the attached cells at 21 days in the primary culture. These results demonstrate that UCB cells cultured with the combination of FGF-1, FGF-2, LIF, SCF, and HGF are capable of producing ALB^+ cells efficiently in vitro.

Expression Profiles of Hepatocyte Lineage Markers

To confirm that the cultured round cells derived from UCB produce the hepatocyte lineage, we examined additional differentiation markers for hepatocyte lineages. RT-PCR analysis revealed that the transcripts of GS, CK-18, and AFP were expressed in the 21-day cultured cells (Fig. 3A). GS mRNA, as well as ALB mRNA, was detected in 9 of 10 samples from different donors, CK-18 mRNA was detected in 4 of 10 samples, and AFP mRNA was seen in 2 of 10 samples. GS is known to be expressed in mature hepatocytes [19, 20], CK-18 is expressed in the hepatocyte lineage [21, 22], and AFP is expressed in hepatic progenitor cells, including oval cells [23, 24]. These previous reports support our findings that the cultured cells had the same characteristics as the hepatocyte lineage. Next, immunofluorescent analysis showed that the cultured cells expressed CK-18 and CK-19. CK-19 is a cell marker for bile duct epithelial cells and hepatic progenitor cells such as oval cells [23-25]. UCB cells in 21-day cultures were positively

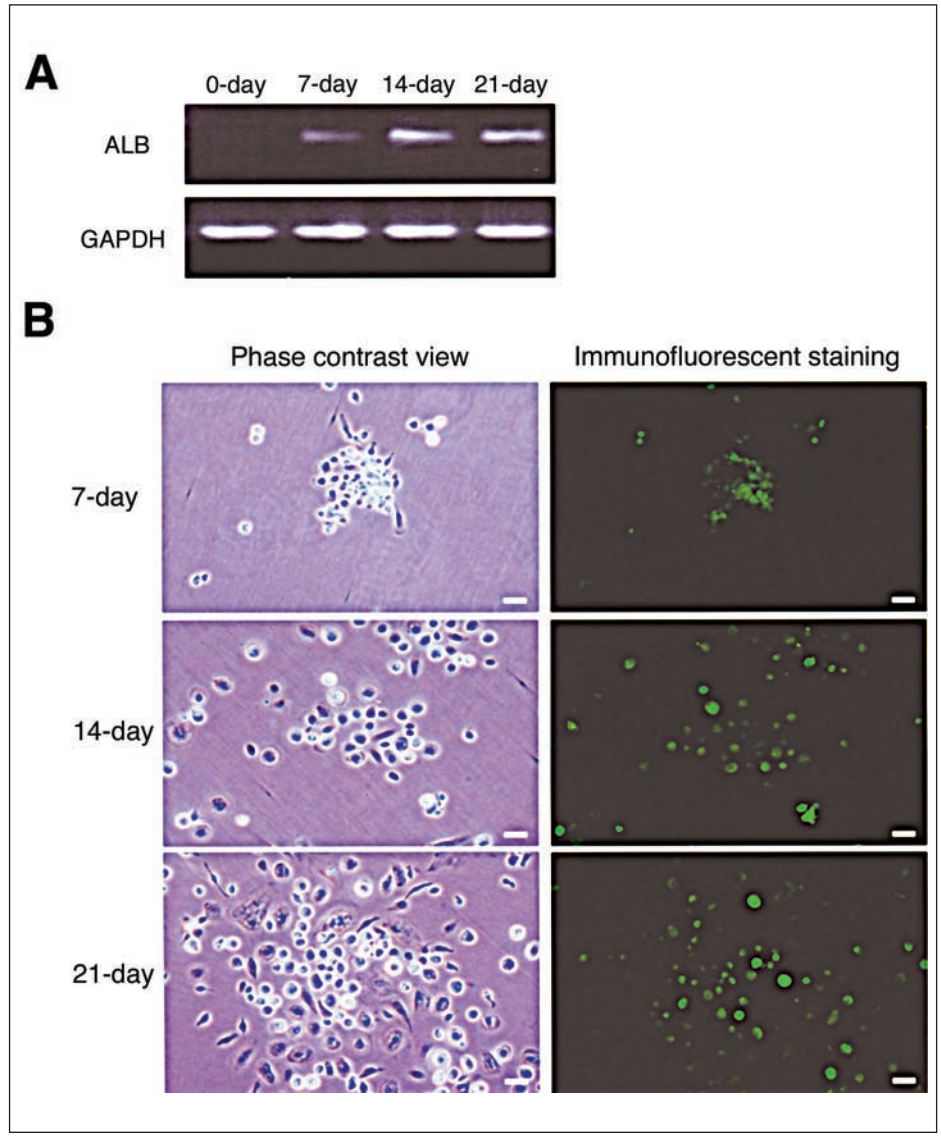

Figure 2. Time-dependent appearance of ALB-producing cells derived from UCB cells in the culture system supplemented with FGF-1, FGF-2, LIF, SCF, and HGF. *A) RT-PCR products of ALB (350 bp) and GAPDH (395 bp) are shown. B) Phase contrast view (left panels) and immunofluorescent staining of ALB with FITC-labeled anti-rabbit IgG antibody (right panels) of cultured UCB cells. Scale bars = 10 μm.*

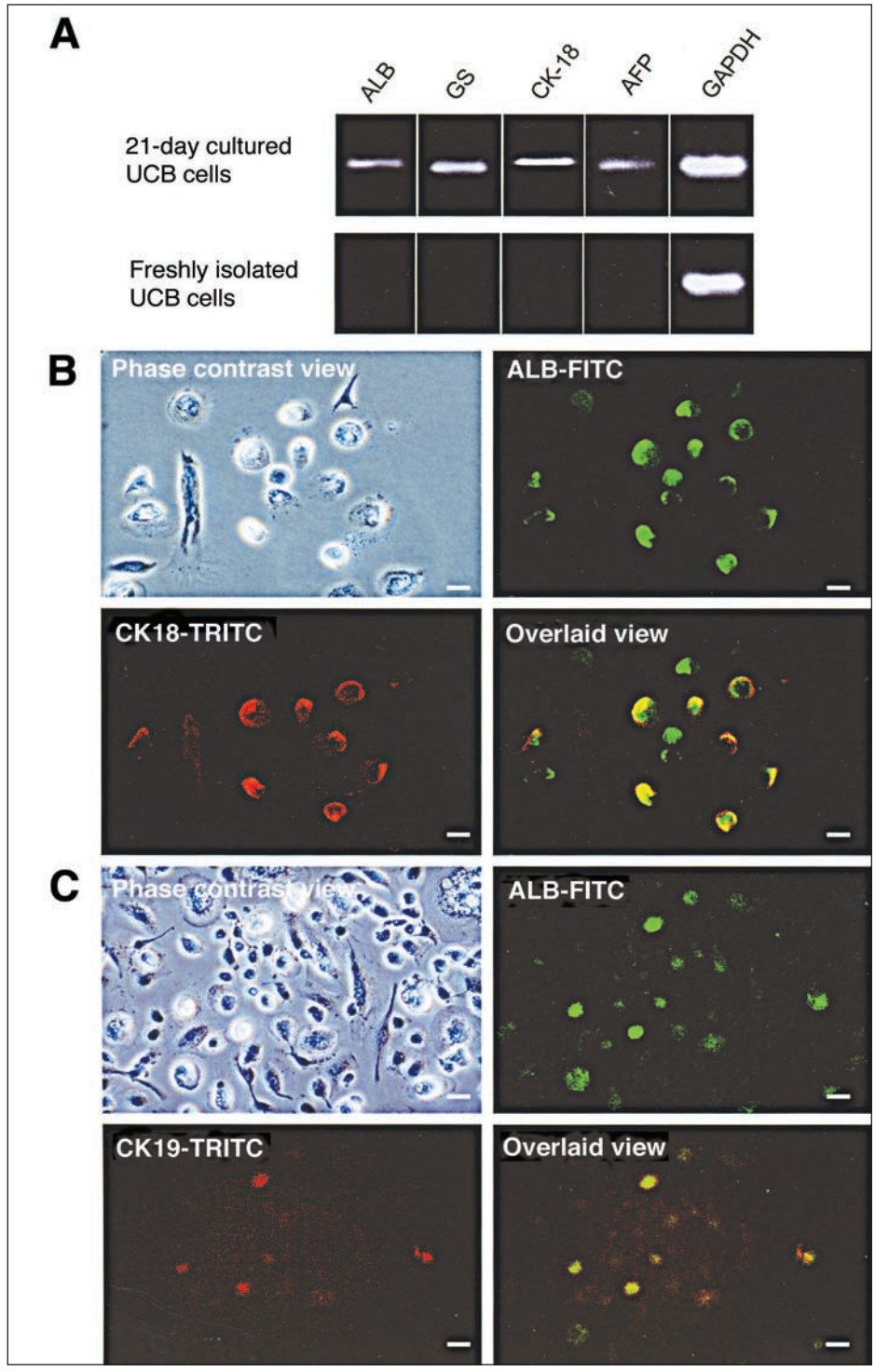

Figure 3. Expression of differentiation markers for hepatocyte lineage in the cultured UCB cells. *A) RT-PCR of transcripts from freshly isolated UCB cells and 21-day cultured cells in the presence of FGF, LIF, SCF, and HGF. The RT-PCR products from transcripts of ALB, GS, CK-18, AFP, and GAPDH were 350 bp, 397 bp, 357 bp, 356 bp, and 395 bp, respectively. B) Dual staining of ALB (green, see Fig. 2) and CK-18 (red; TRITC-labeled anti-mouse IgG antibody). C) The 21-day cultured human UCB cells were dual stained to detect ALB (green) and CK-19 (red; TRITC conjugated with anti-mouse IgG antibody). Scale bars = 10 μm.*

stained for CK-18, and dual-staining analysis showed the presence of ALB in almost all CK-18-expressing cells (Fig. 3B). The percentage of CK-18$^+$ cells ranged from 5%-20% of the attached cells and corresponded to 10%-40% of the ALB$^+$ cells. More importantly, dual-immunostaining analysis indicated that ALB was coexpressed with CK-19 in a portion of cultured round cells (Fig. 3C). CK-19 was detected in 2%-5% of the attached UCB cells, and 5%-10% of the ALB$^+$ cells was also positive for CK-19. Coexpression of ALB and CK-19 demonstrated that these double-positive cells derived from UCB had the same character as bipotential hepatic progenitor cells. Taken together, the expression profiles of ALB, GS, CK-18, AFP, and CK-19 show that various stages of the hepatocyte lineage, including both hepatic progenitor cells and mature hepatocytes, were present in our cultured UCB cells. It is reasonable to consider that UCB cells differentiated into mature hepatocytes via hepatic stem cells in our primary culture system.

Proliferation of Hepatocyte Lineage Cells Derived from UCB Cells

In order to demonstrate that UCB-derived cells expressing ALB were capable of proliferating in our culture system, PCNA expressed in nuclei of proliferating cells [26, 27] was employed as a marker for cell proliferation. Dual-immunostaining analysis showed that some of the ALB-producing cells contained PCNA in their nuclei (Figs. 4A and 4B). The cells expressing both ALB and PCNA accounted for 21% of ALB$^+$ cells; at least one-fifth of ALB-producing cells proliferated in this primary culture system. On the other hand, 25% of ALB$^-$ cells expressed PCNA.

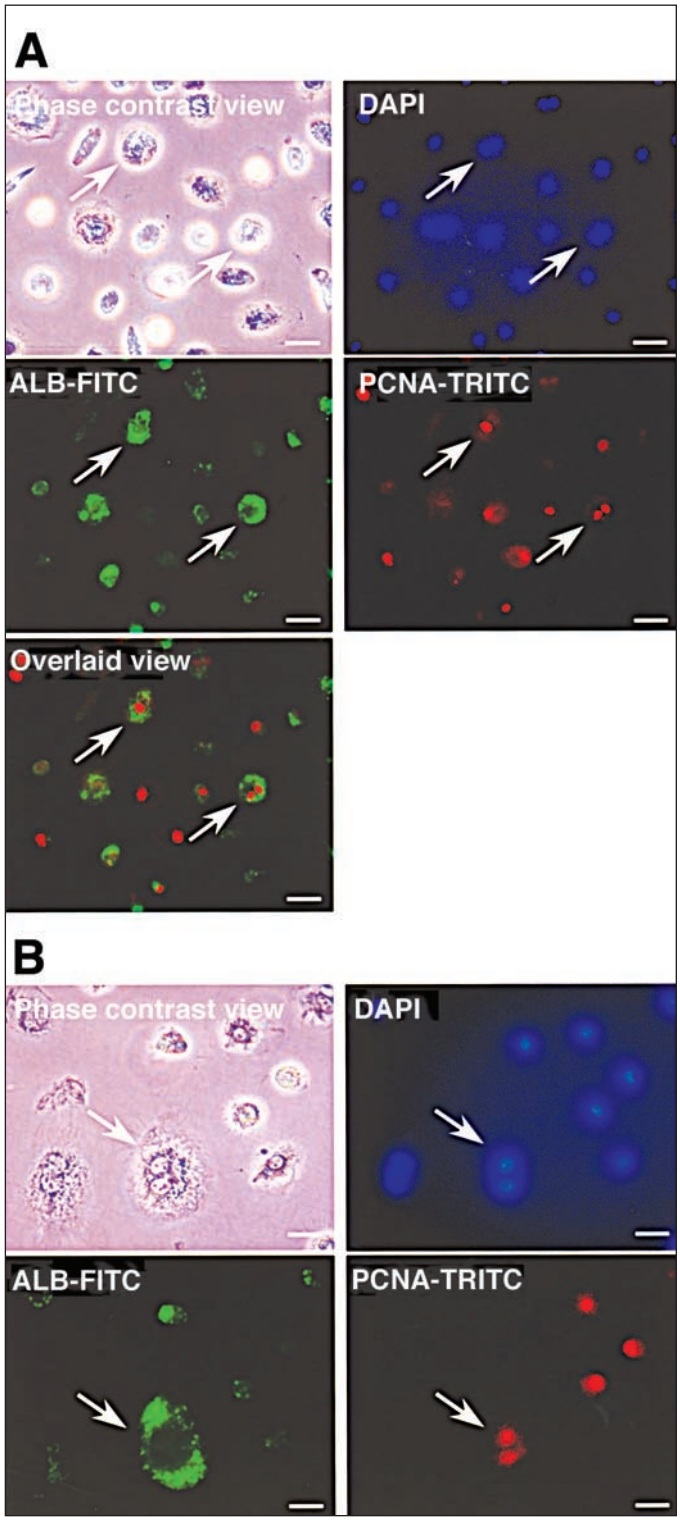

Figure 4. Proliferation of ALB-producing cells derived from UCB cells. *The 21-day cultured UCB cells were dual stained to detect ALB (green, see Fig. 2) and PCNA (red; TRITC conjugated with anti-mouse IgG antibody). The nuclei were counterstained with DAPI (blue). Arrows indicate the cells expressing both ALB and PCNA. A) At lower magnification, the cells expressing both ALB and PCNA accounted for 21% of ALB$^+$ cells, on average. B) Several ALB$^+$ cells exhibited binucleated structure, expressing PCNA. Scale bars = 10 μm.*

These findings show that UCB cells with the ability to produce ALB could proliferate in our culture system.

UCB-Derived Parenchymal Cells as Functional Hepatocytes in the Liver of Recipient Mice

To investigate whether UCB is a potential source of transplantable cells for liver injury, cell transplantation was performed in liver-injured SCID mice. Four groups of mice received transplants of 1×10^7 freshly isolated human UCB cells from a different donor for each group. In conjunction with cell transplantation, recipient livers were injured by administration of 2-AAF and partial hepatectomy. The engraftment and differentiation of human UCB cells in the livers of recipient SCID mice were analyzed at 4, 6, 20, and 55 weeks after cell transplantation.

RT-PCR analysis showed the presence of human ALB mRNA in the livers of the recipient mice (Fig. 5A). Next, we detected inoculated UCB cells by FISH for human X chromosome centromere DNA on liver sections from recipient SCID mice. FISH analysis of human female liver revealed positive signals in the nucleus in about 50% of hepatocytes, whereas no signals were detected in the livers of the control mice (data not shown). Cells with positive signals were observed in the livers of recipient SCID mice sacrificed 20 weeks after transplantation. Most of these were observed in the periportal area of liver lobules (Figs. 5B and 5C). These cells clearly exhibited the morphology of liver parenchymal cells, having large round nuclei and rich cytoplasm. Subsequential immunohistological analysis revealed that FISH-positive cells were also positively stained for human ALB (data not shown).

We traced human UCB-derived parenchymal cells by immunostaining analysis with an anti-human hepatocyte antibody. In human liver samples, almost all hepatocytes and bile duct cells, but not other nonparenchymal cells, were stained with the antibody (Fig. 5D), and no cells were stained in the control mouse liver (Fig. 5E). Figures 5F and 5G show UCB-derived parenchymal cells in the livers of mice sacrificed at 4 weeks. UCB-derived cells were widely distributed in hepatic lobules (Fig. 5F). Clusters of more than 10 parenchymal donor cells were observed only in the livers of the mice sacrificed at 4 weeks (Fig. 5G). Moreover, UCB-derived parenchymal cells were detected in the livers of the mice sacrificed 55 weeks after transplantation (Figs. 5H and 5I). Similarly, some engrafted donor cells were detected in a cluster 55 weeks after inoculation. In 100 random fields, the total number of functional hepatocytes derived from UCB was within 0.1%-1.0% of mouse hepatocytes. The rate of engraftment is likely to be higher in mice sacrificed sooner after transplantation. These data, however, indicated that human hepatocytes derived

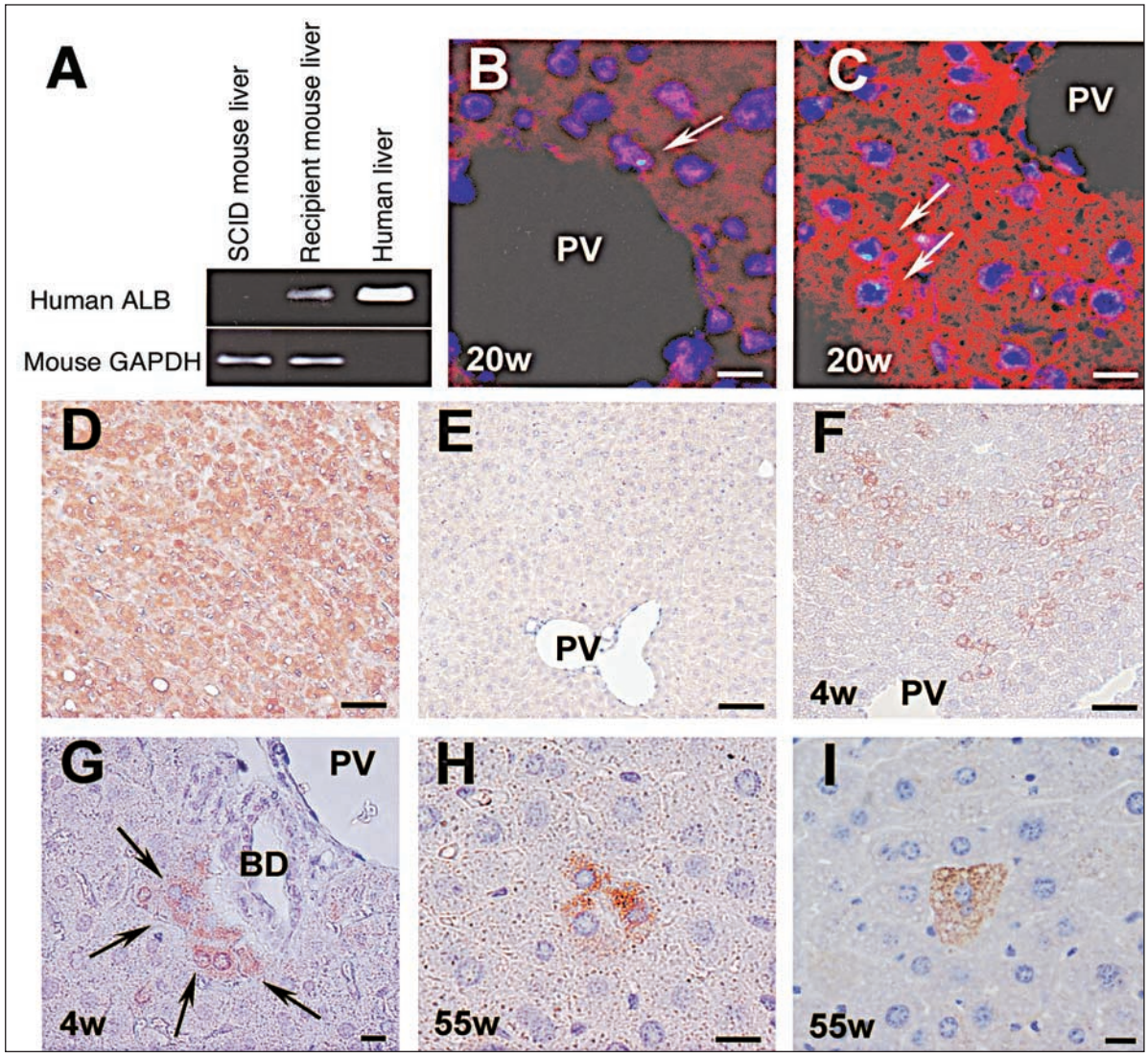

Figure 5. Human UCB-derived hepatocytes in the liver of recipient mice. *A) RT-PCR of human ALB mRNA expressed in the liver of a mouse sacrificed 6 weeks after cell transplantation. The RT-PCR products of human ALB (356 bp) and mouse GAPDH (1,100 bp) are indicated. B, C) Fluorescence images of a liver slice from recipient mice sacrificed 20 weeks after transplantation were obtained using a confocal laser microscope with DAPI (blue; nuclei), FITC (green; X chromosome), and Cy 5 (red; autofluorescence) filters. Arrows indicate positive signals of human X chromosome (green). D) Immunostaining of a human liver section with anti-human hepatocyte mAb (brown). E) Immunostaining of control mouse liver with anti-human hepatocyte mAb. (F-I) Immunohistochemical detection of human hepatocytes in liver slices of recipient mice sacrificed at 4 weeks (F, G) and 55 weeks (H, I) after transplantation. F) Human UCB-derived hepatocytes in the mouse liver were detected specifically by the use of immunoperoxidase staining with anti-human hepatocyte mAb. UCB-derived cells were widely distributed in hepatic lobules. G) A cluster of human UCB-derived parenchymal cells (brown, arrows) was observed in the periportal area in a recipient liver 4 weeks after inoculation. H) UCB-derived hepatocytes were observed in a cluster even 55 weeks after inoculation. I) Some of the engrafted donor cells exhibited binucleated morphology. PV = portal vein; BD = bile duct. Scale bars (B, C) = 10 μm, (D-F) = 50 μm, (G-I) = 10 μm. Original magnification (B, C) ×630, (D-F) ×100, (G-I) ×400.*

from UCB had the ability of long-term engraftment even in xenogeneic conditions.

In order to evaluate the function of these repopulating cells, we analyzed the production of ALB in UCB-derived parenchymal cells. Double-immunofluorescent staining with anti-human hepatocyte and anti-human ALB antibodies demonstrated that UCB-derived parenchymal cells produced human ALB in recipient mice livers. These double-positive cells were observed even 55 weeks after inoculation (Figs. 6A-6F). As generally observed in functionally differentiated hepatocytes of liver, some of the ALB-producing cells derived from human UCB cells also showed a typical binucleated structure (Figs. 6B and 6E). Together with the results of FISH, these findings provide

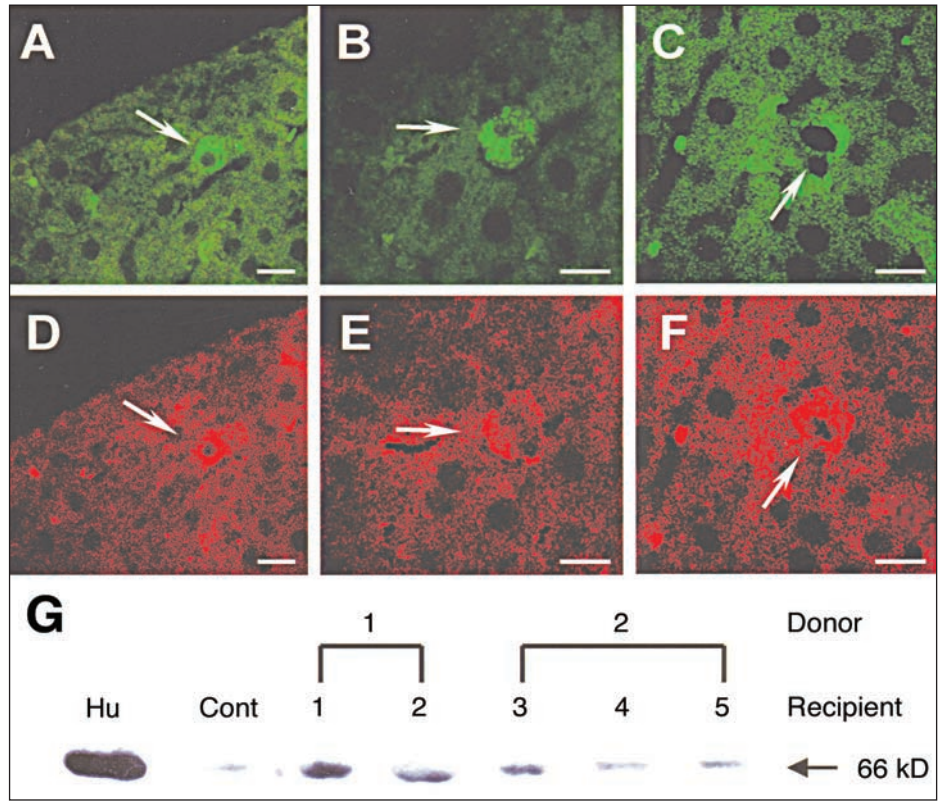

Figure 6. UCB-derived parenchymal cells function as differentiated hepatocytes in the livers of recipient mice. *A-F) The liver slices of recipient mice sacrificed 55 weeks after transplantation were dual stained with FITC-labeled anti-human hepatocyte antibody (green; A-C) and with TRITC-labeled anti-human ALB antibody (red; D-F). Arrows indicate double-positive stained cells. Scale bars = 10 μm. G) Immunoblot analysis of human ALB in the sera of recipient SCID mice. Two groups of mice received grafts of human UCB cells: group 1 (mice 1 and 2) from donor 1 and group 2 (mice 3, 4, and 5) from donor 2. Groups 1 and 2 were sacrificed at 4 and 6 weeks, respectively, after transplantation. Hu indicates human serum, and the amount used was 1/100 that of control (Cont) and transplanted mouse sera. A faint immunoreactive band was detected in control mice.*

direct evidence that UCB cells engrafted in the livers of mice differentiated into functionally mature hepatocytes. Furthermore, we detected human ALB in the sera of recipient mice. The immunoreactive band of control human serum diluted 1:100 was ≥10 times stronger than the faint band of nondiluted serum from the control mice (Fig. 6G). Since the concentration of ALB in the mouse serum of the same strain scarcely changes, the strong immunoreactive bands of sera from recipient mice indicate the presence of human ALB. Human ALB was present in the sera of transplanted SCID mice from all donors. These observations prove that inoculated UCB cells developed into functional hepatocytes, and that UCB-derived hepatocytes may play a role in the support for liver injury.

Discussion

Here, we demonstrated that UCB cells can proliferate hepatocyte lineage cells in the original primary culture system in vitro and that UCB cells differentiate into functionally mature hepatocytes in vivo. To our knowledge, this is the first evidence that UCB contains cells that produce endoderm-proliferating cells. To demonstrate that UCB cells have hepatic competence in vitro, we determined sufficient conditions for the expression of ALB in UCB cells in 75% of cases. Previous reports showed that FGF, LIF, SCF, HGF, and OSM each contribute to the proliferation and/or differentiation of hepatic progenitor cells in a different manner [13-18]. The effects of combinations of these factors on hepatic progenitor cells, however, remain unclear. UCB cells were not induced to express ALB in media containing any one of these factors. We conclude that the combination of FGF-1, FGF-2, LIF, SCF, and HGF is efficient for human UCB cells to generate ALB-producing cells.

The expression profiles of ALB, GS, CK-18, AFP, and CK-19 demonstrated that our cultured UCB cells included cells in various stages of the hepatocyte lineage. Moreover, some of the ALB^+ cells showed the same characteristics as hepatic progenitor cells, such as hepatic oval cells, in the culture system. Transcripts and proteins of AFP and CK-19 in freshly isolated UCB cells were not detectable. Both of these, as well as ALB, are expressed in hepatic progenitor cells [23, 24]. It is unlikely that our isolated UCB cells were contaminated by hepatoblasts or fetal hepatocytes because AFP mRNA, which is abundantly expressed in fetal hepatocytes [28], was not detectable. Therefore, the UCB-derived hepatic progenitor cells in the culture system presumably originated from nonhepatic stem or progenitor cells, and the combined effects of FGF-1, FGF-2, LIF, SCF, and HGF are necessary for this alternation in cell lineage. Our preliminary experiments indicate that neither purified $CD34^+$ nor $CD34^-$ cells alone in 21-day primary culture expressed ALB. By contrast, a mixed culture with both $CD34^+$ cells and $CD34^-$ cells maintained the expression of ALB (data not shown). In other words, coexistence of $CD34^+$ and $CD34^-$ cells was required for the effective appearance of ALB-producing cells in cultured UCB cells,

implying that interaction of these cells is also necessary for this alternation in cell lineage. Thus, the combined effect of FGF-1/FGF-2/LIF/SCF/HGF and the cell-to-cell interaction between $CD34^+$ and $CD34^-$ cells both seem to be required for the conversion of UCB cells into hepatocyte lineage cells in our primary culture system.

Dual-immunostaining analysis of ALB and PCNA showed that 20% of ALB^+ cells maintained the ability to proliferate in our culture conditions (Fig. 4). It is also noteworthy that ALB-producing cells could continue to proliferate after transplantation of UCB cells. Even at 55 weeks after transplantation, there was no formation of tumors, such as teratomas, which can be observed in the transplantation of embryonic stem cells [29, 30]. For these reasons, cultured UCB cells could be a suitable source of cells for transplantation.

We also studied the behavior of human UCB cells inoculated into the mouse liver by tracing the cells with immunohistology and FISH for the human X chromosome. Recently, it was reported that UCB-derived stem cells had BM-repopulating capacity and differentiated into hepatocytes in irradiated NOD/SCID mice without liver injury [31]. It is unclear whether UCB-derived cells function as mature hepatocytes for the support of liver injury. In our experimental model, recipient mice underwent the simultaneous treatments of 2-AAF administration and partial hepatectomy to provide a favorable microenvironment for proliferation and differentiation of the extrinsic cells. As a result, we observed UCB-derived, functionally differentiated hepatocytes 55 weeks after inoculation. Furthermore, we demonstrated the presence of human ALB in the sera of recipient mice, providing direct evidence that transplanted UCB-derived hepatocytes might contribute to the support of injured liver function. In our study, however, the functional hepatocytes appeared at frequencies of 0.1%-1.0% in recipient livers. This low frequency is probably due to the xenogeneic nature of the transplantation, where a considerable number of the inoculated human cells might be rejected, even in SCID mice. Moreover, xenogeneic liver would not provide a satisfactory microenvironment for the proliferation and differentiation of engrafted UCB-derived cells. An alternative explanation is that 2-AAF might affect the proliferation and differentiation of engrafted UCB cells. Further study is necessary to address this issue.

Allogeneic liver transplantation remains the only effective treatment available to patients with liver failure. Because of a serious shortage of liver donors, however, an alternative therapeutic approach is urgently needed. Transplantation of hepatocytes derived from adult or fetal livers is not a candidate for the alternative treatment because the source of such cells is limited to human liver at present [32, 33]. Recently, extrahepatic sources of hepatocyte lineage cells have been explored for use in cell therapy. Embryonic stem cells [29, 30, 34] and BM cells [35-39] have been reported to have the potential to differentiate into multilineage cells, including hepatocytes, in in vitro and in vivo models. However, the clinical application of embryonic stem cells obtained from human fertilized eggs harbors serious ethical problems in many countries. Also, the utilization of human BM as the graft source is restricted by a shortage of healthy donors. Our investigation demonstrates that UCB is a potentially suitable source of cell transplantation for liver injury. UCB will have relevance to the clinical application of cell transplantation as a novel therapeutic option for liver failure, as well as embryonic stem cells and adult-BM-derived cells. When we can successfully cryopreserve freshly isolated UCB cells, produce UCB-derived hepatic stem cells or hepatocytes on a large scale, and utilize them as a source of cell transplantation for decompensated liver diseases, we should be able to overcome the problems of ethics and short supply in allogeneic liver transplantation.

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