

Properties of Hepatocyte-like Cell Clusters from Human Adipose Tissue-Derived Mesenchymal Stem Cells

Hanayuki Okura, M.S.,^{1-4,*} Hiroshi Komoda, M.D., Ph.D.,^{1,5,*} Ayami Saga, M.S.,¹
Aya Kakuta-Yamamoto, B.S.,¹ Yoko Hamada, M.S.,¹ Yuichi Fumimoto, M.D., Ph.D.,¹
Chun Man Lee, M.D., Ph.D.,^{1,3} Akihiro Ichinose, M.D., Ph.D.,⁶
Yoshiki Sawa, M.D., Ph.D.,^{2,3} and Akifumi Matsuyama, M.D., Ph.D.¹

There are only a few reports that describe the hepatocytic differentiation potential of human adipose tissue-derived mesenchymal stem cells (hADMSCs) and no reports that describe the *in vivo* functions of hepatocyte-like cells differentiated from somatic stem cells including hADMSCs. In this study, we established a new method for generation of functional hepatocyte-like cell clusters using floating culture method and induced functional hepatocyte-like cell clusters, which functioned effectively not only *in vitro* but also *in vivo*. The generated hepatocyte-like cell clusters were characterized by gene expression analysis, functional assays, and transplantation into non-obese diabetic severe combined immunodeficiency (NOD-SCID) mouse with chronic liver injury. The generated hepatocyte-like cell clusters expressed various genes normally found on mature hepatocytes. The cell clusters exhibited functional characteristics of hepatocytes: they expressed albumin, secreted urea, had cytochrome P450 activity, could take up low-density lipoprotein, and stored glycogen. Transplantation of these cell clusters into NOD-SCID mouse with chronic liver injury resulted in a significant improvement of serum albumin and total bilirubin levels. In summary, we established a new protocol for efficient induction of hADMSCs into functional hepatocyte-like cell clusters.

Introduction

THE SURVIVAL RATE OF PATIENTS with fulminant hepatic failure ranges from 15% to 25%.¹ Transplantation of hepatocytes may partially solve this problem.² Nevertheless, this treatment still requires a liver from a donor. Accordingly, it will be greatly beneficial if functional hepatocytes could be induced from nonhepatic sources.

Tissue engineering holds promise for the development of stem cell-based therapy of the liver. Many types of stem cells from different sources have been investigated for their ability to differentiate into hepatocytes. Recently, adult somatic stem cells, which can give rise to tissues of more than one germ layer, have been identified.³ The current research focus is on mesenchymal stem cells (MSCs), which are found in human bone marrow (BM),⁴ scalp tissue,⁵ placenta,⁶ and umbilical cord blood.⁷ These stem

cells can be induced into multiple-type lineages.⁸⁻¹² MSCs from the BM and umbilical cord blood have been induced into a hepatic lineage¹³⁻¹⁷; however, it may also prove difficult to obtain sufficient amounts of autologous adult stem cells from these origins. Recently, MSCs have been isolated from adipose tissue.^{18,19} Adipose tissue is an attractive source of human MSCs because it is a safe and abundant source of large amounts of MSCs. Adipose tissue-derived MSCs (ADMSCs) exhibit differentiation potential similar to BM-derived MSCs. Nevertheless, there are a few reports describing the hepatocytic differentiation potential of ADMSCs^{20,21} and the effective functions of these differentiated hepatocyte-like cells not only *in vitro* but also *in vivo*.

In this study, we established a new method for generation of hepatocyte-like cell clusters, which functioned effectively not only *in vitro* but also *in vivo*.

¹Department of Somatic Stem Cell Therapy, Institute of Biomedical Research and Innovation, Foundation for Biomedical Research and Innovation, Kobe, Japan.

²Department of Surgery, Osaka University Graduate School of Medicine, Suita, Japan.

³Medical Center for Translational Research, Osaka University Hospital, Suita, Japan.

⁴Research Fellow of the Japan Society for the Promotion of Science, Tokyo, Japan.

⁵Department of Internal Medicine, National Hospital Organization Chiba Medical Center, Chiba, Japan.

⁶Department of Plastic Surgery, Kobe University Graduate School of Medicine, Kobe, Japan.

*These authors contributed equally to this work.

Materials and Methods

Adipose tissues from human subjects

Excess adipose tissues were resected from the gastro-omental artery during coronary artery bypass graft surgery and gastrectomy in 10 subjects (four men and six women; age, 55 ± 5 years, mean \pm standard error of the mean; range, 40–60 years). All subjects provided informed consent. The protocol was approved by the Review Board for Human Research of the Osaka University Graduate School of Medicine. All subjects fasted for at least 10 h before surgery and none was being treated with steroids. Ten to 50 g of adipose tissues was obtained from each subject.

Isolation of human ADMSCs

Human ADMSCs (hADMSCs) were obtained using the method described by Björntorp *et al.*¹⁸ and Zuk *et al.*,¹⁹ with some modifications as described below.^{22,23} Resected excess adipose tissue was minced and then digested in Hank's balanced salt solution (HBSS; Gibco Invitrogen, Grand Island, NY) containing 0.075% collagenase type II (Sigma-Aldrich, St. Louis, MO) at 37°C for 1 h. Digests were filtered with a cell strainer (BD Bioscience, San Jose, CA) and centrifuged at 800 *g* for 10 min. Erythrocytes were excluded using density gradient centrifugation with Lymphoprep ($d = 1.077$; Nycomed, Oslo, Norway). The cells were then plated using Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen) with 10% defined fetal bovine serum (FBS; Hyclone, Northumberland, United Kingdom) and incubated for 24 h at 37°C. Following incubation, the adherent cells were washed extensively and then treated with 0.2 g/L ethylenediaminetetraacetate (EDTA) solution (Nacalai Tesque, Kyoto, Japan). The resultant suspended cells were replated at a density of 10,000 cells/cm² on human fibronectin (FN)-coated dishes (BD BioCoat) in medium I (60% DMEM-low glucose, 40% MCDB-201 medium [Sigma-Aldrich], 1 \times insulin-transferrin-selenium [Gibco Invitrogen], 1 nM dexamethasone [Sigma-Aldrich], 100 μ M ascorbic acid 2-phosphate [Sigma-Aldrich], 10 ng/mL epidermal growth factor [PeproTec, Rocky Hill, NJ], and 5% FBS).

Cultures of HepG2, normal skin fibroblasts, and primary hepatocytes

Both HepG2 (a hepatoblastoma cell line) and normal skin fibroblasts were cultured with DMEM containing 10% FBS and antibiotic/antimycotic solution. Primary hepatocytes (lot no. ACBRI3716) were purchased from Applied Cell Biology Research Institute (Kirkland, WA) and cultured using the method recommended by the manufacturer. Cells were maintained at 37°C.

Flow cytometric analysis

hADMSCs isolated from adipose tissue were characterized by flow cytometry. Cells were detached and stained with fluorescein isothiocyanate-conjugated mouse monoclonal antibodies to human CD31 (BD Pharmingen, San Diego, CA), CD105 (Ansell, Minneapolis, MN), CD133 (R&D Systems, Minneapolis, MN), and phycoerythrin-conjugated mouse monoclonal antibodies to human CD29, CD34, CD45, CD56, CD73, CD166 (BD Pharmingen), CD44, or CD166

(Ansell). Isotype-identical antibodies served as controls. Further, we incubated cells with mouse monoclonal antibodies against human stage-specific embryonic antigen-4 (SSEA-4), tumor rejection antigen (TRA)-1-60, tumor rejection antigen (TRA)-1-81 (Chemicon International, Temecula, CA), multidrug-resistance transport protein (ABCG-2; BD Pharmingen), or CD117 (BD Pharmingen), with nonspecific mouse antibody used as a negative control. After washing with Dulbecco's phosphate-buffered saline (PBS; Nacalai Tesque), cells were incubated with phycoerythrin-labeled goat anti-mouse immunoglobulin (Ig) antibody (BD Pharmingen) for 30 min at 4°C. After three washes, cells were resuspended in PBS and analyzed by flow cytometry using a FACS-Calibur flow cytometer and CellQuest Pro software (BD Bioscience).

Adipogenic, osteogenic, and chondrogenic differentiation procedures

For adipogenic differentiation, cells were cultured in differentiation medium (Zen-Bio, Durham, NC). After 3 days, half of the medium was changed with adipocyte medium (Zen-Bio) and this was repeated every 2 days. Five days after differentiation, characterization of adipocytes was confirmed by microscopic observation of intracellular lipid droplets by oil red O staining. Osteogenic differentiation was induced by culturing the cells in DMEM containing 10 nM dexamethasone, 50 mg/dL ascorbic acid 2-phosphate, 10 mM β -glycerophosphate (Sigma, St. Louis, MO), and 10% FBS. Differentiation was examined by alizarin red staining. For chondrogenic differentiation, 2×10^5 cells of hADMSCs were centrifuged at 400 *g* for 10 min. The resulting pellets were cultured in chondrogenic medium (α -MEM supplemented with 10 ng/mL transforming growth factor- β , 10 nM dexamethasone, 100 μ M ascorbate, and 10 μ L/mL of 100 \times insulin-transferrin-selenium solution) for 14 days as described previously.²⁴ For alcian blue staining, nuclear counterstaining with Weigert's hematoxylin was followed by 0.5% alcian blue 8GX for proteoglycan-rich cartilage matrix.

Differentiation procedure of hepatocyte-like cell clusters

Our differentiation procedure consisted of three stages. In stage I, hADMSCs were cultured and expanded in medium I for three to four passages. In stage II, the cells were dissociated with trypsin-EDTA and the resulting single cells were suspended in medium II (80% knockout-DMEM [Gibco Invitrogen], 20% defined FBS, 1 mM glutamine, and 1% nonessential amino acids [both from Gibco Invitrogen]). The suspension was placed in an ultralow-attachment culture dish (Hydrocell; purchased from CellSeed, Tokyo, Japan), and the cells self-aggregated into cell clusters within 1 day. The cell clusters were then cultured for additional 2 days. In stage III, after washing extensively with PBS, 2-day-old cell clusters (consisting of 1000 cells on average) were cultured on a Hydrocell dish for 4 weeks in medium III (60% DMEM-low glucose, 40% MCDB-201, 1 nM dexamethasone, 100 μ M ascorbic acid, 10 ng/mL epidermal growth factor, basic fibroblast growth factor [PeproTec, Rocky Hill, NJ], hepatocyte growth factor [PeproTec], and oncostatin M). Finally, 0.1% dimethyl sulfoxide (DMSO;

Nacalai Tesque) was added on the 10th day after induction of differentiation.

Hepatic differentiation procedure on extracellular matrix

After the third passage, hADMSCs were plated on human FN, collagen type I, or Matrigel-coated dishes in medium III. Further, 0.1% DMSO was also added on the 10th day after induction of differentiation.

Quantitative reverse transcription-polymerase chain reaction analysis and western blot analysis

Total RNA was isolated from cells using an RNAeasy kit (Qiagen, Valencia, CA). After treatment with DNase, cDNA was synthesized using Superscript III RNase H-minus Reverse Transcriptase (Invitrogen, Carlsbad, CA). Real-time polymerase chain reaction (PCR) was performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). 20× Assays-on-Demand™ Gene Expression Assay Mix for Albumin (ALB) (Hs00609411_m1), glutamine synthetase (Hs00374213_m1), cytochrome P450 (CYP)1B1 (Hs00164383_m1), cytokeratin 18 (CK18) (Hs01941416_g1), alpha fetoprotein (AFP) (Hs00173490_m1), CYP3A4 (Hs01546612_m1), CYP3A7 (Hs00426361_m1), alpha-1-antitrypsin (Hs01097800_m1), glucose-6 phosphatase (Hs00609178_m1), and glyceraldehyde-3-phosphate dehydrogenase (Hs99999905_m1) were obtained from Applied Biosystems. TaqMan® Universal PCR Master Mix, No AmpErase® UNG (2×) was also purchased from Applied Biosystems. Reactions were performed in triplicate and the mRNA levels were normalized relative to glyceraldehyde-3-phosphate dehydrogenase expression. The ratios were indicated as relative to hepatocytes.

Western blot analysis was performed to detect ALB in undifferentiated and differentiated hADMSC clusters using anti-human ALB antibody (goat polyclonal, bovine, mouse, and pig ALB-adsorbed, affinity purified; Bethyl Laboratories, Montgomery, TX) that does not react with bovine or mouse ALB.

Evaluation of urea synthesis

After washing extensively with PBS, undifferentiated hADMSCs, undifferentiated hADMSC clusters (during the same period of culture with medium stage II), differentiated hADMSCs clusters, HepG2, primary hepatocytes, and normal human fibroblasts were incubated in HBSS containing 5 mM NH₄Cl for 2 h at 37°C to evaluate their capacity for urea synthesis. Each supernatant was collected and the amount of urea was determined using a QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA). Urea synthesis per cell was calculated based on the amount of DNA, which was determined with a DNA Quantification Kit (Primary Cell, Hokkaido, Japan) according to the instructions provided by the manufacturer.

Measurement of CYP activity

Undifferentiated hADMSCs, undifferentiated hADMSC clusters (during the same period of culture with medium stage I), differentiated hADMSC clusters, HepG2, primary hepatocytes, and normal human fibroblasts were cultured for 3 days in the absence or presence of 10 μM rifampicin (Sigma-Aldrich) based on a previous report.²⁵ The medium

was removed and the cells were incubated for 1 h in HBSS supplemented with 100 μM 7-benzyloxy-4-trifluoromethylcoumarin (BFC; Ultrafine, Manchester, United Kingdom). The supernatant was collected from the cultures and absorbance was measured with a spectrofluorometer at excitation and emission wavelengths of 490 and 505 nm, respectively. A standard curve was prepared from serial dilutions of 7-hydroxy-4-trifluoromethylcoumarin (HFC; Ultrafine), and the HFC concentration in each culture medium was calculated based on the standard curve. The CYP activity per cell was calculated relative to the amount of DNA.

Uptake of low-density lipoprotein

Human low-density lipoprotein (LDL; density: 1.019–1.063 g/mL) was isolated by sequential ultracentrifugation from normolipidemic healthy donors, dialyzed against saline-EDTA, and then sterilized by filtration through a 0.2-μm filter. Lipoproteins were labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI; Molecular Probes, Eugene, OR) by incubating the LDL in 0.5% bovine serum albumin/PBS with 100 mL DiI in DMSO (3 mg/mL) for 8 h at 37°C. The lipoproteins were then dialyzed against PBS and filtered before use. To evaluate the uptake of DiI-LDL, differentiated hADMSC clusters were incubated with serum-free DMEM containing 10 μg/mL DiI-LDL for 3 h at 37°C. Finally, the cells were rinsed three times and mounted. Counter staining was performed with 4',6-diamidino-2-phenylindole (DAPI). The slides were examined using a fluorescent microscope (Bioview, Osaka, Japan).

Periodic acid-Schiff staining for glycogen

Differentiated hADMSC clusters were fixed in 4% paraformaldehyde and embedded in paraffin. Samples were cut into 5-μm-thick sections and then oxidized in 1% periodic acid for 5 min, rinsed three times in de-ionized water (dH₂O), treated with Schiff reagent for 15 min, and rinsed in dH₂O for 5 to 10 min. Samples were counterstained with Mayer's hematoxylin for 1 min, rinsed in dH₂O, and examined by light microscopy.

Induction of chronic liver injury in mice and transplantation of differentiated hADMSCs clusters

Six-week-old non-obese diabetic severe combined immunodeficiency (NOD-SCID) mice (Clea Japan, Osaka, Japan) were injected intraperitoneally with carbon tetrachloride (CCl₄; 300 μL/kg body weight) twice a week for 12 weeks to induce chronic liver injury. Differentiated hADMSCs clusters (1.5×10⁶ cells equivalent cell clusters [for differentiated cell clusters] and 5×10⁶ cells for hADMSCs) were transplanted under the left kidney capsule of CCl₄-treated NOD-SCID mice. Seven days after transplantation, the mice were sacrificed and blood samples were collected for determination of serum total bilirubin levels. The left kidneys were removed for histological analysis.

Histological analysis

Native liver and graft-bearing left kidneys obtained from CCl₄-treated mice were fixed immediately with 10% formalin, embedded in paraffin, and processed for staining with

hematoxylin and eosin, or Sirius Red. The left kidneys were also processed for Periodic acid-Schiff (PAS) staining. Differentiated hADMSC clusters and graft-bearing left kidneys were subjected to immunofluorescence staining for ALB and alpha-1-antitrypsin. They were placed into optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan) and immediately frozen. Next, 7 μ m-thick sections were fixed in 4% paraformaldehyde/PBS for 30 min. The fixed sections were then incubated with blocking solution (Blocking One; Nacalai Tesque) for 1 h. The samples were incubated with anti-human ALB antibody (Bethyl Laboratories) followed by Alexa Fluor 546-labeled donkey anti-goat IgG (Molecular Probes) and anti-human alpha-1-antitrypsin antibody (Scy-Tek Laboratories, Logan, Utah), followed by Alexa Fluor 546-labeled donkey anti-rabbit IgG. Counter staining was performed with DAPI. The slides were examined using a fluorescent microscope (Bioview, Tokyo, Japan).

Measurement of serum levels of ALB, alpha-1-antitrypsin, and total bilirubin

Serum levels of mouse ALB and human alpha-1-antitrypsin were determined using an ALB enzyme-linked immunosorbent assay system and human specific alpha-1-antitrypsin enzyme-linked immunosorbent assay system (both from Bethyl Laboratories) according to the instructions supplied by the manufacturer. Serum total bilirubin level was measured using QuantiChrom™ Bilirubin Assay Kit (BioAssay Systems).

Statistical analysis

All data were expressed as mean \pm standard error of the mean. Differences between groups were examined for statistical significance using the Student's *t*-test (SPSS, Chicago, IL). A *p*-value less than 0.05 denoted the presence of a statistically significant difference.

Results

Characterization of hADMSCs

Flow cytometry was used to assess markers expressed by hADMSCs (Supplemental Fig. 1, available online at www.liebertonline.com/ten). The cells were negative for markers of hematopoietic lineage (CD45) and hematopoietic stem cells (ABCG-2, CD34, and CD133). They were also negative for CD31, an endothelial cell-associated marker, and surface antigen c-Kit (CD117). However, they stained positively for several surface markers characteristic of mesenchymal and/or neural stem cells, but not embryonic stem (ES) cells, such as CD29, CD44 (hyaluronan receptor), CD73, and CD105 (endoglin).^{26–28} hADMSCs also were positive for SSEA-4, a marker of ES cells, but generally not adult stem cells.²⁹ hADMSCs did not express other surface markers characteristic of ES or embryonic germ cells (e.g., Tra-1-60 and Tra-1-81).

Next, we examined the adipogenic, osteogenic, and chondrogenic differentiation potentials of hADMSCs. Adipogenic differentiation was confirmed by the accumulation of intracellular lipid droplets that stained positively with oil red O and the amount of oil red O was augmented by induction (Supplemental Fig. 2A, available online at www.liebertonline.com/ten). Next, osteogenic induction was examined by alizarin red S staining and alkaline phospho-

tase (ALPase) activity (Supplemental Fig. 2B, available online at www.liebertonline.com/ten). After 7-day induction for osteogenesis, hADMSCs were stained with alizarin red S, and the intensity of staining increased after 18-day induction. ALPase activity, a marker of osteogenic induction, was augmented with osteogenic induction (Supplemental Fig. 3B, available online at www.liebertonline.com/ten). Third, we examined the chondrogenic potential of hADMSCs. As shown in Supplemental Figure 2C (available online at www.liebertonline.com/ten), hADMSCs were stained with alcian blue after chondrogenic induction with pellet culture. These results indicate that the hADMSCs have MSC properties like BM-derived MSCs.

Quantitative reverse transcription-PCR analysis of immature and mature hepatocyte in clusters of differentiated hADMSCs

To promote the differentiation of hADMSCs to hepatocyte-like cells, we applied the procedure outlined in Figure 1A. In the differentiation protocol, to identify the expression of genes in other endoderm-derived cell types, or mesoderm- or ectoderm-derived cells during differentiation of hADMSCs process, we examined the expression levels of AFP, pdx-1 (pancreas), alpha-cardiac actin (mesoderm), and Sox1 (ectoderm) (Fig. 1B). Next, we used quantitative reverse transcription-PCR to determine whether our procedure resulted in differentiation of hADMSCs into hepatocyte-like cells by examining the expression levels of various markers of immature and mature hepatocytes such as AFP and CYP3A7 (as markers of immature hepatocytes) and CYP3A4, G6P, alpha-1-antitrypsin, ALB, glutamine synthetase, CYP1B1, and CK18 (as markers of mature hepatocytes) (Fig. 2). The mRNA expression levels were also assessed for differentiated hADMSCs on FN, collagen type I, or Matrigel-coated dishes. Differentiated hADMSC clusters by our procedure expressed higher levels of ALB mRNA than other differentiated hADMSCs. The expression levels of ALB, AFP, G6Pase, CYP3A7, and CYP3A4 were same as those of primary hepatocytes. AFP expression levels of primary hepatocytes and the cell clusters were same, but less than that of HepG2. The expression levels of glutamine synthetase, CYP1B1, and CK18 were higher in differentiated hADMSC clusters than other differentiated hADMSCs and HepG2. The mRNA expression level of alpha-1-antitrypsin in differentiated hADMSCs was lower than that of HepG2 and hepatocytes.

In vitro functional characterization of differentiated hADMSC clusters

ALB expression was assessed by western blot analysis and immunofluorescence staining (Fig. 3A, B). The former showed abundant expression of ALB on differentiated hADMSCs, and the latter confirmed these results. ALB- and alpha-1-antitrypsin-expressing cells were clearly detected among the differentiated hADMSCs. The ability to secrete urea was about 12-fold higher for differentiated hADMSCs incubated with NH₄Cl, compared with stage I undifferentiated hADMSCs, and as high as that of HepG2 cells (Fig. 3C). Nonfluorescent BFC is metabolized mainly by the CYP3A family of enzymes and converted to fluorescent HFC. The concentration of HFC in the supernatant was measured after incubation with 100 μ M BFC. CYP activity in differentiated

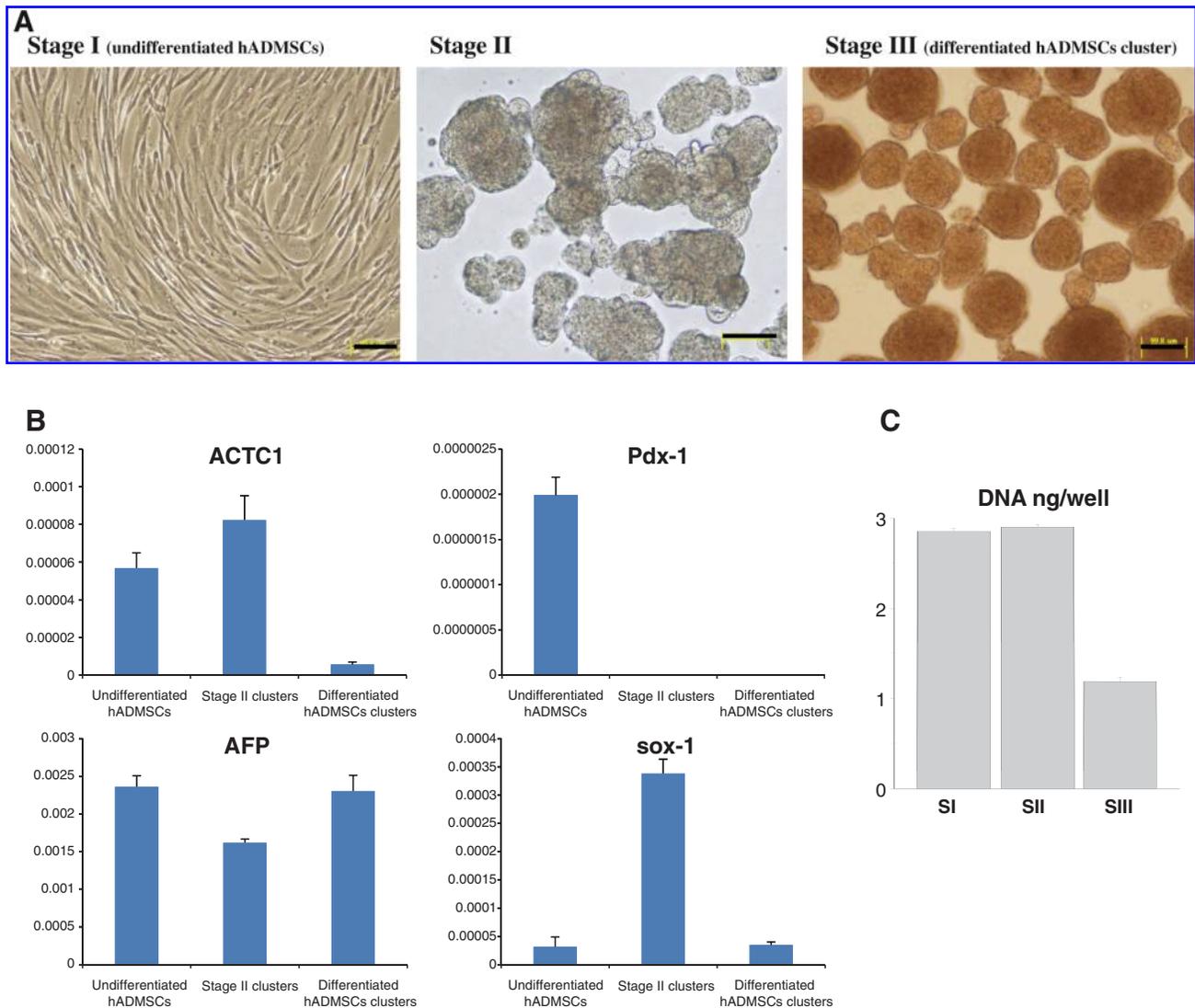


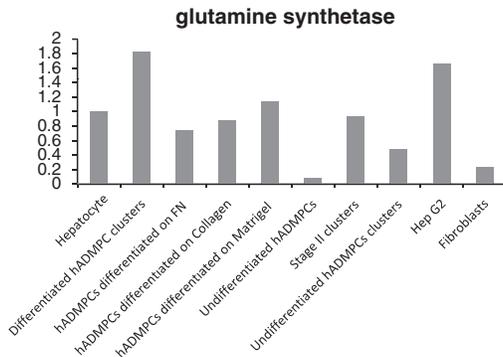
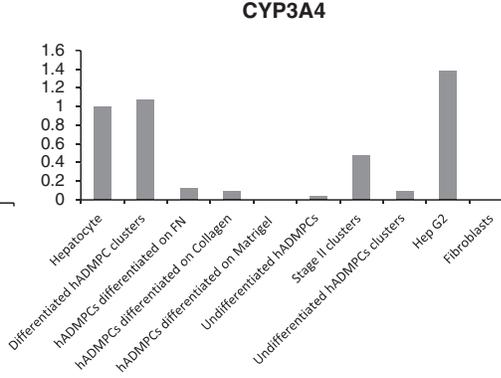
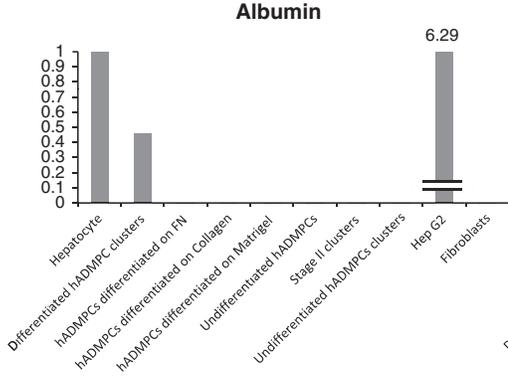
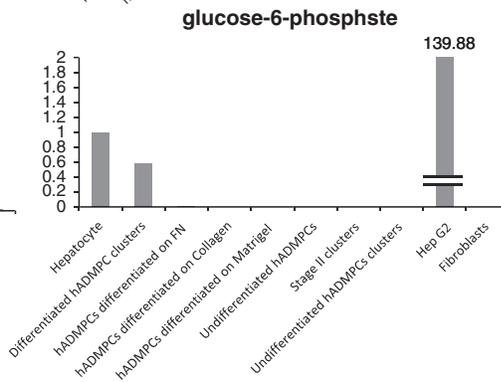
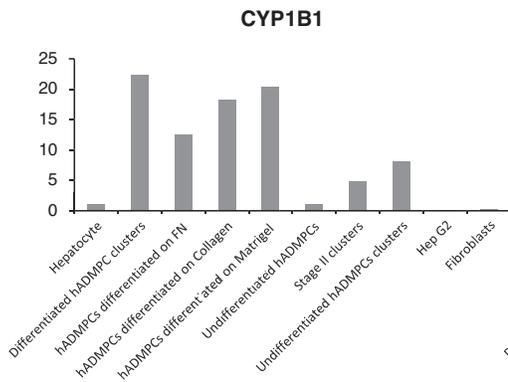
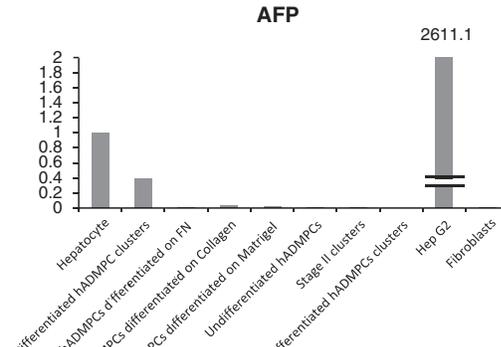
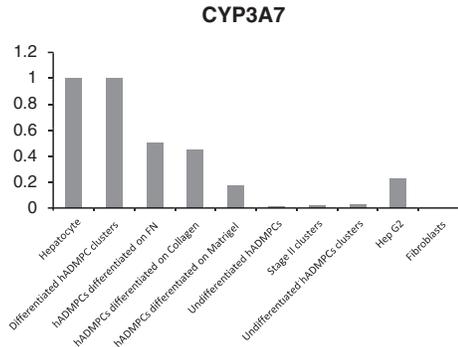
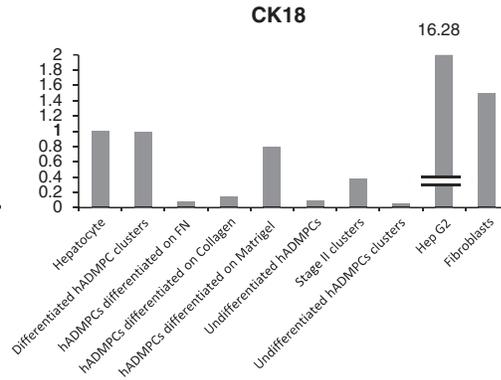
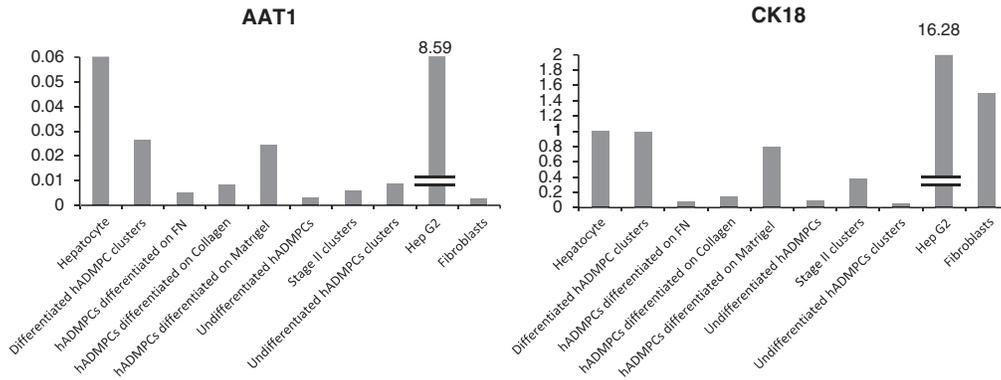
FIG. 1. Hepatocytic differentiation of hADMSCs. (A) General outline of the three-stage differentiation protocol. Stage I: Growth of hADMSCs. Stage II: Formation of cell clusters by culture in low osmotic medium on ultralow-attachment culture dishes. Stage III: Growth factor stimulation by culture of cell clusters with basic fibroblast growth factor, hepatocyte growth factor, and oncostatin M. Dimethyl sulfoxide was added on the 10th day after induction. (B) Trilineage marker expression during the differentiation process. The gene expression levels of alpha fetoprotein (AFP) and pancreas duodenum homeobox 1 (pdx-1) (endoderm markers), actin alpha cardiac muscle (ACTC1) (mesoderm marker), and sex determining region box 1 (Sox1) (ectoderm marker) are indicated. (C) Total number of hepatocytes per input hADMSCs. The total number of hepatocytes that can be generated per input hADMSCs by DNA content. 41.7% of cell content could be generated per input hADMSCs. hADMSCs, human adipose tissue-derived mesenchymal stem cells.

hADMSC clusters was 40-fold higher than that of undifferentiated hADMSCs (Fig. 3D). In addition, CYP activity in differentiated hADMSC clusters increased 2–2.5-folds following preincubation with rifampicin for 3 days. In contrast, no increase in CYP activity was induced in undifferentiated hADMSCs under this condition.

We also assessed LDL uptake by differentiated hADMSC clusters by incubating differentiated hADMSCs with DiI-LDL (Fig. 3E). DiI-LDL was markedly incorporated into the cytoplasmic area of differentiated hADMSCs. Another function of hepatocytes is glycogen production (glyconeogenesis). PAS staining showed glycogen storage in differentiated hADMSCs (Fig. 3F).

Transplantation of differentiated hADMSC clusters into NOD-SCID mice with chronic liver injury

NOD-SCID mice were injected with CCl₄ (300 μL/kg body weight) twice a week for 12 weeks to induce chronic liver injury (Fig. 4A). Control mice were not treated with CCl₄. Histological examination confirmed destruction of acini and fibrosis of the liver in CCl₄-injected mice (Fig. 4B). The serum total bilirubin level in CCl₄-treated mice was significantly higher than in control mice ($p < 0.05$; Fig. 4C). To assess the functionality of differentiated hADMSC clusters *in vivo*, clusters of these cells were transplanted under the kidney capsule of mice with chronic liver injury to exclude possible



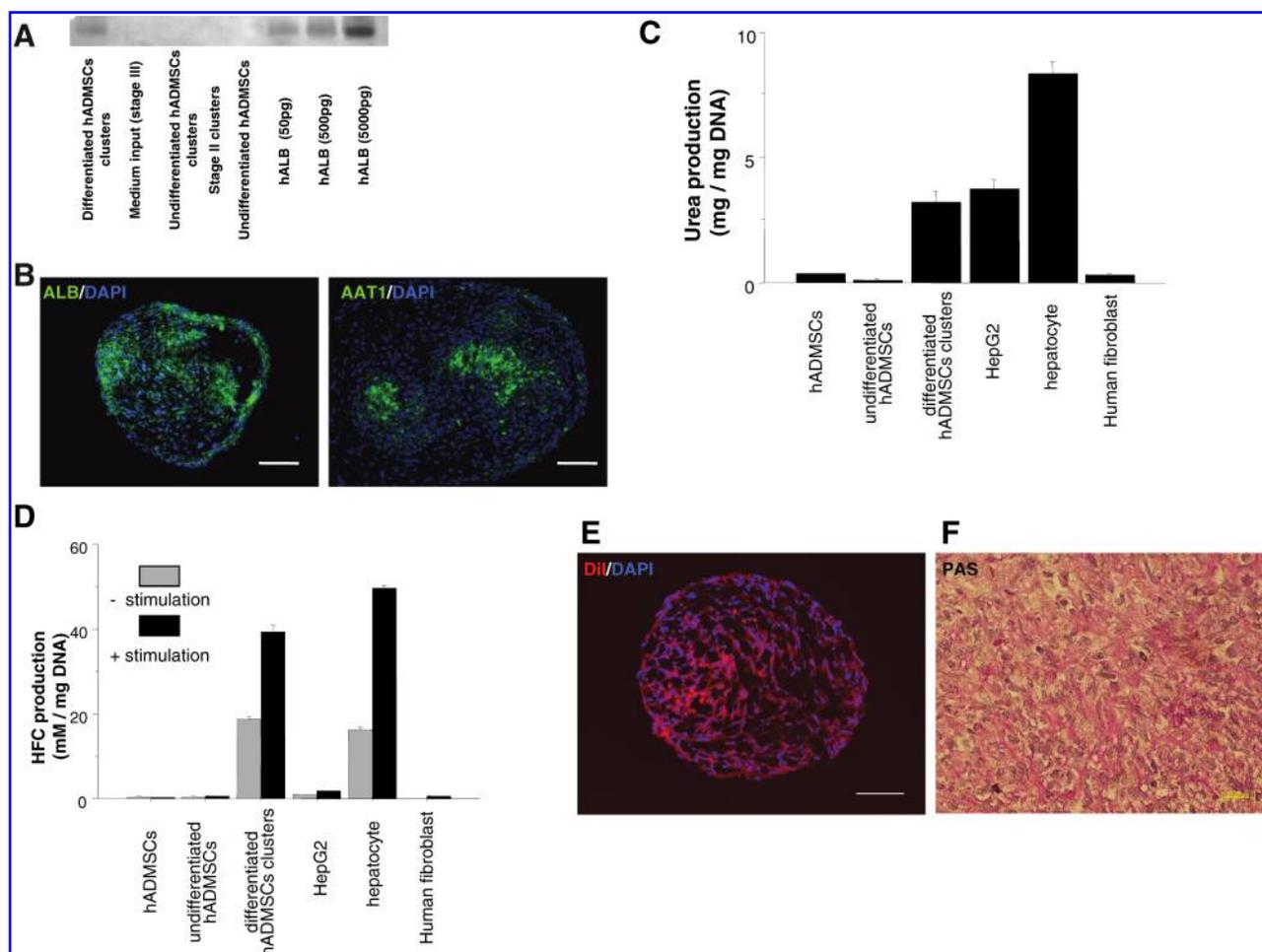


FIG. 3. *In vitro* evaluation of functional properties of hepatocyte-like cell clusters. (A) Detection of ALB and protein in differentiated hADMSCs clusters by western blotting analysis. ALB could be detected only in the conditioned media obtained from stage III clusters, but not in those obtained from undifferentiated hADMSCs, stage II clusters, undifferentiated hADMSCs clusters, or stage III media (medium input). In these assays, 50, 500, and 5000 pg human ALB protein were used as controls. (B) Immunofluorescence staining for ALB (left) and AAT (right) in differentiated hADMSCs clusters. Scale bar = 100 μ m. (C) Urea synthesis by differentiated hADMSCs clusters after incubation with 5 mM NH_4Cl . Urea synthesis per cell was calculated based on the amount of DNA. Data are mean \pm SEM of triplicate experiments. (D) CYP enzyme activity in differentiated hADMSCs clusters, as determined by hydroxylation of 7-benzyloxy-4-trifluoromethoxy-coumarin to HFC. Before incubation with 100 μ M, cells were cultured in the absence (-stimulation) or presence (+stimulation) of 10 μ M/L rifampicin. CYP activity per cell was calculated based on the amount of DNA. Data are mean \pm SEM of triplicate experiments. (E) Low-density lipoprotein uptake by differentiated hADMSCs clusters. Samples were examined by confocal laser scanning microscopy. Scale bar = 100 μ m. (F) Glycogen storage in differentiated hADMSCs clusters, as determined by PAS staining. hALB, human ALB; SEM, standard error of the mean; HFC, 7-hydroxy-4-trifluoromethyl-coumarin; PAS, periodic acid-Schiff; DiI, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine; DAPI, 4',6'-diamidino-2-phenylindole.

cell fusion. Total bilirubin level in mice that underwent transplantation decreased significantly and approached the normal level compared with CCl_4 -treated mice that did not receive transplantation ($p < 0.05$; Fig. 4C). These data are consistent with the presence of the bilirubin transporter,

multidrug resistance-associated protein 2 (MRP2), in differentiated hADMSC clusters (Supplemental Fig. 3). Histological analysis showed engrafted viable cell clusters under the kidney capsule (Fig. 4D), the presence of many human ALB-expressing cells in the graft by immunofluorescence

FIG. 2. Quantitative RT-PCR analysis of the relative expression levels of mRNAs of immature and mature hepatocyte markers. RNAs were prepared from differentiated hADMSCs clusters (stage III), stage II clusters, undifferentiated hADMSCs clusters, hADMSCs differentiated on FN, hADMSCs differentiated on collagen type I, hADMSCs differentiated on Matrigel, undifferentiated hADMSCs, HepG2, and fibroblasts. Levels of AFP and CYP3A7 as markers of immature hepatocytes, and ALB, glutamin synthase, CYP1B1, CK18, CYP3A4, alpha-1-antitrypsin, and glucose-6-phosphatase mRNAs as markers of mature ones were determined by quantitative RT-PCR using Assays-on-Demand™ Gene Expression Assay Mix. The mRNA levels were normalized relative to glyceraldehyde-3-phosphate dehydrogenase expression and data are mean of triplicate experiments. FN, fibronectin; CYP, cytochrome P450; ALB, albumin; AAT, alpha-1-antitrypsin; RT-PCR, reverse transcription-polymerase chain reaction.

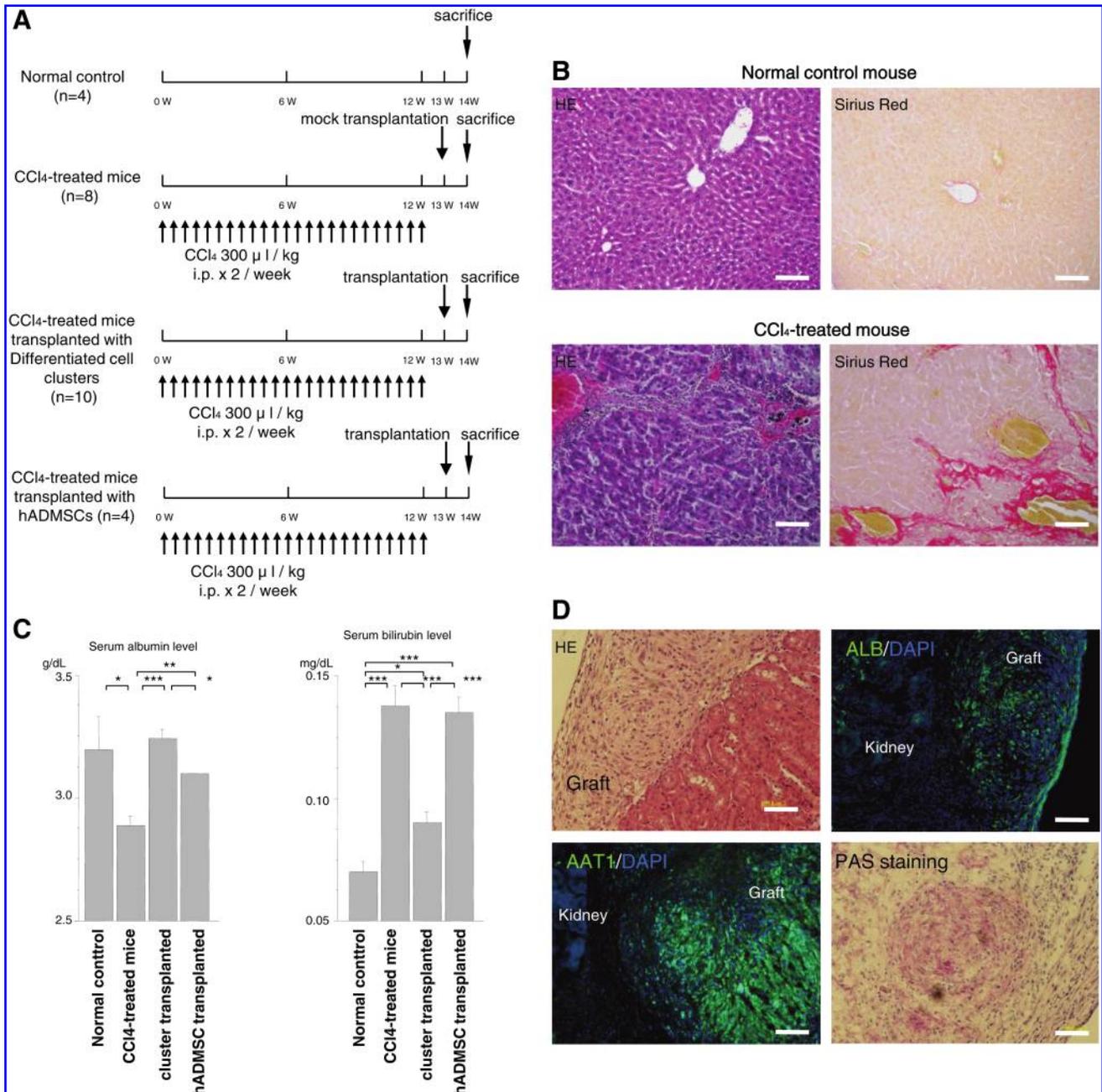


FIG. 4. Transplantation of differentiated hepatocyte-like clusters into non-obese diabetic severe combined immuno deficiency (NOD-SCID) mice with chronic liver injury. **(A)** Experimental design for induction of chronic liver injury and transplantation of differentiated hADMSCs. **(B)** HE staining and Sirius Red staining of liver sections from untreated normal control mice and CCl₄-treated mice. Scale bar = 100 μm. **(C)** Serum ALB and bilirubin levels (mg/dL) in normal control mice ($n = 4$), CCl₄-treated mice ($n = 8$), CCl₄-treated mice transplanted with differentiated hADMSCs clusters, and CCl₄-treated mice transplanted with hADMSCs ($n = 4$) were examined. Data are mean ± SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$). **(D)** Histological analysis of hepatocyte-like cell clusters transplanted under the kidney capsule of CCl₄-treated mice: HE staining, immunofluorescence staining for ALB and AAT, and PAS staining. Scale bar = 100 μm. HE, hematoxylin and eosin; CCl₄, carbon tetrachloride.

staining, and PAS-stained engrafted differentiated hADMSC clusters.

Discussion

There are only few reports describing the hepatocytic transdifferentiation potential of ADMSCs.^{20,21} These stud-

ies described hepatocyte-like cell clusters that differentiate from ADMSCs and continue to display hepatocyte-specific functions. In this study, we generated functional hepatocyte-like cell clusters from hADMSCs that expressed high levels of mature hepatocyte markers such as ALB, glutamine synthetase, CYP1B1, and CK18. These cell clusters exhibited functional characteristics of hepatocytes; they expressed

ALB, secreted urea, had CYP activity, took up LDL, and stored glycogen. Alpha-1-antitrypsin expression was markedly lower than those of hepatocytes and HepG2. Alpha-1-antitrypsin is known as an acute phase protein and stimulation by inflammatory cytokines, such as TNF- α and IL-6, increased its expression by not more than 100-fold in native liver.³⁰ It is possible that the low expression of alpha-1-antitrypsin in regenerated hepatocyte-like clusters is due to the experimental condition, that is, incubation in the absence of inflammatory cytokines.

Transplantation of regenerated hepatocyte-like clusters into NOD-SCID mice with experimentally induced chronic liver failure resulted in the secretion of hepatotrophic factors and augmentation of the function of native resident hepatocytes, as well as improvement of serum total bilirubin and ALB levels. It is possible that in transplanted animals, serum unconjugated bilirubin was converted to conjugated bilirubin by the transplanted hepatocyte-like cell clusters and soluble conjugated bilirubin was excreted in the urine because only conjugated bilirubin can be excreted into urine. It is possible that hepatosupportive factors, which were secreted by the clusters, augmented the function of liver in the transplanted animals.

For effective differentiation into hepatocyte-like cells, we introduced several improvements to the differentiation procedure. This included the formation of cell clusters and growth factor stimulation by culturing the intact cell clusters to allow for cell-to-cell interactions. In somatic stem cell differentiation, signals from the microenvironment such as cell-to-cell contacts are probably important factors that dictate the type of mature functional cells. Previous studies indicated that fetal hepatic cells and BM-derived MSCs could be induced into cells with a hepatocyte phenotype only when the cells were seeded at high density.¹¹ Signals induced at a high cell density, possibly via cell-cell contacts, are suspected to be necessary for hepatocytic differentiation. Further, adult hepatocyte progenitor cells were reported to show high expression levels of mature hepatocyte markers when cultured in spheroids.³¹⁻³³ In our procedure, we used a floating culture and prepared cell clusters to increase cell-cell contacts. In addition, the differentiated hepatocyte-like cell clusters using our method could be transplanted as intact cell clusters. In two previous reports that described the hepatocytic differentiation potential of ADMSCs,^{15,16} hepatocyte-like cells were enzymatically digested to single cells and transplanted. Such transplanted cells may not function effectively. Recently, Ohashi *et al.* developed a cell sheet of primary hepatocytes using temperature-responsive culture dishes to maintain cell-to-cell connections and the presence of adhesion proteins.³⁴ They reported that transplantation of hepatocyte sheets resulted in efficient engraftment and long survival. We transplanted the intact hepatocyte-like cell clusters into animals to allow for cell-to-cell connections and maintain the presence of adhesion proteins. Detachment from extracellular matrix and single-cell dissociation using enzymes such as trypsin or collagenase were not needed. These methodological modifications allowed efficient induction of hADMSCs into functional hepatocyte-like cell clusters both *in vitro* and *in vivo*.

In conclusion, we developed a simple protocol for efficient differentiation of hADMSCs into hepatocyte-like cell clusters. These hepatocyte-like cell clusters exhibited functional

characteristics of hepatocytes. These *in vitro*-introduced hepatocyte-like cells may provide a potentially unlimited source of hepatocyte-like cells without the limitation of immune rejection.

Acknowledgments

This study was supported in part by a grant-in-aid for Akifumi Matsuyama from the Kobe Translational Research Cluster, the Knowledge Cluster Initiative, Ministry of Education, Culture, Sports, Science, and Technology (MEXT) and from the Program for Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), and in part by a grant-in-aid for Yoshiki Sawa from the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

Disclosure Statement

No competing financial interests exist.

References

- Bernuau, J., Rueff, B., and Benhamou, J.P. Fulminant and subfulminant liver failure: definitions and causes. *Semin Liver Dis* **6**, 97, 1986.
- Fox, I.J., Chowdhury, J.R., Kaufman, S.S., Goertzen, T.C., Chowdhury, N.R., Warkentin, P.I., Dorko, K., Sauter, B.V., and Strom, S.C. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. *N Engl J Med* **338**, 1422, 1998.
- Wagers, A.J., and Weissman, I.L. Plasticity of adult stem cells. *Cell* **116**, 639, 2004.
- Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143, 1999.
- Shih, D.T., Lee, D.C., Chen, S.C., Tsai, R.Y., Huang, C.T., Tsai, C.C., Shen, E.Y., and Chiu, W.T. Isolation and characterization of neurogenic mesenchymal stem cells in human scalp tissue. *Stem Cells* **23**, 1012, 2005.
- In't Anker, P.S., Scherjon, S.A., Kleijburg-van der Keur, C., de Groot-Swings, G.M., Claas, F.H., Fibbe, W.E., and Kanhai, H.H. Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. *Stem Cells* **22**, 1338, 2004.
- Bieback, K., Kern, S., Klüter, H., and Eichler, H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. *Stem Cells* **22**, 625, 2004.
- Jiang, Y., Jahagirdar, B.N., Reinhardt, R.L., Schwartz, R.E., Keene, C.D., Ortiz-Gonzalez, X.R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W.C., Largaespada, D.A., and Verfaillie, C.M. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418**, 41, 2002.
- Toma, C., Pittenger, M.F., Cahill, K.S., Byrne, B.J., and Kessler, P.D. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* **105**, 93, 2002.
- Mareschi, K., Novara, M., Rustichelli, D., Ferrero, I., Guido, D., Carbone, E., Medico, E., Madon, E., Vercelli, A., and Fagioli, F. Neural differentiation of human mesenchymal stem cells: evidence for expression of neural markers and eag K⁺ channel types. *Exp Hematol* **34**, 1563, 2006.
- Hong, S.H., Gang, E.J., Jeong, J.A., Ahn, C., Hwang, S.H., Yang, I.H., Park, H.K., Han, H., and Kim, H. *In vitro* differentiation of human umbilical cord blood-derived

- mesenchymal stem cells into hepatocyte-like cells. *Biochem Biophys Res Commun* **330**, 1153, 2005.
12. Sato, Y., Araki, H., Kato, J., Nakamura, K., Kawano, Y., Kobune, M., Sato, T., Miyanishi, K., Takayama, T., Takahashi, M., Takimoto, R., Iyama, S., Matsunaga, T., Ohtani, S., Matsuura, A., Hamada, H., and Niitsu, Y. Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion. *Blood* **106**, 756, 2005.
 13. di Bonzo, L.V., Ferrero, I., Cravanzola, C., Mareschi, K., Rustichell, D., Novo, E., Sanavio, F., Cannito, S., Zamara, E., Bertero, M., Davit, A., Francica, S., Novelli, F., Colomatto, S., Fagioli, F., and Parola M. Human mesenchymal stem cells as a two-edged sword in hepatic regenerative medicine: engraftment and hepatocyte differentiation versus profibrogenic potential. *Gut* **57**, 223, 2008.
 14. Aurich, I., Mueller, L.P., Aurich, H., Luetzkendorf, J., Tislar, K., Dollinger, M.M., Schormann, W., Walldorf, J., Hengstler, J.G., Fleig, W.E., and Christ, B. Functional integration of hepatocytes derived from human mesenchymal stem cells into mouse livers. *Gut* **56**, 405, 2007.
 15. Kuo, T.K., Hung, S.P., Chuang, C.H., Chen, C.T., Shih, Y.R., Fang, S.C., Yang, V.W., and Lee, O.K. Stem cell therapy for liver disease: parameters governing the success of using bone marrow mesenchymal stem cells. *Gastroenterology* **134**, 2111, 2008.
 16. Schwartz, R.E., Reyes, M., Koodie, L., Jiang, Y., Blackstad, M., Lund, T., Lenvik, T., Johnson, S., Hu, W.S., and Verfaillie, C.M. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. *J Clin Invest* **109**, 1291, 2002.
 17. Sgodda, M., Aurich, H., Kleist, S., Aurich, I., König, S., Dollinger, M.M., Fleig, W.E., and Christ, B. Hepatocyte differentiation of mesenchymal stem cells from rat peritoneal adipose tissue *in vitro* and *in vivo*. *Exp Cell Res* **313**, 2875, 2007.
 18. Björntorp, P., Karlsson, M., Gustafsson, L., Smith, U., Sjöström, L., Cigolini, M., Storck, G., and Pettersson, P. Quantitation of different cells in the epididymal fat pad of the rat. *J Lipid Res* **20**, 97, 1979.
 19. Zuk, P.A., Zhu, M., Ashjian, P., De Ugarte, D.A., Huang, J.I., Mizuno, H., Alfonso, Z.C., Fraser, J.K., Benhaim, P., and Hedrick, M.H. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**, 4279, 2002.
 20. Seo, M.J., Suh, S.Y., Bae, Y.C., and Jung, J.S. Differentiation of human adipose stromal cells into hepatic lineage *in vitro* and *in vivo*. *Biochem Biophys Res Commun* **328**, 258, 2005.
 21. Banas, A., Teratani, T., Yamamoto, Y., Tokuhara, M., Takeshita, F., Quinn, G., Okochi, H., and Ochiya, T. Adipose tissue-derived mesenchymal stem cells as a source of human hepatocytes. *Hepatology* **46**, 219, 2007.
 22. Okura, H., Matsuyama, A., Lee, C.M., Saga, A., Kakuta-Yamamoto, A., Nagao, A., Sougawa, N., Sekiya, N., Takekita, K., Shudo, Y., Miyagawa, S., Komoda, H., Okano, T., and Sawa, Y. Cardiomyoblast-like cells differentiated from human adipose tissue-derived mesenchymal stem cells improve left ventricular dysfunction and survival in a rat myocardial infarction model. *Tissue Eng Part C Methods* July 22, 2009. [Epub ahead of print].
 23. Okura, H., Fumimoto, Y., Komoda, H., Yanagisawa, T., Nishida, T., Noguchi, S., Sawa, Y., and Matsuyama, A. Transdifferentiation of human adipose tissue-derived stromal cells into insulin-producing clusters. *J Artif Organs* **12**, 123, 2009.
 24. Alonso, M., Claros, S., Becerra, J., and Andrades, J.A. The effect of type I collagen on osteochondrogenic differentiation in adipose-derived stromal cells *in vivo*. *Cytotherapy* **10**, 597, 2008.
 25. Renwick, A.B., Surry, D., Price, R.J., Lake, B.G., and Evans, D.C. Metabolism of 7-benzoyloxy-4-trifluoromethyl-coumarin by human hepatic cytochrome P450 isoforms. *Xenobiotica* **30**, 955, 2000.
 26. Mitchell, J.B., McIntosh, K., Zvonic, S., Garrett, S., Floyd, Z.E., Kloster, A., Di Halvorsen, Y., Storms, R.W., Goh, B., Kilroy, G., Wu, X., and Gimble, J.M. Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. *Stem Cells* **24**, 376, 2006.
 27. Barry, F., Boynton, R., Murphy, M., Haynesworth, S., and Zaia, J. The SH-3 and SH-4 antibodies recognize distinct epitopes on CD73 from human mesenchymal stem cells. *Biochem Biophys Res Commun* **289**, 519, 2001.
 28. Barry, F.P., Boynton, R.E., Haynesworth, S., Murphy, J.M., and Zaia, J. The monoclonal antibody SH-2, raised against human mesenchymal stem cells, recognizes an epitope on endoglin (CD105). *Biochem Biophys Res Commun* **265**, 134, 1995.
 29. Kannagi, R., Cochran, N.A., Ishigami, F., Hakomori, S., Andrews, P.W., Knowles, B.B., and Solter, D. Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. *EMBO J* **2**, 2355, 1998.
 30. Kalsheker, N., Morley, S., and Morgan, K. Gene regulation of the serine proteinase inhibitors alpha1-antitrypsin and alpha1-antichymotrypsin. *Biochem Soc Trans* **30**, 93, 2002.
 31. Qihao, Z., Xigu, C., Guanghui, C., and Weiwei, Z. Spheroid formation and differentiation into hepatocyte-like cells of rat mesenchymal stem cell induced by co-culture with liver cells. *DNA Cell Biol* **26**, 497, 2007.
 32. Semino, C.E., Merok, J.R., Crane, G.G., Panagiotakos, G., and Zhang, S. Functional differentiation of hepatocyte-like spheroid structures from putative liver progenitor cells in three-dimensional peptide scaffolds. *Differentiation* **71**, 262, 2003.
 33. Yamasaki, C., Tateno, C., Aratani, A., Ohnishi, C., Katayama, S., Kohashi, T., Hino, H., Marusawa, H., Asahara, T., and Yoshizato, K. Growth and differentiation of colony-forming human hepatocytes *in vitro*. *J Hepatol* **44**, 749, 2006.
 34. Ohashi, K., Yokoyama, T., Yamato, M., Kuge, H., Kanehiro, H., Tsutsumi, M., Amanuma, T., Iwata, H., Yang, J., Okano, T., and Nakajima, Y. Engineering functional two- and three-dimensional liver systems *in vivo* using hepatic tissue sheets. *Nat Med* **13**, 880, 2007.

Address correspondence to:

Akifumi Matsuyama, M.D., Ph.D.

Department of Somatic Stem Cell Therapy

Institute of Biomedical Research and Innovation

Foundation for Biomedical Research and Innovation

1-5-4-305 Minatojima-minamimachi

Chuo-ku

Kobe 650-0047

Japan

E-mail: akifumi-matsuyama@umin.ac.jp

Received: March 27, 2009

Accepted: October 19, 2009

Online Publication Date: December 18, 2009

This article has been cited by:

1. Shibashish Giri, Augustinus Bader. 2011. Improved preclinical safety assessment using micro-BAL devices: the potential impact on human discovery and drug attrition. *Drug Discovery Today* . [[CrossRef](#)]
2. Hanayuki Okura , Ayami Saga , Yuichi Fumimoto , Mayumi Soeda , Mariko Moriyama , Hiroyuki Moriyama , Koji Nagai , Chun-Man Lee , Shizuya Yamashita , Akihiro Ichinose , Takao Hayakawa , Akifumi Matsuyama . 2011. Transplantation of Human Adipose Tissue-Derived Multilineage Progenitor Cells Reduces Serum Cholesterol in Hyperlipidemic Watanabe Rabbits. *Tissue Engineering Part C: Methods* 17:2, 145-154. [[Abstract](#)] [[Full Text](#)] [[PDF](#)] [[PDF Plus](#)]