Journal of Cellular Biochemistry 102:52–63 (2007)

In Vitro Differentiation of Mouse Bone Marrow Stromal Stem Cells Into Hepatocytes Induced by Conditioned Culture Medium of Hepatocytes

Ye Chen,[1] Xue-Jun Dong,[2] Guo-Rong Zhang,[2] Jian-Zhong Shao,[1]* and Li-Xin Xiang[1]*

[1]College of Life Sciences, Zhejiang University, Hangzhou 310058, P.R. China
[2]Shaoxing People's Hospital, Shaoxing 312000, P.R. China

Abstract The differentiation potential of adult stem cells has long been believed to be limited to the tissue or germ layer of their origin. However, recent studies have demonstrated that adult stem cells may encompass a greater potential than once thought. In the present study, we examined whether murine bone marrow derived stromal stem cells (BMSSCs) are able to differentiate into functional hepatocytes in vitro. BMSSCs were isolated from murine femora and tibiae, and the mesodermal multilineage differentiation potentials of these cells were functionally characterized. To effectively induce hepatic differentiation, we designed a novel protocol by using hepatocyte-conditioned medium. Hepatic differentiation from mouse BMSSCs was examined by a variety of assays at morphological and molecular levels. Morphologically, mouse BMSSCs became round and epithelioid, binucleated after induction. Differentiated cells were harvested on Days 0, 10, and 20 and subjected to examination of hepatocyte characteristics by reverse transcription-polymerase chain reaction (RT-PCR) and immunocytochemistry. We detected AFP, HNF-3β, CK19, CK18, ALB, TAT, and G-6-Pase at the mRNA and/or protein levels, hepatocyte-like cells by culture in conditioned medium further demonstrated in vitro functions characteristic of liver cells, including glycogen storage, and urea secretion. Moreover, transplantation of the differentiated cells into liver-injured mice partially restored serum albumin level and significantly suppressed transaminase activity. Our findings indicated the transdifferentiation potential of mouse BMSSCs developing into the functional hepatocyte-like cells by conditioned culture medium and, hence, may serve as a model system for the study of mechanisms involved in the transdifferentiation, and a cell source for cell therapy of hepatic diseases. J. Cell. Biochem. 102: 52–63, 2007.

Key words: bone marrow stromal stem cells; transdifferentiation; conditioned medium; hepatocytes; in vitro

Recently, a number of studies readily demonstrated that adult stem cells encompass a plasticity of multiple cell lineages and, hence, promise to have tremendous therapeutic potential in tissue repair and organ regeneration [Wagers et al., 2002; Horwitz, 2003; Turhan, 2003; Wagers and Weissman, 2004; Quesenberry et al., 2005; Bedada et al., 2006]. In bone marrow (BM), there are certain populations of stem cell sources including hematopoietic stem cells (HSCs), bone marrow stromal stem cells (BMSSCs), and multipotent adult progenitor cells (MAPCs) [Muguruma et al., 2003; Wang et al., 2004]. In in vivo studies, cross-sex/cross-strain and whole liver transplantation indicated that the bone marrow would be an alternative source of hepatocyte progenitors [Petersen et al., 1999; Alison et al., 2000; Lagasse et al., 2000; Theise et al., 2000]. It also has been reported that under proper treatments or in a suitable microenvironment, bone marrow-derived cells expressed albumin and a-fetoprotein (AFP) at mRNA level in vitro [Avital et al., 2001; Miyazaki et al., 2002; Okumoto et al., 2003]. These studies have convincingly demonstrated that adult bone marrow contains cells capable of differentiating into liver epithelial cells.

Xue-Jun Dong is the co-first author.

Grant sponsor: Shaoxing Project for Science and Technology; Grant number: 2005141; Grant sponsor: Science and Technology Foundation (Department of Education of Zhejiang Province, P.R. China); Grant number: 20050940.

*Correspondence to: Jian-Zhong Shao, Li-Xin Xiang, College of Life Sciences, Zhejiang University, Hangzhou 310058, Zhejiang Province, P.R. China.
E-mail: shaojz@zju.edu.cn; xianglx@zju.edu.cn

Received 29 June 2006; Accepted 29 December 2006
DOI 10.1002/jcb.21275

BMSSCs (also known as mesenchymal stem cells [Gronthos et al., 2006]), widely studied over the past decade, are readily accessible from bone marrow and have been shown to be capable of differentiating into mesoderm cell lineages such as osteoblasts and adipocytes in vivo and in vitro [Pittenger et al., 1999; Hamada et al., 2005; Luk et al., 2005; Wislet-Gendebien et al., 2005; Zhang et al., 2005]. In the past 3 years, several reports have demonstrated one or more types of mesenchymal cells may rarely acquire the hepatocyte phenotype [Lee et al., 2004; Wang et al., 2004; Hong et al., 2005; Lange et al., 2005; Seo et al., 2005; Ong et al., 2006; Snykers et al., 2006], however, little evidence for mouse BMSSCs differentiating into hepatic cells has been shown yet. So that further studies are needed.

Up to now, the mechanism of adult stem cell transdifferentiation is unclear. Most researchers believe that microenvironment plays an important role in differentiation of stem cells [Watt and Hogan, 2000; Tosh and Slack, 2002], as stem cells have reciprocity with neighboring cells, ECM, and cytokines. The present study aims to examine whether mouse BMSSCs have the potential to differentiate into any hepatocyte-like phenotype when cultured in a hepatic conditioned medium in vitro.

MATERIALS AND METHODS

Experimental Animals

Eight- to ten-weeks-old male ICR mice obtained from the Laboratory Animal Unit of Zhejiang Academy of Medical Sciences (Hangzhou, People's Republic of China) were used in the experiments. Animals were housed under specified pathogen-free conditions. All animal experiments were done in accordance with a legal regulation, which includes approval by a local ethical committee.

Isolation and Long-Term Culture of Stromal Stem Cells From Mouse Bone Marrow

The mice were sacrificed by CO_2 inhalation and femora and tibiae isolated. After dissection of attached muscle and connective tissue from the bones, the marrow was extruded by clipping of the epiphysial ends of the bones and flushing using a 26-gauge needle with Iscove's modified Dulbecco's medium (IMDM, Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (FBS, Hyclone, Rockville, MD), 100 U/ml penicillin (Gibco BRL, Rockville, MD), and 100 μg/ml streptomycin (Gibco BRL) (Medium A) at 37°C and 5% CO_2. After 72 h, non-adherent cells and debris were removed, and the adherent cells were cultured continuously. Cells were harvested by 0.25% trypsin-EDTA (Sigma) when they grew to 90% confluence, and seeded at a density of 5×10^5 cells per 25 cm^2 flask as the first passage. BMSSCs at passage 3 were used for differentiation studies.

Flow Cytometric Analyses of Isolated BMSSCs

To determine the antigen expression profile of the BM-derived adherent cells, cells at passages 3 were harvested by trypsinization for 5 min at 37°C, washed, counted and resuspended in 0.1% BSA/PBS, and incubated with the following primary antibodies: phycoerythrin-conjugated rat monoclonal antibody to mouse CD44 (Caltag Laboratories); FITC-conjugated rat monoclonal antibody to mouse CD45 (Caltag Laboratories); phycoerythrin-conjugated rat monoclonal antibody to mouse CD90 (Caltag Laboratories); monoclonal rat anti-mouse CD105 (eBioscience); and CD11b (eBioscience). Non-conjugated mAbs were stained after washing in 0.1% BSA/PBS with FITC-conjugated anti-rat IgG (1:400, Chemicon). After washing, cell fluorescence signals were determined immediately using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with an argon laser emission of 488 nm. At least 10,000 events were collected. The analysis was performed using Cell Quest Software (Becton Dickinson).

Functional Identification of BMSSCs by Multi-Lineage Differentiation

To verify the multipotential mesenchymal characteristics, the generated cells at passage 3 were analyzed for osteogenic, adipogenic, and myogenic potential. Before inductive media were added, cultures were grown to confluence after which Medium A was replaced with the inductive media.

To induce osteogenic differentiation, cells were daily fed with Medium A in which 10 mM β-glycerophosphate (Sigma), 50 μg/ml ascorbic acid (Sigma), and 10^{-9} M dexamethasone (Sigma) were added for up to 3 weeks. Mineralized deposits were visualized by von Kossa.

To induce adipogenic differentiation, the cells were cultured for 2–4 weeks in Medium A with addition of 5 μg/ml insulin (Sigma) and 10^{-9} M dexamethasone. The medium was refreshed

every 3 days. Adipocytes were easily discerned from the undifferentiated cells by phase-contrast microscopy. To further confirm their identity, cells were washed twice with phosphate-buffered saline (PBS), fixed in 10% formalin neutral buffer solution for 30 min at room temperature and rinsed with distilled water. They were then stained with Oil Red O (six parts 0.6% Oil Red O dye in isopropanol, and four parts water) for 1 h and washed with distilled water.

Myotube formation was induced by the addition of 1.5 μg/ml amphotericin B (Sigma) to the culture medium for approximately 2–3 weeks followed by withdrawal of the fungicide after which the cells were cultured for another 1–2 weeks.

Hepatocyte Isolation and Preparation of Conditioned Medium

Hepatocytes were isolated by two-step collagenase perfusion. Briefly, donor animals received 25 U heparin (Sigma) prior to cell isolation. After cannulation of the portal vein, the liver was perfused with a calcium-free buffer solution, flow 3 ml/min at 37°C for 10 min. Then, the liver was perfused with 0.025% collagenase IV (Invitrogen, Carlsbad, CA), flow 2 ml/min at 37°C for 15 min. The perfused liver was resected, and the cells were released by gentle shaking and collected in 20 ml IMDM. The supernatant cell suspension was filtered using a 200 μm nylon mesh and filtrate was washed twice with PBS by centrifugation at 50*g* for 45 s to remove cell debris, damaged cells, and non-parenchymal cells. After washing, the hepatocytes were cultured in IMDM supplemented with 10% FBS at 5×10^4 cells/cm^2. Forty-eight hours later, the supernates were collected and passed through a 0.25 μm filter. The filtrate was finally defined as hepatocyte-conditioned medium and stored in aliquots at -20°C for future use.

Induction of Hepatogenic Differentiation of BMSSCs by Hepatocyte Conditioned Medium

BMSSCs of passage 3 were inoculated in differentiation medium at 5×10^4 cells/cm^2 on 50 ng/ml type I collagen-coated culture flasks. The differentiation medium consisted of 50% fresh Medium A and 50% hepatocyte-conditioned medium. As a negative control, BMSSCs were cultured in Medium A only. Cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cultures were maintained by medium exchange every 3 days. Cell morphology was observed under an Olympus phase-contrast microscope (CKX31, Olympus Beijing Industry & Technology Limited, Beijing, China).

Identification of Hepatogenic Differentiation

RNA isolation and reverse transcription-polymerase chain reaction. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) was used to determine mRNA transcripts in BMSSCs after treatment for 10 or 20 days. In brief, total RNA was isolated using TRIZOL (Invitrogen) according to the manufacturer's instructions and quantified by UV spectroscopy. One microgram of total RNA was reverse transcribed to complementary DNA using SuperScript III first-strand synthesis system (Invitrogen) with oligo (dT) and random hexamer primers. Gene-specific primers for AFP, HNF3β, CK19, CK18, ALB, TAT, and G-6-p were designed using the Primer Premier software as listed in Table I. PCRs were performed by using Taq DNA polymerase (Gibco BRL). Complementary DNA was amplified at 95°C for 30 s, at annealing temperature for 30 s, and 72°C for 30 s for a total of 35 cycles and final extension at 72°C for 10 min using Gene Amp PCR 9600 (Perkin-Elmer Corporation, Norwalk, CT). The PCR products were fractionated by 1.2% agarose gel electrophoresis and visualized after staining with ethidium bromide under UV illumination. The gel images were captured and analyzed by the Kodak Gel Logic 200 Imaging System (Kodak Molecular Imaging Systems).

Immunofluorescence staining. Cell cultures were washed with PBS twice and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, then permeabilized with cold methanol for 20 min. After several washes with PBS, cells were then incubated at 4°C overnight with following primary antibodies, including rabbit anti AFP (1:100; Dako, Glostrup, Denmark), mouse anti CK19 (1:100; Dako), and sheep anti albumin (1:100; Biodesign, Saco, Maine). The secondary antibodies including FITC-conjugated bovine anti-sheep IgG (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), FITC-conjugated goat anti-rabbit IgG (1:200; Sigma), TRITC-conjugated goat anti-mouse IgG (1:200; Sigma) were used

TABLE I. Primers and Annealing Temperatures Used for RT-PCR

Gene	Sequence (5′-3′)	Product size	Annealing temperature
β-actin-F	TTCCTTCTTGGGTATGGAAT	200 bp	55°C
β-actin-R	GAGCAATGATCTTGATCTTC		
AFP-F	CACTGCTGCAACTCTTCGTA	300 bp	52°C
AFP-R	CTTTGGACCCTCTTCTGTGA		
HNF3β-F	GACCTCTTCCCTTTCTACCG	551 bp	51°C
HNF3β-R	TTGAAGGCGTAATGGTGC		
CK19-F	GTCCTACAGATTGACAATGC	570 bp	55°C
CK19-R	CACGCTGGATCTGTGACAG		
CK18-F	GTTGTCACCACCAAGTCTGC	415 bp	52°C
CK18-R	CCACTCTGCCATCCACGA		
ALB-F	TCTTCGTCTCCGGCTCTG	475 bp	55°C
ALB-R	CTGGCAACTTCATGCAAA		
TAT-F	CTTCAGTCCTGGATGTTCGC	619 bp	55°C
TAT-R	CAGGGATTGGACGGGTTGTT		
G-6-P-F	TCAATCTCCTCTGGGTGGC	602 bp	54°C
G-6-P-R	GGCAAAGGGTGTAGTGTCAAG		

AFP, α-fetoprotein; HNF3β (alternatively called FOXA2), hepatocyte nuclear factor 3β; CK19, cytokeratin 19; CK18, cytokeratin 18; ALB, albumin; TAT, tyrosine aminotransferase; G-6-P, glucose 6-phosphatase.

according to the manufacturer's instructions. The samples were incubated at 37°C for 45 min. After further washings with PBS and counterstained with DAPI solution, the cells were visualized and photomicrographed under a confocal laser-scanning microscope (LSM 510; Carl Zeiss, Inc., Oberkochen, Germany).

Periodic acid-Schiff (PAS) stain for glycogen. Culture dishes containing differentiated cells were fixed in 95% alcohol for 10 min. Samples were then oxidized in 1% periodic acid for 5 min, rinsed three times in deionized (d) H_2O, treated with Schiff's reagent for 15 min, and rinsed in dH_2O for 5 min. Finally, the preparations were assessed under light microscope.

Evaluation of urea synthesis. BMSSCs were plated at 2×10^4 cells/cm^2 on collagen-coated six-well plates in differentiation medium or control medium. After washing extensively with PBS, cells (differentiated at Days 3, 6, 9, 12, 15, 18, and 21) were incubated in 2 ml of serum-free Hanks' buffered salt solution containing 5 mM/L NH_4Cl for 2 h at 37°C. After incubation, the urea concentrations in the supernatant were measured.

Transplantation experiments. Cells cultured in conditioned medium for 3 weeks were detached from the plate by trypsin treatment. ICR mice aged 4 weeks were treated with CCl_4 (5 μl per g (body weight) of a 10% solution in mineral oil injected intraperitoneum, i.p.), and 1×10^6 cells prepared were transplanted by injection into the tail vein. After the transplantation, the mice were treated with CCl_4 twice a week. Mice were killed 7 days after transplant and serum was collected to analyze serum albumin level, serum aspartate aminotransferase (AST), and alanine aminotransferase (ALT). (assays were done by standard automated instrumentation)

Statistical Analysis

All experiments were replicated a minimum of three times; data were expressed as mean ± standard deviation.

RESULTS

Isolation and Characterization of BMSSCs

The BMSSCs-like adherent cells were observed on Day 1–2 after inoculation. By continuous changes of medium, the suspending cells were completely removed from the medium. The adhered cells were fibroblast-like and grew as a whirlpool (Fig. 1A). When the primary culture cells reached confluence on Day 10–12, the cells were sub-cultured at a ratio of 1:3. The sub-cultured cells that reached confluence 8–10 days later were much pure and fibroblast-like (Fig. 1B). Try to rule out the contamination of macrophages and hematopoietic cells which may interfere the results, immunophenotypic analyses was performed. As shown in Figure 2, majority of the adherent fibroblastic cells of passage 3 were negative for CD11b, CD45, Thy-1; and positive for known cell-surface antigens of mesenchymal cells (CD44, CD105) [Baddoo et al., 2003; Meirelles Lda and Nardi, 2003; Rombouts and Ploemacher, 2003; Wang et al., 2006].

In order to examine whether these obtained cells were BMSSCs, differentiation potential of these cells into osteogenic, adipogenic, and myogenic cells were functionally identified. To induce osteoblastic differentiation, cells were cultured in an osteoblastic-inductive medium as described in the "Materials and Methods." In the initial culture, no mineralized cell was detected (data not shown). Three weeks after culturing, some mineralized cells were found using von Kossa staining (Fig. 1C). To further confirm the osteoblastic differentiation, the

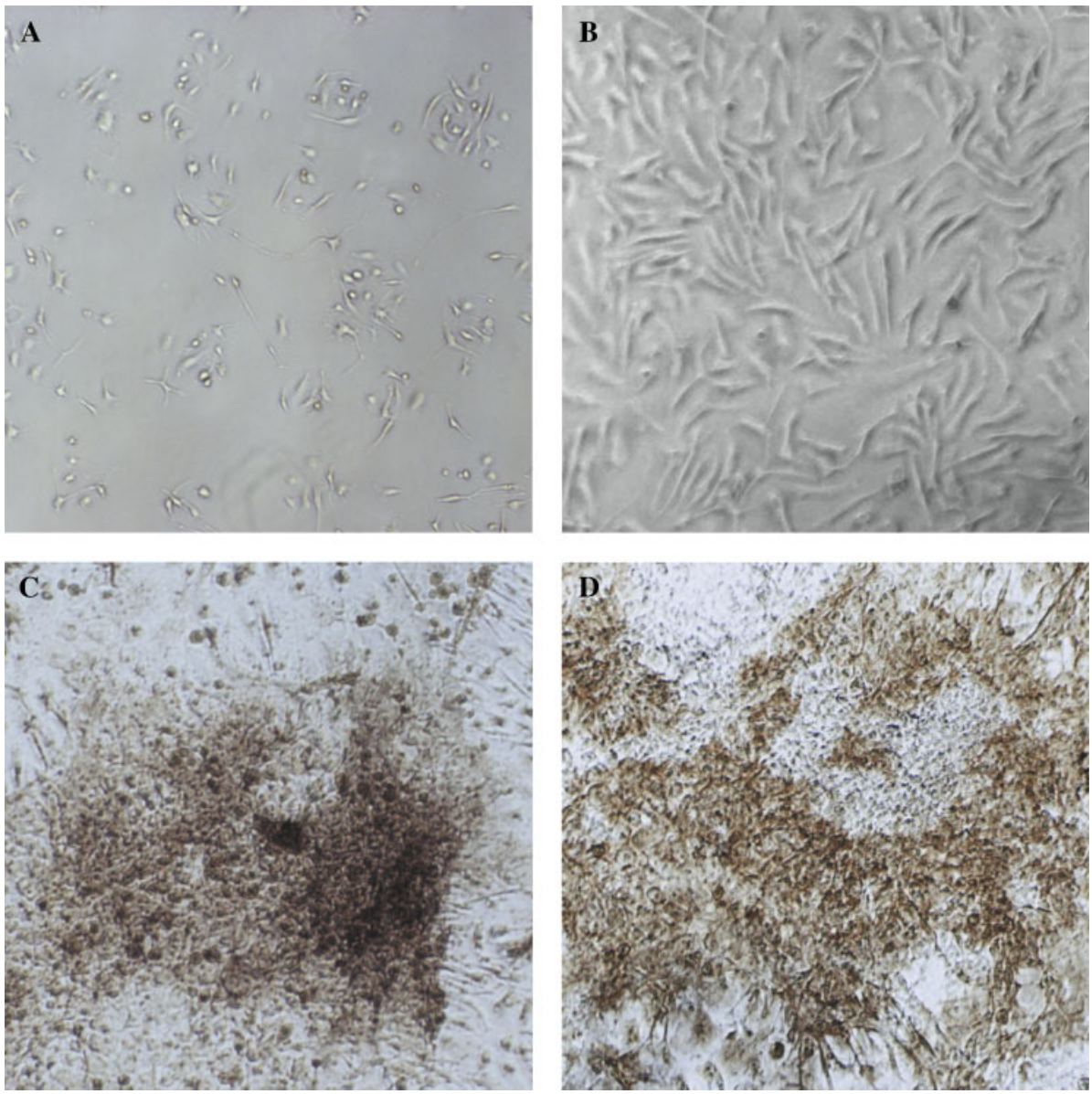

Fig. 1. Photomicrograph of the in vitro differentiation of the 'BMSSCs-like' cells. Cells isolated from ICR mice were expanded in IMDM supplemented with 10% FBS (**A**), the sub-cultured cells reached confluence 8–10 days later were much pure and showed homogeneous fibroblastoid morphology (**B**). Differentiation of mouse 'BMSSCs-like' cells: 3rd passage cells were cultured in differentiation inducing media as described in Materials and Methods. We could observe a trilineage differentiation toward: osteoblasts, the cells were incubated in osteogenic medium for 4 weeks, and mineralized deposits were detected by von Kossa staining (**C**). Concomitantly, cells expressed alkaline phosphatase (**D**); adipocytes, as early as after 1 week of inducement, lipid droplets were detectable, as shown by phase-contrast microscopy (**E**), and lipid accumulation then increased along with the inductive periods. After 3 weeks, lipid accumulation was stained with Oil Red O (**F**); myotubes, under myogenic conditions, myotubes could be observed in this phase-contrast photomicrograph (**G**). (Original magnification: A 40×; B ~ G 100×) [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

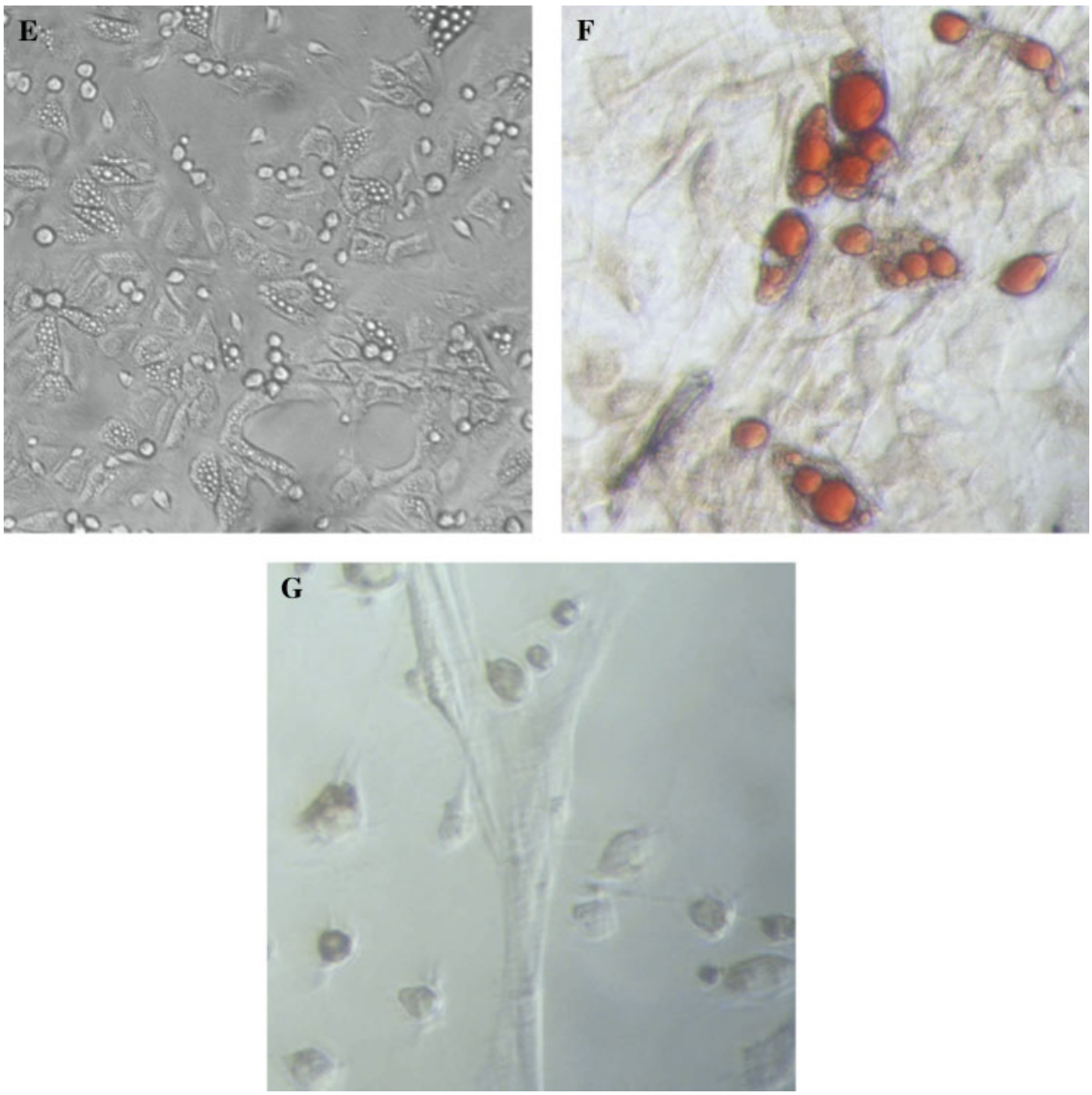

Fig. 1. (*Continued*)

expression of alkaline phosphatase (ALP) was examined (Fig. 1D). To induce adipocytic differentiation, the cells were cultured in an adipocytic-inductive medium as described in the "Materials and Methods." One prominent characteristic of cell differentiation into adipocytes is the accumulation of triglyceride-containing vesicles in the cell cytosol. As early as 1 week after inducement, lipid droplets were detectable, and lipid accumulation thereafter increased along with the inductive periods. Intracellular fat droplets were observed by phase-contrast light microscopy (Fig. 1E) and were chemically stained by Oil Red O (Fig. 1F). We also demonstrated the mesenchymal multilineage differentiation potential of the cells into myotubes (Fig. 1G), albeit in low frequencies.

Morphological Changes of BMSSCs Differentiated into Hepatocyte-Like Cells

After being treated with hepatocyte-conditioned medium, the spindle-shaped morphology of BMSSCs was lost and cells developed into a broadened, flattened morphology. One week post-induction, a retraction of elongated ends was observed with the increasing time of

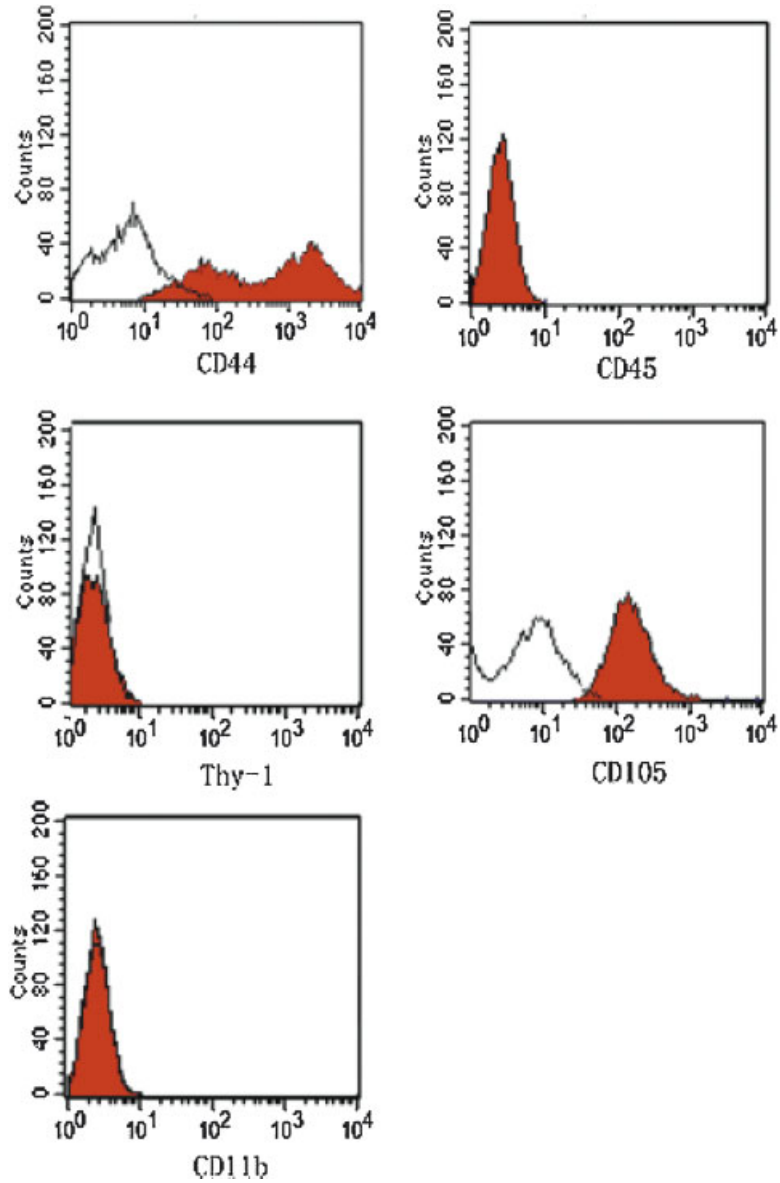

Fig. 2. Flow cytometric analyses of isolated BMSSCs. Cells were harvested and labeled with Abs against CD44, CD45, Thy-1, CD105, CD11b, or control IgGs, and analyzed by FACS. Histograms showing the relative staining intensity of BMSSCs for various cell surface antigens. Cells (98.5%) express CD105, 88.2% of cells express CD44, and 0.2% of cells express Thy-1; none of the cells express CD45 and CD11b. Black line, control immunoglobulin; red line, specific antibody. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

differentiation (Fig. 3A). Two weeks post-induction, the cuboidal morphology of hepatocytes developed (Fig. 3B). After prolonged culture, the hepatocytic morphology further matured with the appearance of abundant granules in the cytoplasm. On Day 20, approximately 75% of the cells were epithelioid with large and rich intracellular structures, and the presence of binucleated cells was observed (Fig. 3C). The size of the differentiated cells was 20–25 μm in diameter, which was consistent with the size of cultured control hepatic cells.

Hepatic Gene and Protein Expression Analysis of the Differentiated Cells

Reverse transcription polymerase chain reaction analysis showed the expression of AFP, HNF3β, and CK19 by Day 10, while TAT, a late marker gene of hepatocytes, was detected by Day 20. Expression of CK18 and ALB was

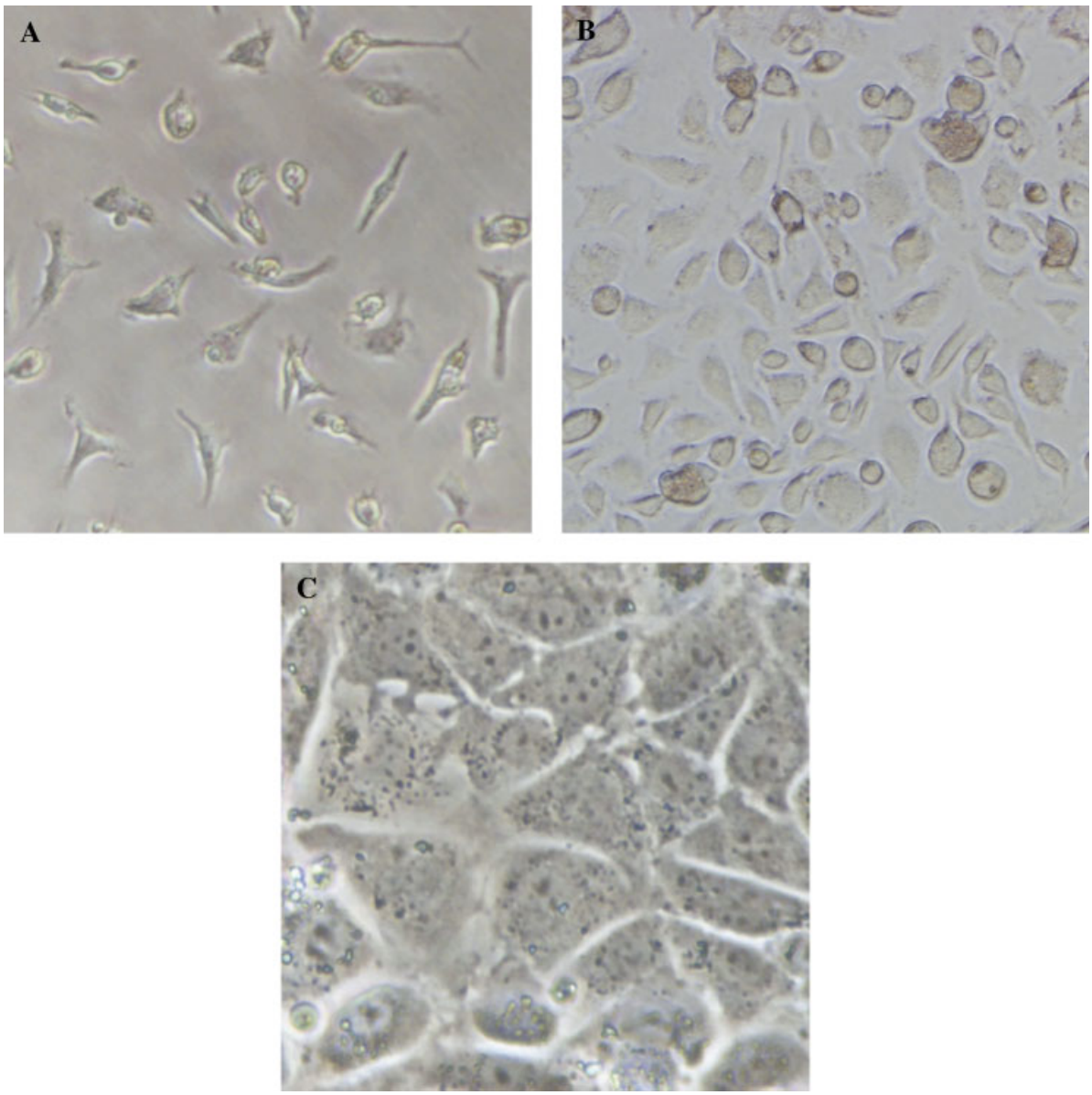

Fig. 3. Morphological observation of BMSSCs differentiated into hepatocyte-like cells. (**A**) BMSSCs induced by hepatocyte-conditioned medium showed a retraction of elongated ends on Day 8; (**B**) Two weeks post-induction, the cuboidal morphology of hepatocytes developed; (**C**) The morphological change was further progressed during the differentiation process, most of treated cells assumed hepatocyte-like morphology with rich intracellular structures on Day 20. (Original magnification: A, B 100×; C 200×) [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

detected at all time points and increased with time of differentiation, whereas undifferentiated BMSSCs expressed none of these genes (Fig. 4). The β-actin housekeeping gene was included in all experiments as an internal control. As differentiation progressed, the protein expression of AFP, CK19, and ALB were detected by indirect immunofluorescence staining, in accordance with the results obtained at the RNA level. Consequently, after 20 days of culture, 82 ± 6, 77 ± 3, and $88 \pm 7\%$ of the cells, respectively, stained positive for these markers (Fig. 5).

Functional Characterization of the Differentiated Cells

Intracellular glycogen accumulation, one feature of adult liver cells, was analyzed by staining the cells with the PAS reagent. The

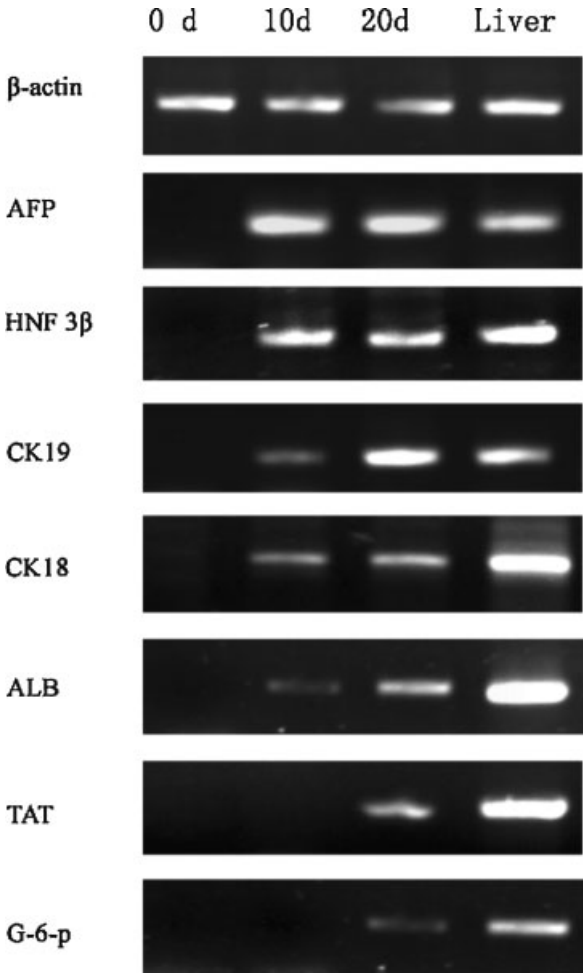

Fig. 4. RT-PCR analyses of the expression of transcriptional factors and lineage-specific markers in the mouse BMSSCs before and after differentiation. BMSSCs were treated with hepatocyte-conditioned medium and total mRNA was extracted for RT-PCR. Mouse β-actin mRNA was used as control for production of cDNA, and liver cells were used as positive control. The results showed that differentiated cells express hepatocyte-specific marker genes at the indicated time points. The experiments were repeated at least thrice and similar findings were observed.

result showed that an accumulation of glycogen was detected in the course of differentiation (Fig. 6A), while no accumulation of glycogen was found in the negative control (data not shown). In addition, we also assessed the urea production, another feature of the liver cells, at various time points throughout the differentiation. The results demonstrated that the differentiated cells produced urea in a time-dependent manner till they reached at the maximum level (Fig. 6B). To demonstrate the hepatic function in vivo, differentiated cells were transplanted in liver injured models. We tested serum ALB, AST, and ALT at 7 days after transplantation (Table II). AST and ALT was increased by CCl_4 treatment, indicating liver parenchymal damage; albumin, which is exclusively synthesized in liver, was decreased after CCl_4 treatment. Transplantation of the differentiated cells into liver-injured mice partially restored their serum albumin level and significantly suppressed transaminase activity.

DISCUSSION

Adult stem cells are an attractive source of cells for tissue therapy, and thought to have developmental potentials or plasticity of multiple cell lineages. BMSSCs, first described by Friedenstein et al. [1976], have attracted intense attention in the past two decades. These cells are capable of clonal expansion in culture, supporting hematopoietic stem cell proliferation, and demonstrating extensive differentiation capacity both in vivo and in vitro [Pittenger et al., 1999; Wang et al., 2004].

For cultures of mature hepatocytes, it has been shown that important stimuli for an adequate maintenance of cellular function in vitro are (1) addition of growth hormones and cytokines, (2) cell-to-matrix contacts, and (3) coculture with other cell types [Shimaoka et al., 1987; Watt and Hogan, 2000; Fiegel et al., 2003]. Thus, an optimal in vitro condition might play a partial role in initiating the liver specific differentiation. Here, we employed conditioned medium of hepatocytes to induce the hepatogenic differentiation. The results showed that mouse bone marrow BMSSCs induced by this medium differentiated into hepatocyte-like cells that expressed primitive and mature marker genes in a time-dependent manner. During the course of differentiation, the fibroblastic morphology of BMSSCs gradually progressed toward the polygonal morphology of hepatocytes and further acquire in vitro functions characteristic of liver cells including albumin production, glycogen storage, and urea secretion. Moreover, transplantation of the differentiated cells into liver-injured mice partially restored serum albumin levels and significantly suppressed transaminase activity.

The nature of the adult stem cells involved and the mechanisms responsible for acquisition of a hepatocyte phenotype are still controversial [Thorgeirsson and Grisham, 2006]. This work suggested that the conditioned medium of

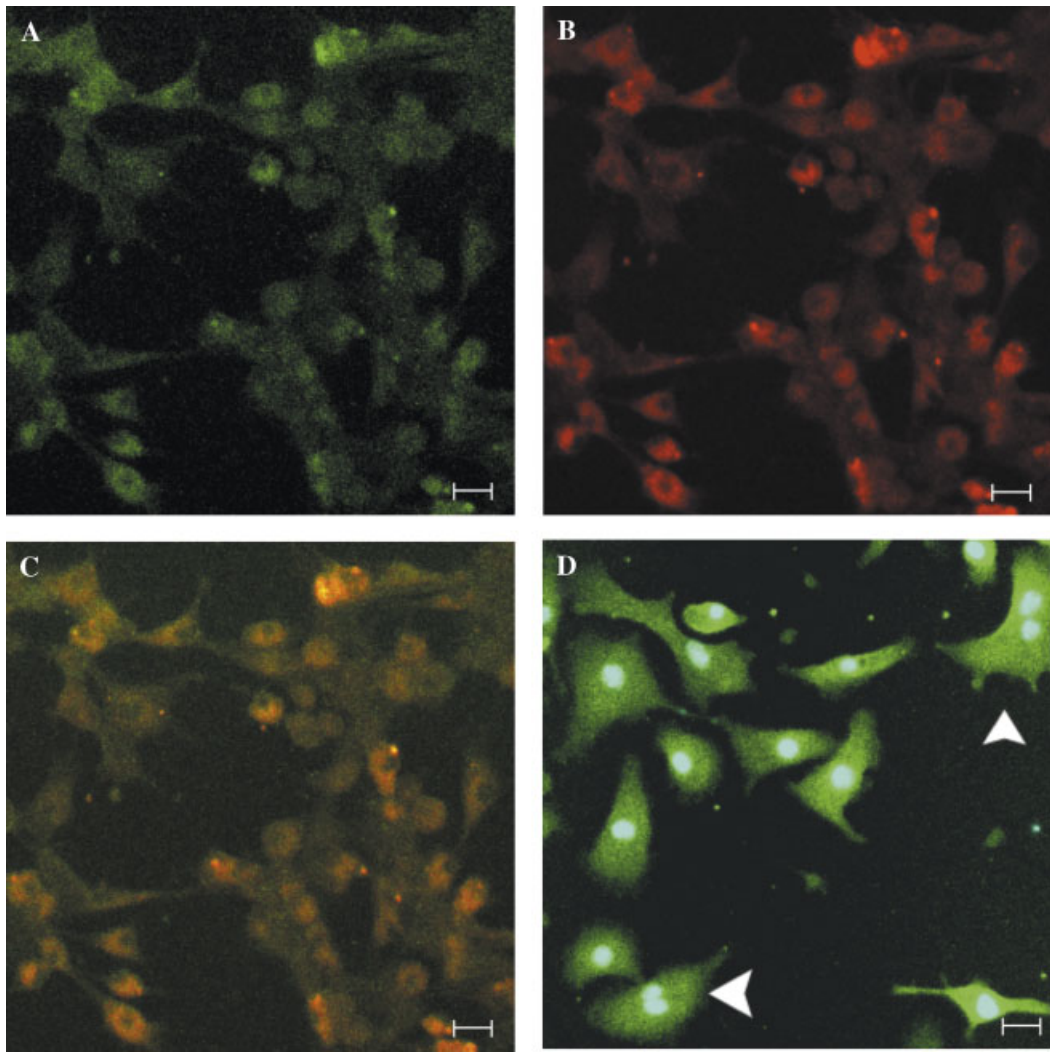

Fig. 5. Immunofluorescence analysis for hepatocyte specific markers of the differentiated cells. The fluorescent images were visualized and photomicrographed under a confocal laser-scanning microscope. The results reveal that hepatocyte-like cells by culture in conditioned medium were stained positive with anti-AFP labeled with FITC (**A**), anti-CK19 with TRITC (**B**); (**C**) figures (A) and (B) are overlaid; (**D**) ALB-positive signals were detected in the cytoplasm of differentiated cells; binucleated cells were noted by white arrows and sections were counterstained with DAPI. (Scale bars represent 20 μm). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE II. Changes in Blood Serum ALT, AST, and Albumin Concentration in Cell-Transplanted Mice

	Normal	7 day CCl_4	7 day CCl_4 & Transplantation
ALT	42 ± 9	308 ± 62	152 ± 25*
AST	96 ± 6	396 ± 90	187 ± 34*
ALB	31.2 ± 1.3	25.3 ± 1.1	27.5 ± 1.5*

Mice were treated with CCl_4 1 day before transplantation. After the transplantation, mice were treated with CCl_4 twice a week. CCl_4-treated mice showed blood chemistry abnormalities consistent with acute liver injury. Each value represents the mean ± SD. By Student's *t*-test, values are significant at $^*P < 0.05$ when comparing mice 7 days after transplantation (7 day CCl_4 & Transplantation) to control mice treated with CCl_4 alone.

hepatocytes has a stimulatory effect for the differentiation of BMSSCs toward liver cells, which was probably mediated by soluble factors. It provided an ideal model system for the study of mechanisms involved in the transdifferentiation from BMSSCs into hepatocytes. Also this system has numerous potential advantages in clinical application: first, there is no problem of limited donors as BMSSCs are readily accessible from bone marrow and can be expanded tremendously in vitro; second, the use of adult stem cells is favorable over embryonic stem cells regarding ethical issues; third, differentiated hepatocyte-like cells can be sustained for

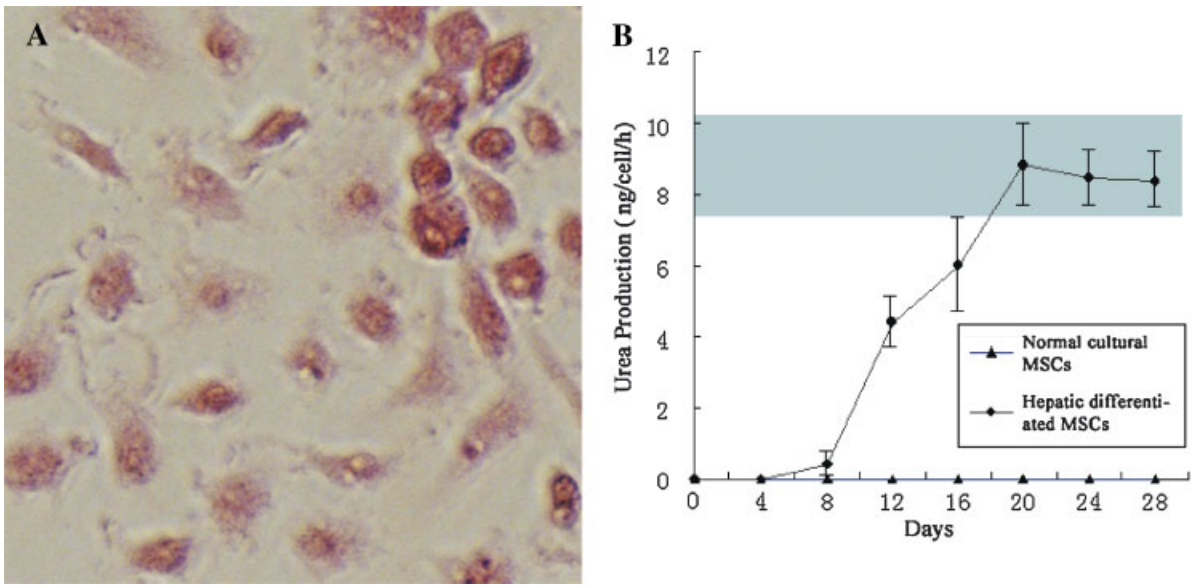

Fig. 6. In vitro functional characterization of hepatocyte-like cells differentiated from BMSSCs. **A:** The polygonal cells had magenta staining in the region on Day 20 post-induction, indicating storage of glycogen. (Original magnification, 100×) **B:** Urea production was measured through a colorimetric assay kit. Differentiated BMSSCs produce urea in a time-dependent manner, whereas no urea was detected in cultural BMSSCs. Gray area represents urea levels that were produced by 4 h-cultured adult mice hepatocytes. Data represent means ± SD, n = 4. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

8 weeks or more with functions, making it an ideal candidate for pharmacological and toxicological studies. We hope that these findings will contribute to the improvement of cell-based therapies for acute and chronic end-stage liver disease. A more detailed characterization study, nevertheless, is needed on the molecular mechanism to uncover the mechanisms that regulate the differentiation of BMSSCs into hepatocytes.

REFERENCES

Alison MR, Poulsom R, Jeffery R, Dhillon AP, Quaglia A, Jacob J, Novelli M, Prentice G, Williamson J, Wright NA. 2000. Hepatocytes from non-hepatic adult stem cells. Nature 406:257.

Avital I, Inderbitzin D, Aoki T, Tyan DB, Cohen AH, Ferraresso C, Rozga J, Arnaout WS, Demetriou AA. 2001. Isolation, characterization, and transplantation of bone marrow-derived hepatocyte stem cells. Biochem Biophys Res Commun 288:156–164.

Baddoo M, Hill K, Wilkinson R, Gaupp D, Hughes C, Kopen GC, Phinney DG. 2003. Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. J Cell Biochem 89:1235–1249.

Bedada FB, Gunther S, Kubin T, Braun T. 2006. Differentiation versus plasticity: Fixing the fate of undetermined adult stem cells. Cell Cycle 5:223–226.

Fiegel HC, Lioznov MV, Cortes-Dericks L, Lange C, Kluth D, Fehse B, Zander AR. 2003. Liver-specific gene expression in cultured human hematopoietic stem cells. Stem Cells 21:98–104.

Friedenstein AJ, Gorskaja JF, Kulagina NN. 1976. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4:267–274.

Gronthos S, Akintoye SO, Wang CY, Shi S. 2006. Bone marrow stromal stem cells for tissue engineering. Periodontology 2000 41 188–195.

Hamada H, Kobune M, Nakamura K, Kawano Y, Kato K, Honmou O, Houkin K, Matsunaga T, Niitsu Y. 2005. Mesenchymal stem cells (MSC) as therapeutic cytoreagents for gene therapy. Cancer Sci 96:149–156.

Hong SH, Gang EJ, Jeong JA, Ahn C, Hwang SH, Yang IH, Park HK, Han H, Kim H. 2005. In vitro differentiation of human umbilical cord blood-derived mesenchymal stem cells into hepatocyte-like cells. Biochem Biophys Res Commun 330:1153–1161.

Horwitz EM. 2003. Stem cell plasticity: The growing potential of cellular therapy. Arch Med Res 34:600–606.

Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L, Wang X, Finegold M, Weissman IL, Grompe M. 2000. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med 6:1229–1234.

Lange C, Bassler P, Lioznov MV, Bruns H, Kluth D, Zander AR, Fiegel HC. 2005. Hepatocytic gene expression in cultured rat mesenchymal stem cells. Transplant Proc 37:276–279.

Lee KD, Kuo TK, Whang-Peng J, Chung YF, Lin CT, Chou SH, Chen JR, Chen YP, Lee OK. 2004. In vitro hepatic differentiation of human mesenchymal stem cells. Hepatology 40:1275–1284.

Luk JM, Wang PP, Lee CK, Wang JH, Fan ST. 2005. Hepatic potential of bone marrow stromal cells: Development of in vitro co-culture and intra-portal transplantation models. J Immunol Methods 305:39–47.

Meirelles Lda S, Nardi NB. 2003. Murine marrow-derived mesenchymal stem cell: Isolation, in vitro expansion, and characterization. Br J Haematol 123:702–711.

Miyazaki M, Akiyama I, Sakaguchi M, Nakashima E, Okada M, Kataoka K, Huh NH. 2002. Improved conditions to induce hepatocytes from rat bone marrow cells in culture. Biochem Biophys Res Commun 298:24–30.

Muguruma Y, Reyes M, Nakamura Y, Sato T, Matsuzawa H, Miyatake H, Akatsuka A, Itoh J, Yahata T, Ando K, Kato S, Hotta T. 2003. In vivo and in vitro differentiation of myocytes from human bone marrow-derived multipotent progenitor cells. Exp Hematol 31:1323–1330.

Okumoto K, Saito T, Hattori E, Ito JI, Adachi T, Takeda T, Sugahara K, Watanabe H, Saito K, Togashi H, Kawata S. 2003. Differentiation of bone marrow cells into cells that express liver-specific genes in vitro: Implication of the Notch signals in differentiation. Biochem Biophys Res Commun 304:691–695.

Ong SY, Dai H, Leong KW. 2006. Inducing hepatic differentiation of human mesenchymal stem cells in pellet culture. Biomaterials 27:4087–4097.

Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP. 1999. Bone marrow as a potential source of hepatic oval cells. Science 284:1168–1170.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.

Quesenberry PJ, Dooner G, Colvin G, Abedi M. 2005. Stem cell biology and the plasticity polemic. Exp Hematol 33: 389–394.

Rombouts WJ, Ploemacher RE. 2003. Primary murine MSC show highly efficient homing to the bone marrow but lose homing ability following culture. Leukemia 17:160–170.

Seo MJ, Suh SY, Bae YC, Jung JS. 2005. Differentiation of human adipose stromal cells into hepatic lineage in vitro and in vivo. Biochem Biophys Res Commun 328:258–264.

Shimaoka S, Nakamura T, Ichihara A. 1987. Stimulation of growth of primary cultured adult rat hepatocytes without growth factors by coculture with nonparenchymal liver cells. Exp Cell Res 172:228–242.

Snykers S, Vanhaecke T, Papeleu P, Luttun A, Jiang Y, Heyden YV, Verfaillie C, Rogiers V. 2006. Sequential exposure to cytokines reflecting embryogenesis: The key for in vitro differentiation of adult bone marrow stem cells into functional hepatocyte-like cells. Toxicol Sci 94: 330–341.

Theise ND, Badve S, Saxena R, Henegariu O, Sell S, Crawford JM, Krause DS. 2000. Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology 31:235–240.

Thorgeirsson SS, Grisham JW. 2006. Hematopoietic cells as hepatocyte stem cells: A critical review of the evidence. Hepatology 43:2–8.

Tosh D, Slack JM. 2002. How cells change their phenotype. Nat Rev Mol Cell Biol 3:187–194.

Turhan AG. 2003. Plasticity of adult stem cells. Transfus Clin Biol 10:103–108.

Wagers AJ, Weissman IL. 2004. Plasticity of adult stem cells. Cell 116:639–648.

Wagers AJ, Sherwood RI, Christensen JL, Weissman IL. 2002. Little evidence for developmental plasticity of adult hematopoietic stem cells. Science 297:2256–2259.

Wang PP, Wang JH, Yan ZP, Hu MY, Lau GK, Fan ST, Luk JM. 2004. Expression of hepatocyte-like phenotypes in bone marrow stromal cells after HGF induction. Biochem Biophys Res Commun 320:712–716.

Wang X, Hisha H, Taketani S, Adachi Y, Li Q, Cui W, Cui Y, Wang J, Song C, Mizokami T, Okazaki S, Li Q, Fan T, Fan H, Lian Z, Gershwin ME, Ikehara S. 2006. Characterization of mesenchymal stem cells isolated from mouse fetal bone marrow. Stem Cells 24:482–493.

Watt FM, Hogan BL. 2000. Out of Eden: Stem cells and their niches. Science 287:1427–1430.

Wislet-Gendebien S, Hans G, Leprince P, Rigo JM, Moonen G, Rogister B. 2005. Plasticity of cultured mesenchymal stem cells: Switch from nestin-positive to excitable neuron-like phenotype. Stem Cells 23:392–402.

Zhang S, Jia Z, Ge J, Gong L, Ma Y, Li T, Guo J, Chen P, Hu Q, Zhang P, Liu Y, Li Z, Ma K, Li L, Zhou C. 2005. Purified human bone marrow multipotent mesenchymal stem cells regenerate infarcted myocardium in experimental rats. Cell Transplant 14:787–798.