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Differentiation of human adipose stromal cells into hepatic lineage in vitro and in vivo

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Abstract

Embryonic stem cells (ES cells), bone marrow-derived mesenchymal stem cells, umbilical cord blood-derived mesenchymal stem cells, and hepatic stem cells in liver have been known as a useful source that can induce to differentiate into hepatocytes. In this study, we examined whether human adipose tissue-derived stromal cells (hADSC) can differentiate into hepatic lineage in vitro. hADSC, that were induced to differentiate into hepatocyte-like cells by the treatment of HGF and OSM, had morphology similar to hepatocytes. Addition of DMSO enhanced differentiation into hepatocytes. RT-PCR and immunocytochemical analysis showed that hADSC express albumin and α-fetoprotein during differentiation. Differentiated hADSC showed LDL uptake and production of urea. Additionally, transplanted hADSC to CCl_4-injured SCID mouse model were able to be differentiated into hepatocytes and they expressed albumin in vivo. Mesenchymal stem cells isolated from human adipose tissue are immunocompatible and are easily isolated. Therefore, hADSC may become an alternative source to hepatocyte regeneration or liver cell transplantation.

Keywords: Human adipose stromal cell; Hepatic lineage differentiation; Hepatocyte growth factor; OncostatinM

Liver diseases are caused by infectious agents [1], autoimmune attack [2], malignant transformation [3], inborn genetic deficiencies [4], or secondary defects [5]. Most liver diseases lead to "hepatocyte dysfunction" with the possibility of eventual organ failure. The replacement of diseased hepatocytes by stem cells or the stimulation of endogenous (mobilized, e.g., from the bone marrow) as well as exogenous (by stem cell infusion from an external source) regeneration by stem cells are the main aims of liver-directed cell therapy. Recent data showed that liver stem cells might be derived from bone marrow. Petersen et al. [6] first identified this phenomenon in rat model of liver injury. Later many researchers have reported similar in vivo and in vitro findings [7–11]. Therefore, bone marrow cells have been hypothesized as the important recruitment source in liver regeneration besides hepatocytes and endogenous liver stem cells [12].

Human bone marrow has a multipotent population of cells capable of differentiating into a number of mesodermal lineages; adipocytes, osteoblasts, and other mesodermal pathways [13,14]. Bone marrow stromal cells (BMSC) also support the proliferation and differentiation of hematopoietic stem cells [15]. BMSC may differentiate into neural tissue, which is derived from embryonic ectoderm [16,17]. Therefore, bone marrow-derived mesenchymal stem cells have been considered as an important source of stem cells for cell therapy. Recently, BMSC have been showed to be differentiated into hepatocyte-like cells [18].

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Mesenchymal stem cells (MSC) exist in tissue sites outside the bone marrow microenvironment. Adipose tissue, like bone marrow, is derived from the mesenchyme and contains a supportive stroma that is easily isolated [19]. These cells have limited self-renewing ability and can be induced to various mesenchymal tissues, including chondrocytes, adipocytes, osteoblasts, myocytes, and endothelial cells [20–23]. We have shown that human adipose tissue-derived stromal cells (hADSC) have similar characteristics with hBMSC in vitro and in vivo [24–26]. However, hADSC have not been demonstrated to be capable of differentiation towards hepatic lineage. In this study, we examined whether hADSC can be induced to undergo morphologic and phenotypic changes consistent with hepatogenic differentiation.

Materials and methods

Isolation and culture of hADSC and HepG2 cell line

hADSC culture. After informed consent, leftover adipose tissues were obtained from four different donors (19 years old; male, 28 years old; female, 36 years old; female, and 55 years old; male) undergoing elective abdominoplasty. To isolate hADSC, adipose tissues were washed with equal volumes of phosphate-buffered saline (PBS), and tissues were digested at 37 °C for 30 min with 0.075% collagenase type I (Sigma). Enzyme activity was neutralized with α-modified Eagle's medium (α-MEM), containing 10% fetal bovine serum (FBS, Bio Whittaker), and centrifuged at 1200*g* for 10 min to obtain a pellet. The pellet was filtered through a 100-μm nylon mesh to remove cellular debris and incubated overnight at 37 °C/5% CO_2 in control medium (α-MEM, 10% FBS, and 1% antibiotic/antimycotic solution). Following incubation, the plates were washed with PBS to remove residual non-adherent red blood cells. The resulting cell population was maintained at 37 °C/5% CO_2 in control medium.

Expansion. hADSC expansion medium consisted of the following: 60% DMEM-LG (Gibco-BRL, Grand Island, NY, USA), 40% MCDB-201 (Sigma) with 1× insulin transferrin-selenium (ITS), 10^{-9} M dexamethasone (Sigma), 10^{-4} M ascorbic acid 2-phosphate (Sigma), 10 ng/ml rhEGF (Daewoong Pharmaceuticals, Korea), 100 U penicillin, 1000 U streptomycin (Gibco-BRL, Grand Island, NY, USA), on fibronectin (FN, Sigma) with 5% FBS (Bio Whittaker). Cells were subcultured at a density of 1000 cells/cm^2.

HepG2 cell culture. Cells were cultured with DMEM (Gibco-BRL, Grand Island, NY, USA) containing 10% FBS (Bio Whittaker) and 1% antibiotic/antimycotic solution. And cells were maintained at 37 °C in an atmosphere of 5% CO2 and 95% room air.

Hepatogenic differentiation

The hADSC between third and fifth passages were plated at 2.5–3×10^4 cells/cm^2 on FN-coated dish in the expansion medium. After 24 h, media were removed, and cells were washed twice with Hanks' balanced salt solution (HBSS, Sigma). Hepatogenic differentiation was induced by culturing hADSC for 3–4 weeks in expansion medium without serum containing 0.1% dimethyl sulfoxide (DMSO, Sigma), 10 ng/ml rhHGF and rhOSM (R&D Systems, Minneapolis, MN, USA).

Immunocytochemistry and immunohistochemistry

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde at 20 °C for 10 min and permeabilized with 0.3% Triton X in PBS for 10 min. Blocking and dilution solution consisted of PBS, 0.1% Triton X, and 10% host serum from species similar to the species in which the primary antibody was raised. Cells were blocked for 1 h, incubated sequentially overnight at 4 °C with antibody against human albumin (ab2406; Abcam) (1:1000), and followed by the treatment of phycoerythrin-coupled donkey anti-rabbit IgG secondary antibody for 40 min. Between each step, cells were washed with PBS.

Immunohistochemistry. Sections for immunohistochemistry of frozen tissues were fixed in 4% paraformaldehyde for 30 min. The sections were air-dried, fixed in 4% paraformaldehyde at −20 °C for 20 min, and incubated in PBS with 2% bovine serum albumin (BSA, Sigma) for 1 h at room temperature to block non-specific binding. Afterwards, sections were incubated overnight in 2% BSA at 4 °C in anti-human albumin antibody (ab2406; Abcam) (1:1000). The sections were washed three times in PBS and were incubated in FITC-conjugated F(ab′)2 fragment donkey anti-rabbit IgG (1:100, Jackson Immuno-Research Laboratories) in BSA for 3 h at room temperature. After being rinsed, they were mounted on glass slides with mounting medium (GVA mount). Specimens were examined on a Zeiss LSM510 confocal imaging system.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from hADSC and HepG2 cells using Tri Reagent (Sigma), reverse transcribed into first strand cDNA using oligo(dT) primer, and amplified by 35 cycles (94 °C, 1 min; 55 °C, 1 min; and 72 °C, 1 min) of PCR using 10 pmole of specific primers. On completion of the PCR, products were examined on 2% agarose gel. GAPDH primers were used as an internal standard, and amplification of GAPDH products was performed at 30 cycles. To compare albumin expression levels between HepG2 cells and differentiated hADSC, albumin products amplified at 30 and 35 cycles were quantitated by an image analyzer (Uvitec, UK).

Primer	Sequence (5′ → 3′)
GAPDH	S: TCCATGACAACTTTGGTATCG
	A: TGTAGCCAAATTCGTTGTCA
α-Fetoprotein	S: CTCGTTGCTTACACAAAGAAAG
	A: ATGGAAAATGAACTTGTCATCA
Albumin	S: TTTGCTCAGTATCTTCAGCAGT
	A: AGTAAGGATGTCTTCTGGCAAT

Uptake of low-density-lipoprotein

The Dil-Ac-LDL staining kit was purchased from Biomedical Technologies (Stoughton, MA, USA) and assay was performed as per manufacturer's instructions.

Determination of urea production

Cell culture media at the indicated times after induction of hepatogenic differentiation were collected, and analyzed for urea and citrulline by methods described by Buga et al. [39]. Briefly, samples for determination of citrulline (170 μl) were added to 30 μl of 50 mM [triethanolamine–HCl (TEA–HCl, pH 7.4) containing 1 U urease and incubated at 25 °C for 15 min to remove the urea. Samples for determination of urea plus citrulline (170 μl) were added to 30 μl of 50 mM TEA–HCl, pH 7.4. To initiate color development, 1 ml of a chromogenic reagent consisting of 1 part 3% wt/vol 2,3-butanedione monoxime in ethanol plus 29 parts of acid mixture consisting of 1 part H_2SO_4, 3 parts H_3PO_4, and 7 parts H_2O was added to all samples including standards. Samples were incubated at 100 °C for 30 min, and optical

densities were measured at 492 nm using spectrophotometer. Standard curves for urea and citrulline (10–500 μM) were run for each assay. Values for citrulline, derived from samples containing urease, were calculated using the citrulline standard curve by standard procedures. Values for urea were obtained by subtracting the citrulline values from values for urea plus citrulline (obtained in the absence of added urease) and calculated using the urea standard curve.

Transplantation of hADSC into NOD/SCID mice

Eight-week-old immunodeficient mice (NOD/SCID, Charles River, MA) were injured by injection of 0.5 ml/kg CCl_4 into intraperitoneal route, and transplanted after 48 h with 1×10^6 hADSC labeled with CM-Dil (Molecular probes) by intravenous injection into a lateral tail vein. As a control, 1×10^6 hADSC labeled with CM-Dil were also injected into normal NOD/SCID mice. At 3 and 10 days after transplantation, mice were perfused with PBS containing 4% paraformaldehyde and various tissues were obtained.

Statistical analysis

Differences in engraftment percentages were calculated using the Kruskal–Wallis test. A value of $p < 0.05$ was considered statistically significant.

Result

Morphologic changes in cultured hADSC treated with HGF, OSM or DMSO

To specify antigenic properties of hADSC, we determined surface protein expression by flow cytometry. hADSC expressed CD29, CD44, CD105, and CD90, but did not express CD34, CD45, CD14, and HLA-DR, as described in our previous study [26]. To determine optimal conditions for hADSC differentiation into hepatocyte-like cells, we tested the effect of different cytokines on morphological changes of hADSC in the fibronectin-coated dishes. Differentiation was induced at 90% confluency of cells after plating. Cell morphology was not changed greatly by the treatment of HGF alone in serum free media for 28 days (Fig. 1A). When cells were differentiated for 28 days in the media containing HGF and OSM, the morphology of hADSC was changed to round shape without cell detachment (Figs. 1B and C). We next determined the effect of DMSO on differentiation of hADSC into hepatocyte-like cells. The addition of 0.1% DMSO into differentiation media at the first day of differentiation induction increased cell detachment from culture dishes (data not shown). However, the addition of 0.1% DMSO at the 10th day after differentiation induction enhanced changes in cell morphology without cell detachment. hADSC treated 0.1% DMSO with OSM and HGF showed a larger and rounder shape than cells treated with OSM and HGF alone (Fig. 1D). Therefore, the following differentiation protocol was used for further experiments. Cells were plated on 5 μg/ml FN-coated dish in FBS-free standard culture media containing

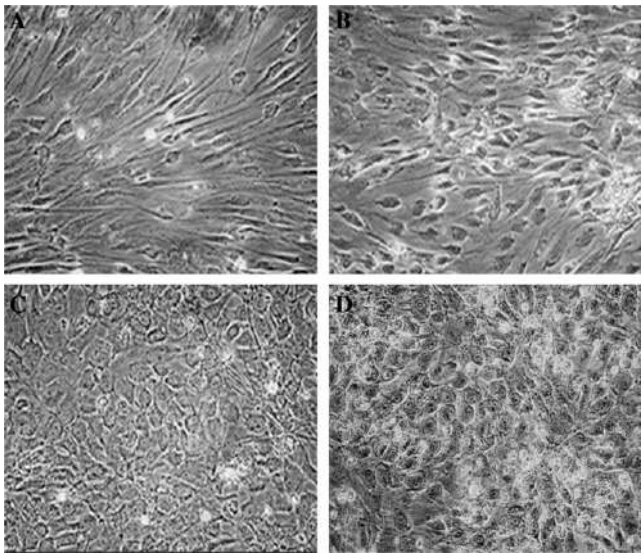

Fig. 1. Morphology of hADSC cultured in serum-free medium with a single or mixed supplements of HGF, OSM or DMSO for 28 days. Magnification, 200×. (A) hADSC cultured in serum free medium (SFM), (B) SFM + HGF (20 ng/ml), (C) SFM + HGF (10 ng/ml) + OSM (10 ng/ml), and (D) SFM + HGF (10 ng/ml) + OSM (10 ng/ml) + 0.1% DMSO.

10 ng/ml HGF and OSM. After 10 days, 0.1% DMSO was added to the media. To obtain proper morphological changes of cells into hepatic lineage, cells were maintained in the differentiation media for 18 days after the addition of DMSO. The protocol was applied to hADSC from four different donors and the identical results were obtained. The following experiments were done in four different samples and the representative data were presented.

Functional characterization of differentiated hADSC into hepatocyte-like cells

To determine whether the cells have functional properties of hepatocytes, total RNA was isolated at 7, 14, 21, and 28 days after differentiation of the hADSC into hepatic lineage and the expression of several hepatic proteins was examined by RT-PCR. Undifferentiated hADSC did not express albumin, but they expressed CK-18 and CK-19. CK-18 and CK-19 expression was not significantly changed by induction of differentiation (data not shown). During differentiation expression of AFP was increased and expression of albumin was induced in hADSC-derived hepatocytes cultured with HGF, OSM, and DMSO at the 7th day after differentiation (Fig. 2). To compare the level of albumin expression in differentiated hADSC with that in HepG2 cells, we quantitated the albumin products relative to GAPDH products by RT-PCR and image analysis. The relative amount of albumin in differentiated hADSC to HepG2 cells was 22.5% and 23.7% at 30 and 35 PCR cycles, respectively (Fig. 3).

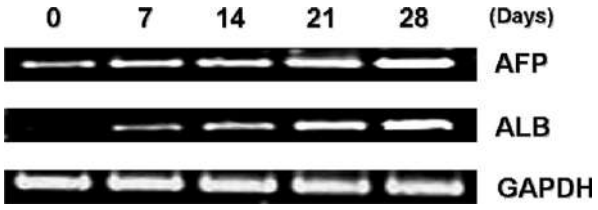

Fig. 2. Determination of hepatic lineage genes by RT-PCR. hADSC were differentiated into hepatic lineage. We isolated total RNA from hepatocyte-like cells at the indicated days after differentiation, and performed RT-PCR by using the specific primers (AFP; ALB, albumin; and GAPDH).

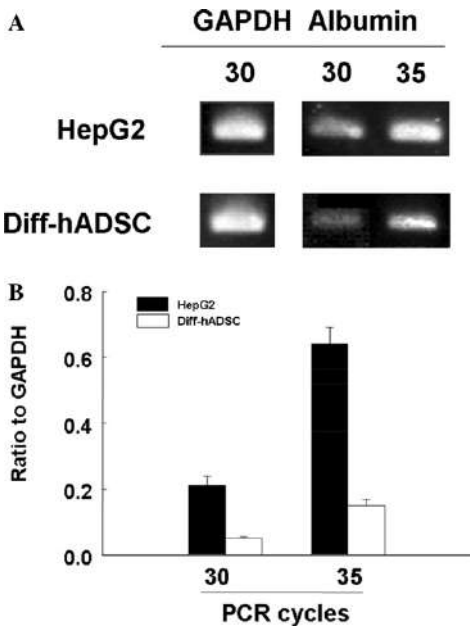

Fig. 3. Comparison of albumin expression in HepG2 cells and differentiated hADSC. (A) Representative photograph of albumin and GAPDH PCR products amplified from HepG2 and differentiated hADSC (Diff-hADSC). (B) Quantitation of PCR products. PCR products of albumin (30 and 35 cycles) and GAPDH (30 cycles) were quantitated by image analyzer. Data represent the relative value to GAPDH signal and means ± SEM ($n = 4$).

To further confirm albumin expression in hADSC-derived hepatocytes, we examined differentiated hADSC by immunocytochemistry. On day 10 after differentiation, 0.1% DMSO was added into the differentiation media. On day 28, cells were fixed and immunostained with anti-albumin antibody. hADSC cultured in media containing 10 ng/ml HGF and OSM showed positive immunostaining to albumin antibody (Figs. 4B and D), whereas control hADSC did not (data not shown).

Uptake of low-density lipoprotein and urea production

Low-density-lipoprotein (LDL) is a lipoprotein that carries cholesterol around the body, for use by various cells and most LDL is metabolized in liver. Therefore,

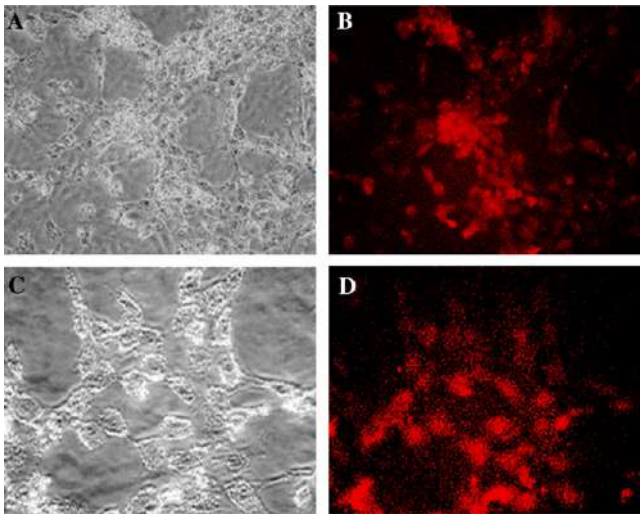

Fig. 4. Immunocytochemical staining of hADSC differentiated for 28 days with anti-albumin antibody. (A) Differentiated hepatocyte-like cells (phase; 200×), (B) human albumin (200×), (C) differentiated hepatocyte-like cells (phase; 400×), and (D) human albumin (400×).

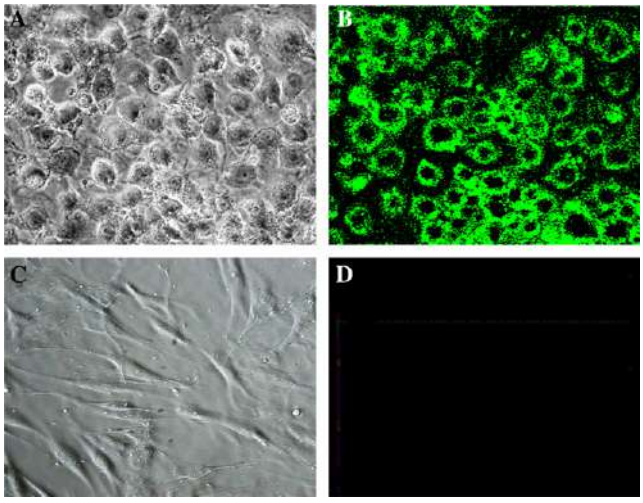

Fig. 5. LDL uptake by hADSC-derived hepatocytes. hADSC differentiated on FN-coated dish for 28 days were incubated with Dil-acil-LDL. (A,C) Phase-contrast photograph; (B,D) (A,B: C,D: undifferentiated hADSC).

we assessed whether hADSC-derived hepatocytes can take up LDL by incubating differentiated hADSC with LDL-Dil-acil. After 28 days of differentiation, most of the hepatocyte-like cells showed the ability to uptake LDL (Figs. 5A and B), whereas undifferentiated hADSC did not (Figs. 5C and D).

We measured whether differentiated hepatocyte-like cells produce and secrete urea at various time points throughout differentiation. Undifferentiated hADSC did not produce urea. After induction of differentiation according to our protocol, urea production by hADSC increased in a time-dependent manner ($n = 4$) (Fig. 6).

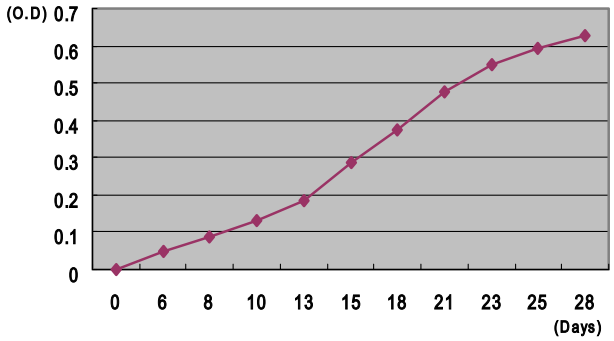

Fig. 6. Urea production by hADSC cultured with HGF, OSM, and DMSO. hADSC were differentiated for 28 days according to the protocol. Urea production was quantitated at 492 nm with a spectrophotometer in four different samples.

Transplantation of hADSC into NOD/SCID mice

To evaluate the engraftment of hADSC into liver, hADSC differentiated for 10 days in hepatogenic medium and undifferentiated hADSC were injected into the tail vein of the control and CCl_4-injected NOD/SCID mice ($n = 10$/group). At 3 and 10 days after transplantation, the distribution of transplanted hADSC in the liver was examined by a fluorescent microscope. The hepatogenic differentiation of transplanted hADSC in the liver of NOD/SCID mice was determined by immunohistochemistry using human albumin antibody. Hematoxylin–eosin staining showed that at least 80% of liver was destroyed at second day after single injection of CCl_4 (Fig. 7A). At 3 days after transplantation, cells were incorporated into the liver and CCl_4-treated NOD/SCID mice, and the number of incorporated cells was significantly higher in the undifferentiated hADSC-transplanted group than in the differentiated hADSC-transplanted group (0.32% vs 0.17%, $p < 0.05$, $n = 5$/group). However, human albumin(+) cells were not detected at 3 days after transplantation. Incorporated hADSC were not observed in the liver of control NOD/SCID mice. At 10 days after transplantation, engrafted CM-Dil-labeled hADSC showed similar morphology of hepatocytes in the liver of CCl_4-treated NOD/SCID mice (Fig. 7B) and expressed human albumin (Figs. 7C and D). The proportion of human albumin(+) hepatocytes to endogenous hepatocytes at 10 days after transplantation was similar between undifferentiated hADSC- and differentiated hADSC-transplanted group (0.12% vs 0.13%, $n = 5$/group).

Discussion

MSC could differentiate into cells of all mesodermal origin, including adipocytes, osteocytes, chondrocytes, myocytes, and endothelial cells [20–23]. Besides these, MSC are also capable of "transdifferentiation" into ectodermal cells, such as neural cells [27,28]. These findings suggest that MSC belong to multipotent adult stem cells. Schwartz et al. [11] isolated a non-hematopoietic stem cell subset ($CD45^-GlyA^-$ in humans or $CD45^-Ter119^-$ in mice) from bone marrow, termed multipotent adult progenitor cells (MAPC). Under

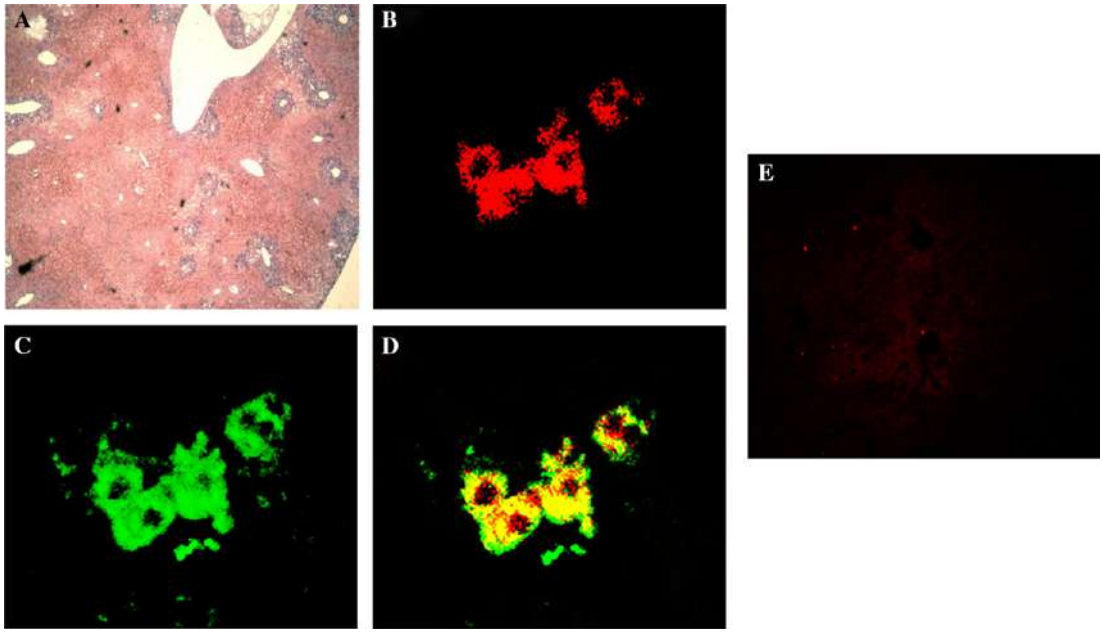

Fig. 7. The engraftment of hADSC into the liver of NOD/SCID mice. (A) CCl_4-induced mouse liver injury model (HE staining, 100×). (B) CM-Dil labeled hADSC were detected in mouse liver at 10 days after transplantation. (C) Immunofluorescence study showed that the engrafted hADSC expressed human albumin. (D) Merged image with (B,C). (E) No detection of transplanted differentiated hADSC in the liver of normal NOD/SCID mice.

appropriate conditions, MAPC were induced into cells with morphological, phenotypic, and functional characteristics of hepatocytes in vitro [11].

Recently, BMSC have been shown to differentiate into hepatocyte-like cells [29]. In this study, we first showed that hADSC can be differentiated into functional hepatocyte-like cells by the treatment of cytokine mixtures. The cytokines to be used in this experiment have been reported to be involved in development and differentiation of hepatocytes. HGF, originally identified and cloned as a potent mitogen for hepatocytes, shows mitogenic, motogenic, and morphogenic activities for a wide variety of cells that express the HGF receptor c-Met, a transmembrane protein possessing an intracellular tyrosine kinase domain. Moreover, HGF plays an essential role in the development and regeneration of the liver [30]. The foregut endoderm is induced to the hepatocyte lineage by acidic FGF (aFGF) and bFGF, both produced by the adjacent cardiac mesoderm, which are required to induce a hepatic fate and not the default pancreatic fate [11]. It has been reported that treatment of the cultures with OSM, a member of the interleukin-6 cytokine family, increased the cell size of hepatocytes and enhanced cell differentiation and formation of bile canaliculi [31]. Although OSM alone had very weak effects on hepatocyte functions, albumin secretion and cytochrome P450IA1/2 capacity were greatly enhanced when combined with nicotinamide or DMSO [32]. DMSO has been known to maintain the functions of adult hepatocytes in vitro [33]. It has been reported that nicotinamide and DMSO remarkably enhanced the emergence of small hepatocytes, and that OSM also synergistically enhanced the selective growth of small hepatocytes and inhibited the growth of blood cell populations [32].

In this study, we established the method for differentiation of hADSC into hepatocytes in vitro. When hADSC were cultured in the media containing HGF and OSM, and 0.1% DMSO was added at the 10th day after induction of differentiation, cells showed morphology of mature hepatocytes and expressed albumin. The differentiated cells showed LDL uptake and urea production, indicating that they have developed hepatocyte function. In our study, morphological changes under phase-contrast microscopy, immunofluorescence analysis for albumin and LDL uptake indicated that most cells differentiated into hepatocyte-like cells, indicating that hepatocyte-like cells differentiated by our protocol are derived from hADSC itself, not from hepatic progenitors present in our hADSC preparation.

The differentiation potential of hADSC into hepatocytes was also supported by in vivo transplantation experiment. Some of hADSC, that were transplanted into CCl_4-induced liver injury model of NOD/SCID, mice, differentiated into hepatocytes and expressed human albumin. It has been reported that bone marrow cells fuse with hepatocytes [34–36]. However, recent reports have indicated that bone marrow cells and hematopoietic stem cells can convert into hepatocytes without fusion [37,38]. The data in this study showed that the percentage of albumin(+) cells among cells engrafted into the mice liver was significantly higher after transplantation of differentiated hADSC than after transplantation of undifferentiated cells, supporting transdifferentiation of hADSC. However, we cannot exclude the involvement of cell fusion in our experimental model, because we did not analyze the karyotype of differentiated cells using in situ hybridization. Future studies will be necessary to resolve these issues.

The proportion of albumin-expressing cells in differentiated hADSC-transplanted group at 10 days after transplantation was similar to the data of undifferentiated cell-transplanted group, although the number of engrafted cells at 3 days after transplantation was lower in differentiated hADSC-transplanted group. These may result from lower survival and higher differentiation of differentiated cells after transplantation. These findings have suggested that manipulation of stem cells in vitro may not enhance therapeutic effects of cell transplantation in vivo. Therefore, the protocol of optimal in vitro manipulation for in vivo transplantation should be carefully determined.

Taken together, we showed that hADSC can be differentiated into functional hepatocytes in vitro, although the mechanisms are not clear. The most important problem is immunorejection in liver cell transplantation. However, autologous stromal cells isolated from human adipose tissue are immunocompatible and are easily isolated. Therefore, we expect that hADSC may become very useful source to hepatocyte regeneration or liver cell transplantation.

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