Original Article

In Vitro Differentiation of Human Bone Marrow Mesenchymal Stem Cells into Hepatocyte-like Cells

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Abstract

Background: Orthotropic liver transplantation (OLT) is the final procedure of both end stage and metabolic liver diseases. Hepatocyte transplantation is an alternative for OLT, but the sources of hepatocytes are limited. Bone marrow mesenchymal stem cells (BM-MSCs) can differentiate into hepatocyte-like cells and are a potential alternative source for hepatocytes. We aimed to investigate the differentiation potential of BM-MSCs into hepatocyte-like cells.

Methods: Human BM-MSCs from a healthy donor were cultured and differentiated into hepatocyte-like cells. We investigated the expression of hepatocyte-specific markers in MSC-derived hepatocyte-like cells (MSC-HLCs) and evaluated their functionality using metabolic assays.

Results: MSC-HLCs expressed hepatocyte-specific markers at both mRNA and protein levels. In addition, the cells had the ability to uptake low density lipoprotein (LDL), clear ammonia, secrete albumin, and store glycogen. MSC-HLCs were transplanted into a familial hypercholesteromia patient.

Conclusion: Human MSCs can be differentiated into partially functional hepatocyte-like cells. Thus, they could be a potential source for cell therapy in liver disorders.

Key words: bone marrow stem cells, differentiation, hepatocyte

Introduction

Annually, many people worldwide die from liver disease. Orthotropic liver transplantation (OLT) is an effective treatment for end stage cirrhosis and liver-based inherited metabolic disorders. However, OLT has several limitations such as a long waiting list, high cost and several major complications.[1] Hepatocyte transplantation is an alternative for OLT in the treatment of liver-based inherited metabolic disorders.[2]

Cell transplantation is less invasive than whole organ transplantation. It can be performed repeatedly, has been used to bridge patients to whole organ transplantation,[3,4] can decrease mortality in acute liver failure,[5,6] and can be used to treat metabolic liver diseases.[7–9] Cell therapy can be defined as the use of living cells to restore, maintain or enhance the function of tissues and organs. The use of isolated, viable cells has emerged as an experimental therapeutic tool in the past decade due to progress in cell biology, and particularly in techniques for the isolation and culture of cells derived from several organs and tissues. However, experimental cell therapy has a longer tradition in hepatology, since it has been known for more than 30 years that isolated hepatocytes infused into the portal vein engraft into the liver cords and express normal cell function. Such a therapeutic strategy has been suggested as an alternative to OLT, which requires major surgery and is limited by the availability of donors.[7,10]

Hepatocyte transplantation could significantly improve metabolic abnormalities present in the animal models of Crigler Nijjar syndrome type 1, Wilson's disease,[11] and hereditary tyrosinaemia type I.[12] Hepatocyte transplantation has lowered serum cholesterol levels by 30% to 60% in rabbit models of homozygous familial hypercholesterolemia.[13] Furthermore, promising results have been obtained from hepatocyte transplantation in human diseases like type 1 Crigler Najjar syndrome,[14] glycogen storage disease type 1a,[15] inherited factor VII deficiency,[16] and argininosuccinate lyase deficiency.[9] Despite positive reports, the application of hepatocyte transplantation in humans is limited to less than 100 cases.[17] The reason for this discrepancy is the success of OLT and limited availability of human hepatocytes. Thus, there is a need to find an easily available cell type equivalent to primary hepatocytes.

Bone marrow (BM) is a reservoir of hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). MSCs have attracted attention as therapeutic tools since they can be obtained with relative ease and expanded in culture. While MSCs have been shown to be capable of mesodermal and neuroectodermal differentiation,[16] they also have endodermal differentiation potential; and their differentiation into hepatocyte-like cells has been reported.[18]

Here, we describe *in vitro* differentiation of allogenic BM-MSCs into functional hepatocyte-like cells. The MSC-derived hepatocyte-like cells (MSC-HLCs) were able to uptake low density lipoprotein (LDL), clear ammonia, make albumin, and store glycogen.

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Materials and Methods

The bone marrow donor

A 25-year-old healthy volunteer male donor was chosen. His lipid profile was normal. His liver enzymes were normal, and serologic tests were negative for hepatitis B and C, human immunodeficiency virus and cytomegalovirus. After signing a written informed consent, 250 mL of BM was obtained from the donor's right and left posterior iliac crest after local anesthesia.

Culture and expansion of MSCs

MSCs were cultured and harvested by the method of Wexler et al.[19] Totally, 250 mL of BM was aspirated with syringes preloaded with 500 IU of preservative-free heparin. Bone marrow mononuclear cells (BM-MNCs) were separated by density gradient centrifugation (Lymphodex, innoTRAin) as previously described with some modifications. Briefly, 35 mL diluted BM was layered on 15 mL Ficoll in a 50 mL tube, centrifuged for 25 min at 200×g. Cells in the interphase layer were diluted and cultured in basal medium that consisted of alpha-modified minimum essential medium (α-MEM, Gibco) supplemented with 10% FBS (HyClone), 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco cell culture, Invitrogen Corporation), and L-glutamine (2 mM, Gibco) at 5% CO_2, 37°C, 95% air humidity and seeded at a density of 1×10^6 cells/cm^2 in 175 cm^2 tissue culture flasks (Nunc). Non-adherent cells were removed after four days, and adherent cells were propagated by feeding twice weekly with supplemented α-MEM. Adherent cells were passaged after additional 12 to 14 days with the use of 0.25% trypsin-EDTA (1 – 3 min, 37°C, Gibco). Cells derived from primary cultures of BM-MNCs were designated as passage 0 and each cycle of reseeded MSCs after trypsinization was considered to be one additional passage. Further culture was performed at defined cell seeding densities with α-MEM supplemented with 10% FBS (4 – 5 passages).[20,21]

Flow cytometry analysis

Surface markers expression of cultured BM-derived MSCs at initial or later passages were analyzed with a flowcytometer (FACSCalibur, Becton Dickinson). Cells were adjusted to 1×10^5 to 2×10^5 cells/mL and blocked with Fc receptor blocking reagent (Miltenyi Biotech, Germany) according to the manufacturer's instructions. Cells were then stained for 30 min at 4°C with fluorochrome-labeled monoclonal antibodies against CD44, CD146, CD11b, CD80, CD117, CD34, CD45, CD73 (all purchased from BD Pharmingen), CD105 (R&D Systems), and CD90 (Dako). Controls were appropriately diluted isotype-matched antibodies (Ebioscience). Data from 10000 events were stored. List mode files were analyzed with computer software (WinMDI, ver. 2.9).[22]

Differentiation potential of MSCs

Osteogenic differentiation was induced by incubation with osteogenic complete medium (DMEM-LG, Gibco) that consisted of ascorbic acid, dexamethasone, 6-glycerol phosphate (all purchased from Sigma), and calcium deposits were visualized by alizarin red.[23]

Adipogenic differentiation was induced by incubation with adipogenic complete medium (DMEM-LG, Gibco) that consisted of ascorbic acid, dexamethasone, indomethacine (all purchased from Sigma), and visualized with oil red O in distilled water for 10 min [3:2 v/v oil red O stock solution (0.5% wt/vol in 99% isopropanol): distilled water].[23]

Hepatocytic differentiation

MSCs were induced to differentiate into hepatocyte-like cells as previously described using hepatocyte growth factor (HGF, R&D Systems) and fibroblast growth factor (FGF-4, R&D Systems).[24] Passage 5 cells were cultured in the presence of liver-specific growth factors were added sequentially (days 0 – 3: basal medium +10 ng/mL FGF-4; days 3 – 6: basal medium + 20 ng/mL HGF; from day 6 on: basal medium 20+ ng/mL HGF, 1×ITS, and 20 μg/L dex). Differentiation media was changed every three days.

Immunofluorescence staining

Cells at days 0, 7, 14, and 40 were rinsed and fixed with 4% paraformaldehyde (Sigma) in phosphate-buffered solution (PBS) at 4°C for 20 min. The cells were permeabilized with 0.2% Triton X100 in PBS, when required. The fixed cells were blocked for 30 min at 37°C with 10% goat serum/PBS-tween-20. Cells were incubated overnight at 4°C in a humidity chamber with the respective primary antibodies: mouse monoclonal anti-albumin (ALB, 1:200, R&D Systems), mouse monoclonal anti-cytokeratin 18 (CK-18, 1:200, Chemicon), AAT (1:500, Abcam), and monoclonal mouse anti-HepPar1 (1:50, DAKO). At the end of the incubation period and washing, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody, goat anti-mouse (1:100; Chemicon) for 30 min at room temperature.

After rinsing, the nuclei were counterstained with propidium iodide (1 mg/mL, Sigma) and cells were then analyzed with a fluorescent microscope (Olympus, BX51, Japan).

Reverse transcription polymerase chain reaction (RT-PCR) analysis

RT-PCR was performed to assess expression of a set of hepatocytic genes. Total RNA was isolated from undifferentiated cells and at varying stages of induction using the Nucleospin RNA II Kit (Macherey-Nagel, Duren, Germany). Before RT, a sample of the isolated RNA was treated with RNase-free DNaseI (EN0521; Fermentas). Standard RT reactions were performed with 1 μg total RNA using random hexamers as primer and a RevertAid First Strand cDNA Synthesis Kit (K1622; Fermentas) according to the manufacturer's instructions. For every reaction set, one RNA sample was prepared without RevertAid M-MuLV reverse transcriptase to provide a negative control in the subsequent PCR. Reaction mixtures, amplification conditions, and sequences of primers were reported previously.[25]

Western blot

Ten micrograms of protein extracted from the cells at different stages was separated by 10% SDS-PAGE electrophoresis using a Mini-PROTEAN 3 electrophoresis cell (Bio-Rad). Proteins were transferred to a PVDF membrane (Bio-Rad) by semidry blotting (Bio-Rad) using Dunn carbonate transfer buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3, and 20% methanol) and membranes were blocked for 1.5 hr using a Western blocker solution (Sigma-Aldrich, W0138) before being incubated with an antibody against ALB (1:300, Sigma). After washing, the membranes were incubated with the peroxidase-conjugated secondary antibody goat anti-mouse horseradish peroxidase (A9044, 1:80, Sigma-Aldrich) and detected with an ECL detection system (Amersham Bioscience).

Periodic acid schiff (PAS) staining for glycogen

Intracellular glycogen was analyzed at day 40 by periodic-acid-schiff staining as previously described.[26]

Uptake of low-density lipoprotein (LDL)

LDL is a lipoprotein that transports cholesterol throughout the body for use by various cells. Most LDL is taken up and metabolized in the liver. Therefore, we assessed whether MSC-HLCs could take up LDL by incubating differentiated MSCs with 1,10-dioctadecyl-10-3,3,30,30-tetramethyl-indo-carbocyanine perchlorate conjugated to acetylated LDL (DiI-Ac-LDL; Biomedical Technologies, Inc.). Cells were harvested, washed with cold PBS and then stained according to the manufacturer's protocol.[26]

Albumin secretion and urea production

Detection of albumin secretion and urea production was performed to confirm hepatic metabolic functions of the differentiated BM-MSCs at different days based on a previously described procedure.[26] Briefly, cells were incubated with medium containing 5 mM NH_4Cl (Sigma) 24 hr before analysis in 5% CO_2 at 37°C. Following this incubation, supernatant was collected and urea concentrations were measured by using a colorimetric assay kit (Pars Azmun, Iran). Albumin production was evaluated using a quantitative enzyme-linked immunoassay kit (Albumin ELISA; Orgentech Diagnostica, Germany) according to the manufacturer's recommendations.

Results

Characterization of BM MSCs

The MSCs grew as a monolayer of large, flat cells and made colony forming unit-fibroblasts (CFU-F, Figure 1A). As the cells approached confluency, they assumed a more spindle-shape, fibroblastic morphology (Figure 2A). The osteogenic and adipogenic differentiation potential of the cells were analyzed (Figures 1B and 1C). Flow cytometric analysis at passages 4 – 6 demonstrated that the cells were negative for CD34, CD86, CD117, CD11b, CD45, CD80 (Figure 1D), cell surface markers associated with lympho-hematopoietic cells, and were positive for CD105 (79%), CD73 (71%), CD44 (87%), CD146 (83%), and CD90 (71%), which was

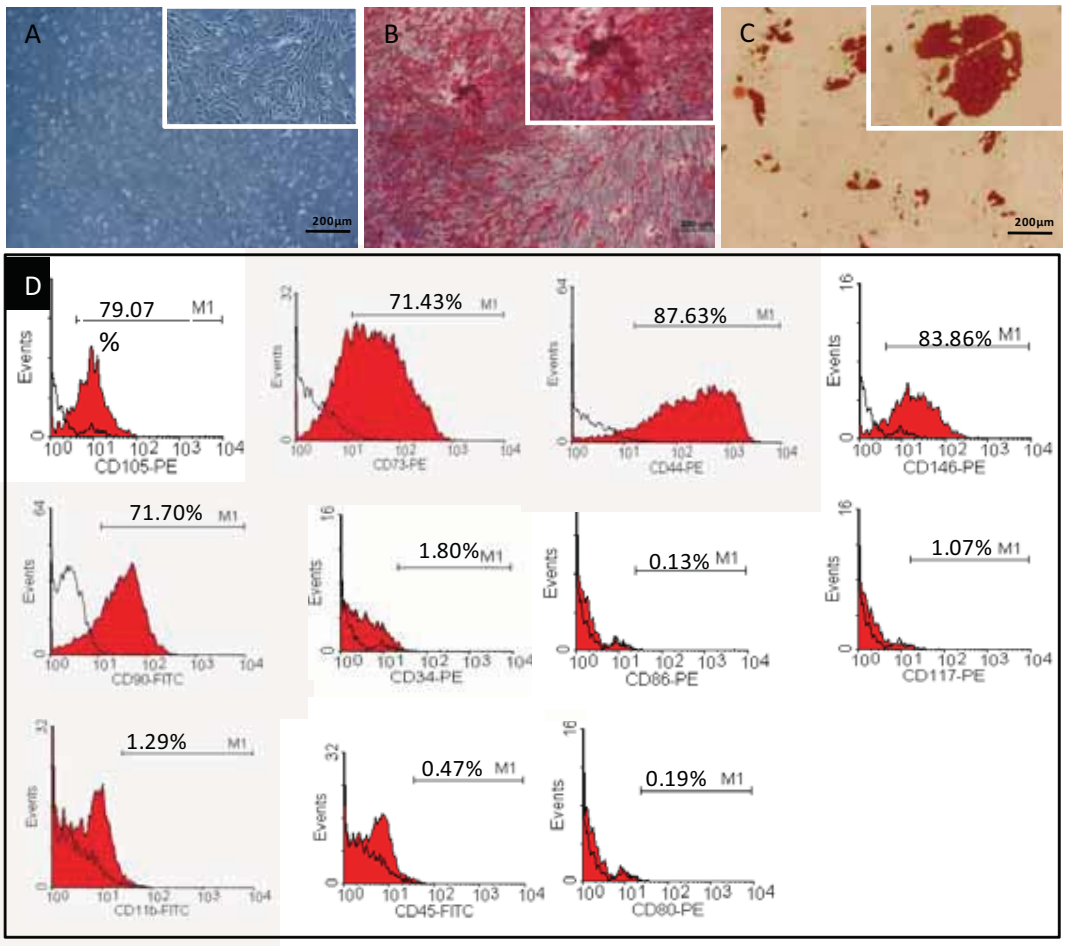

Figure 1. Characterization of cultured human MSCs. At low plating densities, MSCs grew as a monolayer of large, flat cells and made colony forming unit-fibroblasts (CFU-F, lines). **(A)** As the cells approached confluency, they assumed a more spindle-shaped, fibroblastic morphology. Differentiation of MSCs into osteogenic **(B)** and adipogenic **(C)** cells at day 21 after induction showed their multi-lineage potentials for differentiation. Flow cytometric analysis at passage numbers 4 – 5 demonstrated that the cells were positive for CD105, CD73, CD44, CD146, and CD90, but were negative for CD34, CD86, CD117, CD11b, CD45, and CD80 **(D)**.

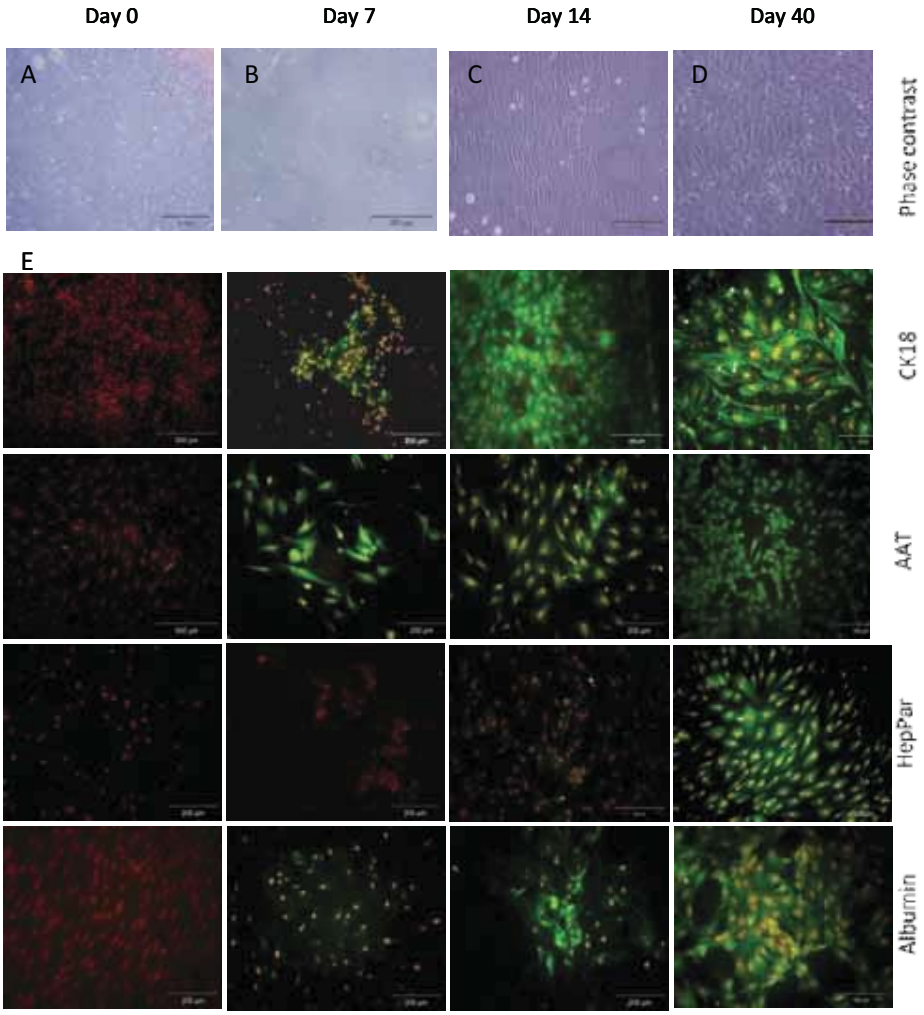

Figure 2. Characterization of MSC-HLCs at phase contrast microscopic **(A-D)** and protein levels upon sequential exposure to liver-specific factors at days 0, 7, 14, and 40 **(E)**. Immunofluorescence staining was performed for FITC conjugated secondary antibodies against anti-cytokeratin 18 (CK18), alpha-1 anti trypsin (AAT), HepPar and albumin. The nucleus was stained with propidium iodide (PI).

consistent with their undifferentiated state (Figure 1D).

Hepatocytic differentiation

We examined the hepatocytic-differentiation potential of MSCs. During the initiation step of hepatic differentiation, the cells showed a remarkable transition from a bipolar fibroblast-like morphology (Figure 2A) to a round epithelial-like shape from days 6 – 7 (Figure 2B). However, at that moment, the cells were still surrounded by spindle-shaped cells. The contraction of the cytoplasm progressed further during maturation; most treated cells became quite dense and round with clear or double nuclei in the late differentiation stage. Small round or oval-shaped cells with a polyhedral structure were visualized at days 14 to 40 (Figures 2C and 2D), which was similar to primary human hepatocytes that underwent morphological changes during hepatic induction. In the next experiments, we examined whether MSCs expressed hepatocyte-specific proteins after hepatic induction. Markers such as CK-18, AAT, heppar antigen (a human hepatocyte-specific antigen) and albumin were analyzed by immunocytochemistry (Figure 2E). Undifferentiated cells did not stain with these four markers.

The temporal expression pattern for a number of hepatocyte-specific genes, such as cytokeratin 18 (CK18), cytokeratin 19 (CK19), alpha-1 anti-trypsin (AAT), tyrosine amino transferase (TAT), LDL receptor (LDLR), and glucose 6-phosphatase (G6P) were analyzed by RT-PCR (Figure 3A). The expression of mature hepatocyte markers such as CK18, CK19, AAT, and TAT were detected during additional differentiation (Figure 3A). To further confirm efficient hepatic induction, we checked the protein expression of ALB by Western blot analysis (Figure 3B). A clear band for the protein was detected in MSC-derived hepatocyte-like cells (MSC-HLC) from day 14. Undifferentiated MSCs did not express detectable levels of albumin as had been observed in RT-PCR analysis (Figure 3A).

We examined whether hepatocyte-derived from MSCs were functionally competent. At 40 days, MSCs were analyzed for their glycogen-storage ability by PAS staining. As shown in Figures 4A and B, the majority of MSC-derived hepatocytes were strongly positive for PAS staining, while undifferentiated MSCs were weakly positive. While undifferentiated MSCs were weakly positive for LDL, after two weeks of hepatic induction almost 20% of the cells incorporated LDL and during maturation (40 days), the majority of induced cells were competent for LDL uptake (Figures 4C and 4D). The cells produced albumin during differentiation and the secreted albumin increased two-fold during 40 days of differentiation (Figure 4E). We examined the ability to detoxify ammonia by their ability to produce urea. Our analyses indicated that

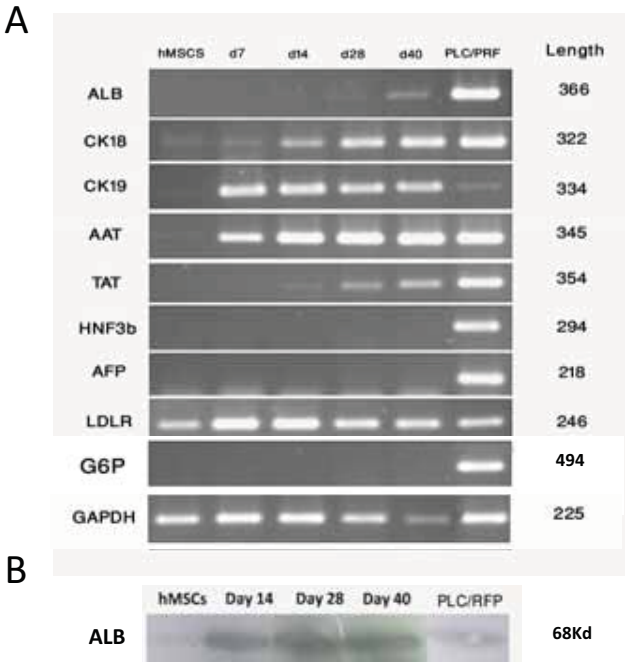

Figure 3. Gene expression analysis of MSC-HLCs upon sequential exposure to liver-specific factors at days 0, 7, 14, and 40 *in vitro*. **(A)** RT-PCR analysis of induced cells. PLC/PRF cell line was used as a positive control. Alb= albumin, CK18= cytokeratin 18, CK19= cytokeratin 19, AAT= alpha-1 anti-trypsin, TAT= tyrosine amino transferase, HNF3β= hepatocyte nuclear-factor 3 beta, AFP= alpha fetoprotein, LDLR= LDL receptor, G6P= glucose 6-phosphatase. GAPDH was used as the internal control. **(B)** Expression of Alb at the protein level as indicated by Western blot.

MSC-derived hepatocytes had the capacity to synthesize urea as an indicator of ammonia metabolism from culture media (Figure 4F).

Infusion of MSC-HLCs into human

We infused a total of 4×10^8 cells [including MSC-HLCs (3.5×10^8) 14 days after the initiation of differentiation induction and undifferentiated MSCs (0.5×10^8)] through the portal vein of a patient with familial hypercholesterolemia. The details of clinical follow up of this patient have previously been reported.[27] In summary, infusion of MSC-HLCs could not significantly improve serum cholesterol levels in this patient.

Discussion

In this report, we described the differentiation of human BM-MSCs into hepatocyte-like cells.

We have demonstrated that differentiated cells functionally behave like hepatocytes. MSC-HLCs have the ability to make albumin, store glycogen, clear ammonia, and uptake LDL.

It has been reported that BM-derived hepatocyte-like cells can partially correct the metabolic abnormality in Gunn rat models of hyperbilirubinemia.[28] Korbling et al. have suggested that BM stem cells can differentiate into liver cells *in vivo*, following BM transplantation.[29] Further reports have debated that finding and demonstrated HSCs acquired liver cell phenotype through fusion with hepatocytes.[30]

However, recent reports have shown that human[31] fibroblasts can reprogram into induced pluripotent cells (iPS) by viral transduction of some key transcription factors specific for embryonic stem cells. Reprogramming human MSCs into iPS cells has also been reported.[32] iPS cells could contribute to all three germ layers after blastocyst injection. These findings have provided proof of concept that somatic cells of mesodermal lineage can differentiate into cells of endodermal lineage.

Schwartz et al. were the first to demonstrate *in vitro* the capacity of BM stem cells to differentiate into hepatocyte-like cells.[33] Their results have been verified by other groups.[34] BM MSCs are a readily available source of cells that proliferate easily. Since human hepatocytes are not readily available, BM MSCs could be a potential source of hepatocytes in cell-based therapies for liver diseases. We differentiated BM MSCs into hepatocyte-like cells; but transplantation of MSC-HLCs was not able to correct the metabolic abnormality of homozygous familial hypercholesterolemia.

Lack of repopulation of the donor cells within the host liver could

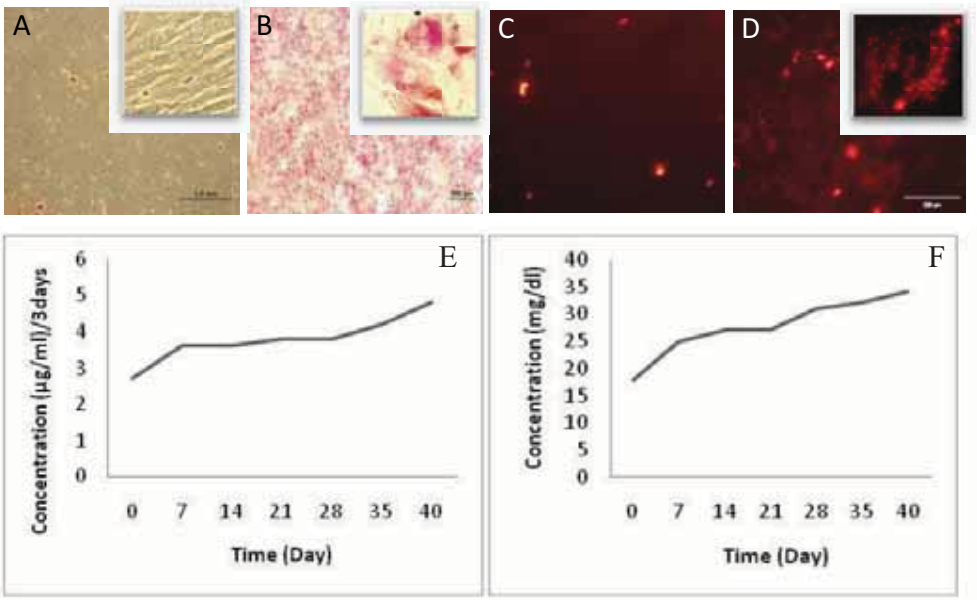

Figure 4. Functional analysis of MSC-HLCs. Glycogen storage of the cells was detected with PAS at day 0 **(A)** and day 40 **(B)**. Glycogen granules become magenta and LDL uptake staining of undifferentiated **(C)** and differentiated MSCs **(D)** at day 40 after treatment *in vitro*. Albumin secretion **(E)**. Urea production **(F)**.

be the most plausible explanation for the lack of clinical improvement of familial hypercholesterolemia after cellular transplantation. We have evaluated this point in another study of MSC transplantation in a mouse model of liver fibrosis. We found MSCs are able to engraft the mouse liver for short term, but they cannot repopulate within the liver and nearly all the donor cells are lost two months after transplantation (Mohamadnejad et al., unpublished data, 2009).

Future animal and human studies are needed to address the potential therapeutic roles of hepatocyte-like cells derived from iPS cells or embryonic stem cells in familial hypercholesterolemia.

In conclusion, we found that human MSCs can differentiate into functional hepatocyte-like cells. Transplantation of allogenic MSC-HLCs is safe and feasible in homozygous familial hypercholesterolemia. However, it does not correct the metabolic abnormality *in vivo*. Future studies should address strategies to improve long-term implantation of MSCs in the host liver.

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