In vitro differentiation of rhesus macaque bone marrow- and adipose tissue-derived MSCs into hepatocyte-like cells

JUNFENG WANG[1,2*], XUFENG FU[2-4*], YAPING YAN[2], SHANSHAN LI[2],
YANCHAO DUAN[2], BRIAUNA MARIE INGLIS[2], WEI SI[2] and BINGRONG ZHENG[4]

[1]Department of Hepatic and Bile Duct Surgery, The First People's Hospital of Yunnan Province, Kunhua Hospital Affiliated to Kunming University of Science and Technology, Kunming, Yunnan 650032; [2]Yunnan Key Laboratory of Primate Biomedical Research, Institute of Primate Translational Medicine, Kunming University of Science and Technology, Kunming, Yunnan 650500; [3]Key Laboratory of Fertility Preservation and Maintenance of Ministry of Education, Department of Biochemistry and Molecular Biology, Ningxia Medical University, Yinchuan, Ningxia 741001; [4]School of Medicine, Yunnan University, Kunming, Yunnan 650091, P.R. China

Abstract. Orthotopic liver or hepatocyte transplantation is effective for the treatment of acute liver injury and end-stage chronic liver disease. However, both of these therapies are hampered by the extreme shortage of organ donors. The clinical application of cell therapy through the substitution of hepatocytes with mesenchymal stem cells (MSCs) that have been differentiated into hepatocyte-like cells (HLCs) for liver disease treatment is expected to overcome this shortage. Bone marrow and adipose tissue are two major sources of MSCs [bone marrow-derived MSCs (BM-MSCs) and adipose tissue-derived MSCs (AT-MSCs), respectively]. However, knowledge about the variability in the differentiation potential between BM-MSCs and AT-MSCs is lacking. In the present study, the hepatogenic differentiation potential of rhesus macaque BM-MSCs and AT-MSCs was compared with the evaluation of morphology, immunophenotyping profiles, differentiation potential, glycogen deposition, urea secretion and hepatocyte-specific gene expression. The results indicated that BM-MSCs and AT-MSCs shared similar characteristics in terms of primary morphology, surface markers and trilineage differentiation potential (adipogenesis, osteogenesis and chondrogenesis). Subsequently, the hepatogenic differentiation potential of BM-MSCs and AT-MSCs was evaluated by morphology, glycogen accumulation, urea synthesis and expression of hepatocyte marker genes. The results indicated that rhesus BM-MSCs and AT-MSCs had hepatogenic differentiation ability. To the best of our knowledge, this is the first report to detect the hepatogenic differentiation potential of rhesus macaque BM-MSCs and AT-MSCs. The present study provides the basis for the selection of seed cells that can trans-differentiate into HLCs for cytotherapy of acute or chronic liver injuries in either clinical or veterinary practice.

Introduction

Liver disease, which includes acute liver injury and chronic liver disease, is a major cause of morbidity and mortality worldwide (1). Chemicals or toxins typically cause acute liver injury, whereas chronic liver disease is stimulated by numerous factors, such as viral hepatitis, alcohol, drugs and metabolic and autoimmune diseases (2). Acute liver injury is a lethal condition characterized by widespread hepatocyte necrosis, acute deterioration of liver function and subsequent multiorgan failure. Chronic liver injury-induced liver fibrosis can lead to the development of liver cirrhosis and hepatocellular carcinoma at the end stage (3). Liver transplantation is currently regarded as one of the most effective treatment options for acute liver injury and end-stage chronic liver injury; however, the extreme shortage of organ donors, high cost of surgery, immunological rejection risk and transplantation complications severely hamper treatment by liver transplantation (4). Alternatively, transplantation of hepatocytes, particularly those isolated from fetal liver, is considered a promising therapy for the treatment of liver diseases (5). Similar problems, such as the shortage of organ donors and the risk of immunological rejection of allogenic hepatocytes, also exist in the clinical application of this treatment (2).

Correspondence to: Professor Wei Si, Yunnan Key Laboratory of Primate Biomedical Research, Institute of Primate Translational Medicine, Kunming University of Science and Technology, 727 Jingming South Road, Kunming, Yunnan 650500, P.R. China
E-mail: siw@lpbr.cn

Professor Bingrong Zheng, School of Medicine, Yunnan University, 2 Cuihu North Road, Kunming, Yunnan 650091, P.R. China
E-mail: zhengbr@ynu.edu.cn

*Contributed equally

Stem cell transplantation, particularly mesenchymal stem cell (MSC) therapy, has shown potential for the treatment of liver diseases (3). MSCs are recognized as promising stem cells for cytotherapy due to their multipotency and paracrine effects. MSCs have been isolated from various tissues, including bone marrow, adipose tissue, placenta, dental pulp, endometrium, perinatal tissues and other mesodermal tissues (6,7). However, there is no consensus on surface markers that identify MSCs from various sources, with the exception that the minimum criteria of MSC markers include positive expression of CD105, CD73, CD44 and CD90, and negative expression of CD45, CD34, CD14 and HLA-DR (8). Studies on animal models have revealed that heterogenic MSCs can ameliorate liver fibrosis and fulminant hepatic failure through paracrine and immunoregulatory effects (9). In veterinarian applications, MSCs rescue animals suffering from acute liver injuries caused by incidents such as accidental ingestion of poison (9-11). The therapeutic mechanisms underlying the effects of MSCs include their multipotent capacity to differentiate into various cell types, including hepatocyte-like cells (HLCs), under appropriate conditions (12). The substitution of hepatocytes with MSCs differentiated into HLCs for liver disease treatment is expected to overcome the shortage of liver donors (13). However, studies have reported that MSCs from various sources or tissues present different cell characteristics, molecular functions and clinical therapeutic effects (14-16).

Bone marrow has commonly been regarded as the most conventional stem cell source in the field of cytotherapy for liver diseases, due to the ability of bone marrow cells to differentiate into HLCs *in vitro* and *in vivo* (17). However, the collection of bone marrow is an invasive procedure that can cause severe pain to the donor, which limits the applicability of bone marrow-derived MSCs (BM-MSCs) for clinical therapy (18). Conversely, adipose tissue is ubiquitous; it is easy to obtain, and the collection procedure is associated with less morbidity and patient discomfort (19). Therefore, the application of adipose tissue-derived MSCs (AT-MSCs) for cellular therapeutic research is feasible and has been shown to be both safe and efficacious in preclinical and clinical studies (19). Although previous studies have reported that AT-MSCs can differentiate into HLCs *in vitro* and *in vivo*, knowledge about the differentiation potential of BM-MSCs and AT-MSCs is lacking (20-22). Only one study has compared the differentiation success rates of equine BM-MSCs and AT-MSCs into HLCs, and the results revealed that BM-MSCs could completely differentiate into HLCs, whereas AT-MSCs failed to fully differentiate (23). An investigation into the differentiation potential of BM-MSCs and AT-MSCs into HLCs will therefore be beneficial for the identification of liver disease stem cell therapies.

Rhesus macaques are one of the most widely used laboratory animals in biomedical research due to their genetic, physiological, behavioral and neurological similarities to humans, and because macaques provide excellent translational validity in preclinical studies (24). The present study therefore aimed to investigate the differentiation potential of rhesus macaque BM-MSCs and AT-MSCs into HLCs *in vitro*, and to provide the basis for selection of seed cells that trans-differentiate into HLCs for cytotherapy of acute or chronic liver injuries in either clinical or veterinary medicine.

Materials and methods

Animals. A total of two male rhesus macaques (age, 2 years) with a body weight of 2-3 kg were used as bone marrow and adipose tissue donors. The rhesus macaques were individually caged in an animal room with a 12/12 h light/dark cycle, and provided with commercial monkey chow, sterile water, fresh fruits and vegetables *ad libitum*. The temperature of the animal room was controlled between 18-26°C and with humidity from 40 to 70%. Animal studies were approved by the Institutional Animal Care and Use Committee of Kunming University of Science and Technology (approval number: LPBR20170201) and were performed in accordance with the Guide for the Care and Use of Laboratory Animals (25).

Preparation of rhesus macaque BM-MSCs and AT-MSCs. BM-MSCs were isolated from the tibias of young rhesus macaques using the procedures described in detail in a previous study (9). AT-MSCs were isolated from the mesenteric adipose tissue of young rhesus macaques. Briefly, the adipose tissues were washed with 75% alcohol and PBS, cut into small pieces(~0.5x0.5 cm) and placed into 10-cm plastic dishes containing Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific) and 1% (v/v) penicillin/streptomycin (Gibco; Thermo Fisher Scientific) in sterile conditions. The adipose tissues were cultured in an incubator at 37°C with a humidified atmosphere of 5% CO_2, which was the same as the BM-MSC culture conditions. The medium was refreshed every 48 h. After 10 days, the primary cell culture was passaged at 80% confluence with 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.).

BM-MSCs and AT-MSCs were resuspended in culture medium at a dilution ratio of 1:3 and expanded on a new plastic petri dish. The morphology, surface markers and differentiation potency of the MSCs were determined at passage 3.

Flow cytometry of the immunophenotype surface markers of MSCs. The procedures were described in detail in our previous study (9). Briefly, $5x10^5$ MSCs were collected, washed and centrifuged (500 x g; 5 min; room temperature) in 500 μl PBS containing 3% FBS (PBSF). MSCs were then resuspended in 100 μl PBSF and incubated with 5 μl (10 μg/μl) antibody markers (Human MSC Analysis kit; cat. no. 562245; BD Biosciences) on ice for 30 min, washed and resuspended in 500 μl PBSF, and examined using flow cytometry (C6; BD Biosciences) and analyzed using using the built-in software.

Evaluation of the differentiation potential of MSCs. These procedures were described in detail in our previous study (9). Briefly, for adipogenic differentiation, BM-MSCs and AT-MSCs were seeded into 24-well plates ($8x10^4$ cells per well), cultured for 12 h and treated with adipogenic differentiation medium (Gibco; Thermo Fisher Scientific, Inc.) for 7 days; the medium was refreshed every 3 days. Adherent cells were stained red with 60% Oil Red O (Sigma-Aldrich; Merck KGaA) for 1 min at room temperature. For osteogenic differentiation, BM-MSCs and AT-MSCs were seeded into 24-well plates ($4x10^4$ cells per well), cultured for 12 h and treated with

osteogenic differentiation medium (Gibco; Thermo Fisher Scientific, Inc.) for 21 days; the medium was refreshed every 3 days. Osteogenic differentiation was confirmed by 0.2% Alizarin Red staining for 5 min at room temperature. For chondrogenic differentiation, $2x10^5$ MSCs were collected in 15-ml centrifuge tubes and cultured with chondrogenic differentiation medium (Gibco; Thermo Fisher Scientific, Inc.) for 21 days; the medium was refreshed every 3 days. The chondroid pellets were sectioned (8 μm) with a freezing microtome. The slices were stained with 1% toluidine blue for 3 min at room temperature and were captured using a light microscope (Olympus Corporation) at a x50 magnification.

Hepatogenic differentiation protocol. For hepatogenic differentiation, the commonly used three-step protocol was applied, which includes serum-free culture, differentiation and maturation steps (26). Firstly, passage 3 cells were seeded on a 6-well plastic plate at a density of $2x10^4$/cm^2 and cultured in serum-deprived medium containing 20 ng/ml epidermal growth factor (EGF; PeproTech EC Ltd.) and 10 ng/ml basic fibroblast growth factor (bFGF; PeproTech EC Ltd.) for 2 days. Hepatogenic differentiation was sustained for 7 days, and the cells were cultured in differentiation medium consisting of DMEM supplemented with 10% FBS, 10 ng/ml bFGF, 0.6 mg/ml nicotinamide (PeproTech EC Ltd.) and 20 ng/ml hepatocyte growth factor (HGF; PeproTech EC Ltd.). At the maturation step, the cells were cultured in maturation medium consisting of DMEM supplemented with 10% FBS, 20 ng/ml oncostatin M (PeproTech EC Ltd.), 1 μM dexamethasone (Beijing Solarbio Science & Technology Co., Ltd) and 50 μg/ml insulin-transferrin-selenium (ITS; PeproTech EC Ltd.) for 2 weeks. The culture medium was changed every 3 days.

Hepatocyte isolation. Whole liver was obtained from a 2-year-old rhesus macaque following euthanasia with intravenous sodium pentobarbital at 100 mg/kg body weight, according to the Guide for the Care and Use of Laboratory Animals Primary monkey hepatocytes were isolated using a modified multipoint puncture perfusion technique (27). Briefly, the liver tissue samples were flushed with 38˚C perfusion buffer (NaCl, 9.3 g/l; KCl, 0.5 g/l; HEPES, 2.4 g/l; EGTA, 0.95 g/l) via repetitive multipoint puncture with a 10-ml sterile syringe until the liver changed to an off-white color and the perfusion buffer turned clear. The tissue was then continuously perfused with a prewarmed digestion buffer solution (perfusion buffer with 0.05% collagenase IV). After sufficient digestion, the liver tissue was mechanically disrupted using ophthalmic scissors and an operating knife. The chopped tissue was suspended in DMEM (containing 10% FBS and 1% penicillin/streptomycin) and repeatedly pipetted up and down prior to gentle shaking. The hepatocyte suspension was divided into equal aliquots, which were then filtered through a 500-μm strainer and centrifuged at 50 x g for 2 min at 4˚C. The suspension was removed, added to red blood cell lysis buffer (Beyotime Institute of Biotechnology) and incubated for 5 min at room temperature before centrifugation at 50 x g for 2 min at 4˚C. Sedimentary cells were washed with DNaseI solution (Beyotime Institute of Biotechnology) and filtered through a 250-μm nylon membrane, and the cells were harvested by low-speed centrifugation at 50 x g for 5 min at 4˚C. Finally, the hepatocytes were seeded on 6-well plates at a density of $2x10^4$ cells/cm^2 and cultured with high-glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C and 5% CO_2. Due to their inability to proliferate, hepatocytes were cultured for 5 days before testing in the present study.

Periodic acid-Schiff (PAS) staining for hepatic glycogen detection. PAS staining is a typical technique used to detect hepatocyte function (28). The functional features of HLCs were assessed for glycogen deposition using a PAS staining kit (Beijing Leagene Biotech Co., Ltd). The cell culture medium was removed from the plates, and the cells were rinsed with PBS three times. Subsequently, the cells were fixed in methyl alcohol (99% purity) for 10 min at room temperature. After being washed three more times with PBS, the cells were oxidized for 15 min with 1% periodic acid and washed three times with deionized water, then stained with Schiff's reagent at room temperature for 20 min. After being washed three times with PBS, the cells were stained with Mayer's hematoxylin (room temperature) for 1 min. Finally, the dye was washed off with PBS prior to further evaluation under a light microscope (Olympus Corporation) at a magnification of x50.

Urea production assay. The urea production test is one of the most widely used methods to detect the function of hepatocytes (28). The culture media from MSCs and HLCs were changed every 3 days normally and collected 3-day culture before the timepoints (0, 9, 16 and 23 days), and the culture media from hepatocytes were changed at 2 days and collected after a 3-day culture. All culture media were assessed for urea production using a urease method kit (Beijing Leagene Biotech Co., Ltd.; cat.no: TC1165), according to the manufacturer's protocol. Briefly, urease working solution, phenol coloring solution and urea test solution were mixed with standard urea and with the samples, which were measured at 540 nm with a 96-well microplate reader following incubation at 37˚C for 30 min. Subsequently, the concentrations of the samples were calculated. Three independent urea samples were assessed at each time point and each assay was repeated three times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from monkey hepatocytes and HLCs differentiated from monkey BM-MSCs and AT-MSCs using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The precipitated RNA was solubilized in sterile diethylpyrocarbonate-treated water (Sangon Biotech), and cDNA was then synthesized using a Prime-Script RT reagent kit (Takara Biotechnology Co., Ltd) at 37˚C for 5 min and 85˚C for 5 sec. Quantification of specific genes was performed using SYBR® Premix Ex Taq™ II kit (Takara Biotechnology Co., Ltd) and CXF real-time PCR system (Bio-Rad Laboratories, Inc.) at particular condition (Stage 1: 95˚C for 30 sec; stage 2: 95˚C for 3 sec; 60˚C for 30 sec and repeat for 40 cycles). All experiments were performed in triplicate, and the data were analyzed using the $2^{-\Delta\Delta Cq}$ method (29). The gene-specific primers were commercially synthesized (Sangon Biotech), and sequence information is shown in Table I.

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Forward primer sequences (5'-3')	Reverse primer sequences (5'-3')
GAPDH	ACGGATTTGGTCGTATTGG	GCTCCTGGAAGATGGTGAT
CK-18	GCCCGCTATGCCCTACAGAT	TTCACTGACACCATTCTTTCG
HNF-4α	CCACGGGCAAACACTACGG	TGGACGGCTTCCTTCTTCAT
ALB	AAGGCTTGGTGCTGGTT	GTTCGGGTTGTCATCTTTGT
CYP3A4	AAAAGAAAGTCGCCTCAAAGA	GAAGGAAAGAACACTGCTGGT
CYP7A1	TTTCCAGTGCCTCCCTCAAC	GGTAGTCTTTGTCTTCCCGTTTT

CK-18, keratin 18; CYP3A4, cytochrome P450 family 3 subfamily A member 4; CYP7A1, cytochrome P450 family 7 subfamily A member 1; HNF-4α, hepatocyte nuclear factor-4α.

Data analysis. All data are presented as the mean ± SD. Statistical significance among groups was analyzed using repeated measures ANOVA with a post-hoc paired t-test with Bonferroni's correction. Statistical significance within groups at time-point was analyzed using Student's t-test. P<0.05 was considered to indicate a statistically significant difference. Statistically analyze and histograms were generated using GraphPad Prism 5 (GraphPad Software, Inc.).

Results

Characterization of BM-MSCs and AT-MSCs. BM-MSCs and AT-MSCs were obtained from bone marrow and adipose tissue. To evaluate whether the expanded cells were genuine MSCs, the characterization of BM-MSCs and AT-MSCs at passage 3 was performed. Both cell types were compared according to morphology, immunophenotyping profiles and trilineage differentiation potential. During primary culture, BM-MSCs and AT-MSCs adhered to the plastic dishes in a scattered manner and exhibited similar morphology to each other. Cells appeared fibroblast-like, elongated and spindle-shaped with a single nucleus (Fig. 1A).

The BM-MSCs and AT-MSCs could differentiate into adipocytes (Fig. 1B), osteocytes (Fig. 1C) and chondrocytes *in vitro* (Fig. 1D). After the induction of adipogenic differentiation, numerous neutral lipid droplets stained with Oil Red O were observed in the cytoplasm of BM-MSCs and AT-MSCs. After the induction of osteogenic differentiation, the cells presented an aggregation of micronodules or calcium deposits that were stained by Alizarin Red. The chondrogenic differentiation of both types of MSCs was observed using an Alcian Blue stain.

The immunophenotyping profiles of BM-MSCs and AT-MSCs were analyzed by flow cytometry. The results revealed that both BM-MSCs and AT-MSCs expressed high levels of the positive markers CD44, CD90, CD73 and CD105, but did not express the negative markers CD45, CD34, CD11b, CD19 and human leukocyte antigen-DR (HLA-DR; Fig. 2A and B). No differences were observed between BM-MSCs and AT-MSCs using a t-test (Fig. 2C).

Morpholog yand glycogen deposition of BM-MSCs and AT-MSCs during differentiation into HLCs. During the differentiation of BM-MSCs and AT-MSCs into HLCs,
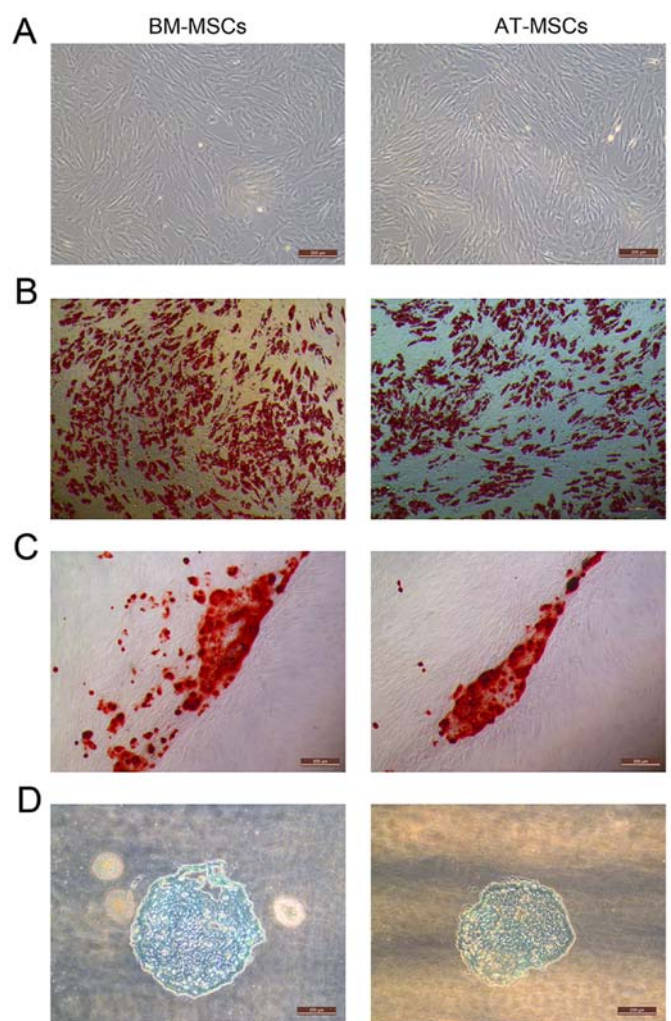

Figure 1. Morphology and trilineage differentiation potential of rhesus macaque BM-MSCs and AT-MSCs. (A) Fibroblast-like morphology of rhesus macaque BM-MSCs and AT-MSCsat passage 3. (B) Adipogenic differentiation (Oil Red O staining). (C) Osteogenic differentiation (Alizarin Red staining). (D) Chondrogenic differentiation (Alcian Blue staining). Scale bar: 200 μm. AT-MSCs, adipose tissue-derived mesenchymal stem cells; BM-MSCs, bone marrow-derived mesenchymal stem cells.

BM-MSCs (Fig. 3A) gradually changed from spindle and fibroblast-like cells to round or polygonal epithelioid cells. These changes were also observed in AT-MSCs (Fig. 3C).
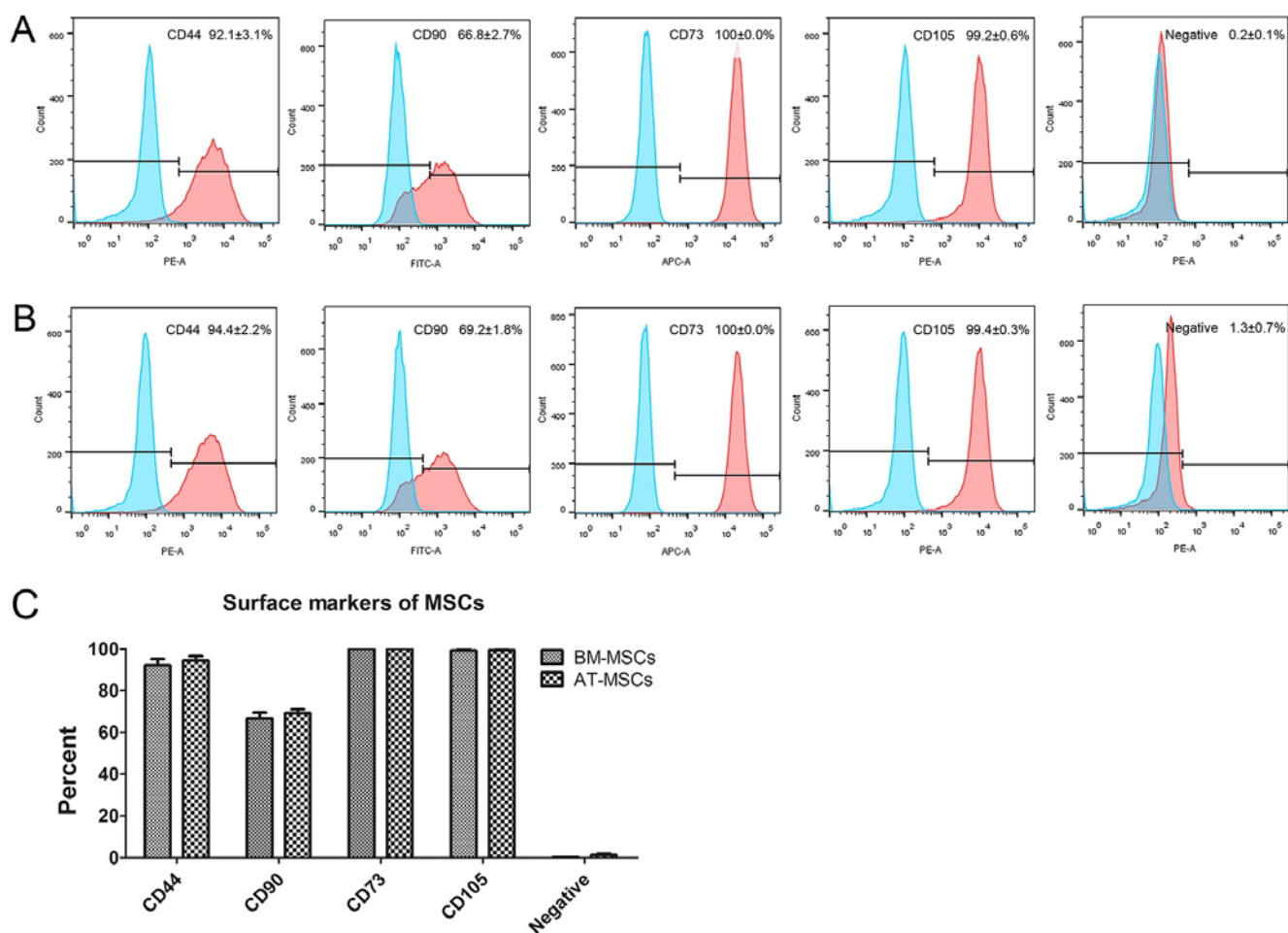
Figure 2. Immunophenotyping profiles of BM-MSCsand AT-MSCs. Surface marker expression of (A) BM-MSCs and (B) AT-MSCs analyzed by flow cytometry. (C) Semi-quantitativeprofilecomparison of surface markers between BM-MSCs and AT-MSCs. AT-MSCs, adipose tissue-derived mesenchymal stem cells; BM-MSCs, bone marrow-derived mesenchymal stem cells.

At day 0, before hepatogenic differentiation induction, BM-MSCs and AT-MSCs exhibited similar fibroblast-like morphology. However, a greater number of flattened and polygonal cells were observed in the BM-MSC group than in the AT-MSC group on day 9. On days 16 and 23, the induced MSCs showed epithelioid and cuboidal shapes, which were similar to the morphology of the primary hepatocytes of the control group (Fig. 3E). In addition, the presence of deposited glycogen was determined by PAS staining, to further characterize the glycogen deposition function of HLCs differentiated from BM-MSCs and AT-MSCs. After 23 days of hepatogenic differentiation induction, magenta-stained glycogen was detected in the differentiated cells but not in the undifferentiated cells. The PAS intensity of HLCs differentiated from BM-MSCs (Fig. 3B) was higher than that of HLCs differentiated from AT-MSCs (Fig. 3D) on days 16 and 23. The level of staining was similar to the PAS staining of the hepatocytes derived from liver tissue that had been cultured for 5 days (Fig. 3F). These results suggested that the morphology of HLCs differentiated from BM-MSCs and AT-MSCs was similar to primary hepatocytes. Moreover, the HLCs differentiated from both BM-MSCs and AT-MSCs exhibited the hepatic function of glycogen deposition and could be stained with PAS.

Urea secretion in HLCs differentiated from BM-MSCs and AT-MSCs. Urea assays detected the urea secretion function of HLCs differentiated from BM-MSCs and AT-MSCs at various time points (days 0, 9, 16 and 23), while urea secretion in the culture medium of hepatocytes derived from liver tissue cultured for 3 days was tested as a control. The urea production of the BM-MSCs and AT-MSCs gradually increased over the culture time (days 9, 16 and 23) during the HLC differentiation process compared to the undifferentiated BM-MSCs and AT-MSCs (Fig. 4), and the urea secretion function of differentiated hepatocytes from BM-MSCs was superior to that of AT-MSCs ($P < 0.05$). These results showed that HLCs differentiated from rhesus macaque BM-MSCs and AT-MSCs possessed the hepatic function of urea secretion.

RT-qPCR analysis of hepatocyte marker expression. To further investigate the differentiation potential and hepatocyte function of BM-MSCs and AT-MSCs differentiated into HLCs, the mRNA expression levels of the hepatocyte markers, albumin (ALB; Fig. 5A), keratin 18 (CK-18; Fig. 5B), hepatocyte nuclear factor-4α (HNF-4α; Fig. 5C), cytochrome P450 family 7 subfamily A member 1 (CYP7A1; Fig. 5D) and cytochrome P450 family 3 subfamily A member 4 (CYP3A4;
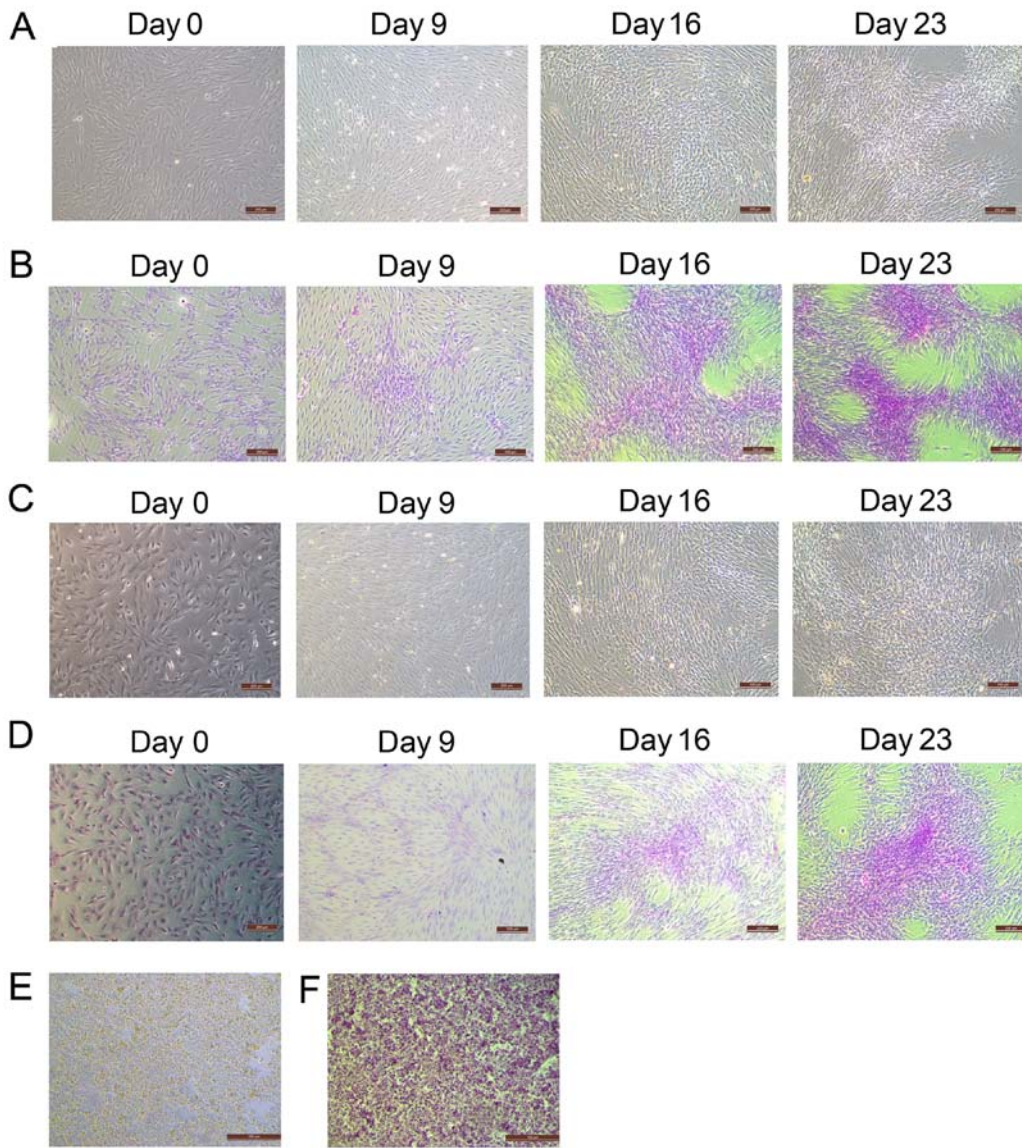
Figure 3. Morphology and glycogen deposition changes during the differentiation of BM-MSCs and AT-MSCs into HLCs. (A) Morphological changesand (B) glycogen deposition of BM-MSCs differentiating into HLCs that were cultured for 23 days (n=3). (C) Morphological changesand (D) glycogen deposition of AT-MSCs differentiating into HLCs that were cultured for 23 days (n=3). (E) Morphologyand (F) glycogen deposition of hepatocytes isolated from liver tissue and cultured for 5 days (n=3). Scale bars: (A-D) 200 μm; (E and F) 100 μm. AT-MSCs, adipose tissue-derived mesenchymal stem cells; BM-MSCs, bone marrow-derived mesenchymal stem cells; HLCs, hepatocyte-like cells.

Fig. 5F), in BM-MSCs and AT-MSCs during the HLC differentiation process were evaluated by RT-qPCR analysis. In these assays, hepatocytes derived from liver tissue cultured for 5 days were used as a positive control. During the HLC differentiation process, the expression of these differentiated hepatocyte makers in BM-MSCs and AT-MSCs increased from day 0 to day 23. In the process of BM-MSCs differentiation into HLCs, the expression of ALB, CYP7A1 and CYP3A4 was significantly different on days 9-23 compared with expression at day 0. CK-18 and HNF-4α expression was significantly different on days 16 and 23 compared with at day 0. In the process of AT-MSC differentiation, the expression of ALB was significantly different on days 9-23 compared with that on day 0; HNF-4α, CYP7A1 and CYP3A4 expression was significantly different on days 16 and 23, and CK-18 expression was significantly different on day 23 (Fig. 5).
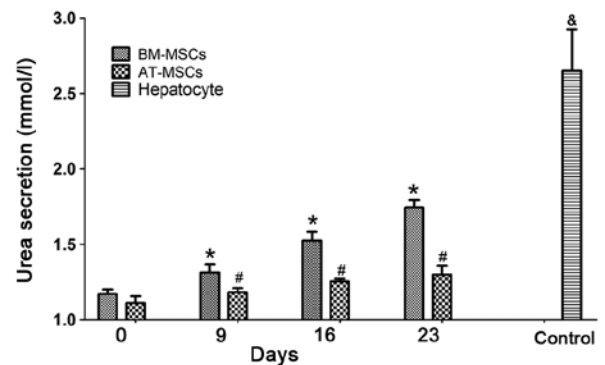

Figure 4. Urea secretion of BM-MSCs and AT-MSCs differentiated into HLCs. After a 23-day culture, the urea secretion of BM-MSCs and AT-MSCs in 3-day culture medium was detected (n=3). *P<0.05 BM-MSCs vs. day 0, #P<0.05AT-MSCs vs. day 0. &denoteshepatocyte samples vs. BM-MSCs and AT-MSCs. AT-MSCs,adipose tissue-derived mesenchymal stem cells; BM-MSCs, bone marrow-derived mesenchymal stem cells; HLCs, hepatocyte-like cells.
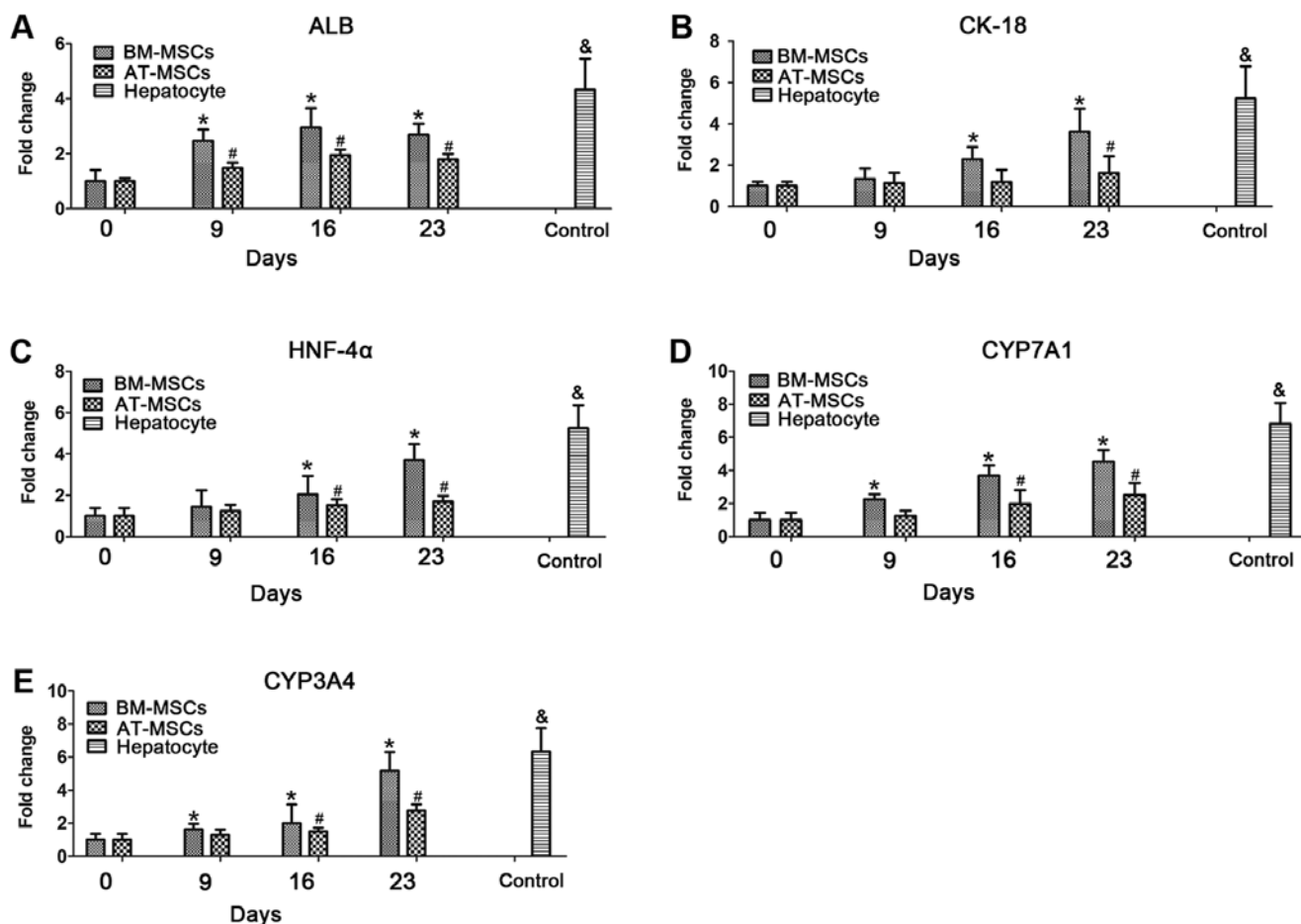
Figure 5. Hepatocyte-specific gene expression was detected in BM-MSCs and AT-MSCs during the HLC differentiation process. The mRNA expression of the hepatocyte-specific genes ALB (A), CK-18 (B), HNF-4α (C), CYP7A1 (D) and CYP3A4 (E) was analyzed on days 0, 9, 16 and 23 by reverse transcription-quantitative polymerase chain reaction and normalized to GAPDH expression. Control, collected hepatocytes after 5 days (n=3). *P<0.05 BM-MSCs vs. day 0, #P<0.05 AT-MSCs vs. day 0. &denotes hepatocyte samples vs. BM-MSCs and AT-MSCs. ALB, albumin; AT-MSCs, adipose tissue-derived mesenchymal stem cells; BM-MSCs, bone marrow-derived mesenchymal stem cells; CK-18, keratin 18; CYP3A4, cytochrome P450 family 3 subfamily A member 4; CYP7A1, cytochrome P450 family 7 subfamily A member 1; HLCs, hepatocyte-like cells; HNF-4α, hepatocyte nuclear factor-4α.

Discussion

The extreme shortage of liver donors hampers clinical therapy with orthotopic liver or hepatocyte transplantation for patients with end-stage liver diseases. MSCs will likely continue to be used in future clinical applications; therefore, the differentiation of MSCs into HLCs as an alternative source of seed cells, such as hepatocytes, shows considerable promise to overcome the problem of organ donation shortage in liver disease therapy. Previous studies have reported that MSCs derived from various tissues exhibit different cell characteristics, molecular function and clinical therapeutic effects (1,15). Our previous study investigated whether MSCs could improve liver fibrosis. The results indicated that transplanted MSCs could migrate to the liver and that the paracrine effects of MSCs may play an important role in vivo (9). To understand the therapeutic effect of MSCs on liver disease therapy, it is essential to determine whether MSCs can be successfully differentiated into HLCs. Currently, human MSCs isolated from various tissue sources, including bone marrow (30), adipose tissue (31), umbilical cord blood (32) and menstrual blood (33), have been proven to have the potential to differentiate into HLCs under suitable induction conditions. A previous study reported that equine MSCs

derived from peripheral blood, adipose tissue and bone marrow had different hepatogenic differentiation efficiency (23). The tissue-specific differentiation potency of human MSCs derived from perinatal tissues, including the amnion, chorion and umbilical cord, has been revealed through adipogenic, osteogenic and chondrogenic differentiation, and the discovery of the innate tissue-specific differentiation potency of various types of MSCs will be helpful in choosing the appropriate cell sources for better outcomes in specific diseases (34).

Although rhesus macaques have genetic and physiological similarities with humans, and are widely used as a laboratory animal (24), the differentiation potency of BM-MSCs and AT-MSCs remains unclear in this important species. Therefore, the differentiation potencies of rhesus BM-MSCs and AT-MSCs into HLCs were analyzed in the present study; this comparison may help to identify an optimal seed cell for liver disease therapy either in preclinical or veterinary applications.

In the present study, BM-MSCs and AT-MSCs were isolated from age-matched rhesus macaques and cultured based on conventional plastic adherence. According to the International Society for Cellular Therapy definition of MSCs (35), both rhesus BM-MSCs and AT-MSCs exhibited the characteristics of homogeneous fibroblast-like adherent cells, positively

expressed MSC markers and negatively expressed hematopoietic markers, and exhibited trilineage differentiation potential into osteoblasts, adipocytes and chondroblasts. Using a cytokine induction cocktail *in vitro* is one strategy for the differentiation of stem cells into HLCs. Culture medium containing various combinations of cytokines and growth factors, such as HGF, EGF, bFGF, oncostatin M (OSM), ITS, nicotinamide and hexadecadrol have been shown to augment the differentiation of MSCs into functional HLCs (26,33,36,37). Mou *et al* (33) successfully induced menstrual blood-derived MSC differentiation into HLCs using an HGF, FGF-4, OSM, dexamethasone and ITS premix. Ayatollahi *et al* (38) used a combination of insulin growth factor-1 and liver-specific factors, including HGF, OSM and dexamethasone, to differentiate human BM-MSCs into HLCs. Shi *et al* (37) induced hair follicle MSCs differentiation into hepatocytes by treating the cells with L-glutamine and activin A and then culturing the cells with bone morphogenetic protein-4, FGF-4, HGF, OSM and dexamethasone for maturation. In the present study, a modified three-step protocol (serum-free culture for 2 days, differentiation for 7 days and maturation for 14 days) was used to induce rhesus MSC differentiation into HLCs. The results confirmed that BM-MSCs and AT-MSCs of rhesus macaques have the potential to differentiate into HLCs. Compared with the isolated hepatocytes from rhesus macaque liver tissue, the HLCs differentiated from BM-MSCs and AT-MSCs not only displayed hepatocyte morphology but also exhibited mature hepatocyte-specific functions, including glycogen deposition, urea production and hepatocyte-related gene expression. Xu *et al* (22) compared mouse AT-MSCs and BM-MSCs *in vitro* by using a one-step culture protocol (continuously culturing in the same medium for 10 days) with a cocktail containing HGF, FGF4, OSM, EGF, acidic FGF, bFGF, dexamethasone, ITS, vitamin C and nicotinamide, and did not observe differences between the two types of mouse MSCs. Pennington *et al* (23) detected differences in hepatogenic differentiation efficiency by comparing various tissue-derived equine MSCs; the results indicated that the difference between BM-MSC and AT-MSC differentiation into HLCs may be protocol-, duration-, and species-dependent.

The typical functional assays for hepatocyte identification are glycogen deposition, urea production, albumin secretion and low-density lipoprotein uptake assays. In addition, hepatocyte-related gene expression is widely used in the detection of MSCs differentiating into HLCs (39-41). In the present study, HLCs differentiated from both BM-MSCs and AT-MSCs acquired the functions of glycogen deposition, urea secretion and expression of hepatocyte-related genes (including CK-18, HNF-4α, ALB, CYP3A4 and CYP7A1) in the differentiation process. The differentiation of rhesus macaque BM-MSCs into HLCs occurred prior to that of AT-MSCs in the culture timeline. The HLCs morphology appeared first at 9 days in BM-MSCs, which showed clustered and globular cells, which were similar to primary hepatocytes from liver tissue. The function of differentiated hepatocytes from BM-MSCs was superior to that of AT-MSCs, due to the differences shown in the glycogen deposition and urea secretion assays. This superiority of BM-MSCs was also indicated in hepatocyte-related gene expression after 23 days. The ALB, CK-18, and HNF-4α genes are regarded as mature hepatocyte markers, and the major cytochrome P450 forms,

including CYP3A4 and CYP7A1, are highly expressed during hepatocyte differentiation (26,42-44). In the present study, the expression levels of ALB, CK-18, HNF-4α, CYP3A4 and CYP7A1 in BM-MSCs were higher than those in AT-MSCs after 23 days of differentiation into HLCs. These results indicated that the differentiation potential of BM-MSCs into HLCs is better than that of AT-MSCs in rhesus macaques. Although AT-MSCs are easier to harvest from donors than BM-MSCs and the harvest procedure causes much less pain for donors, the differentiation potential of AT-MSCs into HLCs is inferior to that of BM-MSCs in rhesus macaques according to glycogen deposition, urea secretion assays and gene expression detection. Previous studies have reported that the HLC differentiation potential of cells derived from various tissues may be dependent on the species and methods (23,26). The present results are consistent with these previous studies and indicated that different species require specific methods for MSC differentiation into HLCs, which will be necessary to study further in the future.

In conclusion, rhesus macaque BM-MSCs and AT-MSCs have the potential to differentiate into HLCs, which was confirmed by morphology, glycogen deposition, urea secretion and hepatocyte-related gene expression using a modified three-step protocol of culture for 23 days. To the best of our knowledge, this study is the first to report the potential of BM-MSCs and AT-MSCs differentiating into HLCs in rhesus macaques and indicates that the potential of various tissue-derived MSCs differentiating into HLCs may be species- and method-dependent. These results will be beneficial to improve hepatocyte differentiation protocols for MSCs to ensure high efficiency for preclinical cytotherapy of liver diseases as well as veterinary medicine.

Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Author's contributions

JW, XF and YY carried out the experiments, SL, YD and BMI performed sampling and analysed the data, XF, WS and BZ designed the experiments and wrote the manuscript. BMI and BZ revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal studies were approved by the Institutional Animal Care and Use Committee of Kunming University of Science and Technology (approval number: LPBR20170201).

Patient consent for publication

Not applicable.

Competing interests

Thecauthors declare that they have no competing interests.

References

1. Alfaifi M, Eom YW, Newsome PN and Baik SK: Mesenchymal stromal cell therapy for liver diseases. J Hepatol 68: 1272-1285, 2018.
2. Michalopoulos GK: Hepatostat: Liver regeneration and normal liver tissue maintenance. Hepatology 65: 1384-1392, 2017.
3. Jalan R, Yurdaydin C, Bajaj JS, Acharya SK, Arroyo V, Lin HC, Gines P, Kim WR, Kamath PS and World Gastroenterology Organization Working Party: Toward an improved definition of acute-on-chronic liver failure. Gastroenterology 147: 4-10, 2014.
4. Nasralla D, Coussios CC, Mergental H, Akhtar MZ, Butler AJ, Ceresa CDL, Chiocchia V, Dutton SJ, García-Valdecasas JC, Heaton N, et al: A randomized trial of normothermic preservation in liver transplantation. Nature 557: 50-56, 2014.
5. Yu Y, Fisher JE, Lillegard JB, Rodysill B, Amiot B and Nyberg SL: Cell therapies for liver diseases. Liver Transpl 18: 9-21, 2012.
6. Zhao L, Chen S, Shi X, Cao H and Li L: A pooled analysis of mesenchymal stem cell-based therapy for liver disease. Stem Cell Res Ther 9: 72, 2018.
7. Chen Y, Shao JZ, Xiang LX, Dong XJ and Zhang GR: Mesenchymal stem cells: A promising candidate in regenerative medicine. Int J Biochem Cell Biol 40: 815-820, 2008.
8. Lv FJ, Tuan RS, Cheung KM and Leung VY: Concise review: The surface markers and identity of human mesenchymal stem cells. Stem Cells 32: 1408-1019, 2014.
9. Fu X, Jiang B, Zheng B, Yan Y, Wang J, Duan Y, Li S, Yan L, Wang H, Chen B, et al: Heterogenic transplantation of bone marrow-derived rhesus macaque mesenchymal stem cells ameliorates liver fibrosis induced by carbon tetrachloride in mouse. Peer J 6: e4336, 2018.
10. Marx C, Silveira MD and Beyer Nardi N: Adipose-derived stem cells in veterinary medicine: Characterization and therapeutic applications. Stem Cells Dev 24: 803-813, 2015.
11. Shi D, Zhang J, Zhou Q, Xin J, Jiang J, Jiang L, Wu T, Li J, Ding W, Li J, et al: Quantitative evaluation of human bone mesenchymal stem cells rescuing fulminant hepatic failure in pigs. Gut 66: 955-964, 2017.
12. Vezzani B, Pierantozzi E and Sorrentino V: Mesenchymal stem cells: From the perivascular environment to clinical applications. Histol Histopathol 7: 1235-1246, 2018.
13. Nicolas CT, Hickey RD, Chen HS, Mao SA, Lopera Higuita M, Wang Y and Nyberg SL: Concise review: Liver regenerative medicine: From hepatocyte transplantation to bioartificial livers and bioengineered grafts. Stem Cells 25: 42-50, 2017.
14. Barlow S, Brooke G, Chatterjee K, Price G, Pelekanos R, Rossetti T, Doody M, Venter D, Pain S, Gilshenan K and Atkinson K: Comparison of human placenta- and bone marrow-derived multipotent mesenchymal stem cells. Stem Cells Dev 17: 1095-1107, 2008.
15. Heo JS, Choi Y, Kim HS and Kim HO: Comparison of molecular profiles of human mesenchymal stem cells derived from bone marrow, umbilical cord blood, placenta and adipose tissue. Int J Mol Med 37: 115-125, 2016.
16. Strioga M, Viswanathan S, Darinskas A, Slaby O and Michalek J: Same or not the same? Comparison of adipose tissue-derived versus bone marrow-derived mesenchymal stem and stromal cells. Stem Cells Dev 21: 2724-2752, 2012.
17. Sgodda M, Aurich H, Kleist S, Aurich I, König S, Dollinger MM, Fleig WE and Christ B: Hepatocyte differentiation of mesenchymal stem cells from rat peritoneal adipose tissue in vitro and in vivo. Exp Cell Res 313: 2875-2886, 2007.
18. Oliver K, Awan T and Bayes M: Single-versus multiple-site harvesting techniques for bone marrow concentrate: Evaluation of aspirate quality and pain. Orthop J Sports Med 5: 232596711772439, 2017.
19. Mizuno H, Tobita M and Uysal AC: Concise review: Adipose-derived stem cells as a novel tool for future regenerative medicine. Stem cells 30: 804-810, 2012.
20. Aurich H, Sgodda M, Kaltwasser P, Vetter M, Weise A, Liehr T, Brulport M, Hengstler JG, Dollinger MM, Fleig WE and Christ B: Hepatocyte differentiation of mesenchymal stem cells from human adipose tissue in vitro promotes hepatic integration in vivo. Gut 58: 570-581, 2009.
21. Sun J, Yuan Y, Qin H, Ying C, Liu W, Zhang J, He Y and Liu Z: Serum from hepatectomized rats induces the differentiation of adipose tissue mesenchymal stem cells into hepatocyte-like cells and upregulates the expression of hepatocyte growth factor and interleukin-6 in vitro. Int J Mol Med 31: 667-675, 2013.
22. Xu LJ, Wang SF, Wang DQ, Ma LJ, Chen Z, Chen QQ, Wang J and Yan L: Adipose-derived stromal cells resemble bone marrow stromal cells in hepatocyte differentiation potential in vitro and in vivo. World J Gastroenterol 23: 6973-6982, 2017.
23. Pennington MR, Curtis TM, Divers TJ, Wagner B, Ness SL, Tennant BC and Van de Walle GR: Equine mesenchymal stromal cells from different sources efficiently differentiate into hepatocyte-like cells. Tissue Eng Part C Methods 22: 596-607, 2016.
24. Fu X, Yan Y, Li S, Wang J, Jiang B, Wang H, Duan Y, Tan T, Gao F, Gong D, et al: Vitrification of rhesus macaque mesenchymal stem cells and the effects on global gene expression. Stem Cells Int 2017: 3893691, 2017.
25. Jiang B, Fu X, Yan L, Li S, Zhao D, Wang X, Duan Y, Yan Y, Li E, Wu K, et al: Transplantation of human ESC-derived mesenchymal stem cell spheroids ameliorates spontaneous osteoarthritis in rhesus macaques. Theranostics 9: 6587-6600, 2019.
26. Khanjani S, Khanmohammadi M, Zarnani AH, Akhondi MM, Ahani A, Ghaempanah Z, Naderi MM, Eghtesad S and Kazemnejad S: Comparative evaluation of differentiation potential of menstrual blood- versus bone marrow-derived stem cells into hepatocyte-like cells. PLoS One 9: e86075, 2014.
27. Li Y, Wang Y, Wu Q, Li L, Shi Y, Bu H and Bao J: Comparison of methods for isolating primary hepatocytes from mini pigs. Xenotransplantation 23: 414-420, 2016.
28. Sarvandi SS, Joghataei MT, Parivar K, Khosravi M, Sarveazad A and Sanadgol N: In vitro differentiation of rat mesenchymal stem cells to hepatocyte lineage. Iran J Basic Med Sci 18: 89-97, 2015.
29. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(Delta Delta C(T)) method. Methods 25: 402-408, 2001.
30. Pournasr B, Mohamadnejad M, Bagheri M, Aghdami N, Shahsavani M, Malekzadeh R and Baharvand H: In vitro differentiation of human bone marrow mesenchymal stem cells into hepatocyte-like cells. Arch Iran Med 14: 244-249, 2011.
31. Al Battah F, De Kock J, Vanhaecke T and Rogiers V: Current status of human adipose-derived stem cells: Differentiation into hepatocyte-like cells. ScientificWorldJournal 11: 1568-1581, 2011.
32. Yu J, Cao H, Yang J, Pan Q, Ma J, Li J, Li Y, Li J, Wang Y and Li L: In vivo hepatic differentiation of mesenchymal stem cells from human umbilical cord blood after transplantation into mice with liver injury. Biochem Biophys Res Commun 422: 539-545, 2012.
33. Mou XZ, Lin J, Chen JY, Li YF, Wu XX, Xiang BY, Li CY, Ma JM and Xiang C: Menstrual blood-derived mesenchymal stem cells differentiate into functional hepatocyte-like cells. J Zhejiang Univ Sci B 14: 961-972, 2013.
34. Kwon A, Kim Y, Kim M, Kim J, Choi H, Jekarl DW, Lee S, Kim JM, Shin JC and Park IY: Tissue-specific differentiation potency of mesenchymal stromal cells from perinatal tissues. Sci Rep 6: 23544, 2016.
35. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj and Horwitz E: Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8: 315-317, 2006.
36. Hu C and Li L: In vitro culture of isolated primary hepatocytes and stem cell-derived hepatocyte-like cells for liver regeneration. Protein Cell 6: 562-574, 2015.

37. Shi X, Lv S, He X, Liu X, Sun M, Li M, Chi G and Li Y: Differentiation of hepatocytes from induced pluripotent stem cells derived from human hair follicle mesenchymal stem cells. Cell Tissue Res 366: 1-11, 2016.

38. Ayatollahi M, Soleimani M, Tabei SZ and Kabir Salmani M: Hepatogenic differentiation of mesenchymal stem cells induced by insulin like growth factor-1. World J Stem Cells 3: 113-121, 2011.

39. Borhani-Haghighi M, Talaei-Khozani T, Ayatollahi M and Vojdani Z: Wharton's jelly-derived mesenchymal stem cells can differentiate into hepatocyte-like cells by hepg2 cell line extract. Iran J Med Sci 40: 143-151, 2015.

40. Wang B, Li W, Dean D, Mishra MK and Wekesa KS: Enhanced hepatogenic differentiation of bone marrow derived mesenchymal stem cells on liver ECM hydrogel. J Biomed Mater Res A 106: 829-838, 2018.

41. Zhang YN, Lie PC and Wei X: Differentiation of mesenchymal stromal cells derived from umbilical cord Wharton's jelly into hepatocyte-like cells. Cytotherapy 11: 548-558, 2009.

42. Khanjani S, Khanmohammadi M, Zarnani AH, Talebi S, Edalatkhah H, Eghtesad S, Nikokar I and Kazemnejad S: Efficient generation of functional hepatocyte-like cells from menstrual blood-derived stem cells. J Tissue Eng Regen Med 9: E124-E134, 2015.

43. Yang XF, Ren LW, Yang L, Deng CY and Li FR: In vivo direct reprogramming of liver cells to insulin producing cells by virus-free overexpression of defined factors. Endocr J 64: 291-302, 2017.

44. Sa-ngiamsuntorn K, Wongkajornsilp A, Kasetsinsombat K, Duangsa-ard S, Nuntakarn L, Borwornpinyo S, Akarasereenont P, Limsrichamrern S and Hongeng S: Upregulation of CYP 450s expression of immortalized hepatocyte-like cells derived from mesenchymal stem cells by enzyme inducers. BMC Biotechnol 11: 89, 2011.