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*

JIANGYANG SUN[1*], YUFENG YUAN[1*], HAIQUAN QIN[1], CHENGCHENG YING[2], WEIJUN LIU[1], JIN ZHANG[1], YUEMING HE[1] and ZHISU LIU[1]

Departments of [1]General Surgery, [2]Urology Surgery, Zhongnan Hospital of Wuhan University, Wuhan, Hubei 430071, P.R. China

Received October 27, 2012; Accepted December 19, 2012

DOI: 10.3892/ijmm.2013.1257

Abstract. Adipose tissue-derived mesenchymal stem cells (AT-MSCs) are an attractive alternative for clinical application due to their minimally invasive accessibility and availability in the body. However, the hepatic differentiation efficiency of AT-MSCs is insufficient for therapeutic application and the role of extrahepatic stem cells in liver regeneration remains poorly understood. This study was designed to investigate the effects of serum from rats subjected to 70% partial hepatectomy (PH) on the differentiation ability of rat AT-MSCs *in vitro*, and to explore the potential role of AT-MSCs *in vivo* following PH injury. Results showed that AT-MSCs treated with serum collected from rats 24 h after 70% PH differentiated into hepatocyte-like cells, resembled hepatocyte-like cells with round or polygonal shape, expressed α-fetoprotein, secreted albumin, synthesized urea and acquired cytochrome P450 type 3A4 enzyme activity, and upregulated the expression of interleukin (IL)-6 and hepatocyte growth factor (HGF) transiently *in vitro*, although the hepatic differentiation efficiency was extremely low. AT-MSC transplantation after 70% PH ameliorated liver injury and promoted liver regeneration, but did not increase the serum levels of IL-6 and HGF *in vivo*. This result suggests that the therapeutic effect of AT-MSCs *in vivo* after 24 h of 70% PH does not increase IL-6 and HGF expression.

Correspondence to: Dr Zhisu Liu, Department of General Surgery, Zhongnan Hospital of Wuhan University, Wuhan, Hubei 430071, P.R. China
E-mail: liuzhisu53@163.com

*Contributed equally

Abbreviations: PH, partial hepatectomy; AFP, α-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DAPI, 4',6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; IL, interleukin; mRNA, messenger RNA; MSC, mesenchymal stem cell; PBS, phosphate-buffered saline; RT-PCR, reverse transcription polymerase chain reaction; CYP3A4, cytochrome P450 type 3A4

Key words: mesenchymal stem cells, interleukin 6, hepatocyte growth factor, hepatic differentiation, partial hepatectomy, transplantation

Introduction

Accumulating evidence suggests that adipose tissue-derived mesenchymal stem cells (AT-MSCs) possess the ability to transdifferentiate into other cell types including hepatocytes, similar to bone marrow-derived stem cells (BMSCs). Previous studies have shown that AT-MSCs differentiate into hepatocyte-like cells by culture with appropriate culture conditions, such as various cytokine mixtures (1-5). However, the efficacy of the hepatic differentiation of AT-MSCs is insufficient for therapeutic application and the role of extrahepatic stem cells in liver regeneration remains poorly understood. A successful differentiation is generally achieved by the step-by-step addition of growth factors, cytokines, and hormones, which emulate the sequence of events occurring during *in vivo* hepatogenesis (6). Therefore, studies have been conducted to explore novel means of facilitating differentiation of MSCs into hepatocytes. At present, no induction method can completely represent or mimic the pathological environment of the various types of liver diseases, as there is a marked regulation and complicated interaction *in vivo* under certain pathological environments. For example, liver regeneration after partial hepatectomy (PH) injury is an extremely complex and well-orchestrated phenomenon (7). PH triggers a sequence of events that proceed in an orderly manner and are observed from the first 5 min to 5-7 days, and induces rapid induction of >100 genes not expressed in normal liver (8). A number of cytokines are upregulated during acute liver injury, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), hepatocyte growth factor (HGF), transforming growth factor-α (TGF-α), macrophage inflammatory protein-2 (MIP-2), stem cell factor (SCF), and various other cytokines (9-11).

Changes in the concentrations of the above signaling molecules in the plasma likely account for the fact that the signals for liver regeneration are transmitted by blood in pairs of parabiotic rats (12) or isolated hepatocytes in the

adipose tissue when the orthotopic liver is subjected to partial hepatectomy (13). By mimicking the injured liver microenvironment, Wang *et al* (14) isolated BMSCs to induce differentiation of these cells into hepatocyte-like cells with HGF *in vitro*. Yang *et al* also reported that serum from liver radiofrequency ablation (RFA)-treated rats induced the differentiation of BMSCs into hepatic progenitor cells more efficiently compared with HGF (15), suggesting that serum under certain pathological environments may provide the ideal induction conditions to study unknown events that BMSCs may be involved in *in vivo*. Therefore, the present study aimed to determine whether serum from hepatectomized rats is able to induce the differentiation of AT-MSCs into hepatocyte-like cells, and to explore the possible role of AT-MSCs *in vivo* during liver regeneration.

Materials and methods

Materials. Low-glucose Dulbecco's modified Eagle's medium (L-DMEM), fetal bovine serum (FBS) (both were from Invitrogen Life Technologies, Carlsbad, CA, USA), and 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA; Sino-American Company, Shanghai, China) were used for cell cultures. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.

Experimental animals. Sixty-five male Sprague-Dawley (SD) rats weighing 140-160 g were obtained from the Laboratory Animals Center of Wuhan University (Wuhan, Hubei, China). Experiment procedures were carried out in accordance with the Code of Ethics of the World Medical Association.

Isolation and culture of AT-MSCs. AT-MSCs were isolated from the groin adipose tissue of SD rats. The capillaries of the adipose tissue were removed and washed three times with phosphate-buffered solution (PBS) under aseptic conditions. The adipose tissue was then minced into 3-5 mm sections using scissors and scalpels prior to digestion using 0.1% collagenase solution for 50 min in a water bath at 37˚C. The mixture was centrifuged at 1,500 rpm for 8 min at room temperature (25˚C), and the supernatant containing adipose tissue was discarded. After the collected cells were washed three times with PBS (1,500 rpm, 8 min), they were resuspended in L-DMEM containing 15% FBS, 1% L-glutamine, and 1% penicillin and streptomycin. Cells ($1x10^6$ cells/ml)were seeded in culture flasks (T25; Corning Inc., Corning, NY, USA) and maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C. When the cells reached 80-90% confluence, they were collected with 0.25% Trypsin-EDTA and diluted 1:2 or 1:3 at each passage.

Flow cytometry (FACS) analysis. AT-MSCs of passage 3 (P3) were analyzed using a FACSCalibur (FC500) Flow Cytometer (Becton-Dickinson, San Jose, CA, USA). PE-conjugated CD34 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and fluorescein isothiocyanate (FITC)-conjugated CD44, CD45 and CD90 (AbD Serotec) antibodies were employed.

Differentiation potential of AT-MSCs. AT-MSCs from P3 to passage 5 (P5) were seeded into 6-well plates at $3x10^4$ cells/cm^2 and grown to 90% confluence for the differentiation studies mentioned above. The cells were treated with each induction medium for at least two weeks, changing the medium twice a week.

Adipogenic differentiation protocol. The adipogenic medium consisted of L-DMEM supplemented with 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 200 μM indomethacin, 10 μg/ml bovine insulin, and 1% penicillin and streptomycin.

Osteogenic differentiation protocol. Osteogenic medium consisted of L-DMEM supplemented with 10% FBS, 0.1 M dexamethasone, 0.3 mM ascorbic acid, and 10 mM β-glycerol phosphate.

Hepatic differentiation protocol. Hepatic differentiation was induced over a period of two weeks based on the published differentiation protocols (16) with minor modifications. The hepatic differentiation medium consisted of L-DMEM supplemented with 10% FBS, 10 μg/ml transferrin, 10 μg/ml insulin, 0.1 mM ascorbic acid, 0.1 μM dexamethasone, 10 ng/ml HGF (PeproTech; Rocky Hill, NJ, USA), 10 ng/ml bFGF (PeproTech), and 10 ng/ml OSM (PeproTech).

Immunofluorescence analysis. On days 7 and 14, samples of cultured cells were rinsed and fixed with 4% paraformaldehyde at 4˚C for 30 min. The cells were permeabilized with 0.1% Triton X-100 for 15 min. After blocking the fixed cells for 30 min at 37˚C with 1% bovine serum albumin (ALB), the cells were incubated overnight at 4˚C in a humidity chamber with the respective primary antibodies: rabbit polyclonal anti-ALB (1:100) and goat polyclonal anti-α-fetoprotein (AFP; 1:100) (both from Santa Cruz Biotechnology, Inc.). The cells were then incubated with the corresponding secondary antibody: rhodamine (TRITC)-conjugated donkey anti-rabbit IgG (1:100) and FITC-conjugated Affinipure donkey anti-goat IgG (1:100) (both from Proteintech Group, Inc., Chicago, IL, USA) for 30 min at room temperature. After rinsing, the nuclei were counterstained with 5 μg/ml 4',6-diamidino-2-phenylindole (DAPI; Beyotime, China). The cells were then visualized with a fluorescence microscope (Olympus BX51, Japan), and the images were analyzed using Image-Pro Plus 6.0.

Hepatic basal function evaluation of the differentiation cells. Cells differentiated on days 0, 3, 6, 9, 12, 15, 18, 21 and 24 were extensively washed with PBS, and then incubated with L-DMEM containing 5 mM NH_4Cl overnight (12 h) in 5% CO_2 at 37˚C prior to analysis. The supernatant was collected and the urea concentration was measured using a colorimetric assay kit (BioVision, USA) according to the manufacturer's instructions.

ALB production was evaluated on days 0, 6, 12, 18 and 24 using a quantitative enzyme-linked immunoassay kit (rat albumin ELISA; ALPCO Diagnostics, Salem, NH, USA) according to the manufacturer's instructions.

Cytochrome P450 type 3A4 (CYP3A4) enzyme activity was assessed by measurement of luciferase activity with the P450-Glo™ CYP3A4 Assay (Luciferin-PFBE; Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, the cells were cultured in L-DMEM supplemented with 1 mmol/l phenobarbital (Sigma) for 48 h with a daily medium change. The cells were incubated at

Table I. Primer sequences and product sizes.

Gene name	Primer sequences		Product size (bp)	T(˚C) annealing
IL-6	Sense	5'-GCCTTCTTGGGACTGATGT-3'	541	61
	Antisense	5'-TGAGTTGGATGGTCTTGGT-3'		
HGF	Sense	5'-ACATCACTCCCGAGAACTT-3'	520	61
	Antisense	5'-AAACTAACCATCCACCCTAC-3'		
GAPDH	Sense	5'-CAGTGCCAGCCTCGTCTCAT-3'	595	65
	Antisense	5'-AGGGGCCATCCACAGTCTTC-3'		

IL-6, interleukin-6; HGF, hepatocyte growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

37˚C in L-DMEM supplemented with 50 μmol/l luciferin PFBE (150 μl/well). After 3 h of incubation, 50 μl of medium was transferred in a opaque white 96-well plate and mixed with 50 μl of luciferin detection reagent to initiate luminescent reaction. After 20-min incubation at room temperature, luminescence was measured with a Victor 3 luminometer (Perkin-Elmer, Boston, MA, USA).

Establishment of 70% PH and collection of post-hepatectomy serum. The 70% PH procedure was performed through a midline abdominal incision (17) under anesthesia with sodium pentobarbital (35-40 mg/kg, i.m. n=5). For the sham operation (n=5), a similar surgical approach was used but only a portion of the xiphoid was excised. Approximately 24 h after surgery, blood samples were collected from the ventricle. The blood samples were placed at 4˚C for 2 h and centrifuged at 3,000 rpm for 10 min (4˚C). The upper layer of each blood sample was aliquoted into microcentrifuge tubes and stored at -80˚C for future use. Surgery was performed using sterile surgical techniques. Surgical procedures were performed between 8 and 11 a.m. to minimize the influence of diurnal rhythms on subsequent hepatocyte proliferation.

AT-MSCs treated with post-hepatectomy serum. P3-P5 AT-MSCs were induced to differentiate under the following conditions: i) FBS group, L-DMEM supplemented with 10% FBS; ii) serum group, L-DMEM supplemented with 70% PH serum to a final concentration of 10%; iii) HGF group, induction composition is the same as the hepatic differentiation medium; iv) Sham group, L-DMEM supplemented with serum from rats that had undergone the sham operation to a final concentration of 10%. On days 7 and 14, the cell type was identified. The abovementioned induction experiments were repeated using three sets of serum (the serum collected from one rat representing one set).

AT-MSC transplantation following PH. Approximately 24 h after 70% PH, ~1x10^6 cells/rat in 200 μl of PBS were transfused into the tail vein with a 30-gauge needle for >1 min (AT-MSC group, n=12). Rats treated with PBS alone (PBS group, n=12) and untreated rats (untreated group, n=12) served as the control groups. The animals were sacrificed at 48 and 72 h after PH (n=6 for each time-point in all treatment groups). Blood samples were collected from the ventricle and the residual livers were harvested and weighed, and then properly preserved for the subsequent procedures.

IL-6 and HGF concentration analysis by ELISA. On days 3 and 6 of the cell culture, the culture medium was removed and the cells were washed twice with PBS and replenished with serum-free L-DMEM. After 24 h, the culture supernatant was harvested and centrifuged at 3,000 rpm for 10 min at 25˚C. The concentrations of IL-6 and HGF were measured following the manufacturer's instructions using commercial ELISA kits (Quantikine Mouse IL-6 ELISA kit and Mouse HGF Duoset ELISA Development kit; Biotechnology Systems). The cells in the plate were harvested by 0.25% trypsin-EDTA digestion, and the number of cells was determined by hematocytometer counts. The total RNA was then isolated.

Reverse transcription polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated from AT-MSCs cultured in each group for mRNA expression analysis. cDNA synthesis and the polymerase chain reaction (PCR) were performed using the ReverTra Ace Reverse Transcriptase kit (Toyobo Co., Ltd., Tokyo, Japan) and the Hot Start PCR kit (Takara), respectively, following the manufacturer's instructions. The PCR primer sequences and conditions are shown in Table I. The PCR reaction was 30 cycles.

Assessment of liver functions. The serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and ALB, were measured using a multiple serum biochemical analyzer (Hitachi, Japan).

Liver regeneration rate. The total body weight of each rat that fasted was weighed before it was sacrificed. The residual liver was then excised and weighed. The ratio of the residual liver weight to the whole body weight was utilized instead of the weight of the residual liver alone.

Histology and immunohistochemistry. For conventional morphological evaluation, liver tissue was fixed with 10% formalin, embedded with paraffin, sectioned, and stained with hematoxylin and eosin using standard histological techniques. The sections were also immunostained for Ki-67 according to the manufacturer's instructions. The samples were coun-

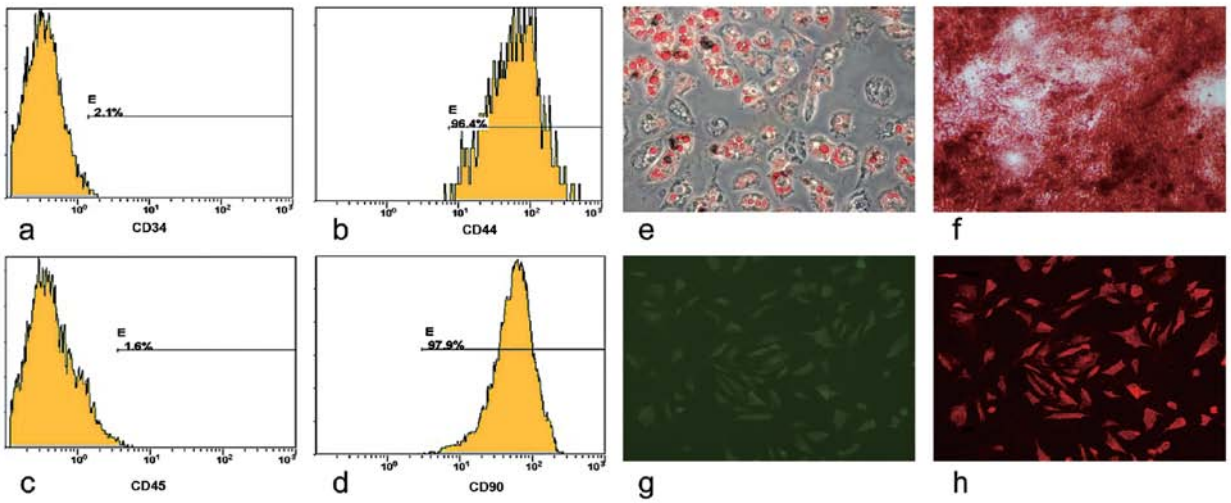

Figure 1. Cell surface markers and multi-lineage differentiation of isolated AT-MSCs. AT-MSCs were positive for (b) CD44 and (d) CD90 and negative for (a) CD34 and (c) CD45. (e) Adipogenic differentiation (Oil Red O staining, x200). (f) Osteogenic differentiation (Alizarin-Red staining, x200). (g and h) Hepatic differentiation, AFP (green) and ALB (red). Immunofluorescent staining shows the cells on the slide (x400).

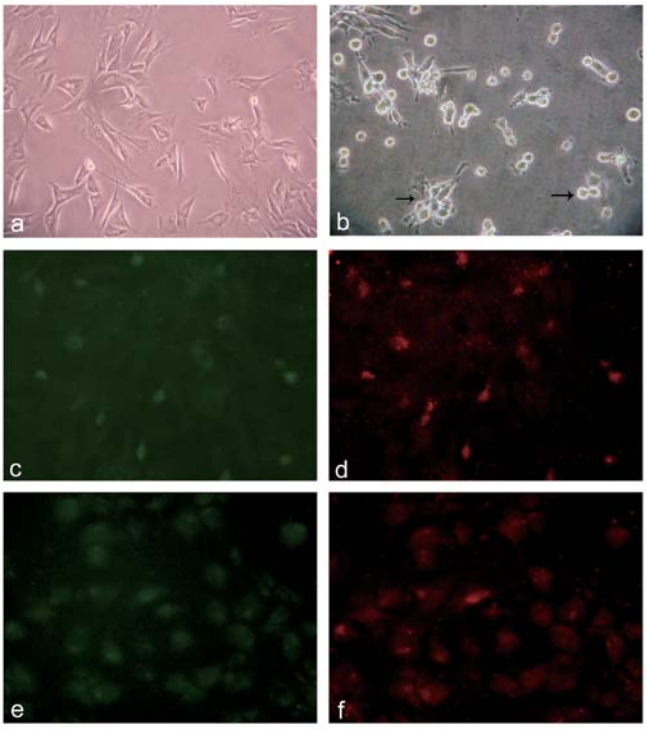

Figure 2. AT-MSCs were treated for two weeks with post-hepatectomy serum. Two weeks later, some of the spindle-shaped AT-MSCs gradually transformed into hepatocyte-like round or polygonal cells, the ratio of nucleoplasm decreased, adhesion ability weakened and was associated with a reduced number of cells in the serum group at the time of medium change. (a) Prior to induction, (b) two weeks after induction in the serum group. (e and f) More positive cells were observed in the HGF group compared with (c and d) the serum group. (Positive cell ratio was 53.12±0.76 vs. 24.35±2.0, P<0.001). The positive cells were counted in at least five randomly selected high-power fields (x400) per slide.

terstained with hematoxylin. The Ki-67-positive cells were counted in at least five randomly selected high-power fields (x400) per slide.

Statistical analysis. Data are presented as the mean ± standard deviations. Statistical significance was calculated using the SPSS 19.0 program. The Student's t-test was used for the pairwise comparisons and one-way ANOVA was used for the multiple comparisons. P<0.05 was considered statistically significant.

Results

Characterization of AT-MSCs. The FACS analysis demonstrated that AT-MSCs expressed CD44 and CD90 (Fig. 1b and d), but not CD34 and CD45 (Fig. 1a and c). Two weeks after their exposure to adipogenic and four weeks after exposure to osteogenic induction medium, intracellular lipid droplets (Fig. 1e) and extracellular calcium phosphate precipitates (Fig. 1f) were observed using Oil Red O and Alizarin-Red staining. Two weeks after the induction of hepatic differentiation, AFP (green) and ALB (red) were observed using immunofluorescence (Fig. 1g and h).

Morphological observations and immunofluorescence staining of AT-MSCs cultured with post-hepatectomy serum. After culturing the cells with the serum for one week, some spindle-shaped cells transformed into round or polygonal hepatocyte-like cells gradually, the ratio of the nucleoplasm decreased, and adhesion ability weakened and was associated with a reduced number of cells in the serum group at the time of the medium change (Fig. 2a and b). By contrast, the cells in the FBS and Sham groups maintained the spindle-shaped phenotype of AT-MSCs. Following the two-week induction, the green fluorescence of the FITC-labeled AFP and the red fluorescence of the TRITC-labeled ALB were observed both in the serum and HGF groups (Fig. 2c-f). However, more positive cells were observed in the HGF group compared with the serum group (positive cell ratio, 53.12±0.76 vs. 24.35±2.0, P<0.001).

ALB secretion, urea synthesis and CYP3A4 enzyme activity. The cells produced ALB during differentiation and the

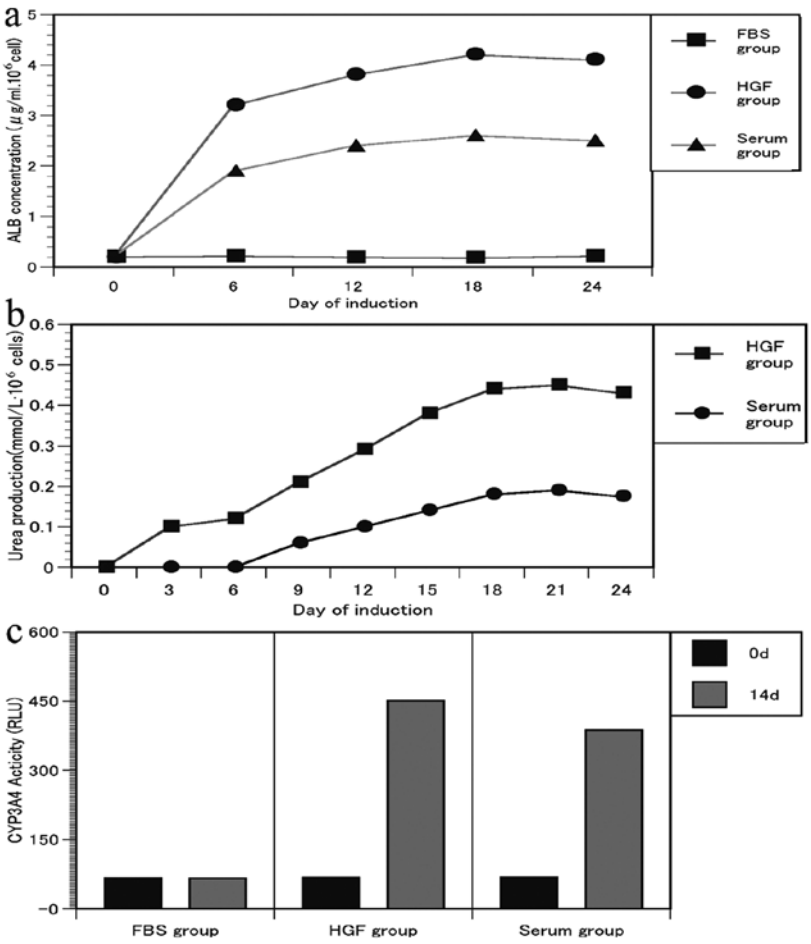

Figure 3. Functional characterization of AT-MSC-derived hepatocytes. Differentiation efficiency was evaluated at the functional level. (a) The cells in the HGF and serum groups produced ALB during differentiation and the secreted ALB increased 2-fold during the 24 days of differentiation. Urea levels gradually increased until day 21 in a time-dependent manner in the HGF and serum groups. (b) AT-MSCs in the FBS and Sham groups did not produce urea. (c) Activity of the CYP3A4 enzyme was significantly increased in differentiated AT-MSCs after 14 days of culture under differentiating conditions compared with undifferentiated AT-MSCs. The changes identified suggest that the AT-MSCs had undergone hepatocyte maturation.

secreted ALB increased 2-fold during the 24 days of differentiation (Fig. 3a). ALB was almost not detected in supernatants of the FBS group.

Urea production was present and the urea levels gradually increased until day 21 in a time-dependent manner in the HGF and serum groups (Fig. 3b). Cells in the FBS and Sham groups did not produce urea (data not shown).

Activity of the CYP3A4 enzyme was detected in differentiated AT-MSCs and was significantly enhanced when cells were cultured with 1 mmol/l phenobarbital compared with undifferentiated AT-MSCs (Fig. 3c). These changes suggest that the AT-MSCs had undergone hepatocyte maturation.

mRNA expression and cytokine concentration in culture supernatant. An increase in IL-6 and HGF levels was detected (day 3) in the culture supernatant in the HGF and serum groups (Fig. 4b) ($P<0.01$) compared with the FBS and Sham groups. However, these levels significantly decreased on day 6 ($P<0.05$), coinciding with the changes in mRNA expression (Fig. 4a).

Effect of AT-MSC transplantation on liver function after 70% PH. Approximately 24 h after AT-MSC transplantation, the ALT and AST levels decreased to a level lower than that of the PBS and untreated groups, and ~48 h after the transplantation, this change became more pronounced (Fig. 5a and b) ($P<0.001$). Simultaneously, ~48 h after AT-MSC transplantation, the ALB level in the AT-MSC group significantly increased compared with that in the PBS and untreated groups (Fig. 5c) ($P<0.01$). Thus, AT-MSC transplantation attenuated hepatocyte injury and improved the protein synthesis ability of the hepatocytes.

IL-6 and HGF concentration in serum after AT-MSC transplantation. After 70% PH, the serum levels of HGF and IL-6 in the PBS and untreated groups peaked within 24 h, gradually decreased, and then reached normal levels after 72 h (Fig. 5d and e). However, the HGF and IL-6 levels in the AT-MSC group were significantly decreased after 24 h of the transplantation ($P<0.01$) compared with the PBS and untreated groups. This decrease was even closer to that of their normal levels, showing that AT-MSC transplantation altered HGF and IL-6 expression *in vivo*.

Histological examination of livers. Approximately 24 h after 70% PH, the hepatic sinusoidal structure was well preserved in

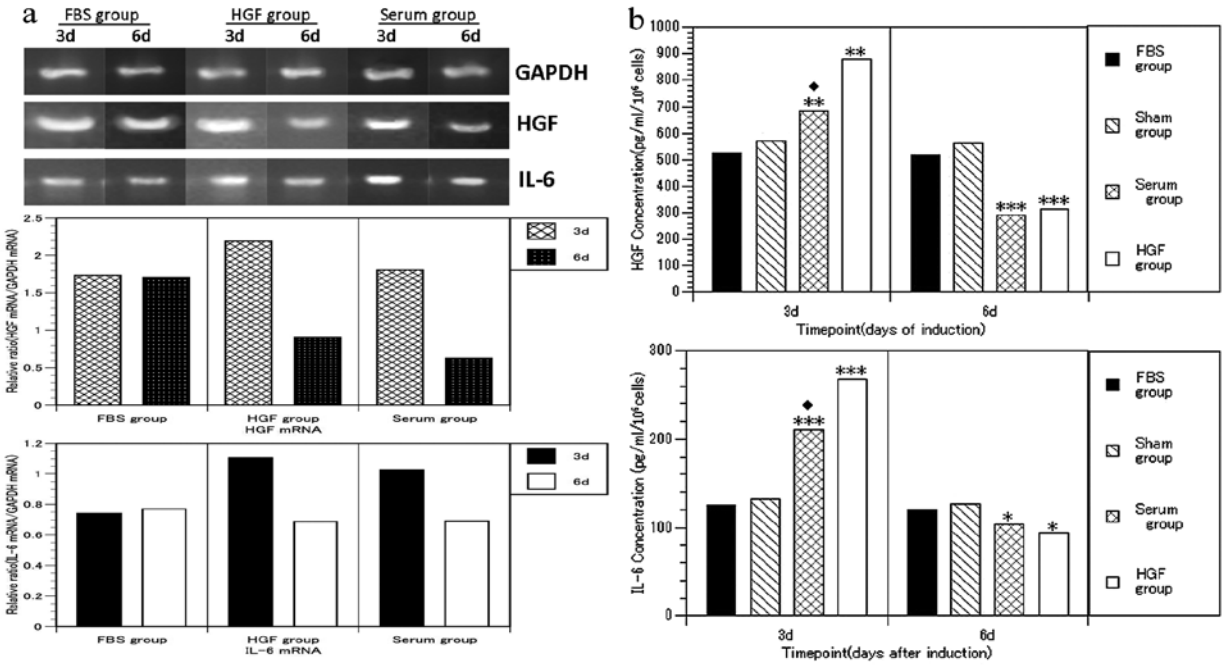

Figure 4. mRNA expression and HGF and IL-6 concentration in culture supernatant. (a) Total RNA of AT-MSCs was harvested 7 days (d7) and 14 days (d14) during induction and analyzed by RT-PCR for cytokine genes/growth factors. GAPDH was used as an internal reference to determine the expression of the FBS, HGF and PH groups. (b) HGF and IL-6 concentration change in the supernatant of culture cells. A transient increase (day 3) was noted in the culture supernatant in the HGF and serum groups. (***P<0.001, **P<0.01, *P<0.05, compared with other groups, respectively; ♦P<0.01, serum group compared with HGF group).

all the samples, and inflammation or necrosis was not present. Histopathologic examination showed vacuolar degeneration (Fig. 6a, arrows) and mild central vein dilatation (Fig. 6a and c, asterisks). These features are commonly observed after liver resection. Approximately 24 h after AT-MSC transplantation, the injured livers were found to have less vacuolar degeneration than those in the PBS group (Fig. 6b), and the changes were more apparent 48 h after the transplantation (Fig. 6d). Approximately 48 h after 70% PH, all the groups exhibited a similar proliferation rate, although the proliferation rate 72 h after 70% PH was significantly increased (Fig. 6e and f) in the AT-MSC group compared with the PBS group (Fig. 6g; Ki-67-positive ratio, 48.07±0.88 vs. 35.33±0.81, P<0.001), and the residual liver weight was also increased in the AT-MSC group (Fig. 6h) (P<0.01).

Discussion

The 70% PH model is a classical model for studying liver regeneration, in which many cytokines and growth factors that favor liver regeneration are released into the blood via the injured liver tissue. Different studies have suggested that various paracrine factors secreted by injured liver tissue have an important role in stem cell recruitment and engraftment. Those chemokines allow MSCs to enter the circulation prior to returning to the injured liver (18-20). In the present study, serum from PH-induced acute liver injury was used as an agent to induce differentiation of AT-MSCs for the first time. As a result, these cells resembled hepatocyte-like cells with round or polygonal shape, expressed α-fetoprotein, secreted ALB, synthesized urea, and acquired CYP450 3A4 enzyme activity, showing that these cells partially function as mature hepatocytes. However, the hepatic differentiation efficiency of AT-MSCs in the PH serum group was significantly lower than that in the HGF group. This result was different to that obtained by Yang *et al* (15) who showed that the serum collected after 24 h of 70% PH did not exhibit appropriate culture conditions to promote the hepatic differentiation of AT-MSCs. It is known that in a typical wound healing scenario, the injury to the tissue results in disruption of capillary vascular networks and extravasation of blood, accompanied by local release of coagulation factors, platelets and growth factors (21). This does not occur after 70% PH. Various changes in hepatic blood flow patterns and oxygen partial pressure occur following the surgical removal of three liver lobes without damage to the two residual lobes. The two types of pathological processes of liver injury (PH and RFA injury) render the composition in the serum markedly different, leading to significantly different induction results, although the detailed mechanism of action remains to be elucidated.

The therapeutic effect of MSC transplantation is promising. However, studies have demonstrated that the rate of MSCs differentiating into liver cells *in vivo* was low (22,23). Previous studies have suggested that <3% of transplanted MSCs are attributed to the total organ mass (24-26). Moreover, even induced hepatocyte-like cells expressing a certain number of markers do not necessarily indicate that these cells possess similar functions to liver cells. MSCs act as 'trophic mediators' (27-32), which by secretion of bioactive factors act as either immunosuppressors or promoters of regeneration (29,33,34). A previous study has also shown that AT-MSCs produce significantly more HGF, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor

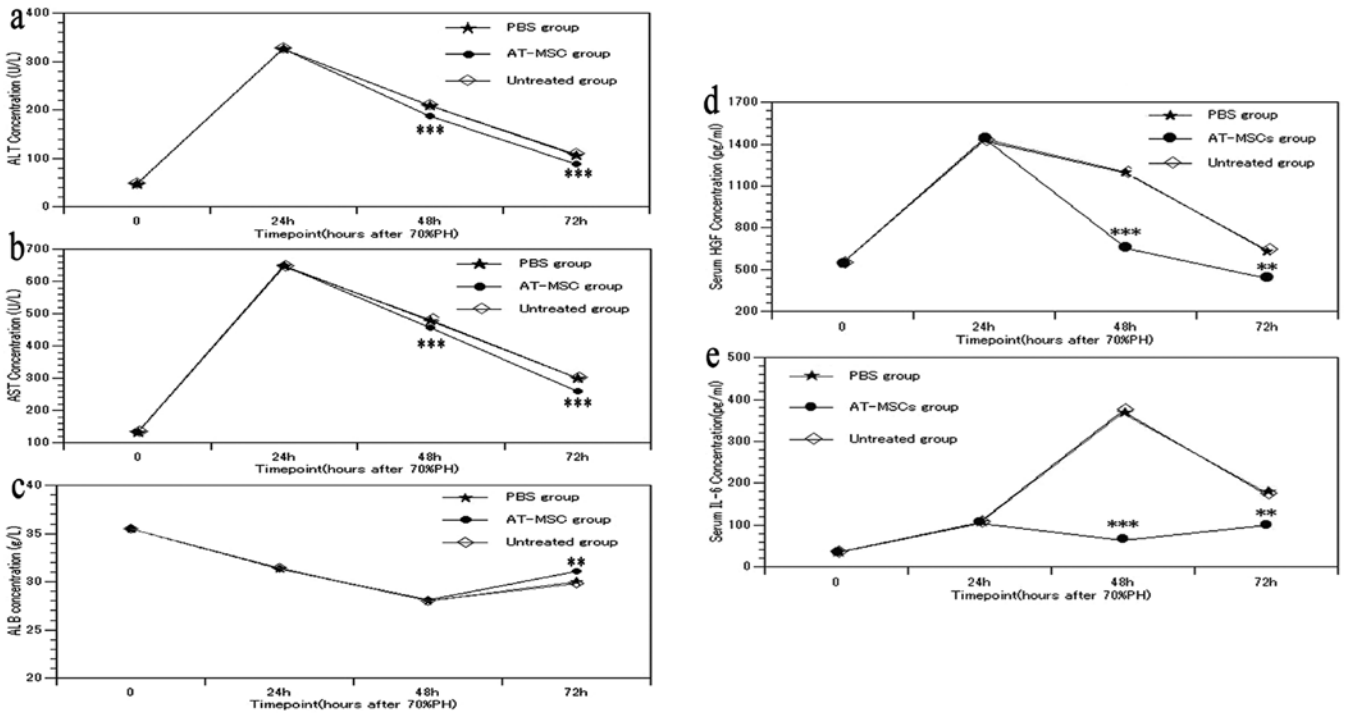

Figure 5. Liver function test and concentration of cytokines in post-hepatectomy serum at different time-points after 70% PH. The levels of (a) ALT and (b) AST were slightly decreased in the AT-MSC group at 48 h after 70% PH. (c) ALB levels were markedly increased in the AT-MSC group at 72 h after 70% PH. (***P<0.001, **P<0.01, compared with the PBS and untreated groups, respectively). (d) HGF and (e) IL-6 levels in the AT-MSC group were significantly decreased 24 h after the transplantation. (***P<0.001, **P<0.01, *P<0.05, compared with the PBS and untreated group, respectively).

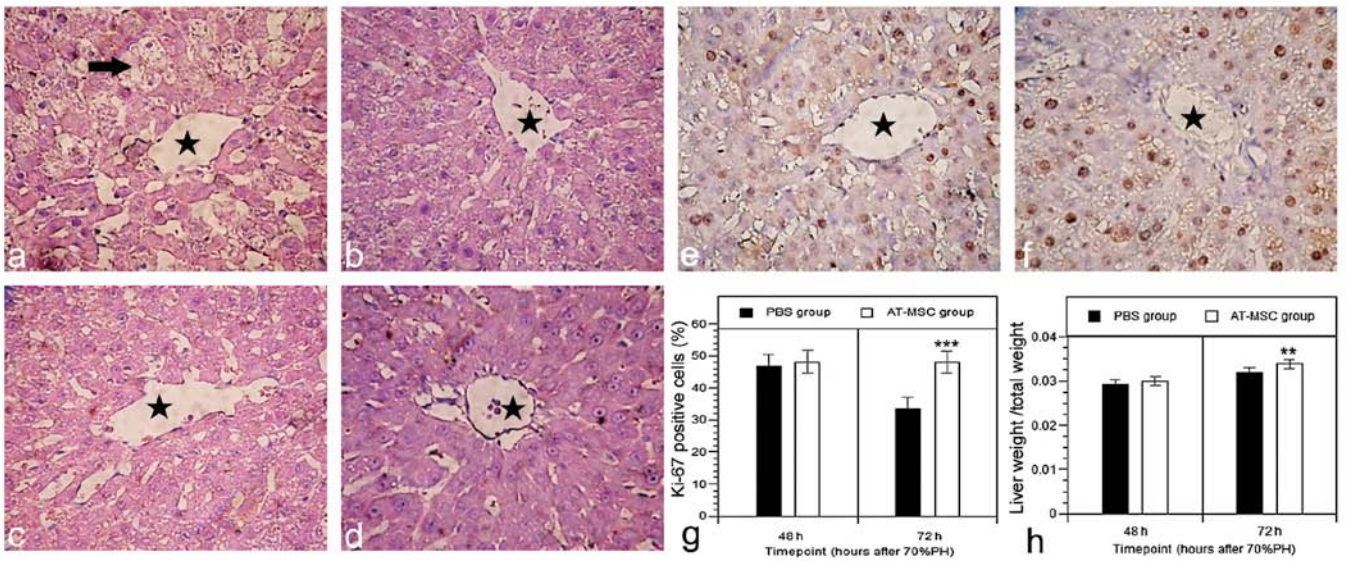

Figure 6. Histological examination at different time-points following AT-MSC administration with (a and d) hematoxylin and eosin staining (x400). The arrow shows vacuolar degeneration of hepatocytes, the star shows the central vein. (c) Twenty-four hours after AT-MSCs transplantation, (d) the injured livers were found to have less vacuolar degeneration, and the changes were more apparent 48 h after the transplantation compared with those in the PBS group. (a and b) Twenty-four and 48 h after PBS administration. (e and f) Immunohistochemistry staining of Ki-67 at 48 h after AT-MSCs administration (x400). The number of (g) Ki-67-positive cells and ratio of (h) residual liver weight to the whole body weight (***P<0.001, **P<0.01 compared with the PBS group, respectively.).

(GM-CSF), and IL-6 compared with BMSCs and normal human dermal fibroblasts (NHDFs) (35). HGF is an important factor in the process of differentiation from BMSCs to hepatocytes and osteoblasts (36,37), IL-6 is necessary and sufficient for enhanced MSC proliferation, and inhibits adipogenic and chondrogenic differentiation (38). Moreover, an increase in the mRNA expression levels of IL-6 was observed when BMSCs were co-cultured with hepatocytes from GaIN-induced injured liver (39). Therefore, we examined the expression of HGF and IL-6 during the induction process.

In this study, we found that AT-MSCs briefly upregulated the mRNA expression of HGF and IL-6 when cultured with the post-hepatectomy serum. The concentration of HGF and IL-6 in the supernatant, as detected by ELISA, also conformed with the change of the mRNA expression. Previous studies have shown that HGF is a potent mitogen *in vivo*, and that an increasing HGF expression benefits liver injury attenuation (40,41). It has also been reported that implantation of the human HGF-expressing MSCs, improves liver regeneration, reduces mortality in rats, and has a potent anti-fibrotic effect on the small-for-size liver transplantation model (42). Thus, the upregulated expression of HGF and IL-6 may be the effector molecules involved in the promotion of liver regeneration *in vivo*. To validate our hypothesis, we transplanted AT-MSCs into the rat tail vein at 24 h after 70% PH to observe the concentration changes of HGF and IL-6 *in vivo*. We found that AT-MSC transplantation significantly improved liver function and facilitated liver regeneration, but did not increase the serum levels of HGF and IL-6 as expected. By contrast, these results showed that a reduced concentration of HGF and IL-6 may be more beneficial for liver regeneration and function recovery, which is a notewothy finding to explain the reduction in HGF and IL-6 concentrations following AT-MSC transplantation.

However, the reason for the concentration of HGF and IL-6 transiently increasing from 24 to 48 h after the transplantation remains to be verified, as well as whether other cytokines/growth factors are involved in the regeneration process. To clarify the aforementioned issues, more detailed studies are required. Recently, a new study (43) has demonstrated that the acutely transplanted neural stem/progenitor cells (NSPCs) had beneficial effects on spinal cord injury, particularly neuroprotection and neurohumoral secretion, whereas their *in situ* secretory activity differed significantly from that predicted *in vitro*. Authors of that study found that in the pathological environment, overall transcriptional activity, external signal transduction and neural differentiation of engrafted NSPCs were significantly suppressed. Those results emphasize the vulnerability of engrafted stem cells to environmental force, while emphasizing the importance of *in situ* analysis in advancing the efficacy and safety of stem cell-based therapies (43).

In conclusion, we have shown that serum from hepatectomized rats after 24 h of surgery triggered the differentiation of AT-MSCs into hepatocyte-like cells, although the hepatic differentiation efficacy was extremely low. AT-MSCs upregulated the expression of HGF and IL-6 when cultured with the post-hepatectomy serum. However, they did not increase the serum levels of HGF and IL-6 when transplanted *in vivo* as we expected. This finding should therefore be studied further. However, there were limitations to our study. One was that we did not observe whether AT-MSCs are able to migrate into the injury liver and differentiate into hepatocytes. Another one was the concentration of the growth factors and cytokines in the blood markedly altered during the liver regeneration process, thus, serum from hepatectomized rats after 24 h of their operation did not exhibit the entire change in blood after 70% PH. Thus, AT-MSCs may act through multiple mechanisms to coordinate a marked integrated response to liver regeneration after 70% PH *in vivo*. Therefore, the processes by which these cells act to decrease the serum levels of HGF and IL-6, as well as the mechanisms by which they act to reverse hepatocyte damage and promote liver regeneration *in vivo* remains to be investigated.

Acknowledgements

This study was supported by the Fundamental Research Funds for the Central Universities.

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