Yi Guan Jian decoction may enhance hepatic differentiation of bone marrow-derived mesenchymal stem cells via SDF-1 *in vitro*

LIN-LIN FU[1], BING-YAO PANG[1], YING ZHU[1], LING WANG[2], AI-JING LENG[3] and HAI-LONG CHEN[4]

[1]Department of Infectious Disease, The First Affiliated Hospital, Dalian Medical University, Dalian, Liaoning 116011; [2]Department of Digestive Disease, Gansu Provincial Hospital, Lanzhou, Gansu 730000; Departments of [3]Chinese Medicine and [4]General Surgery, The First Affiliated Hospital, Dalian Medical University, Dalian, Liaoning 116011, P.R. China

Received November 30, 2015; Accepted December 12, 2016

DOI: 10.3892/mmr.2017.6888

Abstract. A previous study reported that Yi Guan Jian (YGJ) may increase the proliferation and differentiation of hepatic oval cells in a rat liver cirrhosis model. The aim of the present study was to investigate the effect and mechanism of action of YGJ on inducing hepatic differentiation in bone marrow-derived mesenchymal stem cells (BM-MSCs) via stromal-cell derived factor-1 (SDF-1). Murine BM-MSCs were isolated with whole bone marrow adherence, then identified by immunocytochemical staining and flow cytometry. Passage 2 cells were divided into 8 groups and their differentiation was induced by cell factors added to the medium, including hepatocyte growth factor (HGF), SDF-1 and YGJ. Each of the cell factors was used alone and any two or three of them were combined to establish different cell microenvironments in the different treatment groups. Albumin (ALB) was selected as a hepatocellular marker and cytokeratin-18 (CK-18) as a cholangiocellular marker. The protein and mRNA expression levels of ALB and CK-18 were used to determine the differentiation of BM-MSCs using immunocytochemical staining, western blotting and reverse transcription-quantitative polymerase chain reaction on days 7, 14, 21 and 28 during induction. The relative expression levels of ALB and CK-18 resulted in time-dependent increases in the groups supplemented only with HGF, SDF-1 or YGJ. Combination treatment of any two HGF, SDF-1 and YGJ led to a higher expression of ALB and CK-18 compared with only one cell factor treatment. Additionally, when all three were used in a combined treatment the expression levels of ALB and CK-18 occurred at an earlier time and was higher overall. Therefore, the present study suggested that YGJ had an effect on inducing hepatic differentiation in BM-MSCs via SDF-1 and may act in a synergistic manner with HGF and SDF-1.

Correspondence to: Professor Ying Zhu, Department of Infectious Disease, The First Affiliated Hospital of Dalian Medical University, 222 Zhongshan Road, Xigang, Dalian, Liaoning 116011, P.R. China
E-mail: zhuyingsh52@126.com

Professor Hai-Long Chen, Department of General Surgery, The First Affiliated Hospital of Dalian Medical University, 222 Zhongshan Road, Xigang, Dalian, Liaoning 116011, P.R. China
E-mail: hailongchen2007@hotmail.com

Key words: Yi Guan Jian, bone marrow-derived mesenchymal stem cells, stromal-cell derived factor-1, hepatocyte growth factor, hepatic differentiation

Introduction

The liver is the largest substantive gland in the human body. Liver transplantation is an effective method to treat end-stage liver diseases, including liver cirrhosis and liver failure caused by severe injury (1). However, this procedure is expensive, liver donors are finite and immunological rejection occurs frequently, which all limit the effectiveness of organ transplantation (2,3). Therefore, it is vital to investigate liver tissue engineering further.

Bone marrow-derived mesenchymal stem cells (BM-MSCs) are adult stem cells, which have the capacity to differentiate into multiple cells crossing layer boundaries and have the ability to self-repair (4,5). Additionally, it is possible to avoid immunological rejection by autotransplantation. BM-MSCs have a stable genotypic milieu. Therefore, they may be used for various clinical practices as tissue engineering seed cells. However, the quantity of BM-MSCs is low *in vivo*. Further investigation is required to improve the efficacy of the isolation and culture of BM-MSCs.

Hepatic differentiation can include differentiation into hepatocytes and biliary cells. In the present study, albumin (ALB) was selected as a hepatocellular marker and cytokeratin-18 (CK-18) as a cholangiocellular marker. The expressions of ALB and CK-18 were used to indicate the level of hepatic differentiation.

The stromal-cell derived factor-1 (SDF-1)/C-X-C motif chemokine receptor 4 (CXCR4) signaling pathway is crucial for the recruitment of BM-MSCs to the injured liver and differentiating into hepatocytes *in vivo*. SDF-1 is a chemotactic factor and may act as a pivotal cell chemokine. CXCR4 is the specific receptor of SDF-1, which is expressed on the surface of multiple stem or progenitor cells, including BM-MSCs and may direct cell migration through binding SDF-1 (6).

Yi Guan Jian (YGJ) is a traditional Chinese formula used to treat diseases induced by reduced liver health, and

previous studies have demonstrated that YGJ may reverse liver cirrhosis (7,8). However, the effect of YGJ on BM-MSCs remains to be elucidated. The present study hypothesized that YGJ may influence the hepatic differentiation of BM-MSCs through SDF-1; however, the molecular mechanism behind this remains to be elucidated.

Therefore, the aim of the present study was to investigate the effect of the YGJ decoction on inducing hepatic differentiation of BM-MSCs by isolating and culturing murine BM-MSCs *in vitro* and investigate the association between YGJ and SDF-1. The present study may provide an experimental basis for clinical transplantation of stem cells.

Materials and methods

Cell source. BM-MSCs were harvested from the femurs, tibias and humeri of 200 male Kunming mice (age, 4 to 5 weeks; weight, 18±2 g), bred in the specific pathogen free conditions in the Center of Dalian Medical University (Dalian, China) [license no, SCXK (Liao) 2008-0002]. The mice were housed in a pathogen-free environment at room temperature (22±1˚C) on a 12 h light/dark cycle. All procedures and animal experiments were approved by the Animal Care and Use Committee of Dalian Medical University.

Preparation of YGJ decoction. YGJ is a traditional Chinese formula used for nourishing yin and dispersing stagnated liver, which was initially recorded in 'Xu Ming Yi Lei An' written by Zhi-xiu Wei of the Qing dynasty (9). In the present study, the YGJ decoction was composed of *Glehnia littoralis* F. Schmidt ex Miq. (voucher no. 120801), *Ophiopogon japonicus* (Thunb.) Ker Gawl. (voucher no. 120801), *Angelica sinensis* (Oliv.) Diels. (voucher no. 120801), *Rehmannia glutinosa* (Gaertn.) Libosch. ex Fisch. & C. A. Mey. (voucher no. 120801), *Lycium barbarum* L. (voucher no. 120806), *Melia toosendan* Siebold & Zucc. (voucher no. 120806). These were obtained from the Department of Chinese Medicine of the First Affiliated Hospital of Dalian Medical University, where the specimens were also maintained. The herbs were decocted with water and the liquid was maintained at 4˚C in the dark.

Preparation of YGJ drug serum. Normal Kunming mice were administered with 0.016 ml/g/day YGJ herbal extract orally for 3 days, 2 times per day. On the third day, 1 h following administration, blood (100 ml) was withdrawn from the eye, centrifuged at 1,131 x g for 20 min at room temperature. The supernatant serum was collected in a tube and sterilized with a sterile syringe filter (cat no. SLGP033RB; EMD Millipore, Bedford, MA, USA) and inactivated at 56˚C for 30 min prior to storage at -20˚C.

Isolation, culture and subculture of BM-MSCs. Mice were sacrificed by cervical dislocation. The fascia and muscle were removed from the femurs, tibias and humeri, with end of the bones cut and the bone marrow extruded with Dulbecco's modified Eagle's medium (DMEM)/F12 solution. Next, the bone marrow aspirate was collected and centrifuged at 377 x g for 5 min at room temperature. The cell pellet was resuspended at $1x10^9$ cells/l in 5 ml fresh DMEM/F12 supplemented with 15% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 100 U/ml penicillin-streptomycin (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and were maintained in a humidified incubator at 37˚C with 5% CO_2, marked as passage 0. The initial medium was changed following 72 h in order to remove non-adherent hematopoietic cells. The medium was replaced with fresh media every 3-4 days. Finally, cells were observed and photographed using Eclipse TS100 inverted microscope (Nikon Corporation, Tokyo, Japan).

When the cells reached 80-90% confluence, they were harvested with 0.25% trypsin containing 0.02% EDTA (Gibco; Thermo Fisher Scientific, Inc.) and then passaged at the dilution of 1:2 for one to three rounds (passage 1 to passage 3).

Identification of BM-MSCs using immunocytochemical staining. BM-MSCs from passage 2 were harvested and seeded at $1x10^5$ cells/cm^2 in a 6-well plate and the medium was replaced every 3 to 4 days. When the cells grew to confluence, the cells were fixed by 4% paraformaldehyde for 15 min at room temperature, washed three times with phosphate-buffered saline (PBS), and incubated in 3% H_2O_2 for 15 min, rinsed with PBS three times and blocked with goat serum (ZSGB-BIO, Beijing, China) for 30 min at room temperature. The cells were then incubated with the primary antibody rabbit anti-mouse CD90 (1:200; cat no. bs-0778R; BIOSS, Beijing, China) at 4˚C overnight. The following day, cells were washed with PBS and then incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1:1,000; cat. no. ZB-2301; OriGene Technologies, Inc., Beijing, China) for 30 min at 37˚C. Following rinsing with PBS, the cells were stained with 3,3-diaminobenzidine (DAB) for about 5 min in the dark, next hematoxylin staining was performed. The slides were observed under the inversion phase contrast microscope (Leica Microsystems, Inc., Buffalo Grove, IL, USA).

Flow cytometry. Passage 2 BM-MSCs were obtained and centrifuged at 377 x g for 5 min at room temperature, the supernatant was removed and the cell pellet was rinsed with PBS and centrifuged at 377 x g for 5 min at room temperature. Cells were seeded at a density of $1x10^7$ cells/ml and were incubated with fluorescein isothiocyanate-rabbit anti-mouse CD90 antibody (1:20; cat no. 11-0900-81; eBioscience, Inc., San Diego, CA, USA) and phycoerythrin-rabbit anti-mouse CD34 (BioLegend, Inc., San Diego, CA, USA) for 30 min at 4˚C away from light. Quantification was performed by FACS Vantage flow cytometer with CellQuest software v.4.0 (BD Biosciences, Franklin Lakes, NJ, USA).

Grouping and induction of BM-MSCs in vitro. Passage 2 cells were harvested and seeded at $1x10^5$ cells/cm^2 in 6-well plates. When 70-80% confluence was reached, the cells were divided into 8 groups to induce the differentiation for 28 days: Negative control (NC) group, 15% FBS; hepatocyte growth factor (HGF) group, 20 ng/ml HGF (PeproTech, Inc., Rocky Hill, NJ, USA); SDF-1 group, 50 ng/ml SDF-1 (PeproTech, Inc.); HGF + SDF-1 group, 20 ng/ml HGF and 50 ng/ml SDF-1; YGJ group, 20% YGJ drug serum; YGJ + HGF group, 20% YGJ drug serum and 20 ng/ml HGF; YGJ + SDF-1 group, 20% YGJ drug serum and 50 ng/ml SDF-1, YGJ + HGF + SDF-1 group,

20% YGJ drug serum, 20 ng/ml HGF and 50 ng/ml SDF-1. The extent of cell differentiation was monitored on days 7, 14, 21 and 28.

Identification of differentiated BM-MSCs. The negative control group was used to observe the normal cell growth process and the remaining groups were used to observe cell proliferation process in different induction conditions. At 7, 14, 21 and 28 days following induction, cells were incubated with the primary antibodies at 4˚C overnight as follows: Rabbit anti-mouse ALB (1:200; cat no. bs-2256R-HRP; BIOSS), rabbit anti-mouse CK-18 (1:200; cat no. bs-1339R-HRP; BIOSS), followed by DAB staining (10).

Western blotting analysis. Western blotting was performed at 7, 14, 21 and 28 days following induction, the total protein of each group was extracted in ice-cold lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) containing the proteinase inhibitor phenylmethylsulfonyl fluoride (100:1; Nanjing KeyGen Biotech, Co., Ltd., Nanjing, China) and subjected to 10% SDS-PAGE, then transferred to polyvinylidene fluoride membranes (EMD Millipore). The membranes were blocked with 5% non-fat milk, then incubated with the primary antibodies as follows: Anti-mouse ALB (1:2,000; cat no. 16475-1-AP; Proteintech Group, Inc.; Wuhan Sanying Biotechnology, Wuhan, China) and anti-mouse CK-18 (1:500; cat no. 10830-1-AP; Proteintech Group, Inc.; Wuhan Sanying Biotechnology) at 4˚C overnight. Washed with PBS with 0.1%Tween-20, the membranes were incubated with HRP-IgG secondary antibody (1:10,000; cat. no. ZB-2301; OriGene Technologies, Inc.) for 1 h at 37˚C. The immunoreactive bands were detected using an enhanced chemiluminescence system (Advansta, Inc., Menlo Park, CA, USA) and analyzed by AlphaView software v3.4.0.0 (ProteinSimple, San Jose, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA in the treatment groups was isolated with the TaKaRaMiniBEST Universal RNA Extraction kit (TakaraBio, Inc., Otsu, Japan) at 7, 14, 21 and 28 days following induction. The cDNA was synthesized according to the manufacturer's protocol. qPCR was performed on a StepOnePlusReal-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the SYBR® Premix Ex Taq™ (Clontech Laboratories, Inc., Mountainview, CA, USA). Optimal reaction conditions were 95˚C for 30 sec, 60˚C for 30 sec, 40 cycles. The target genes were ALB and CK-18, β-actin was used as a reference gene. Cq SYBR was calculated using the $2^{-\Delta\Delta Cq}$ method (11). All the primers were designed and synthesized by Bioneer Corporation (Daejeon, Korea).

Primer sequences were as follows: β-actin, F 5'-CCTAGCACCATGAAGATCAAGATCAT-3' and R 5'-ATCTGCTGGAAGGTGGACAGTGA; ALB, F 5'-GCATTGGTCTCATCTGTCCGTC-3' and R 5'-CAAGTTCCGCCCTGTCATCT; CK18, F 5'-ACCACCAAGTCTGCCGAAATC-3' and R 5'-CTGCTCCATCTGTGCCTTGTAT-3'.

Statistical analysis. SPSS version 22.0 (IBM SPSS, Armonk, NY, USA) was used for statistical analysis. Data are presented as the mean ± standard deviation and comparisons among groups were performed using one-way analysis of variance followed by a Cochran's q test for post-hoc analysis. Student's t-test was used to compare the difference of two groups. $P<0.05$ was considered to indicate a statistically significant difference.

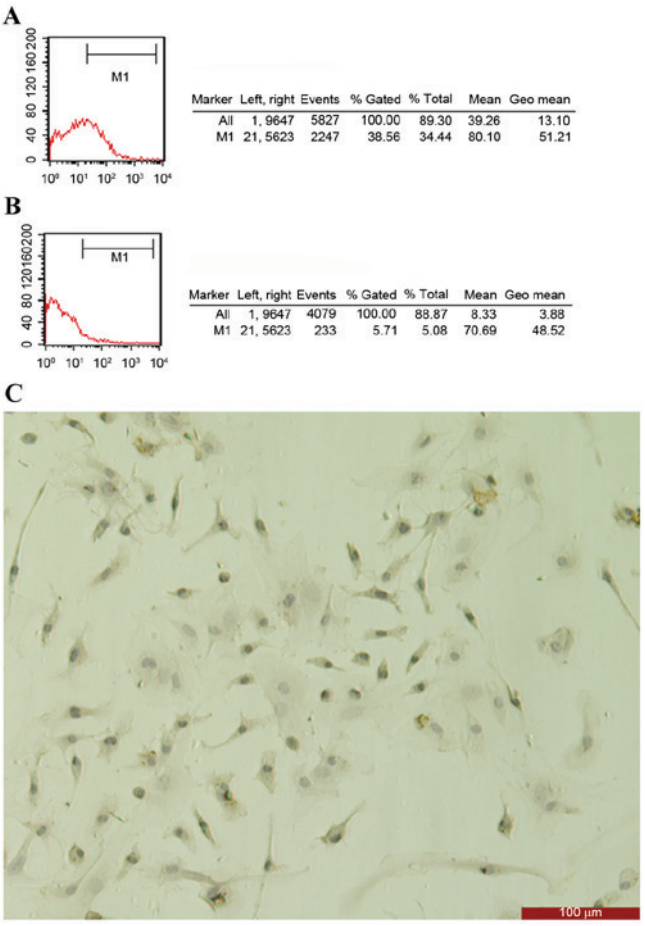

Figure 1. Identification of surface biomarkers of BM-MSCs. Flow cytometry of (A) CD90 and (B) CD34. (C) Immunocytochemical staining for expression of CD90 of BM-MSCs. Magnification, x200. BM-MSCs, bone marrow-derived mesenchymal stem cells.

Results

Characteristics of BM-MSCs. Cells were isolated from whole bone marrow adherent cultures. BM-MSCs were round-shaped in appearance and floating on the surface at the time of isolation from the bone marrow. Following 4 h the cells began to adhere to the culture plate. At 24 h almost all of the cells were adherent. At 72 h, the adherent cells developed elliptical, polygonal and spindle shapes. With extended incubation most cells developed into a spindle shape. Following 12-14 days, the cultured cells reached ~80% confluency. The cells became relatively homogeneous in appearance following subculture and had an elongated and spindle shape.

Identification of surface biomarker. BM-MSCs positively expressed CD90 (38.56%) and negatively expressed CD34 (5.71%). In addition, positive CD90 expression was observed in BM-MSCs using immunocytochemical staining, as brown staining was evident in the cytoplasm and near the nucleus (Fig. 1).

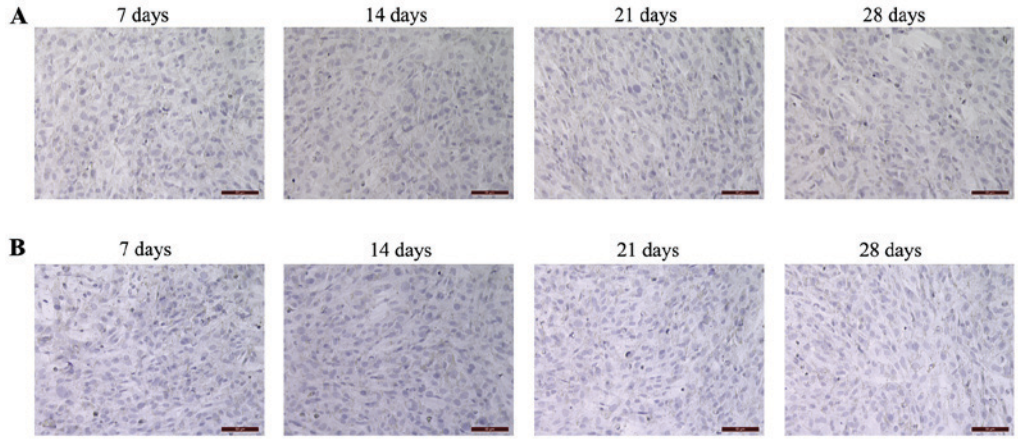

Figure 2. Immunostaining for (A) ALB and (B) CK-18 immunostaining at different induction times in the NC group. Magnification, x200; scale bars,50 μm. ALB, albumin; CK-18, cytokeratin-18; HGF, hepatocyte growth factor; NC, negative control group.

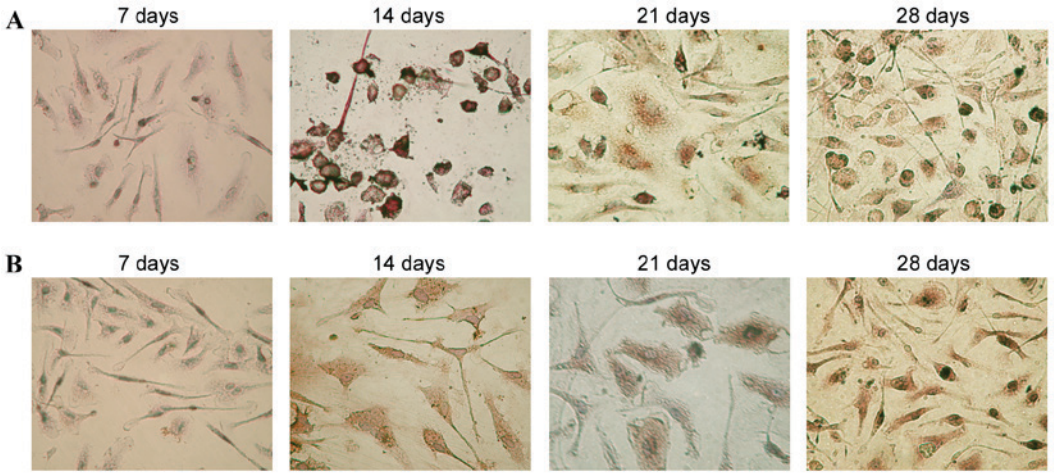

Figure 3. Immunostaining for (A) ALB and (B) CK-18 immunostaining at different induction times in the HGF group. Magnification, x200. ALB, albumin; CK-18, cytokeratin-18; HGF, hepatocyte growth factor.

Morphology of BM-MSCs during induction. Cells in the NC group were spindle-shaped, whereas cells in the remaining groups were larger in size, with increased cytoplasm, a larger nucleus, retractile antenna, a polygonal or elliptical shape, all of which were morphological characteristics of hepatocyte-like cells. This was observed on day 14 post-induction in the HGF, SDF-1 and YGJ groups, on day 8 in the YGJ + HGF and YGJ + SDF-1 groups, on day 7 in the HGF + SDF-1 group and day 5 in the YGJ + HGF + SDF-1 group.

Identification of post-induction BM-MSCs. The NC group did not stain positive for ALB expression (Fig. 2A). In the HGF (Fig. 3A), SDF-1 (Fig. 4A) and YGJ (Fig. 5A) groups positive staining was observed on day 14 following induction, with the cytoplasm stained brown, and more evident near the nucleus and the number of positively stained cells increased gradually. In the HGF + SDF-1 (Fig. 6A), YGJ + HGF (Fig. 7A), YGJ + SDF-1 (Fig. 8A) and HGF + SDF-1 + YGJ groups (Fig. 9A) positive staining was detected at day 7 post-induction. The NC group did not stain positive for CK-18 expression (Fig. 2B). The positive staining of other groups was similar to that observed for ALB (Fig. 3B-9B). As no positive staining was observed for ALB or CK-18 in the NC group by immunocytochemistry (Fig. 2), the expression levels of ALB and CK-18 were not verified by western blotting or RT-PCR.

Western blotting. The protein expression levels of ALB at different time in the HGF group increased gradually and on day 28 were significantly higher compared with that of day 21 ($P<0.05$). In the SDF-1 group ALB significantly increased from day 14 to day 28 ($P<0.05$). In the HGF + SDF-1 group ALB increased from day 14 until day 21 ($P<0.05$). In the YGJ group ALB expression significantly increased from day 14 when compared with day 7 ($P<0.05$). In the YGJ + HGF and YGJ + SDF-1 groups ALB expression significantly increased from day 21 when compared with day 14 ($P<0.05$). In the YGJ + HGF + SDF-1 group ALB expression increased gradually from day 14 to day 28 ($P<0.05$; Table I, Fig. 10A).

Comparison of relative expression levels of ALB in the differentiated groups. The expression levels of ALB in the YGJ + HGF group were significantly higher compared with the HGF group at day 21 ($P<0.05$; Fig. 10A). The ALB expression in the YGJ + SDF-1 group was significantly higher compared

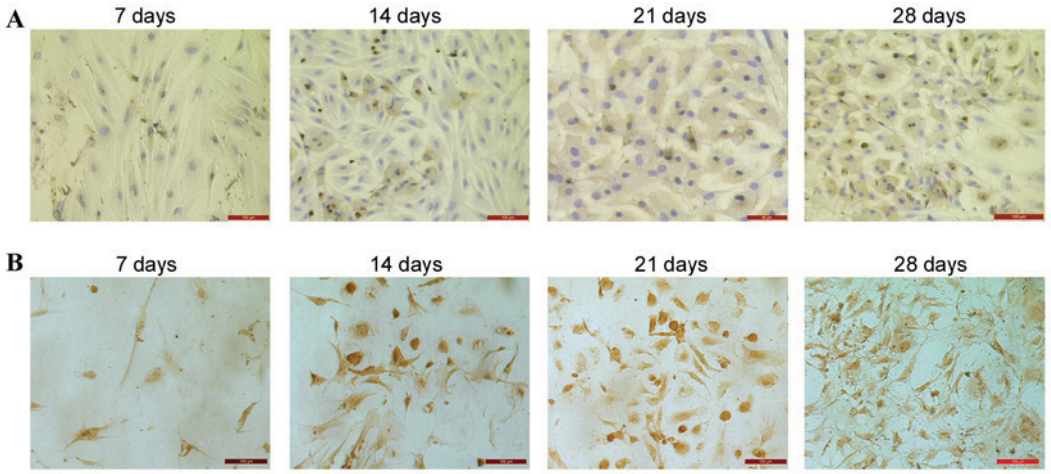

Figure 4. Immunostaining for (A) ALB and (B) CK-18 immunostaining at different induction time in the SDF-1 group. Magnification, x200. ALB, albumin; CK-18, cytokeratin-18; SDF-1, stromal-cell derived factor-1.

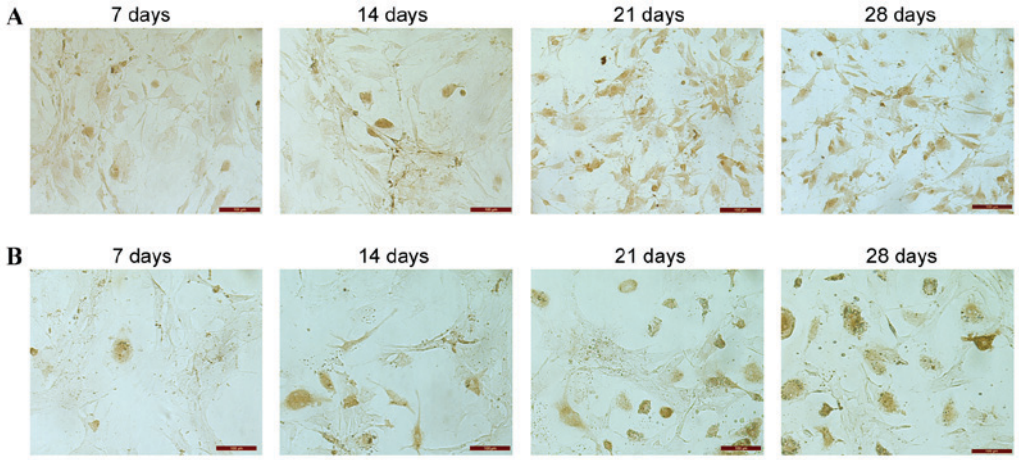

Figure 5. Immunostaining for (A) ALB and (B) CK-18 immunostaining at different induction times in the YGJ group. Magnification, x200. ALB, albumin; CK-18, cytokeratin-18; YGJ, Yi Guan Jian.

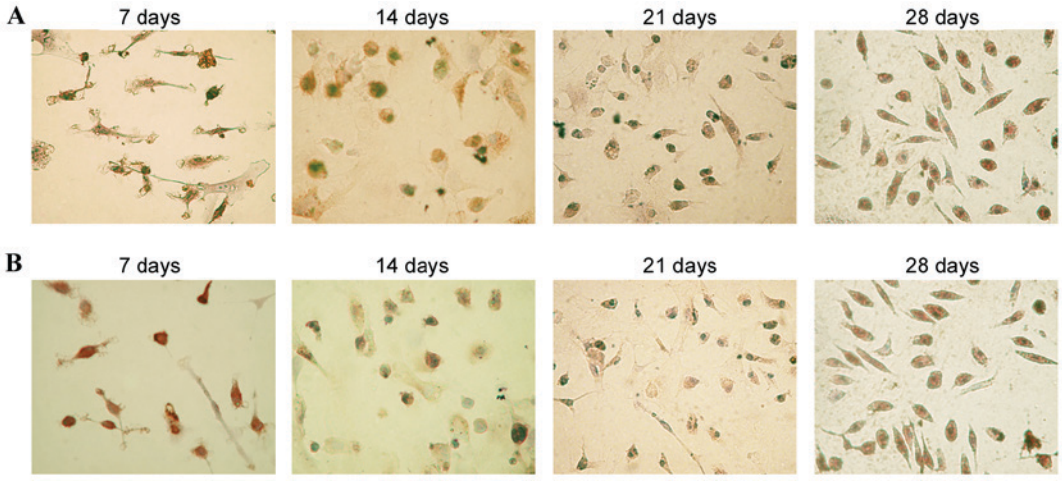

Figure 6. Immunostaining for (A) ALB and (B) CK-18 immunostaining at different induction times in the HGF + SDF-1 group. Magnification, x200. ALB, albumin; CK-18, cytokeratin-18; HGF, hepatocyte growth factor; SDF-1, stromal-cell derived factor-1.

with the SDF-1 group from day 7 to 28 ($P<0.05$). In the YGJ + HGF + SDF-1 group ALB expression was significantly higher compared with the HGF + SDF-1 group from day 14 to 28 day ($P<0.05$). YGJ + HGF vs. YGJ and YGJ + SDF-1 vs. YGJ, expression of ALB was higher in the former ($P<0.05$) at day 7 and day 14. In addition, there were statistically

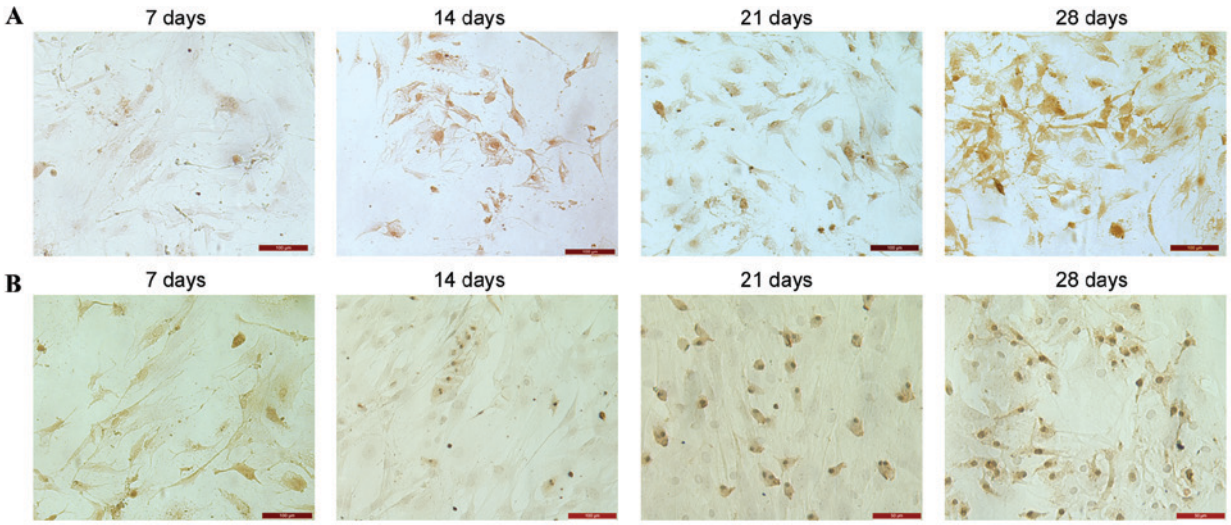

Figure 7. Immunostaining for (A) ALB and (B) CK-18 immunostaining at different induction times in the YGJ + HGF group. Magnification, x200. ALB, albumin; CK-18, cytokeratin-18; YGJ, Yi Guan Jian; HGF, hepatocyte growth factor.

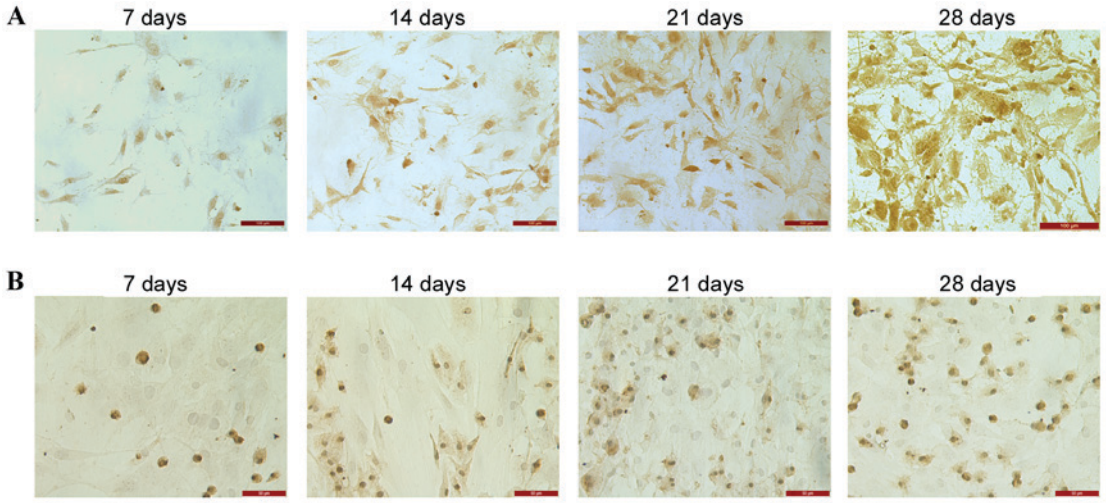

Figure 8. Immunostaining for (A) ALB and (B) CK-18 immunostaining at different induction times in the YGJ + SDF-1 group. Magnification, x200. ALB, albumin; CK-18, cytokeratin-18; YGJ, Yi Guan Jian; SDF-1, stromal-cell derived factor-1.

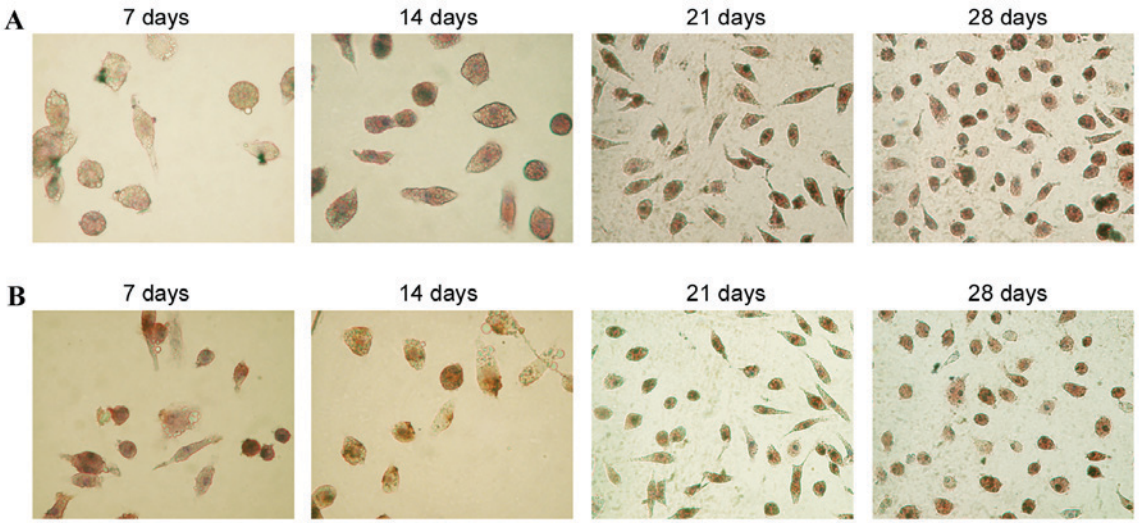

Figure 9. Immunostaining for (A) ALB and (B) CK-18 immunostaining at different induction times in the YGJ + HGF + SDF-1 group. Magnification, x200. ALB, albumin; CK-18, cytokeratin-18; YGJ, Yi Guan Jian; HGF, hepatocyte growth factor; SDF-1, stromal-cell derived factor-1.

Table I. Relative expression of ALB in differentiated groups by western blotting (mean ± standard deviation).

Group	7 days	14 days	21 days	28 days
HGF	0.33±0.014	0.435±0.014	0.51±0.01	1.515±0.092[a]
SDF-1	0.431±0.031	0.723±0.067[a]	0.849±0.092[a]	1.415±0.014[a]
HGF + SDF-1	0.447±0.041	0.988±0.147[a]	1.555±0.172[a]	1.835±0.144
YGJ	0.452±0.04	1.145±0.09[a]	2.143±0.088	1.685±0.357
YGJ + HGF	0.136±0.004[e]	0.578±0.011[e]	1.386±0.01[a,b]	2.222±0.03
YGJ + SDF-1	0.138±0.006[c,f]	0.156±0.026[c,f]	1.431±0.289[a,c]	2.111±0.06[c]
YGJ + HGF + SDF-1	0.769±0.04[g,h,i]	2.286±0.038[a,d,g,h,i]	2.411±0.002[a,d,g,h,i]	2.826±0.014[a,d,g,h,i]

[a]P<0.05 14 days vs. 7 days, 21 days vs. 14 days, 28 days vs. 21 days, [b]P<0.05 YGJ + HGF vs. HGF, [c]P<0.05 YGJ + SDF-1 vs. SDF-1, [d]P<0.05 YGJ + HGF + SDF-1 vs. HGF + SDF-1, [e]P<0.05 YGJ + HGF vs. YGJ, [f]P<0.05 YGJ + SDF-1 vs. YGJ, [g]P<0.05 YGJ + HGF + SDF-1 vs. YGJ, [h]P<0.05 YGJ + HGF + SDF-1 vs. YGJ + HGF, [i]P<0.05 YGJ + HGF + SDF-1 vs. YGJ + SDF-1. ALB, albumin; HGF, hepatocyte growth factor; SDF-1, stromal-cell derived factor-1; YGJ, Yi Guan Jian.

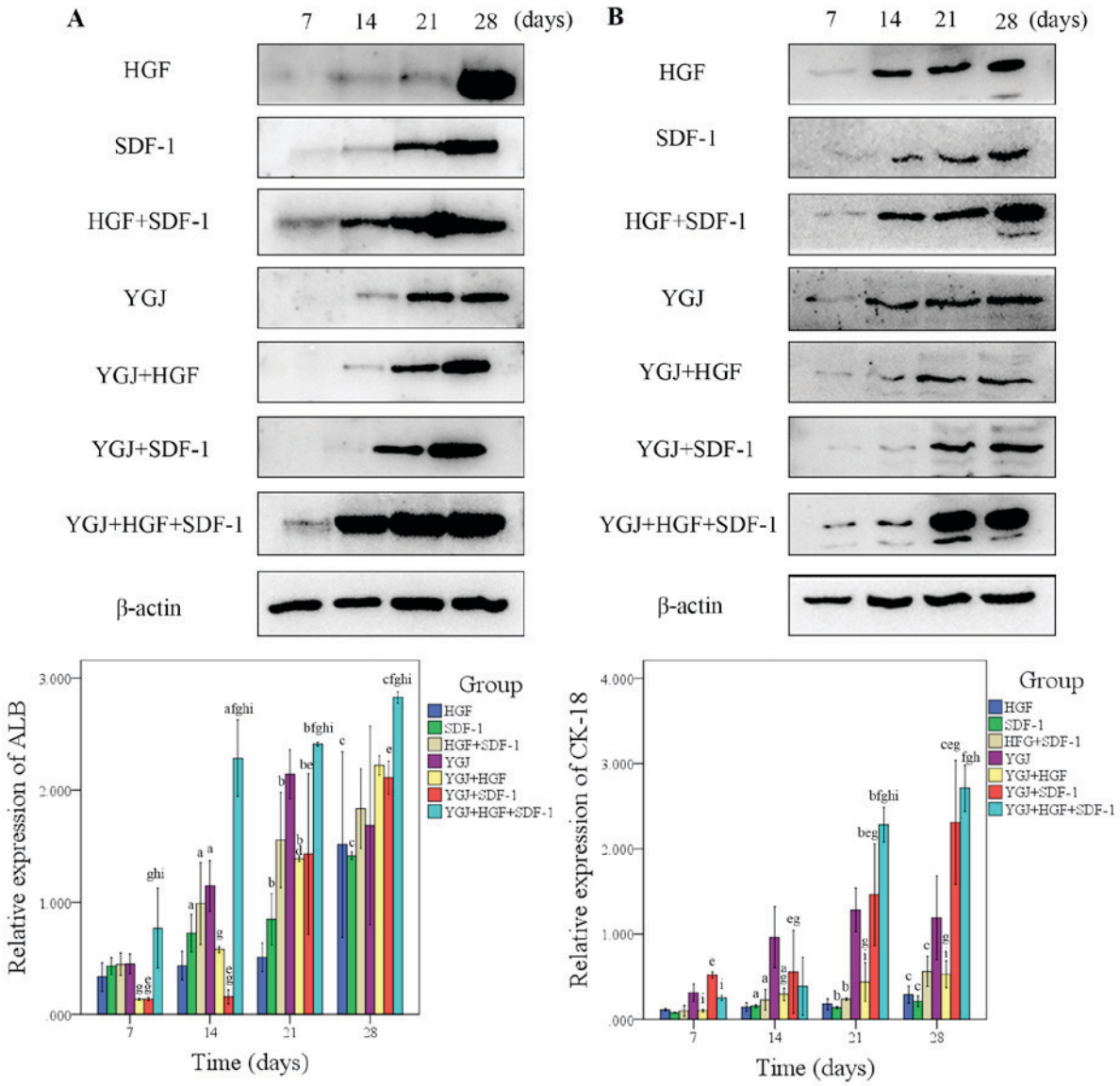

Figure 10. Expression levels of (A) ALB and (B) CK-18 in different treatment groups. Data are presented as the mean ± standard deviation. [a]P<0.05 14 days vs. 7 days; [b]P<0.05 21 days vs. 14 days; [c]P<0.05 28 days vs. 21 days; [d]P<0.05 YGJ + HGF vs. HGF; [e]P<0.05 YGJ + SDF-1 vs. SDF-1; [f]P<0.05 YGJ + HGF + SDF-1 vs. HGF + SDF-1; [g]P<0.05 YGJ + HGF, YGJ + SDF-1 and YGJ + HGF + SDF-1 vs. YGJ; [h]P<0.05 YGJ + HGF + SDF-1 vs. YGJ + HGF; [i]P<0.05 YGJ + HGF + SDF-1 and YGJ + HGF vs. YGJ + SDF-1. ALB, albumin; CK-18, cytokeratin-18; HGF, hepatocyte growth factor; SDF-1, stromal-cell derived factor-1; YGJ, Yi Guan Jian.

Table II. Relative expression of cytokeratin-18 in differentiated groups determined using western blotting (mean ± standard deviation).

Group	7 days	14 days	21 days	28 days
HGF	0.113±0.061	0.143±0.2	0.177±0.256	0.287±0.41[a]
SDF-1	0.075±0.003	0.154±0.007[a]	0.139±0.005[a]	0.212±0.025[a]
HGF + SDF-1	0.099±0.007	0.23±0.013[a]	0.238±0.001[a]	0.562±0.2[a]
YGJ	0.312±0.041	0.963±0.145[a]	1.283±0.104[a]	1.119±0.198
YGJ + HGF	0.102±0.007[i]	0.293±0.03[a,d]	0.439±0.092[d,i]	0.527±0.064[d,i]
YGJ + SDF-1	0.52±0.015[b]	0.557±0.197[b,e]	1.463±0.24[a,b,e]	2.31±0.293[a,b,e]
YGJ + HGF + SDF-1	0.251±0.113[h]	0.387±0.138[f]	2.281±0.083[a,c,f,g,h]	2.712±0.109[e,f,g]

[a]P<0.05 14 days vs. 7 days, 21 days vs. 14 days, 28 days vs. 21 days, [b]P<0.05 YGJ + SDF-1 vs. SDF-1, [c]P<0.05 YGJ + HGF + SDF-1 vs. HGF + SDF-1, [d]P<0.05 YGJ + HGF vs. YGJ, [e]P<0.05 YGJ + SDF-1 vs. YGJ, [f]P<0.05 YGJ + HGF + SDF-1 vs. YGJ, [g]P<0.05 YGJ + HGF + SDF-1 vs. YGJ + HGF, [h]P<0.05 YGJ + HGF + SDF-1 vs. YGJ + SDF-1, [i]P<0.05 YGJ + HGF vs. YGJ + SDF-1. HGF, hepatocyte growth factor; SDF-1, stromal-cell derived factor-1; YGJ, Yi Guan Jian.

significant differences between YGJ + HGF + SDF-1 and YGJ (P<0.05), YGJ + HGF + SDF-1 and YGJ + HGF (P<0.05), and YGJ + HGF + SDF-1 and YGJ + SDF-1 (P<0.05) between day 7 to 28, with the former being the group with the higher level of expression (Table I, Fig. 10A).

Relative expression levels of CK-18. Expression of CK-18 in the HGF group was significantly higher on day 28 compared with day 21 (P<0.05). In the SDF-1 and HGF + SDF-1 groups, CK-18 expression level increased from day 14 to day 28 (P<0.05). In the YGJ group, CK-18 expression increased from day 14 to day 21 (P<0.05). In the YGJ + HGF group, CK-18 expression levels significantly increased from day 14 when compared with day 7 (P<0.05). In the YGJ + SDF-1 group, CK-18 expression increased from day 21 to day 28 (P<0.05). The YGJ + HGF + SDF-1 group exhibited significantly increased CK-18 expression from day 21 when compared with day 14 (P<0.05; Table II, Fig. 10B).

Comparison of relative expression of CK-18 in the differentiated groups. No significant difference was identified between CK-18 expression levels in the YGJ + HGF and HGF groups (P>0.05). CK-18 expression level in the YGJ + SDF-1 group was significantly higher when compared with the SDF-1 group from day 7 to day 28 (P<0.05). CK-18 expression level was significantly higher in the YGJ + HGF + SDF-1 group when compared with the HGF + SDF-1 group at day 21 and day 28 (P<0.05). For YGJ + HGF vs. YGJ, YGJ + SDF-1 vs. YGJ and YGJ + HGF + SDF-1 vs. YGJ, the former was significantly higher than the latter (P<0.05) at days 14, 21 and 28. YGJ + HGF + SDF-1 was significantly higher than YGJ + HGF at days 21 and 28 (P<0.05), YGJ + HGF + SDF-1 was significantly higher than YGJ+SDF-1 at days 7 and 21 (P<0.05), and YGJ + HGF was lower than YGJ + SDF-1 (P<0.05) at days 7, 21 and 28 (Table II; Fig. 10B).

ALB mRNA expression level. ALB expression level in the HGF and SDF-1 groups significantly increased on day 28 (P<0.05). In the HGF + SDF-1 group ALB expression increased at day 14 when compared with day 7, and increased at day 21 when compared with day 14 (P<0.05). In the YGJ group ALB expression significantly increased at day 21 when compared with day 14 (P<0.05). In the YGJ + HGF, YGJ + SDF-1 and YGJ + HGF + SDF-1 groups ALB expression level significantly increased when comparing day 14 vs. day 7, day 21 vs. day 14 and day 28 vs. day 21 (P<0.05).

Comparison of ALB mRNA expression level in the differentiated groups. The relative ALB mRNA expression level in the YGJ + HGF group was significantly higher when compared with the HGF group at day 21 (P<0.05). For YGJ + SDF-1 vs. SDF-1, YGJ + HGF vs. YGJ, YGJ + SDF-1 vs. YGJ, and YGJ + HGF + SDF-1 vs. YGJ, the former was significantly higher than the latter from day 7 to 28 (P<0.05). For YGJ + HGF + SDF-1 vs. HGF + SDF-1, YGJ + HGF + SDF-1 vs. YGJ + HGF, and YGJ + HGF + SDF-1 vs. YGJ + SDF-1, the former was significantly higher than the latter from day 14 to 28 (P<0.05), whereas YGJ + HGF was significantly lower than YGJ + SDF-1 at days 14 and 28 (P<0.05; Table III).

CK-18 mRNA expression level. CK-18 mRNA expression level in the HGF and SDF-1 groups increased at day 28 when compared with day 21 (P<0.05). In the HGF + SDF-1, YGJ + SDF-1 and YGJ + HGF + SDF-1 groups, CK-18 mRNA expression increased from day 14 when compared with day 7, and day21 when compared with day 14 (P<0.05). No significant difference was identified when comparing days 7, 14, 21 and 28 in the YGJ group (P>0.05). CK-18 expression was significantly increased in the YGJ + HGF group from day 14 when compared with day 7 (P<0.05).

Comparison of relative expression of CK-18 mRNA among differentiated groups. Relative expression of CK-18 mRNA in the YGJ + HGF group was significantly higher when compared with the HGF group at day 21 (P<0.05). CK-18 mRNA expression level was significantly higher in the YGJ + SDF-1 group when compared with the SDF-1 group from day 7 to 28 (P<0.05). CK-18 mRNA expression level was significantly higher in the YGJ + HGF + SDF-1 group when compared with the HGF + SDF-1 group from day 14 to 28

Table III. Relative expression of albumin mRNA in differentiated groups by reverse transcription-polymerase chain reaction (mean ± standard deviation).

Group	7 days	14 days	21 days	28 days
HGF	1.495±0.035	4.387±2.83	4.529±3.704	15.609±3.881[a]
SDF- 1	6.624±3.982	25.296±17.473	22.466±6.146	87.369±20.778[a]
HGF + SDF-1	79.022±15.458	125.588±9.208[a]	189.298±30.494[a]	195.594±38.057
YGJ	437.258±92.11	457.901±70.327	827.487±51.741[a]	788.599±23.209
YGJ + HGF	988.247±84.168[e]	1277.435±70.592[a,e,i]	3499.887±189.488[a,b,e]	4516.83±300.24[a,e,j]
YGJ + SDF-1	932.631±356.961[c,f]	2364.43±199.63[a,c,f]	4038.367±902.747[a,c,f]	6410.877±308.084[a,c,f]
YGJ + HGF + SDF-1	1327.049±218.4[g]	3121.382±218.31[a,d,g,h,i]	5175.584±195.39[a,d,g,h,i]	7287.218±152.7[a,d,g,h,i]

[a]P<0.05 14 days vs. 7 days, 21 days vs. 14 days, 28 days vs. 21 days, [b]P<0.05 YGJ + HGF vs. HGF, [c]P<0.05 YGJ + SDF-1 vs. SDF-1, [d]P<0.05 YGJ + HGF + SDF-1 vs. HGF + SDF-1, [e]P<0.05 YGJ + HGF vs. YGJ, [f]P<0.05 YGJ + SDF-1 vs. YGJ, [g]P<0.05 YGJ + HGF + SDF-1 vs. YGJ, [h]P<0.05 YGJ + HGF + SDF-1 vs. YGJ + HGF, [i]P<0.05 YGJ + HGF + SDF-1 vs. YGJ + SDF-1, [j]P<0.05 YGJ + HGF vs. YGJ + SDF-1. HGF, hepatocyte growth factor; SDF-1, stromal-cell derived factor-1; YGJ, Yi Guan Jian.

Table IV. Relative expression of CK-18 mRNA in differentiated groups was determined using reverse transcription-polymerase chain reaction (mean ± standard deviation).

Group	7 days	14 days	21 days	28 days
HGF	7.582±3.513	15.832±10.249	14.887±6.05	51.218±7.639[a]
SDF- 1	10.874±0.483	39.935±19.534	32.068±13.29	218.19±73.026[a]
HGF + SDF-1	11.321±1.483	56.123±2.086[a]	114.687±16.63[a]	139.799±22.902
YGJ	11.594±2.859	22.276±6.883	31.241±7.749	24.28±6.353
YGJ + HGF	77.635±33.438[e]	229.726±19.703[a,e]	251.298±115.635[b,e]	254.506±13.688[e,j]
YGJ + SDF-1	12.422±7.402[c,f]	101.982±16.213[a,c,f]	319.306±120.409[a,c,f]	332.784±12.412[c,f]
YGJ + HGF+SDF-1	82.767±2.41[g,i]	218.667±7.356[a,d,g,i]	330.883±20.416[a,d,g,i]	424.935±60.09[d,g,h,i]

[a]P<0.05 14 days vs. 7 days, 21 days vs.14 days, 28 days vs. 21days, [b]P<0.05 YGJ + HGF vs. HGF, [c]P<0.05 YGJ + SDF-1 vs. SDF-1, [d]P<0.05 YGJ + HGF + SDF-1 vs. HGF + SDF-1, [e]P<0.05 YGJ + HGF vs. YGJ, [f]P<0.05 YGJ + SDF-1 vs. YGJ, [g]P<0.05 YGJ + HGF + SDF-1 vs. YGJ, [h]P<0.05 YGJ + HGF + SDF-1 vs. YGJ + HGF, [i]P<0.05 YGJ + HGF + SDF-1 vs. YGJ + SDF-1, [j]P<0.05 YGJ + HGF vs. YGJ + SDF-1. HGF, hepatocyte growth factor; SDF-1, stromal-cell derived factor-1; YGJ, Yi Guan Jian.

(P<0.05). For YGJ + HGF vs. YGJ, YGJ + SDF-1 vs. YGJ, YGJ + HGF + SDF-1 vs. YGJ, and YGJ + HGF + SDF-1 vs. YGJ + SDF-1, the former was significantly higher than the latter from day 7 to 28 (P<0.05). YGJ + HGF + SDF-1 was significantly higher than YGJ + HGF at day 28 (P<0.05), YGJ + HGF was significantly lower than YGJ + SDF-1 at day 28 (P<0.05) (Table IV).

Discussion

There are three types of bone marrow stem cells: hematopoietic, endothelial progenitor and mesenchymal stem cells (12). BM-MSCs are important pluripotent stem cells (13) and are able to differentiate into various cell types and maintain self-renewal activity (4), which possess a prospective application in case of organ injury (14-16). Previous studies have demonstrated that BM-MSCs may differentiate into hepatocytes under specific conditions (17-20), have a histocompatibility and stable genotypic milieu, which may be a potential treatment for patients with end-stage liver diseases. It is crucial to isolate and culture BM-MSCs efficiently, as the cells occur in limited numbers *in vivo* and account for 0.001-0.01% of total karyocytes in the bone marrow (21,22). Previous studies have demonstrated that CD90 (+) and CD34 (-) BM-MSCs (23,24) may be harvested with high activity, purity and negligible quantity by isolating cells with whole bone marrow adherence (25).

The differentiation of BM-MSCs is affected by the cell microenvironment and cell factors including HGF (26), fibroblast growth factors (27), interleukin 6 (28) and epidermal growth factors (29). In the present study, various induced factors, including HGF, SDF-1 and YGJ drug serum were added to the culture medium in order to observe the differentiation of BM-MSCs.

HGF is a mitosis promoter of normal hepatocytes, which is primarily secreted by Kupffer cells and sinusoidal endothelial cells. C-met is the receptor of HGF and is a type of receptor tyrosine kinase. C-met is also expressed on the surface of BM-MSCs (30). The HGF-C-met axis participates in the directed migration of BM-MSCs and directs BM-MSCs to HGF-rich areas. Injured liver secretes HGF (5,31,32), which directs the migration of MSCs, this in turn facilitates

the hepatic differentiation of BM-MSCs. Additionally, this chemotaxis may be enhanced by SDF-1 (5). Consequently, BM-MSCs may be induced to differentiate into hepatocytes when HGF concentration has been increased in the culture medium.

BM-MSCs may migrate to injured tissues following a SDF-1 gradient and differentiate into homologous parenchymal cells when the SDF-1 concentration is reduced in the bone marrow and increased in peripheral blood (2). In the present study, SDF-1 was supplemented into the culture medium, which simulated an extracellular environment with a high SDF-1 concentration. Therefore, this concentration likely led to SDF-1 binding to CXCR4 and promoted the differentiation of BM-MSCs.

Previous studies have identified that YGJ may repair liver function by recruiting BM-MSCs (33), and reverse liver fibrosis (6,7). Additionally, a previous study demonstrated that YGJ had an impact on inducing differentiation of hepatic oval cells into hepatocytes and biliary epithelial cells (34).

Hepatic differentiation can include differentiation into hepatocytes and biliary cells. The present study selected ALB and CK-18 as the unique markers for identification of mature hepatocytes and biliary cells. ALB and CK-18 expression levels were high at day 28 in the HGF and SDF-1 groups. ALB and CK-18 expression levels were significantly higher in the HGF + SDF-1 group at day 14, which demonstrated a synergistic effect of HGF and SDF-1.

The expression level of ALB significantly increased on day 14 and CK-18 expression was increased on day 21 in the YGJ group. In the HGF + YGJ and SDF-1 + YGJ groups, a significant increase of ALB and CK-18 expression was observed on day 14. In the HGF + SDF-1 + YGJ group, cell morphology changes were observed on day 5. At the same time of induction, for YGJ + HGF vs. HGF, YGJ + SDF-1 vs. SDF-1, and YGJ + HGF + SDF-1 vs. HGF + SDF-1, the expression levels of ALB and CK-18 were significantly increased in the former compared with that of the latter, thus suggesting that YGJ decoction had a capacity to induce the hepatic differentiation of BM-MSCs. Additionally, YGJ + HGF vs. YGJ, YGJ + SDF-1 vs. YGJ, and YGJ + HGF + SDF-1 vs. YGJ, the expression of ALB and CK-18 of the former was higher than that of the latter, which suggested that YGJ may act in conjunction with other cytokines to facilitate the differentiation. The present study demonstrated the effect of YGJ enhancing the differentiation of BM-MSCs, accomplished via SDF-1.

It was determined that for YGJ + HGF + SDF-1 vs. YGJ + HGF, and YGJ + HGF + SDF-1 vs. YGJ + SDF-1, expression of ALB and CK-18 of the former was higher than that of the latter, suggesting that combining YGJ decoction with HGF + SDF-1 promoted the hepatic differentiation of BM-MSCs. Additionally, the relative expression of ALB and CK-18 in the YGJ + HGF group was lower compared with the YGJ + SDF-1 group, which demonstrated SDF-1 was more effective in inducing the differentiation of BM-MSCs than HGF.

In conclusion, YGJ decoction may enhance BM-MSC differentiation into hepatocytes and biliary cells. SDF-1 was also able to facilitate the differentiation of BM-MSCs. It is possible that YGJ enhanced differentiation of BM-MSCs via SDF-1. However, further investigations should aim at identifying the elements in the SDF-1/CXCR4 pathway. Additionally, as YGJ is composed of the water extract of six herbs and is likely to contain numerous impurities that may lead to unnecessary side-effects on BM-MSCs. Future studies should focus on identifying the active ingredients and their molecular mechanisms of action.

Acknowledgements

The present study was supported by the National Natural Science foundation of China (grant nos. 81273925 and 81673728). The authors would like to thank the Center Laboratory of the First Affiliated Hospital of Dalian Medical University (Dalian, China) for the experimental technical support.

References

1. O'Grady J: Modern management of acute liver failure. Clin Liver Dis 11: 291-303, 2007.
2. Lei Y, Liu Z, Han Q, Kang W, Zhang L and Lou S: G-CSF enhanced SDF-1 gradient between bone marrow and liver associated with mobilization of peripheral blood CD34+ cells in rats with acute liver failure. Dig Dis Sci 55: 285-291, 2010.
3. Baertschiger RM, Serre-Beinier V, Morel P, Bosco D, Peyrou M, Clément S, Sgroi A, Kaelin A, Buhler LH and Gonelle-Gispert C: Fibrogenic potential of human multipotent mesenchymal stromal cells in injured liver. PLoS One 4: 6657, 2009.
4. Amado LC, Saliaris AP, Schuleri KH, St John M, Xie JS, Cattaneo S, Durand DJ, Fitton T, Kuang JQ, Stewart G, *et al*: Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. Proc Natl AcadSci USA 102: 11474-11479, 2005.
5. Son BR, Marquez-Curtis LA, Kucia M, Wysoczynski M, Turner AR, Ratajczak J, Ratajczak MZ and Janowska-Wieczorek A: Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. Stem Cells 24: 1254-1264, 2006.
6. Askari AT, Unzek S, Popovic ZB, Goldman CK, Forudi F, Kiedrowski M, Rovner A, Ellis SG, Thomas JD, DiCorleto PE, *et al*: Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet 362: 697-703, 2003.
7. Lin HJ, Chen JY, Lin CF, Kao ST, Cheng JC, Chen HL and Chen CM: Hepatoprotective effects of Yi Guan Jian, an herbal medicine, in rats with dimethylnitrosamine-induced liver fibrosis. J Ethnopharmacol 134: 953-960, 2011.
8. Chen W, Chen JY, Tung YT, Chen HL, Kuo CW, Chuang CH, Chong KY, Mao FC and Chen CM: High-frequency ultrasound imaging to evaluate liver fibrosis progression in rats and Yi Guan Jian herbal therapeutic effects. Evid Based Complement Alternat Med 2013: 302325, 2013.
9. Wei ZX: Xu Ming Yi Lei An, 1770 (In Chinese).
10. Chen Z, Sun M, Yuan Q, Niu M, Yao C, Hou J, Wang H, Wen L, Liu Y, Li Z and He Z: Generation of functional hepatocytes from human spermatogonial stem cells. Oncotarget 7: 8879-8895, 2016.
11. 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.
12. Herzog EL, Chai L and Krause DS: Plasticity of marrow-derived stem cells. Blood 102: 3483-3493, 2003.
13. Jin SZ, Liu BR, Xu J, Gao FL, Hu ZJ, Wang XH, Pei FH, Hong Y, Hu HY and Han MZ: Ex vivo-expanded bone marrow stem cells home to the liver and ameliorate functional recovery in a mouse model of acute hepatic injury. Hepatobiliary Pancreat Dis Int 11: 66-73, 2012.
14. Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G and Mavilio F: Muscle regeneration by bone marrow-derived myogenic progenitors. Science 279: 1528-1530, 1998.
15. Hassink RJ, Brutel de la Rivière A, Mummery CL and Doevendans PA: Transplantation of cells for cardiac repair. J Am Coll Cardiol 41: 711-717, 2003.

16. Mezey E, Chandross KJ, Harta G, Maki RA and McKercher SR: Turning blood into brain: Cells bearing neuronal antigens generated in vivo from bone marrow. Science 290: 1779-1782, 2000.
17. Ayatollahi M, Soleimani M, Tabei SZ and Kabir Salmani M: Hepatogenic differentiation of mesenchymal stem cells induced by insulin like growth factor-I. World J Stem Cells 3: 113-121, 2011.
18. Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS and Goff JP: Bone marrow as a potential source of hepatic ovel cells. Science 284: 1168-1170, 1990.
19. Theise ND, Badve S, Saxena R, Henegariu O, Sell S, Crawford JM and Krause DS: Derivitation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology 31: 235-240, 2003.
20. Hong H, Chen JZ, Zhou F, Xue L and Zhao GQ: Influence of serum from liver-damaged rats on differentiation tendency of bone marrow-derived stem cells. World J Gastroenterol 10: 2250-2253, 2004.
21. Ohgushi H and Caplan AI: Stem cell technology and bioceramics: From cell to gene engineering. J Biomed Mater Res 48: 913-927, 1999.
22. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S and Marshak DR: Multilineage potential of adult human mesenchymal stem cells. Science 284: 143-147, 1999.
23. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, *et al*: Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418: 41-49, 2002.
24. Polisetti N, Chaitanya VG, Babu PP and Vemuganti GK: Isolation, characterization and differentiation potential of rat bone marrow stromal cells. Neurol India 58: 201-208, 2010.
25. Friedenstein AJ, Chailakhyan RK and Gerasimov UV: Bone marrow osteogenic stem cell: In vitro cultivation and transplantation in diffusion chambers. Cell Tissue Kinet 20: 263-272, 1987.
26. Oh SH, Miyazaki M, Kouchi H, Inoue Y, Sakaguchi M, Tsuji T, Shima N, Higashio K and Namba M: Hepatocyte growth factor induces differentiation of adult rat bone marrow cells into a hepatocyte lineage in vitro. Biochem Biophys Res Commun 279: 500-504, 2000.
27. Schwartz RE, Reyes M, Koodie L, Jiang Y, Blackstad M, Lund T, Lenvik T, Johnson S, Hu WS and Verfaillie CM: Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. J Clin Invest 109: 1291-1302, 2002.
28. Lam SP, Luk JM, Man K, Ng KT, Cheung CK, Rose-John S and Lo CM: Activation of interleukin-6-induced glycoprotein 130/signal transducer and activator of transcription 3 pathway in mesenchymal stem cells enhances hepatic differentiation, proliferation, and liver regeneration. Liver Transpl 16: 1195-1206, 2010.
29. Martínez-Palacián A, del Castillo G, Herrera B, Fernández M, Roncero C, Fabregat I and Sánchez A: EGFR is dispensable for c-Met-mediated proliferation and survival activities in mouse adult liver oval cells. Cell Signal 24: 505-513, 2012.
30. Neuss S, Becher E, Wöltje M, Tietze L and Jahnen-Dechent W: Functional expression of HGF and HGF receptor/c-met in adult human mesenchymal stem cells suggests a role in cell mobilization, tissue repair, and wound healing. Stem Cells 22: 405-414, 2004.
31. Kaido T, Yamaoka S, Seto S, Funaki N, Kasamatsu T, Tanaka J, Nakamura T and Imamura M: Continuous hepatocyte growth factor supply prevents lipopolysaccharide-induced liver injury in rats. FEBS Lett 411: 378-382, 1997.
32. Xue F, Takahara T, Yata Y, Kuwabara Y, Shinno E, Nonome K, Minemura M, Takahara S, Li X, Yamato E and Watanabe A: Hepatocyte growth factor gene therapy accelerates regeneration in cirrhotic mouse livers after hepatectomy. Gut 52: 694-700, 2003.
33. Liu WL, You HJ and Che NC: The promotion of Yiguanjian on the recovery of liver fibrosis through mesenchymal stem cells: An experimental study. Global Traditional Chinese Med 7: 401-405, 2014.
34. Zhu Y and Liu P: Effect of Yi Guan Jian on proliferation and differentiation of hepatic oval cells in rat liver cirrhosis model set up by dimethylnitrosamine. J Dalian Med University 33: 11-16, 2011 (In Chinese).