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Original Article

Effects of human umbilical cord mesenchymal stem cells in the treatment of CCl_4-induced liver cirrhosis

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Abstract: Mesenchymal stem cells (MSCs), a population of multipotent progenitors capable of differentiating into adipogenic, osteogenic, and hepatogenic lineages with low immunogenicity, can be induced to differentiate into liver cells and can also repair the injured liver. Human umbilical cord mesenchymal stem cells (hucMSCs) provide a novel source of MSCs. We obtained hucMSCs from human umbilical cord tissue, and cultured them in serum free medium. The cultured cells showed morphological and functional characteristics of hucMSCs and showed potential to differentiate into different kinds of cells, especially hepatic-like cells. In mouse models of liver cirrhosis, alpha-smooth muscle actin (α-SMA) expression and the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were decreased after the transplantation of hucMSCs, resulting in the amelioration of liver cirrhosis and restoration of liver function. In a co-culture of hucMSCs and hepatic stellate cells (HSCs), α-SMA expression indicated that hucMSCs suppressed HSC activation indirectly. Our results indicated that downregulation of cyclinD1 expression and cell cycle stagnation at G0/G1 phase may be mechanisms involved in the suppression of HSC activation. These results demonstrate that hucMSCs can ameliorate liver cirrhosis by affecting the cell cycle of HSCs. Thus, hucMSCs are a promising source of cell therapy for liver cirrhosis.

Keywords: Human umbilical cord mesenchymal stem cells, induced differentiation, hepatic stellate cells, liver cirrhosis

Introduction

Liver cirrhosis, followed by chronic end-stage liver disease, is a major cause of morbidity and mortality worldwide; however, its treatment remains a global challenge. The most successful tangible solution is liver transplantation [1], but its extensive use in the clinic is restricted due to the paucity of liver donors and the high costs [2]. Several studies have found that MSCs express no MHC class II antigens associated with graft versus host disease, and mesenchymal stem cells derived from exosomes can replace autologous bone marrow stem cells. These two properties make MSCs a promising source for cell therapy. Recently, mesenchymal stem cells (MSCs) from the adult bone marrow were utilized in basic and clinical medical research, but their clinical application is hampered by their scarcity, as they account for only 0.001%~0.01% of the total nucleated cells [3]. Human umbilical cords function not only as a conduit for the transport of materials between the mother and the fetus, but also contain MSCs (human umbilical stem cells (hucMSCs)), which have higher self-renewal ability and lower immunogenicity [4, 5] compared to bone marrow MSCs, making the umbilical cord an attractive source of cells for liver transplantation. Unfortunately, human umbilical cords are routinely discarded after delivery, without ethical consideration.

Research from China and other countries worldwide has shown that hucMSCs play a direct or indirect, but critical role in anti-inflammatory activity, anti-fibrosis, inhibition of hepatocyte apoptosis, and stimulation of liver cell regeneration. Thus, hucMSCs provide an ideal source for cell therapy of liver disease [6-8]. Some

studies have confirmed that hucMSCs can be induced to expand and maintain their stemness in vitro by using conditional serum free medium of appropriate defined chemical composition. Moreover, the expanded hucMSCs retain their immunologic properties, and hematopoietic supportive functions, and can be induced to differentiate into osteocytes and hepatic-like cells [9], suggesting that the in vitro expanded huMSCs can be employed for cell therapy and biomedical research.

In this study, we isolated fresh hucMSCs in vitro and cultured them in conditioned serum free medium to obtain an abundance of hucMSCs. Further, the cultured hucMSCs demonstrated a potential for differentiation into a multitude of cell types, suggesting that these cells could provide a source for cell therapy in liver disorders. Next, we transplanted these hucMSCs in a mouse model of liver cirrhosis, which resulted in a decrease in the cirrhotic index and down-regulation of α-SMA expression, as well as a decrease in serum AST and ALT levels. Liver cirrhosis was ameliorated, and liver function was restored in the mice. Most interestingly, co-culture of hucMSCs with hepatic stellate cells (HSCs) in vitro verified that hucMSCs could influence cell cycle progression as well as the expression of the proteins associated with HSCs. These results show that the hucMSCs reverse liver cirrhosis by at least two mechanisms: by secretion of one or more factors that can affect HSCs; and by differentiation into hepatic-like cells.

Materials and methods

Isolation of hucMSCs

This study was approved by the Research Ethics Committee of the People's Liberation Army 117th Hospital. The hucMSCs were collected from full-term delivered infants and processed within the optimal period of 6 h, then cut into 3 pieces. Blood vessels (2 veins and 1 artery) were stripped. Umbilical cords were rinsed twice in phosphate-buffered saline (PBS), digested overnight with 0.2% collagenase I, and incubated at 37°C, 5% CO_2. After centrifugation at 3000 rmp/20 min, at the digested umbilical cords were plated in serum free DMEM medium and incubated at 37°C, 5% CO_2. The medium was changed every 2 days after initial plating. When well-developed colonies of MSC-like cells reached 80% confluence, cultures were trypsinized with 0.25% trypsin-EDTA (Gino Biological) and cells were split into new Petri dish for further expansion.

Cell viability tested by CCK-8 assay

Cell viability was determined by CCK-8 assay. Cells were seeded in 96-well flat bottom microtiter plates at a density of 2×10^4/mL. At particular time points during 1 day (d)-7 d, medium was changed, and 10 l CCK-8 solution was added to each well. Then the cells were incubated 1 h at 37°C, 5% CO_2. The growth curve was followed by measuring optical density (value of OD) at 450 nm on a microplate reader (Synergy HT, Bio-Tek, USA).

Cell cycle analysis by flow cytometry

Pharmingen annexin V-FITC Apoptosis Detection Kit I (BD Bioscience, USA) was used to detect cell cycle stages and estimation of cells in each phase was done as per according to the manufacturer's instructions. Cells were seeded in a 6 cm dish. After attachment overnight, cells were washed twice with PBS and the medium was replaced. All cells including the floating cells in the culture medium were harvested. The cells were resuspended in ice-cold binding buffer at a concentration of 1×10^6 cells/mL. One hundred microliters of cell suspension was mixed with 5 l PI. The mixture was incubated for 15 min at room temperature in the dark and then analyzed by FACS Calibur Flow Cytometer (BD Biosystems, Heidelberg, Germany).

Immunophenotyping of MSC-like cells by flow cytometry

The hucMSCs were trypsinized and suspended at a concentration of 1×10^6 cells/ml in phosphate buffered saline (PBS) containing 0.1% FBS, and then stained with antibodies against MSC markers (CD29, CD44, CD90, CD105 and CD34, CD45) (Santa Cruz Biological). After 30 min, the cells were washed and resuspended in Cell Fix (BD). Flow cytometry was performed.

Differentiation of hucMSCs in vitro into adipogenic, osteogenic and hepatic-like cells

The hucMSCs were cultured in a medium containing osteogenic or adipogenic reagents. We used hucMSCs cultured in regular medium as

Table 1. Specific primers for target and control genes

Target/Control gene	Primer sequence (5)	Annealing temperature (°C)	Amplification size (bp)
AFP	For: TGCAGCCAAAGTGAAGAGGGAAGA	58	317
	Rev: CATAGCGAGCAGCCCAAAGAAGAA		
ALB	For: ATCTGTCTGCCA TCCTGAACC	58	306
	Rev: ATTGTGCGAAGTCACCCATCA		
GAPDH	For: CAAGGTCATCCATGACAACTTTG	56	500
	Rev: GTCCACCACCCTGTTGCTGTAG		

For, forward; Rev, reverse.

the control. After 3 weeks of induction, the cells were stained using Oil Red O or Alizarin Red to detect the presence of neutral lipid vacuoles in adipocytes or calcium deposition in osteocytes, respectively.

Real-time polymerase chain reaction

Total RNA was extracted with the Trizol reagent according to the manufacturer's instructions (Invitrogen). The cDNA was synthesized using the SuperScript II RT kit according to the manufacturer's instructions (Invitrogen). Primers to generate specific products (α-fetoprotein (AFP), and albumin (ALB)) were designed as shown in **Table 1**. Primers were produced by Shanghai Bio-Engineering Company. GAPDH served as an internal control. All reactions were performed in triplicate. RNA isolated from hucMSCs at each time point was reverse transcribed and amplified with the following primers shown in **Table 1**. Levels are expressed relative to matched control samples from the same time point.

After amplification, the reaction products were detected by agarose gel electrophoresis (AGE). For each sample, the quantity of specific hepatic marker was expressed by gray level ratio with amount of DNA from GAPDH.

Immunohistochemistry

Cells were fixed in 4% formaldehyde for 30 min and rinsed 1-2 times with distilled water, followed by incubation with Protein Block 5% BSA for 20 min, then incubated with cytokeratin-18 (CK-18, 1:200; Invitrogen), or AFP (1:200, Invitrogen) in 5% BSA overnight at 4°C. Cells were rinsed thrice with PBS (pH 7.2-7.6) for 5 min each time and then incubated with anti-mouse secondary antibodies for 20 min. Rinsed thrice with PBS for 10 min cytoplasmic staining was observed under inverted microscope after dyeing with hematoxylin (1:1000).

Immunofluorescence

Cells were cultured for 14 days and washed thrice with PBS for 2 min each time, fixed for 10 min with 4% paraformaldehyde, and then again washed thrice with PBS for 2 min each time. The cells were then collected, fixed and permeabilized 15 min with 0.1% Triton X-100, incubated 30 min with 1% BSA at 37°C, incubated with albumin (ALB, 1:250; Bethyl Laboratories) specific antibody overnight at 4°C, washed twice with PBS for 5 min each time, and then incubated with diluted secondary antibody for 1 h at 37°C, and washed thrice with PBS for 5 min each time. Stained cells were mounted on slides with anti-fluorescent mounting medium. Pictures were taken with fluorescence microscope.

Periodic acid-Schiff staining

Cells were fixed with 10% formaldehyde oxidized in 1% periodic acid (Sigma-Aldrich) for 10 min and rinsed twice with water. Afterward, cells were treated with the Schiff's reagent for 10 min and rinsed with water.

Therapeutic effect of hucMSCs on carbon tetrachloride (CCl_4)-induced liver cirrhosis

All experimental procedures were in accordance with the Chinese legislation regarding experimental animals. Mice were disposed according to the regulation of Ministry of Science and Technology, Guidance of good disposal for experimental animals. The regimen for CCl4 intoxication was performed during 6 weeks. Balb/c mice (18-22 g) were administered with an intraperitoneal injection of CCl4 at 0.125 ml/kg doses in olive oil, 3 times a week for 6 weeks. One hundred-microliter hucMSC suspensions were intravenously injected via the tail at weeks 5 and 6. The control groups

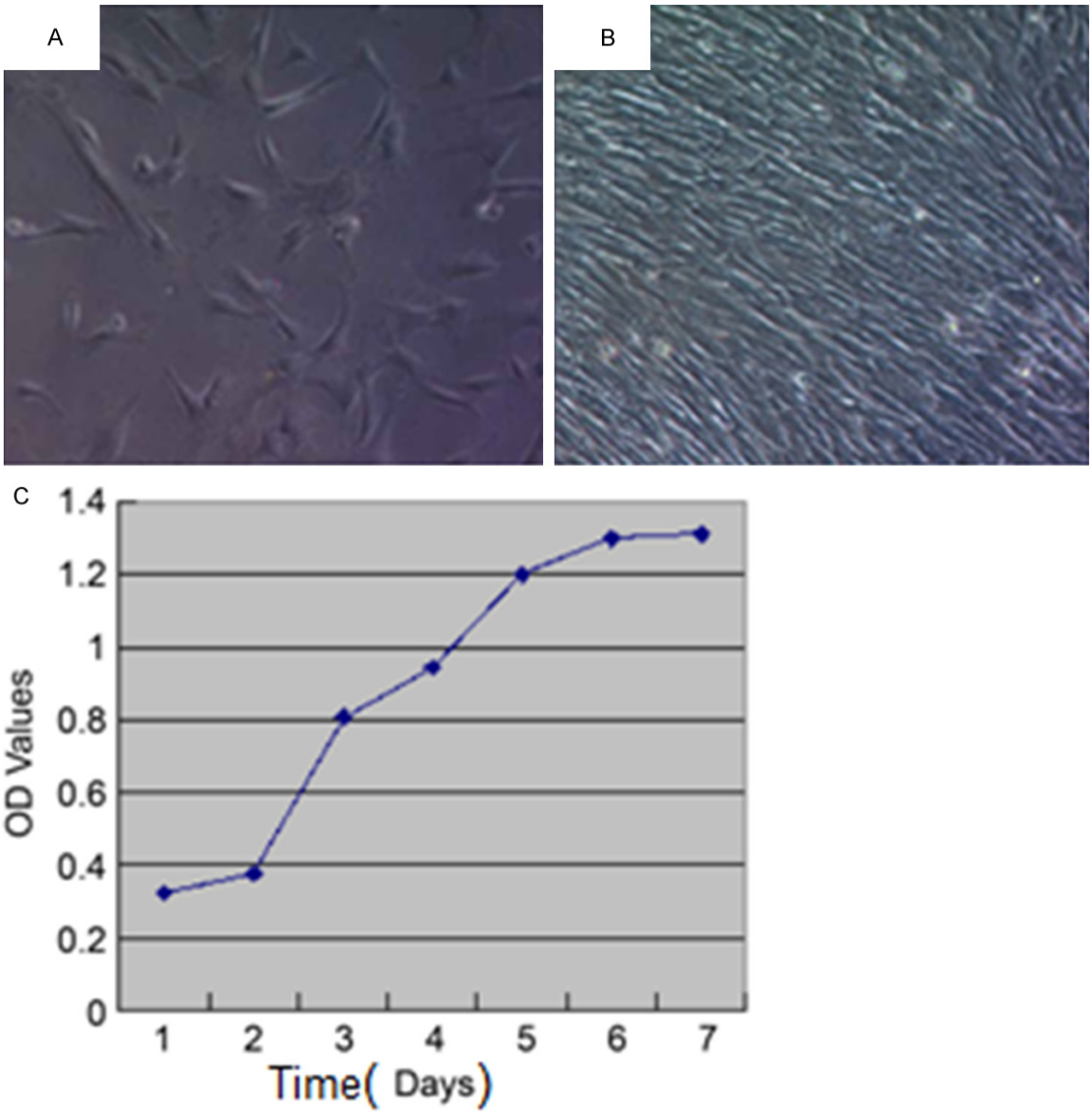
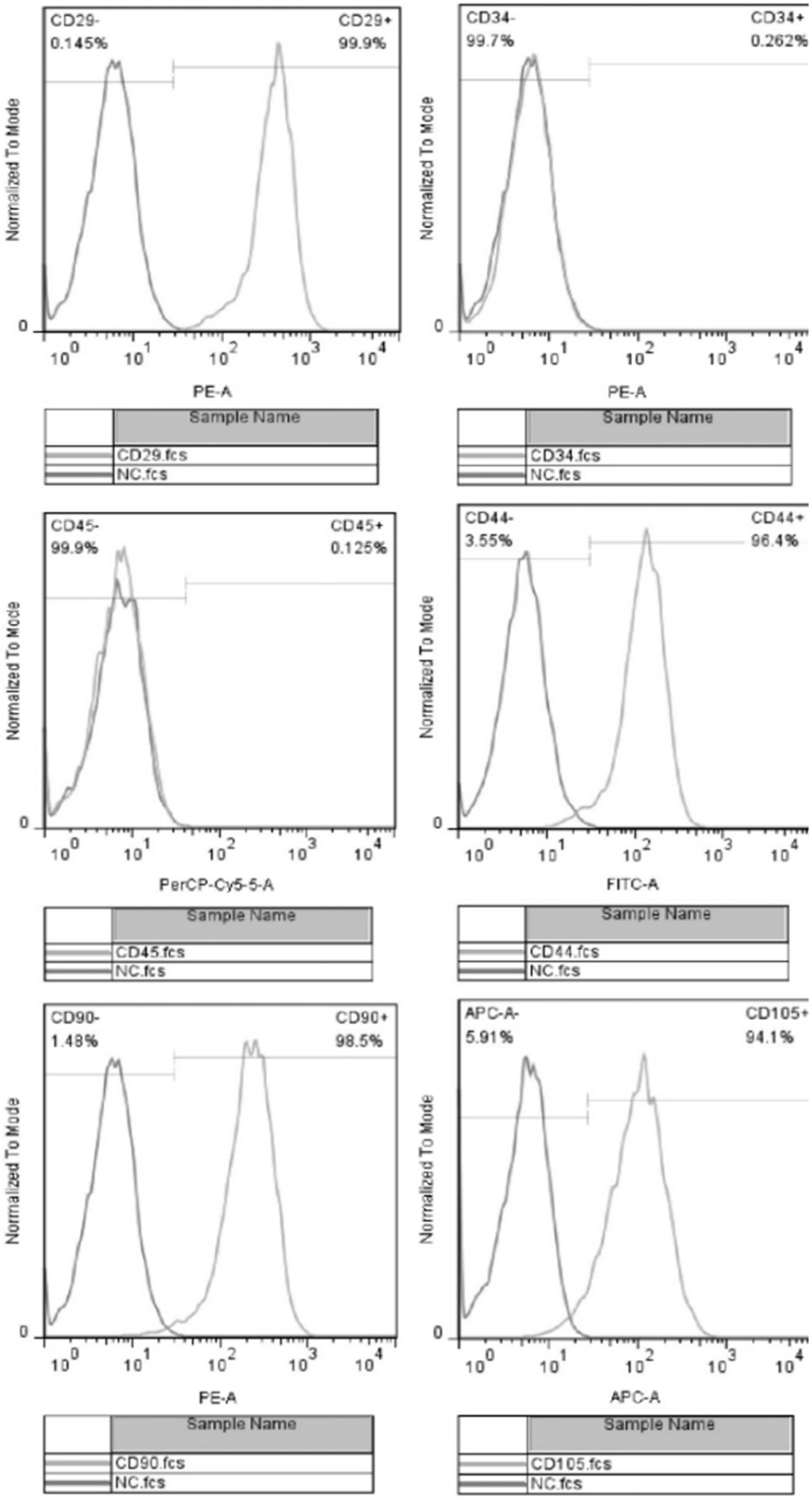


Figure 1. Characteristics of hucMSCs. A. Morphology of primitive hucMSCs at 4 d of culture. B. Morphology of 3rd passage hucMSCs at 3 d of culture (100 ×). C. Cell viability of hUCMSCs via CCK-8 assay at particular points during 1 d-7 d. Optical density (value of OD) was measured on a microplate reader (Synergy HT, Bio-Tek, USA) at 450 nm. Growth curve with OD value was performed. The experiments were performed in triplicate.

were injected with equivalent volume of PBS. Six weeks later blood samples were collected, liver tissue were excised and pathological changes were observed. Serum AST and ALT levels in mouse blood were measured with Toshiba automatic biochemical analyzer.

Co-culture of HSCs with hucMSCs

HSCs were isolated and expanded according to methods reported by Chen Xiaoling (Chen Xiaoling, article in Chinese) and then directly co-cultured with hucMSCs. Mouse HSCs were cultured in Transwell cell dish with two chambers, hucMSCs occupied the upper chamber and HSCs occupied the lower chamber, where the two kinds of cells share a common culture medium without physical contact. The hucMSCs at 2×10^5 cells/well and HSCs at 2×10^5 cells/well were cultured in the co-culture group. For the control group, the lower layer (below the transmembrane of the Transwell) consisted of 2×10^5 HSCs/well, with the upper layer consisting of 10% fetal bovine serum containing DMEM medium. Cell cycles were analyzed after incu-


CD29-
0.145%
CD29+
99.9%
Normalized To Mode
PE-A
Sample Name
CD29.fcs
NC.fcs
CD34-
99.7%
CD34+
0.262%
Normalized To Mode
PE-A
Sample Name
CD34.fcs
NC.fcs
CD45-
99.9%
CD45+
0.125%
Normalized To Mode
PerCP-Cy5-5-A
Sample Name
CD45.fcs
NC.fcs
CD44-
3.55%
CD44+
96.4%
Normalized To Mode
FITC-A
Sample Name
CD44.fcs
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CD90-
1.48%
CD90+
98.5%
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Sample Name
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NC.fcs
APC-A-
5.91%
CD105+
94.1%
Normalized To Mode
APC-A
Sample Name
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NC.fcs

Figure 2. Identification of human umbilical cord-mesenchymal stem cells (hucMSCs). Flow cytometry analysis of the surface markers in hucMSCs of CD29, CD44, CD90, CD105 and CD34, CD45.

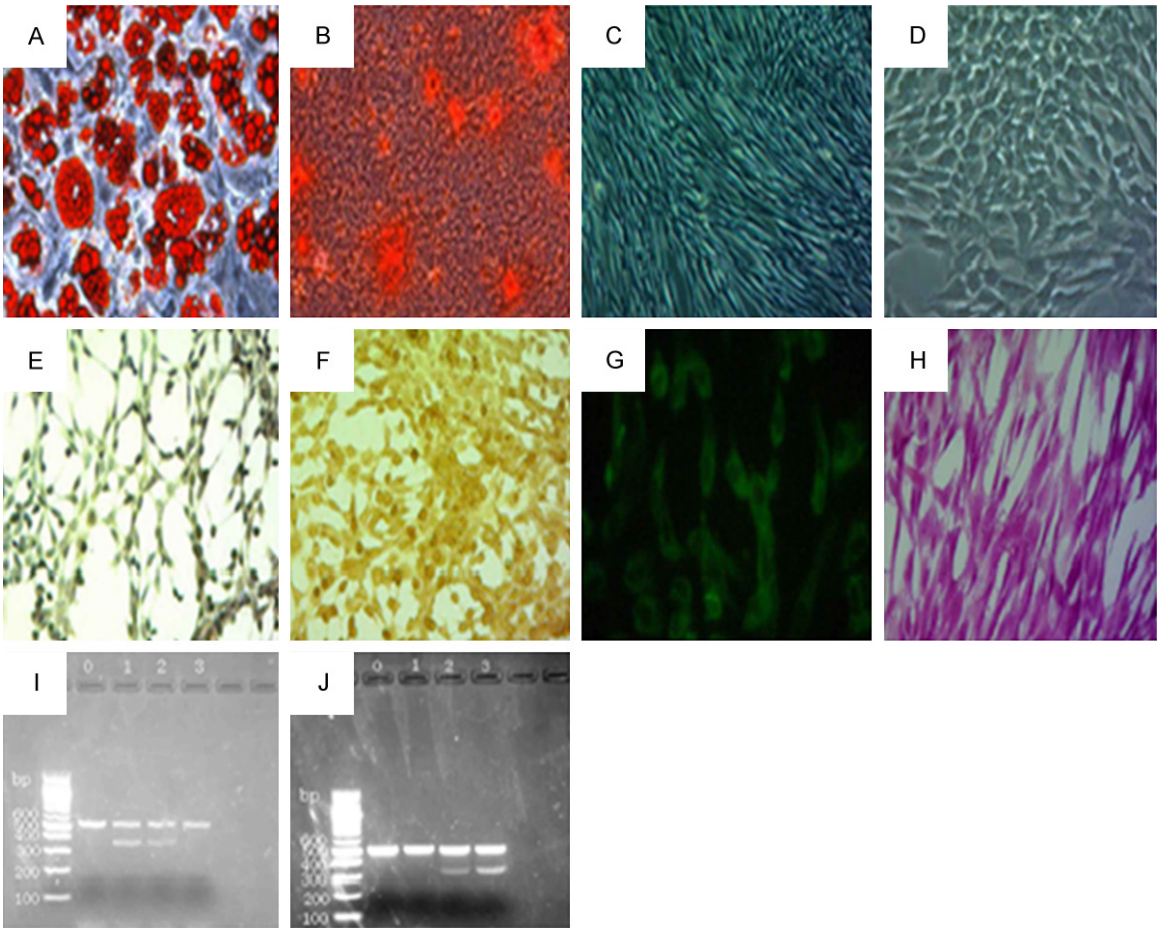

Figure 3. HucMSCs can be differentiated induced into adipogenic, osteogenic and hepatocyte-like cells. A. Result of Oil-Red-O staining detection formation of intracytoplasmic lipid droplets in hucMSCs cultures grown with osteogenic or adipogenic reagents for 3 weeks (100 ×). B. Results of Alizarin Red staining detection abundance of orange density nodules of calcium deposits in hucMSCs cultures grown with osteogenic or adipogenic reagents for 3 weeks (100 ×). D. Induced differentiation to hepatocyte-like cells, 2 weeks (200 ×). C. Control group, 2 weeks (100 ×). E. AFP immumohistochemical staining of hucMSCs at induced culturing for 7 d (100 ×). F. CK18 immumohistochemical staining of hucMSCs at induced culturing for 14 d (100 ×). G. ALB immunofluorescent staining of hepatocyte-like cells (100 ×) at induced culturing for 14 d. H. PAS staining of hepatocyte-like cells at induced culturing for 20 d (200 ×). I. Expression of AFP gene tested by RT-PCR analysis of hucMSCs when cultured for 7 d, 14 d, 21 d. (M stands for marker, 0 stands for the control group, 1 stands for cultured 7 d, 2 stands for cultured for 14 d, 3 stands for cultured 21 d. J. The expression of ALB gene tested by RT-PCR analysis of hucMSCs when cultured for 7 d, 14 d, 21 d. (M stands for marker, 0 stands for the control group, 1 stands for cultured 7 d, 2 stands for cultured for 14 d, 3 stands for cultured 21 d.

bating for 24 h, 48 h, and 72 h. CyclinD1 and α-SMA (alpha-Smooth Muscle Actin) expression was tested by western blotting.

Statistical analysis

Statistical analysis was performed using SPSS 16.0, and the results were expressed as values of mean ± standard deviation (SD). Analysis of variance was used to analyze variance among all groups. We performed independent sample's t-test, and the statistical significance was set at $P < 0.05$.

Results

Characteristics of hucMSCs

To observe the characteristics of hucMSCs, we collected colonies of hucMSCs after 4 days of

culture in serum free culture medium. The isolated hucMSCs started to grow, and partial cells of diverse types extended pseudopodia (**Figure 1A**), eventually reaching confluence as fibroblast forms. Cells stretched and stuck to the bottom of the Petri dish, divided rapidly and fused upon reaching 90% confluence after 3 d in culture in culture. The cells were strongly adhesive, formed swirling colonies and multiplied rapidly, characteristics similar to those found in other studies [9], and consistent with the three appraisal standards for MSCs stipulated by the international commission on cell therapy [10] (**Figure 1B**). CCK-8 assay revealed that the cells had the characteristic S growth curve of normal cells. One to two days after inoculation, the cells entered an adaptive period followed by a rapidly growing phase at 3-5 d, then ceased growing (**Figure 1C**).

Expression of hucMSC markers

Flow cytometric analysis revealed hucMSCs highly expressed CD29, CD44, CD90 and CD105 (which are generally considered as markers of MSCs) with expression levels of 99.9%, 96.4%, 98.5%, 94.1%, but CD34 (surface marker of hematopoietic precursor cell) and CD45 (human leucocyte antigen) were not expressed in the hucMSCs [11] (**Figure 2**). Based on the above results, these cells were confirmed as MSCs rather than a source of hematopoietic stem cells.

Induction of the differentiation of the hucMSCs into osteogenic, adipogenic, and hepatic-like cells

To investigate the capacity of the progenitor hucMSCs to differentiate into multiple cell types, we cultured hucMSCs with osteogenic or adipogenic reagents, which resulted in their osteogenic and adipogenic differentiation. Adipogenic differentiation was demonstrated by the formation of intracytoplasmic lipid droplets that stained with Oil Red O after 3 weeks; cells gradually deformed from the shuttle type to a circular shape (**Figure 3A**). The secretion of extracellular precipitate induced by osteogenic differentiation increased and resulted in an abundance of orange density nodules, which were identified as calcium deposits by Alizarin Red staining after 3 weeks (**Figure 3B**). Furthermore, the hucMSCs were differentiated into hepatic-like cells at 14 d in culture (**Figure 3C**). We examined AFP, CK-18, PAS, and ALB expression levels (**Figure 3E-H** respectively) by immunostaining of the hepatic-like cells formed after 7-20 d in culture. RT-PCR analyses of AFP and ALB gene expression (**Figure 3I** and **3J** respectively) showed that AFP transcription appeared at 7 d and 14 d, while ALB transcription appeared at 14 d, with the transcription increasing with time. Together, these data indicate that the hucMSCs were successfully differentiated into liver cells after induction in culture.

Transplantation with hucMSCs ameliorated CCl_4-induced mouse liver cirrhosis

To identify their therapeutic potential, hucMSCs were transplanted into mice at week 5 and 6 after CCl4-induced liver cirrhosis. Hematoxylin and eosin (HE) staining shows that the fibrotic area of hucMSCs treated group is much less than the control group (**Figure 4A**, **4B**). According to our IHC results, α-SMA expression is down regulated in hucMSCs treated group compared to the control group (**Figure 4C**, **4D**). As shown, ALT ($P < 0.05$) and AST ($P < 0.05$) levels declined after hucMSCs transplantation compared with the control group which indicates the hucMSCs transplantation decreases serum ALT and AST levels (**Figure 4E**), confirming that hucMSCs mediate liver protection.

Modulation of the cell cycle by hucMSCs co-cultured with HSCs

To identify the regenerative ability of hucMSCs on HSCs, we compared the cell cycle upon co-culture of hucMSCs with HSCs (hucMSCs+ HSCs) with that observed in single culture groups. G0/G1-S phase checkpoint is the key in determining cell cycle progression. Flow cytometry results showed there was no obvious inhibition of cell cycle progression of the HSCs ($P > 0.05$) at 24 h. However, when we compared the co-culture group with the control group (HSCs) at 48 h, cells in G0/G1 phase were found to be significantly ($P < 0.05$), while cells in the S phase were lower ($P < 0.05$) in the co-culture group. Furthermore, when we compared the co-culture group with the control group at 72 h, cells in G0/G1 phase were significantly higher ($P < 0.05$), while cells in the S phase were lower (**Figure 5A**) in the co-culture group. Quantitative flow cytometry (FCM) analysis revealed 78% cells in the G0/G1 phase of the cell cycle, which is in agreement with the slow cell cycle characteristics of stem cells ($P < 0.05$) (**Figure 5B**).

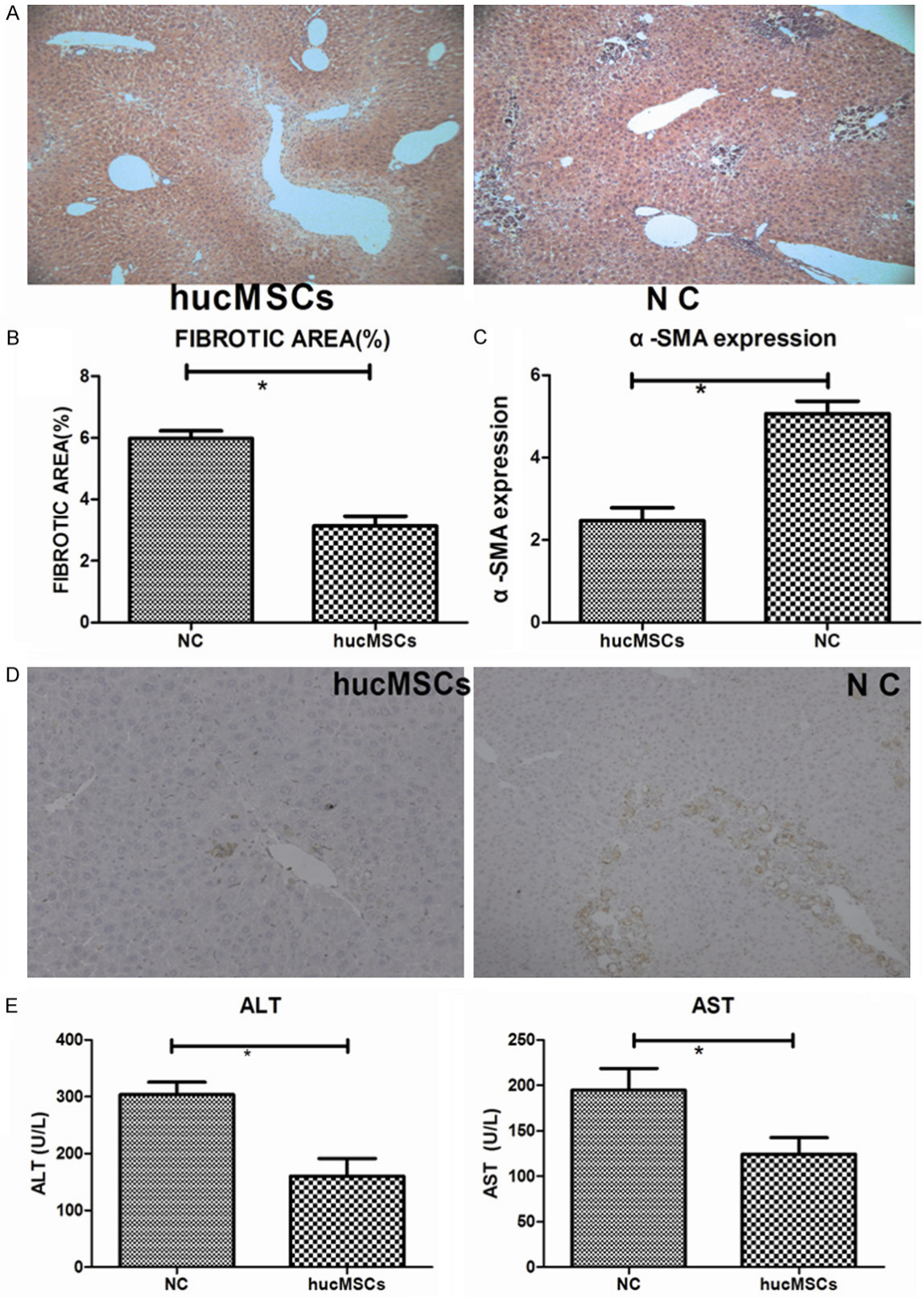

Figure 4. After hucMSCs transplantation, the morphology of the fibrotic area was observed via Masson Dyeing (A; magnification ×4) and the percentage of fibrosis (B) was determined. The expression of α-SMA (C and D; magnification ×20) was also evaluated. (E) the serum expression levels of ALT, AST were evaluated. *P<0.05, **P<0.01.

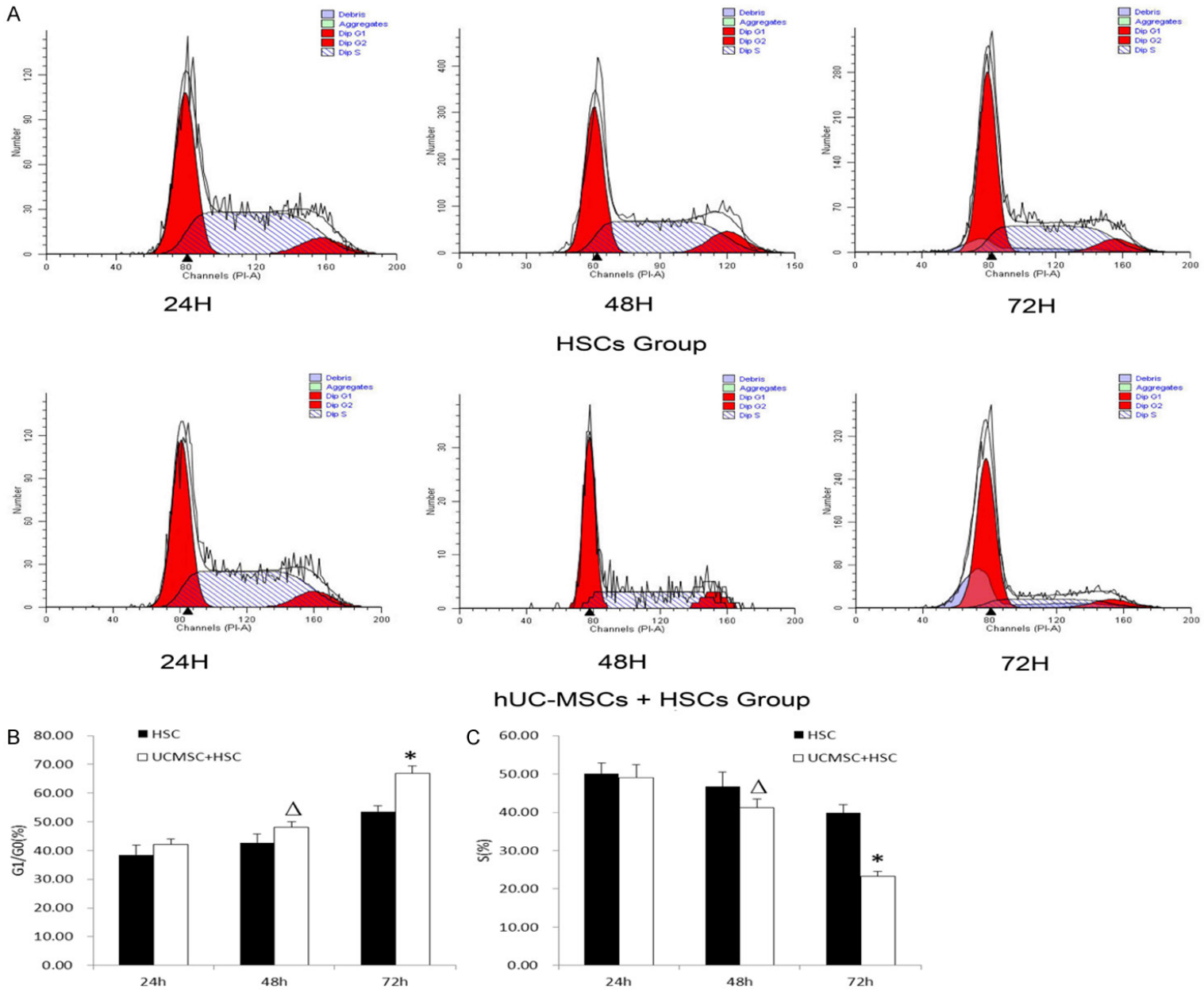
A
Debris
Aggregates
Dip G1
Dip G2
Dip S
Number
Channels (PI-A)
24H
48H
72H
HSCs Group
hUC-MSCs + HSCs Group
B
HSC
UCMSC+HSC
G1/G0(%)
24h
48h
72h
C
S(%)

Figure 5. Flow Cytometry detect the cell cycle. A. HSCs group and HSCs+hucMSCs group, respectively cultured for 24 h, 48 h, 72 h. B. Quantitative analyses changes of cell cycle G0/G1 phase ; *P < 0.05. C. Quantitative analyses changes of cell cycle S phase ; *P < 0.05. The experiments were performed in triplicate.

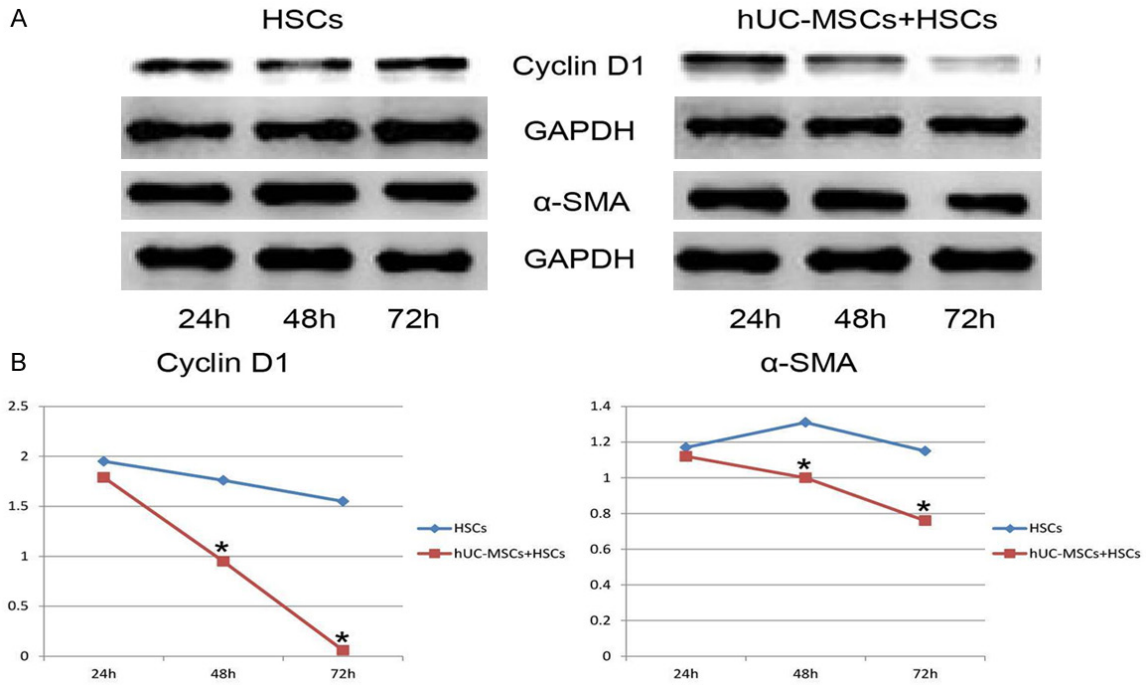

Figure 6. hucMSCs decreased expression of cyclinD1 and α-SMA. A. Detection of CyclinD1 and α-SMA expression by western blotting; B. Representative results of CyclinD1 and α-SMA expression were measured by density analysis of western blot bands. *P < 0.05 and *P < 0.01. Value are expressed as the Mean ± SD of three independent experiments.

The use of hucMSCs decreased the expression of cyclinD1 and α-SMA

Alpha-smooth muscle actin is a reliable marker expressed upon hepatic stellate cell activation, which precedes liver fibrosis [12]; therefore, a decrease in α-SMA expression is expected upon liver transplantation and/or protection. CyclinD1 controls the G0/G1-S phase checkpoint and is expressed at an early stage of G1 [13]. Cells passing through the G0/G1-S checkpoint largely depend on the accumulation of cyclinD1 in the G1 phase. We discovered that cyclinD1 and α-SMA levels began to decline at 48 h and were obviously lower than that in the control group at 72 h (P < 0.01) in the co-culture of hucMSCs with HSCs; However, in the first 24 h of culture, there was no significant difference in the levels of both proteins compared to that in the control group. In the control group, no change in cyclinD1 or α-SMA levels was seen at 24 h, 48 h, or 72 h (**Figure 6A**). Differential expression of cyclinD1 and α-SMA was measured by quantitative densitometric analysis of western blot bands (**Figure 6B**). The decline in cyclinD1 and α-SMA levels that began at 48 h in co-culture, with a significant decrease in both proteins at 72 h; this suggests that hucMSCs suppressed HSC activation indirectly. The decline in cyclinD1 expression further suggests that hucMSCs may inhibit HSC activation and proliferation by downregulating cyclinD1 expression.

Discussion

Mesenchymal stem cells have been suggested as an alternative to liver transplantation because of their capacity to differentiate into mature hepatocytes, but their clinical application is hampered by the scarcity of MSCs and the difficulties posed in propagating and differentiating them. Compared to MSCs, hucMSCs isolated from full-term infants possess higher

self-renewal ability and low immunogenicity [14, 15]. Collagenase digestion of the umbilical cord has been used to quickly acquire a large number of adherent, growing, primitive cells with good cell vitality; these cells have been confirmed to show no significant changes in their immunophenotype following collagenase digestion and continued culturing [16]. Our studies have confirmed that hucMSCs can be induced to expand and maintain their stemness in vitro by using conditioned serum free medium of defined chemical composition.

Several studies have confirmed the potential of MSCs to differentiate into liver cells [17]. One of the markers expressed in differentiating hepatic precursor cells, but not in mature hepatocytes, is AFP. We analyzed AFP expression in cultured hucMSCs and found that AFP was expressed at 7 d after inducing differentiation; the expression reduced at 14 d and was absent at 21 d, thus confirming that AFP was expressed in immature precursors of hepatocytes when differentiation was induced in cultured hucMSCs. On the other hand, ALB is expressed by liver parenchyma cells, with its expression gradually increasing as the liver cells mature. Accordingly, we found that ALB gene transcription was detected at 14 d in culture after inducing differentiation, and increased with time. Detection of these markers in cultured cells confirms the successful differentiation of hucMSCs grown in serum-free culture into liver cells following induction of differentiation [18].

Tsai et al. [19] reported that hucMSCs co-cultured with CCl4-induced mouse liver cells could remarkably alleviate mouse liver fibrosis and reduce serum ALT, α-SMA, and TGF-β1 expression. In our experiments, following transplantation with hucMSCs, mice subjected to CCl4-induced liver cirrhosis showed remarkably lower serum ALT and AST levels compared to that in the control group, indicating that transplanting with hucMSCs protected liver tissue and function. However, there is a risk of vascular thrombosis upon transplantation with large number of cells, as the transplanted cells can circulate through the lung endothelial barrier on entering peripheral venous blood [20]. While this imposes a challenge on the treatment of liver injury using hucMSCs, studies have reported that transplantation with 106 cells produced a significant positive effect on liver repair [21].

Cell cycle regulation is realized by controlling cyclin-CDK compound activation through orderly phosphorylation and dephosphorylation of cyclin and CDK [22]. The cyclin-CDK compound activation is regulated by CKI, which suppress the formation of cyclin-CDK compounds and inhibits cell proliferation [23, 24]. CyclinD1 factors control the G0/G1-S phase checkpoint, which is a key determinant in cell cycle progression [13, 25]. CyclinD1 is expressed at the early stage of G1 as an initiator of the cell cycle and plays a vital role in G1/S transition [26]. The number of cells passing through the checkpoint largely depends on the accumulation of cyclinD1 in G1 phase. Blocking the expression of cyclinD1 prevents cells from progressing from the G1 phase to S phase. We found that upon co-culture of hucMSCs and HSCs for 48 h, cyclinD1 expression began to decrease compared to that in the control group, and significantly reduced at 72 h. Concomitantly, cells stagnated at the G0/G1 phase, and exerted an inhibitory effect on rat HSC proliferation.

Expression of α-SMA by HSCs indicates their activation [27], which is accompanied by enhanced expression of the c-myb gene and activation of the NF-κB transcription factor. In HSCs, c-myb protein interacts with the regulatory region of the α-SMA gene, resulting in expression of the α-SMA protein [28]. Suppressing and/or reducing the expression of α-SMA can inhibit the activation of HSCs to slow down liver fibrosis [29, 30]. We therefore investigated the mechanism that inhibited HSC proliferation and activation through hucMSCs. HUCMSCs can promote the apoptosis of HSCs and reduce the expression of the HSC activation-associated marker α-SMA. Our results show that co-culturing hucMSCs with HSCs for 48 h results in a decrease in α-SMA expression, with the expression decreasing significantly at 72 h; this indicated that hucMSCs suppressed HSC activation, albeit indirectly.

In summary, our studies demonstrate that hucMSCs can be induced to differentiate into hepatic-like cells. However, hucMSCs may not differentiate into liver cells as effectively in vivo as in vitro. The number of cells used for transplantation in clinical trials is not consistent with improvement based on the degree of liver function index observed. When we transplanted hucMSCs in mice suffering acute liver injury induced by CCl4, their serum ALT and AST levels, which are sensitive indicators of liver cell

damage, remarkably reduced compared to that in the control group. In addition, α-SMA and cyclinD1 expression, which are involved in HSC activation and cell proliferation, respectively, also decreased with time following hucMSC transplantation. This indicates inhibition of HSC activation, which is known to precede liver fibrosis. Our results provide preliminary evidence that hucMSC transplantation can accelerate the repair of acute liver injury and protect liver tissue. However, these protective effects are contingent on various complicated factors, warranting further investigations into the mechanisms involving acute liver injury and repair.

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Disclosure of conflict of interest

None.

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