The differentiation of MSCs into functional hepatocyte-like cells in a liver biomatrix scaffold and their transplantation into liver-fibrotic mice

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1. Introduction

Liver transplantation remains the definitive treatment option for end-stage liver disease. However, the surgical complications, chronic rejections, critical shortage of donor organs and high cost of this procedure have sparked tremendous interest in finding new treatments [1]. Cell-based therapies, such as cell transplantation and bioartificial liver devices, have emerged as alternative therapies [2,3]. Because of the difficulties associated with obtaining autologous hepatic tissue and maintaining the phenotype of the primary hepatocytes in culture, the scarcity of human hepatocytes remains a serious roadblock for the development of cell-based therapies [4]. Therefore, the promise of a renewable supply of functional hepatocytes from an alternative source represents an important goal in current studies.

Stem cells are considered an alternative cell source for functional hepatocytes. Increasing evidence suggests that the differentiation of stem cells into hepatocytes is achieved in the appropriate microenvironment following stimulation with hepatic growth factors [5–7]. Mesenchymal stem cells (MSCs) are a type of adult stem cell and, compared to hepatocytes, are more suitable for cell therapy because of their adequate availability, easy accessibility, rapid proliferation, multipotent differentiation, and successful integration and immunological tolerance in the host tissue [8]. Meanwhile, MSCs could be derived from a patient’s own tissues rather than blastocyst or embryos, and are considered more appropriate for clinical use [9]. In addition, it has been shown that the transplantation of MSCs or MSC-derived hepatocyte-like cells improves the liver function in rodents [10,11] or patients [12] suffering from liver damage. However, the several traditional protocols used to date have had limited success, and these hepatocyte-like cells exhibit only a portion of the markers and functions of normal hepatocytes.

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functions of primary hepatocytes. Therefore, further investigations are needed to optimize the direct differentiation protocol and the culture conditions for MSCs to yield mature hepatocyte-like cells that are fully functional.

Recent major advances in stem cell biology have produced a biologically inspired in vitro cellular microenvironment for guiding stem cell growth, differentiation and functional assembly [13]. To mimic the biological environment in vivo, a dynamic three-dimensional (3D) environment comprised of biomaterial scaffolds and bioreactors has been used for stem cell differentiation; the scaffolds provide structural templates for cell attachment and proliferation, whereas the bioreactors provide environmental control [14]. Moreover, a key factor in the cellular environment is the extracellular matrix (ECM), which includes numerous chemical and biophysical cues for stem cell differentiation, depending on the extracellular matrix (ECM) components and preserved mechanical properties [16].

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Recently, bioscaffolds derived from the decellularized organ/tissue matrix have been used to successfully support the growth and function of several cell types, such as hepatocytes [17,18] and endothelial cells [15], because of the preserved "spatial" array of matrix components [20]. Furthermore, studies have emphasized that the differentiation of stem/progenitor cells is lineage restricted by the tissue-specific biomatrix scaffold. The differentiation of human hepatic stem cells into mature cells is more efficient in the decellularized liver biomatrix, as demonstrated by marker gene expression and function analysis [21]. Ng et al. demonstrated that the intact ECM components and preserved mechanical properties of the decellularized heart directed the differentiation of stem cells into the cardiac lineage [20]. The combined use of MSCs and decellularized allogeneic artery conduits achieved satisfactory nerve regeneration and functional restoration in vivo, which was associated with the transplanted cells maintaining their acquired Schwann cell phenotype [22]. This study investigates whether decellularized liver promotes the hepatic differentiation of MSCs.

We hypothesized that the 3D bioscaffold derived from decellularized rat liver provides a microenvironment mimicking the native liver, and promotes the hepatic differentiation of murine MSCs into high yields of mature hepatocytes in vitro. In this study, a modified dynamic culture system was used to control the environmental conditions. To determine the differentiation stages of the MSCs, RT-PCR of the endoderm and hepatocyte genes, immunostaining of the hepatocyte markers, and ultrastructural and functional analyses were performed. Furthermore, the therapeutic potential and cell derivation of the pre-differentiated cells in vitro was investigated in vivo following the intravenous administration of the cells in a mouse model of chronic liver injury.

2. Materials and methods

2.1. Animals

The Institutional Animal Use and Care Committee of the Fourth Military University (China) approved this research, and this protocol complied with the Guide for the Care and Use of Laboratory Animals. Male Fisher 344 rats, aged 8–12 weeks and weighing between 250 and 300 g, were used for the liver harvests. Green fluorescent protein (GFP)-transgenic C57BL/6 mice, aged 6–8 weeks, were used for the isolation of the primary hepatocytes and bone marrow-derived MSCs. Wild-type male C57BL/6 mice, weighing between 20 and 25 g and aged 6–8 weeks, were used as the liver-injured models that underwent cell transplantation. The animals were maintained in a 12 h light/dark cycle with free access to standard laboratory feed and water.

2.2. Preparation of decellularized rat liver

After the rats were euthanized, a sub-phrenic abdominal incision was made to visualize the liver. First, the portal vein was cannulated using an 18-gauge (18G) cannula, and the vein was injected with 5 ml of 0.9% saline containing 200 U of heparin (Sigma, St. Louis, MO). The vena cava and the remaining attachments were dissected, and the harvested liver was frozen at −80 °C overnight. Next, the frozen liver was thawed at 4 °C, and the cannula in the portal vein was attached to a pump (LongerPump® BT100-2 peristaltic pump with LongerPump® YZ1515xs pump head and 16G tubing, Baoding, China). The decellularized liver was obtained by a modified protocol previously reported [23]. Briefly, the liver was initially perfused with deionized water for 30 min, followed by 0.25% trypsin/0.038% ethylenediaminetetraacetic acid (Invitrogen, Carlsbad, CA) in phosphate-buffered saline (PBS) at 37 °C for 30 min, and perfusion with 1% nonylphenolpolyethoxylethanol (NP-40, Sigma) in deionized water for 4 h. For further decellularization and delipidation, the liver was perfused with PBS for 2 h. After the decellularization procedure, the decellularized liver was frozen, lyophilized in a FreeZone 6 L Freeze Dry System (Labconco, USA), and sealed at −20 °C.

2.3. Isolation and cultivation of cells

2.3.1. Primary mouse hepatocytes

Primary hepatocyte cultures, derived from the GFP-transgenic mice, were isolated using collagenase perfusion and were plated on rat-tail collagen as described previously [24]. The hepatocytes were cultured using the following defined, serum-free hepatocyte media: William’s E Media (Sigma) supplemented with 10 nM HEPES (Sigma), 100 U/ml penicillin/streptomycin, 5 ng/ml selenium, 5 µg/ml transferin, and 1% Linoleic acid-albumin (all from Sigma). The cells were maintained in a standard CO2 (5%) cell incubator (HEPA class-100, Thermo, USA) at a constant temperature of 37 °C, and the hepatocyte media was replenished every other day.

2.3.2. MSCs derived from mouse bone marrow

The MSCs were harvested by flushing the diaphysis of the femurs and tibias from the GFP-transgenic C57BL/6 mice using Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) medium (HyClone) containing 15% fetal bovine serum (FBS, Gibco). Following filtration through a 200 mesh screen and repeated pipetting, the single-cell suspension was plated into 25 cm² tissue culture flasks (TCF). The non-adherent cells were removed after 72 h by rinsing and replacing the medium in the flasks. At 60–70% confluence, the cells were passaged at a ratio of 1:2 by the addition of an equal volume of 0.25% trypsin-EDTA (Invitrogen). The medium was replaced at least every 2–3 days. The cells were characterized using flow cytometry at passage 3 and were used at passage 3–6.

2.4. Cell seeding and dynamic culture

The decellularized whole livers were cut into discs measuring 30 mm in diameter and 3-mm thick. The biomatrix scaffolds were sterilized overnight in PBS containing 100 U/ml penicillin/streptomycin and amphotericin B (Sigma) and were placed in 6-well culture plates. The cells of approximately 1 x 10⁷ (resuspended at a concentration of 5 x 10⁶ cells/ml) were dropped directly onto the surface of each scaffold and additional vacuum/aeration cycles, controlled by injection needles, were performed to help the cells absorb. The scaffolds were surrounded by DMEM/F12 medium and were placed in a cell incubator for 4–6 h to allow cell adherence. Next, the scaffolds were maintained in static culture or were moved into the perfusion chambers of the modfed dynamic culture system, as described previously by Salion et al [25]. For the dynamic culture, the medium was recycled at a flow rate of 0.5, 1, 2, 4, or 6 ml/min, regulated using a peristaltic pump. For the static culture, the cells were seeded directly onto the TCF or the cell-loaded scaffolds and were maintained in 6-well tissue culture plates. The medium was replenished every other day. The cell proliferation was determined by quantifying the amount of dsDNA in each scaffold using the Quant-it™ PicoGreen® dsDNA assay (Invitrogen), and the cell apoptosis level was assessed using the TMR Red in Situ Death Detection Kit (Roche, Indianapolis, IN) in accordance with the manufacturer’s instructions. In addition, hematoxylin and eosin (H&E) stain and scanning electron microscopy (SEM) analysis were performed at each specific time point.

2.5. Hepatic differentiation of MSCs in vitro

To induce hepatic differentiation, serum-free Iscove’s modified Dulbecco’s Medium (IMDM, HyClone) supplemented with a combination of growth factors (GF) (described previously by Lee et al [5] with minor modifications) was used for the MSCs in the dynamic cultured scaffold (DCS) and the TCF. Briefly, prior to the two-step induction protocol, the cells were serum-deprived for two days in IMDM medium supplemented with 20 ng/ml epidermal growth factor (EGF, Sigma) and 10 ng/ml basic fibroblast growth factor (bFGF, Sigma). The induction protocol was as follows: step one, the MSCs were treated with differentiation medium consisting of IMEM medium supplemented with 20 ng/ml hepatocyte growth factor (HGF, Sigma), 10 ng/ml bFGF, and 0.01 g/l nicotinamide (Sigma) for 7 days; step two: the MSCs were induced with maturation medium, which consisted of IMEM supplemented with 20 ng/ml oncostatin M (Sigma), 1 µmol/l dexamethasone (Sigma), and 20 ng/ml transforming growth factor beta (TGF-β, Sigma). At day 7, the cells were harvested and stained for marker gene expression analysis.
50 mg/ml insulin-transferrin-selenium premix (ITS−, Sigma) for 2 weeks. As a control, IMDM supplemented with 50 mg/ml ITS− in the absence of GF was used for both the DCS and the TCF.

2.6. RNA extraction and RT-PCR analysis

At various stages during the culture in the DCS and TCF, the total RNA was extracted from the differentiated cells using TRIzol reagent (Invitrogen) in accordance with the manufacturer’s protocol. The total RNA was reverse-transcribed, and cDNA was synthesized using SuperScriptIII reverse transcriptase (Invitrogen). The cDNA samples were subjected to polymerase chain reaction (PCR) amplification using the mouse-specific primers (Sangon Biotechnology Co., Shanghai, China) listed in Supplemental Table S1. The glyceraldehydes-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as an endogenous internal control. The amplification protocol included 3 min at 95 °C, followed by 45 cycles of 10 s at 95 °C for denaturation and 45 s at 60 °C for annealing, and a final extension at 72 °C for 10 min. The PCR reactions were performed in triplicate. The PCR products were analyzed by gel electrophoresis on 1.5% agarose gels stained with ethidium bromide (10 μg/ml, Sigma) and were visualized and photographed on a UV transilluminator (Uvivid, UK). The undifferentiatedMSCs and primary hepatocytes were used as the negative or positive controls.

2.7. Flow cytometry analysis

The cells were harvested by digestion with 0.02% trypsin/EDTA and were fixed in 4% paraformaldehyde for 40 min. The cells were rinsed with PBS containing 5% bovine serum albumin (BSA, Thermofisher) for 30 min and were incubated either with direct PE-conjugated antibodies for 45 min or with primary antibodies overnight. This step was followed by three washes with PBS and incubation with PE-conjugated secondary antibody for 4 h in the dark at room temperature. Finally, the cells were washed three times in PBS and were resuspended in 200 μl PBS. The mouseMSCs that reached a concentrationof 5 × 10⁷ cells at passage 3 were incubated with the following PE-conjugated antibodies: anti-mouse/rat CD90.1 (1:200, eBioscience), anti-mouse/rat CD105 (1:200, eBioscience), anti-mouse/rat CD29 (1:200, Biolegend, San Diego, CA), anti-mouse CD45 (1:200, Biolegend), anti-mouse CD34 (1:200, Biolegend) and anti-mouse CD80 (1:200, Biolegend). The differentiatedMSCs were incubated with primary antibodies (1:200) against alpha-fetoprotein (AFP, Santa Cruz Biotechnology, Santa Cruz, CA), albumin (ALB, Santa Cruz, CA), α-smooth muscle actin (α-SMA, 1:500, Santa Cruz) antibodies diluted in antibody dilution solution. The differentiatedMSCs cultured with and without the presence of GF. At the end of the procedure, the slides weresubjected to deep anesthesia, and the venousblood andliver tissue were harvested for analysis. The serum ALB and AST levels were determined using a chemistry analyzer (AU4000, Olympus, Tokyo, Japan). The in vivo bio-imaging was performed using a NightOwl imaging system and the WinLight software (Berthold, Germany).

2.8. Hepatocyte-specific function assays

2.8.1. AFP, ALB and urea production

The conditioned media from the differentiatedMSCs cultured with and without exogenous GF in the TCF and DCS were collected at days 0, 7, 14 and 21 and were frozen at −70 °C until used for assaying. The conditioned media were assayed for AFP and ALB production using a chemistry analyzer (AU4000, Olympus, Japan). The differentiatedMSCs from each condition were incubated with 1 ml medium containing 5 mM NH4Cl (Sigma) for 24 h in 5% CO₂ at 37 °C on days 0, 7, 14 and 21. Following incubation, the supernatants were collected and the urea concentrations were measured using a colorimetric assay kit (BD Corporation, San Diego, CA). The primary hepatocytes were used as a positive control. The production of AFP, ALB and urea was presented in term of pg/cell/day.

2.8.2. Periodic acid-Schiff (PAS) staining

The glycogen storage in the inducedMSCs in the DCS in the presence and absence of GF was determined using the PAS kit (Jiancheng, Nanjing, China) in accordance with the manufacturer’s instructions. The undifferentiatedMSCs and primary hepatocytes were used as the negative and positive controls, respectively.

2.8.3. Indocyanine green (ICG) and low-density lipoprotein (LDL) uptake

For the ICG uptake analysis, the cellular and tissue sections were deparaffinated and stained using H&E stain, Masson’s trichrome stain or picrosirius red. For the immunofluorescence analysis, the cellular and tissue sections were fixed in 4% paraformaldehyde and were permeabilized using 100 μl cold acetone. The samples were blocked using 5% BSA and were incubated overnight at 4 °C with the primary monoclonal antibodies (1:400, Abcam, CA, UK), collagen IV (1:400, Abcam), thrombin (1:400, Abcam), laminin (1:400, Abcam), AFP (1:400), ALB (1:400), CCl₄ (1:400), and alpha-smooth muscle actin (α-SMA, 1:500, Santa Cruz) antibodies diluted in antibody dilution solution. The excess primary antibody was removed by washing three times in PBS, and the samples were incubated with PE-conjugated secondary antibody (1:1000) at room temperature for 4 h. In addition, the sections were counter-stained using 4’,6-diamidino-2-phenylindole (DAPI, Sigma) to label the nucleus. The slides were mounted in propidium iodide (Sigma) containing mounting media for visualization using a confocal microscope (FV-1000, Olympus, Japan).

2.10. Cell collection from dynamic cultured scaffold

To harvest the differentiated cells cultured in DCS, the bioscaffolds were taken out of the perfusion chambers, washed twice with PBS, chopped and digested with 1 mg/ml collagenase type II (Gibco) for 25 min at 37 °C. After filtration through a 200 mesh screen and repeated pipetting, the cells were washed twice with PBS and the single-cell suspension in PBS was prepared for flow cytometry analysis and cell transplantation.

2.11. Animal experimental model and cell transplantation

To induce liver fibrosis, 10% CCl₄ (Sigma) dissolved in olive oil (Sigma) was injected intraperitoneally at a dose of 2 ml/kg body weight, three times per week for up to 12 weeks [26]. After 12 weeks, 3 × 10⁶ cells were infused slowly via the tail vein (MSC, MSC-GF and MSC-DCS treatment groups). Following transplantation, the mice wasanesthetized for six additional weeks (18 injections) to enable the transplanted cells to engraft and differentiate. In the normal (n = 15) and sham treatment (n = 15) groups, CCl₄ was not administered. In the sham group, the mice were treated with the vehicle (olive oil) alone (three times per week for 18 weeks). In the CCl₄ group (n = 20), the mice were injected with CCl₄ alone without cell transplantation. In the MSC group (n = 15), the mice were administered the undifferentiatedMSCs. In MSC-GF group (n = 15), the mice were administered the differentiatedMSCs cultured in the DCS in the presence of GF. At the end of the procedure, the mice were sacrificed under deep anesthesia, and the venous blood and liver tissue were harvested for analysis. The serum ALB and AST levels were determined using a chemistry analyzer (AU4000, Olympus, Tokyo, Japan). The in vivo bio-imaging was performed using a NightOwl imaging system and the WinLight software (Berthold, Germany).

2.12. Immunohistology and immunofluorescence staining

For the histological examinations, the fresh and decellularized livers were fixed in 10% buffered formalin, were dehydrated and embedded in paraffin. The liver sections were deparaffinized and stained using H&E stain, Masson’s trichrome stain or picrosirius red. For the immunofluorescence analysis, the cellular and tissue sections were fixed using 4% paraformaldehyde and were permeabilized using 100 μl cold acetone. The samples were blocked using 5% BSA and were incubated overnight at 4 °C with anti-Cd90.1 antibody or the primary monoclonal antibodies (1:400, Abcam, CA, UK), collagen IV (1:400, Abcam), thrombin (1:400, Abcam), AFP (1:400), ALB (1:400), CCl₄ (1:400) and alpha-smooth muscle actin (α-SMA, 1:500, Santa Cruz) antibodies diluted in antibody dilution solution. The excess primary antibody was removed by washing three times in PBS, and the samples were incubated with PE-conjugated secondary antibody (1:1000) at room temperature for 4 h. In addition, the sections were counter-stained using 4’,6-diamidino-2-phenylindole (DAPI, Sigma) to label the nucleus. The slides were mounted in propidium iodide (Sigma) containing mounting media for visualization using a confocal microscope (FV-1000, Olympus, Japan).

2.13. Western blot analysis

Portions of the livers were collected and placed in ice-cold homogenization buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X, and protease inhibitor cocktail) and were homogenized before centrifugation. Aliquots of the supernatant were used to determine protein concentration by a colorimetric assay kit (BD Corporation, San Diego, CA). The primary hepatocytes were used as a positive control.

2.14. Statistical analysis

All data are expressed as the mean ± SD (standard deviation). Statistical significance was determined using SPSS 12.0 software (SPSS, San Rafael, CA, USA). Analysis of variance followed a Student’s t-test was used to determine significant difference between the control and test group. For analysis of multiple groups, the P-
values were adjusted using the Bonferroni method and p-values less than 0.05 were considered significant. All procedures were performed by blinded investigators.

3. Results

3.1. Characterization of decellularized rat liver

The whole-organ decellularization was achieved through the initial physical treatments and the portal perfusion using NP-40 and other reagents. After 8 h of decellularization of the rat livers, the liver parenchyma became transparent, and the acellular scaffold retained the gross shape of the liver (Fig. 1A). The H&E staining revealed the pink eosinophilic staining typical of collagen, whereas the basophilic staining typical of cellular nuclear material was not observed (Fig. 1B). The Masson’s trichrome staining confirmed these results, and the homogeneous blue staining revealed the retention of collagen in the biomatrix (Fig. 1B). The immunostaining for the four ECM proteins, collagen type I, collagen type IV, fibronectin and laminin, indicated that both the structural and basement membrane components of the ECM were retained and were similar to the native liver (Fig. 1B). The lack of DAPI staining in the biomatrix confirmed the absence of cells (Fig. 1B). The SEM revealed the honeycomb structure of the decellularized rat livers (Fig. 1C, left lower panel). The data demonstrate that the decellularization process removed all the residual blood, cells, and other soluble components from the liver.

To test the cytotoxicity of the scaffold, the primary hepatocytes derived from the GFP-transgenic mice were seeded into the liver matrix discs and cultured in static. The hepatocyte-loaded scaffolds were sectioned on day 4 and day 7. The H&E staining and SEM demonstrated that the hepatocytes were adhered to the decellularized scaffold (Fig. 1C). We determined the population of hepatocytes according to the increased number of cells per field (≈ 5.1 x 10^5 and 8.8 x 10^5 cells/scaffold on day 4 and 7). To confirm that the scaffold provided a 3D rather than a two-dimensional (2D) environment, the bioscaffold seeded with the GFP-labeled hepatocytes was examined using laser scanning

Fig. 1. Decellularization and characterization of rat decellularized liver. (A) General appearance of the rat liver during the decellularization process at different time points. (B) Histology and immunofluorescence of normal liver (top panel) and decellularized liver (bottom panel). (C) H&E staining (top panel) and SEM analysis (bottom panel) of decellularized liver before and after recellularization with hepatocytes on day 4 and day 7. (D) The three-dimensional scaffolds reseeded with the GFP-positive hepatocytes were counterstained using collagen I and DAPI on day 7. Scale bars A = 10 mm; B, C (i–iv), D = 50 μm; C (v, vi) = 5 μm.
confocal microscopy. The results demonstrated that the hepatocytes resided inside the scaffold as well as on the surface (Fig. 1D).

3.2. Compatibility of MSCs in dynamic cultured bioscaffold

The MSCs were isolated from the bone marrow of the GFP-transgenic mice. In the first passage (P1), the bone marrow-derived cells observed in the bright field were of various sizes and emitted heterogeneous levels of green fluorescence when observed under the fluorescence microscope (Fig. 2A, top panel). After the third passage (P3), the cells exhibited a homogeneous fibroblast-like morphology and the GFP signal intensity was uniform among the cells (Fig. 2A, bottom panel). Flow cytometry analysis was performed to characterize the surface markers of the cultured cells. Most cells expressed the standard MSC surface markers CD90 (91.8 ± 5.7%), CD105 (93.5 ± 3.0%) and CD29 (89.2 ± 4.8%), whereas the cells were negative for CD45 (7.6 ± 2.1%), CD80 (5.8 ± 2.1%) and CD34 (7.4 ± 2.1%). The results suggest that the surface phenotype of the cells is consistent with those reported in the literature for MSCs (Fig. 2B,C), i.e., MSCs were generated successfully and could be used in the following experiments.

To optimize the flow rate for the cell attachment and population of the bioscaffold, the cell number and apoptosis in the DCS were assessed. Assuming that the cell number is proportional to the amount of DNA, the DNA quantification was determined on day 4 and day 7. The dsDNA amount of the cells at a flow rate of 4 ml/min increased significantly compared to culturing in the TCF or DCS at a flow rate of 0.5, 1, 2 and 6 ml/min (Fig. 2C). The percentage of TUNEL− cells in each group indicated that less apoptotic cells were observed in the DCS at 2 ml/min and 4 ml/min flow rates than at other flow rates (Fig. 2D). Based on these results, we used a flow rate of 4 ml/min for further experiments. The proliferation of the MSCs cultured in the TCF or DCS was also analyzed in the presence and absence of GF. On day 21, the dsDNA amounts of the cells in the DCS (19.0 ± 3.2 and 13.2 ± 1.8 µg/scaffold in the absence and presence of GF) were significantly more than in the TCF (15.8 ± 2.1 and 6.3 ± 1.4 µg/scaffold in the absence and presence of GF), irrespective of the presence of GF.

3.3. Gene and protein expression of MSC-derived cells

The mRNA expression of the octamer-binding transcription factor 4 (Oct4) in the DCS without GF decreased slowly over 21 days, whereas a significant reduction in the mRNA expression of Oct4 was detected in the MSCs cultured in the TCF and DCS in the presence of GF, suggesting the loss of pluripotency or stemness during the differentiation of the MSCs (Fig. 3). The mRNA expression of the liver-generated plasma proteins AFP and ALB in the cells cultured in the TCF or DCS in the presence of GF increased significantly in a time-dependent manner and on day 21, was slightly elevated in the DCS in the absence of GF compared to the TCF in the absence of GF. Compared to the MSCs in the TCF in the absence of GF, the cells cultured under the other three conditions exhibited a significant increase in the expression of selected hepatic-enriched transcription factors, such as forkhead box A1 (FOXA1), constitutive androstane receptor (CAR) and hepatic nuclear factor 4 alpha (HNF4α), a transcription factor that is vital for hepatic nuclear receptors. In the DCS in the presence of GF, the mRNA expression of hepatic functional marker genes and hepatic metabolic enzymes, such as transthyretin (TTR), α-1-antitrypsin (A1AT) and glucose-6-phosphatase (G6P), as well as members of the cytochrome P450 subunits CYP3A4 and CYP7A1, was detected earlier than in the TCF in the presence of GF. Additionally, the liver-specific gene expression profiles in the differentiated MSCs in DCS in the presence and absence of GF were compared. The differentiated MSCs without the GF expressed endodermal-specific genes, such as AFP, TTR, A1AT, ALB and CYP7A1 but not G6P and CYP3A4 on day 21.

To confirm the in vitro hepatic differentiation of the MSCs, we analyzed the expression of CD90 (a marker of undifferentiated MSCs), AFP (the definitive marker of the hepatic endoderm or early hepatic differentiation) and ALB (the most abundant protein synthesized by functional hepatocytes) using immunofluorescence staining (Fig. 4A). The cells in the TCF cultured in the absence of GF stained positive for CD90 but negative for AFP and ALB. However, a portion of the cells in the DCS cultured in the absence of GF stained positive for AFP (12.0 ± 2.8%) and ALB (5.2 ± 4.2%) on day 21. Using the exogenous GF, the percentage of AFP and ALB-positive cells increased to 38.1 ± 9.2% and 15.3 ± 5.6% in the TCF and 52.3 ± 11.0% and 24.5 ± 9.6% in the DCS, respectively, whereas CD90-positive cells were not detected.

To determine the exact percentage of cells expressing hepatic proteins, the MSCs-derived cells were analyzed for the expression of AFP and ALB as well as cytoketatin (CK7, CK8, CK18 and CK19) using flow cytometry analysis (Fig. 4B). AFP percentages in TCF, DCS, TCF-GF and DCS-GF were 3.8 ± 3.5, 12.3 ± 2.8, 38.4 ± 9.2 and 52.5 ± 11.0% respectively; ALB percentages in TCF, DCS, TCF-GF and DCS-GF were 1.6 ± 2.4, 5.5 ± 4.2, 15.1 ± 5.6 and 24.8 ± 9.6% respectively; CK-7 percentages in TCF, DCS, TCF-GF and DCS-GF were 4.4 ± 3.2, 9.6 ± 3.9, 33.4 ± 4.4 and 42.2 ± 7.4% respectively; CK-8 percentages in TCF, DCS, TCF-GF and DCS-GF were 4.0 ± 2.7, 11.3 ± 4.4, 28.0 ± 6.3 and 32.1 ± 13.0% respectively; CK-18 percentages in TCF, DCS, TCF-GF and DCS-GF were 6.2 ± 4.2, 6.0 ± 2.8, 16.9 ± 3.3 and 24.9 ± 8.7% respectively; CK-19 percentages in TCF, DCS, TCF-GF and DCS-GF were 5.7 ± 3.7, 10.1 ± 3.5, 24.5 ± 4.8 and 37.9 ± 12.3% respectively. The data suggested that the percentage of AFP-, CK7- and CK19-positive cells in the DCS were significantly higher than in the TCF in the absence and presence of GF (Fig. 4C).

3.4. Functional analysis and ultrastructure of differentiated MSCs

The production of AFP and ALB was not detected in the cells cultured in the TCF in the absence of GF, whereas both proteins were detected at low levels in the cells cultured in the DCS in the absence of GF. In the presence of GF, the secretion of AFP increased steadily in both the TCF and DCS and reached a peak on day 21, and significant differences in the levels of AFP were not observed between the cells cultured in the TCF and DCS on day 21 (Fig. 5A). In the presence of GF, the production of ALB in the cells cultured in the DCS was significantly higher than in the cells cultured in the TCF, and an unexpected decrease in ALB production was observed on day 21 in the TCF cultured cells (Fig. 5B). The urea production in the cells in the DCS was significantly higher than in the TCF cultured cells in the presence of GF on days 14 and 21 (Fig. 5C). The PAS staining of the MSCs-derived cells cultured in the DCS was used to determine the glycogen storage on day 21 (Fig. 5D). Compared to the undifferentiated MSCs, a small number of positive cells exhibiting a pink or red-purple cytoplasm was detected in the DCS (Fig. 5D, black arrowhead), whereas a higher number of positive cells was detected when exogenous GF were added. In addition, the uptake of ICG and LDL was investigated; this assay determines the glycogen storage on day 21 (Fig. 5D). The uptake capacity of the hepatocyte-like cells derived from MSCs cultured in the DCS in the presence of GF was notably higher than the cells cultured in the DCS in the absence of GF.

To evaluate the morphological and structural characteristics of the undifferentiated and fully differentiated MSCs, the cells cultured in the DCS in the presence of GF were examined using SEM
Fig. 2. The characterization of GFP-positive MSCs and scaffold compatibility in the dynamic culture system. (A) The morphology of the MSCs in the first (P1) and third (P3) passage observed under bright field and fluorescence microscopy, respectively. (B) Surface molecule characterization of the MSCs analyzed by flow cytometry after incubation with PE-conjugated antibodies (CD90, CD105, CD29, CD45, CD80 and CD34). (C) The exact surface-molecule expression levels are shown in the column chart. (D) DNA quantification of the MSCs in the scaffold on day 4 and 7 of the static and dynamic culture with different flow rates. (E) DAPI and TUNEL staining of the MSCs in the dynamic cultured scaffold (DCS) on day 7 with a flow speed of 1 (i), 2 (ii), 4 (iii) and 6 (iv) ml/min. (F) DNA content analysis of the MSCs seeded on tissue culture flasks (TCF) and DCS in the presence and absence of growth factors (GF) on day 0, 7, 14, and 21. \( * P < 0.05; \) \( \ast P < 0.05 \), DCS compared to TCF; \( \overset{\ast}{*} P < 0.05 \), DCS-GF compared to TCF-GF. Scale bars A, E – 50 µm.
and TEM on days 0, 14, and 21 (Fig. 6). The cells in the DCS on day 0 exhibited a spindle-like morphology with few microvilli (Fig. 6Ai) and contained a high nuclear (N)–to–cytoplasm (C) ratio (Fig. 6Bi) with very few cytoplasmic structures (Fig. 6Bii). The whole-cell micrograph of the differentiated MSCs on days 14 and 21 revealed a polygonal morphology with larger microvilli (Fig. 6Aii), a significantly reduced nuclear (N)–to–cytoplasm (C) ratio and an abundance of cytoplasmic structures (Fig. 6Biii–vii). The micrograph of a portion of a single cell revealed an abundance of rough/smooth endoplasmic reticulum (Fig. 6Biv), mitochondria (Fig. 6Bvii), and especially glycogensomes (markers of mature hepatocytes) in the cytoplasm (Fig. 6Bvii, white arrow). The images suggest that within 3 weeks, the ultrastructures of the MSCs cultured in the DCS in the presence of GF were transformed from those characteristic of stem cells to those characteristic of mature hepatocytes.

3.5. Morphology and functional evaluation of injured liver

The animals in the normal and sham control groups survived the observation period. The survival of the animals in the three groups that underwent cell transplantation was significantly higher than in the group treated with CCl4 (Fig. 7A). The percentage survival in the MSC group was significantly lower than in the MSC-GF and MSC-DCS groups, between which there was no notable difference. The histological evaluations in the CCl4-injured mice demonstrated that compared with the normal and sham mice, the liver fibrosis increased significantly and was characterized by the collagen deposition and fibrotic septum formation starting in the portal areas (as demonstrated by the Sirius red staining in Fig. 7B), and the coagulation necrosis of hepatocytes and the infiltration of inflammatory cells (as demonstrated by the H&E staining in Fig. 7D). After transplantation of the MSC-DCS, the injured livers exhibited the maximal restoration of live architecture with thinner or absent septal fibrosis and decreased collagen deposition. In contrast, the hepatic fibrosis decreased to a lesser extent in the mice transplanted with the MSC-TCF and the undifferentiated MSCs, also indicated by the percentage of the fibrosis area (Fig. 7C). Moreover, compared with the mice in the MSC group and the CCl4 group, the mice receiving the MSC-DCS and the MSC-GF exhibited optimal improvement of liver function, as demonstrated by the peripheral blood ALB and AST levels (Fig. 7E,F).

3.6. Engraftment and derivation of transplanted cells

To trace the transplanted cells, the liver sections were examined using in vivo bio-imaging and immunofluorescence analyses. The GFP-positive cells were detected in the background of the mouse liver as early as 2 h after cell transplantation (Fig. 8A, black arrowhead). On day 14, transplanted cells were mainly located around the portal tracts and interlobular connective tissue and extremely few of cells moved toward the central region of hepatic lobular (Fig. 8B, black arrowhead). The GFP signals were still detectable in the livers 6 weeks after the cell injection, as shown in Fig. 8C. The GFP intensity of the livers in the MSC-DCS group was significantly higher than in the MSC-GF and MSC groups (Fig. 8D). These results were confirmed by the percentage of GFP-positive cells detected after nuclear staining with DAPI (Fig. 8E,F). The immunofluorescence staining in the MSC-DCS group revealed a low percentage of double labeled GFP+/ALB+ and GFP+/CK19+ cells in the host livers (3 ± 0.8% and 1.5 ± 0.6%, respectively), whereas higher percentage of double GFP+/ALB+ cells compared with the MSC-GF (2.4 ± 0.7%) and the MSC (0.9 ± 0.5%) groups (Fig. 8F,G). The expression of α-SMA decreased significantly in the livers transplanted with either cell type. The α-SMA expression in the MSC-DCS group was
significantly lower than in the other two groups (Fig. 9A). Double GFP<sup>+</sup>/α-SMA<sup>+</sup> cells were not detected in any of the groups. The expression of ALB and HGF proteins in both pre-differentiated MSC groups was higher than in the MSC group and the CCl<sub>4</sub> group, whereas there was no significant difference in ALB expression between the MSC-GF and the MSC-DCS groups (Fig. 9B,C).

4. Discussion

In this study, a natural 3D scaffold from the decellularized liver matrix was used to optimize the differentiation of MSCs to functional hepatocytes. Our data demonstrate that the 3D biomatrix scaffold in dynamic culture with optimal flow rate promoted significantly better cell proliferation compared to the biomatrix scaffold cultured in static or the monolayer static culture system. In addition, the biomatrix scaffold, either on its own or in combination with hepatic GF, induced the lineage-specific differentiation of MSCs into hepatocyte-like cells. Moreover, after the systemic transplantation into a mouse model of CCl<sub>4</sub>-induced liver fibrosis, when compared with undifferentiated MSCs or MSCs differentiated using GF alone, the pre-differentiated MSCs produced using the bioscaffold method combined with GF in vitro facilitated the
survival of the mice, liver restoration and the long-term functional hepatic integration in vivo.

In a living organism, cell renewal and differentiation are coordinated with the changing environment characterized by spatial and temporal gradients of multiple factors via interactions with neighboring cells, which is difficult to achieve under standard 2D culture conditions [13]. One approach is to develop a 3D culture system mimicking the microenvironment of the native tissue. Whole-organ decellularization is an attractive technique for the preparation of a natural biomatrix scaffold. To date, the potential application of this technique has been demonstrated successfully for a number of organs, including the heart [27], lung [28], liver [17–19,21], kidney [29] and bladder [30]. Because of the preserved native ECM and 3D porous structure, the biomatrix scaffold has an advantage over other scaffolds and has been investigated for its potential to interact with stem cells and to control their behavior [20–22]. In this study, we developed a unique protocol based on NP-40 perfusion combined with physical and enzymatic techniques to prepare the decellularized liver, creating a translucent liver matrix within a short period in which the porous architecture and partial ECM of the original organ was preserved. The amount of dsDNA retained on the decellularized liver was determined to verify that the minimum criteria [31] for decellularization were satisfied. The results showed that the residual dsDNA was less than 50 ng per mg ECM dry weight and was more consistent in the different liver lobes using the NP-40 detergent compared with other more potent detergents (e.g., SDS and Triton X-100) (data not shown).

It is definitely that the biomatrix scaffolds used in present study provided a 3D microenvironment for the loaded cells, which was
demonstrated initially by the engraftment and population of the GFP-labeled hepatocytes within the scaffold. After the MSCs seeded in the DCS, the results of DNA analysis suggested that the dynamic culture at a flow rate of 4 ml/min promoted cell growth as compared with the static culture. The static culture and dynamic culture with the low flow rate inhibited the cell proliferation due to the severely limited oxygen and nutrient diffusion within the 3D bioscaffold which were essential for the cell viability, while the appreciated dynamic culture medium enhanced mass transport, ensures continuous nutrition of the cells and removed waste products [13]. The high flow rate induced massive cell apoptosis because of the extreme shear stresses imparted by the medium perfusion. It was worth noting that the shear levels within the system should be optimized for cell growth and differentiation [32]. Different studies have reported the inconsistent effects of the flow rate on stem cell differentiation [33,34], which may be explained in terms of differences in the cell types and bioreactors. We adopted 4 ml/min as the flow rate for further experiments based on the results of the cell growth analysis. However, further investigations will be needed to determine if this flow rate is optimal for cell differentiation.

To understand the effects of the native ECM on cell growth and differentiation, the MSCs were seeded into the biomatrix scaffold and cultured under dynamic conditions without GF. When compared with the static cultivation in TCF, the results of DNA analysis revealed that the DCS provided the seeded MSCs with an excellent environment that was supportive of cell proliferation, as indicated by a previous report [15]. As the absence of GF, the cells in the DCS slowly lost their “stemness” (decreased expression of Oct4), by contrast, exhibited an increase in the mRNA expression of hepatic-enriched transcription factors (FOXA1, CAR and HNF4α), plasma proteins (AFP and ALB), and biotransformation enzymes (TTR and A1AT). The protein expression and hepatic-specific functions confirmed the hepatic differentiation of the MSCs in the DCS culture. However, these cues of hepatic differentiation had not been detected in the MSCs cultured in the TCF. These results suggested that the liver biomatrix scaffold exhibited the independent inductive potential for the differentiation of MSCs into cells of hepatic lineage. The induction might be contributed to the specific composition and ultrastructural characteristics of the biomatrix scaffold derived from liver. Numerous studies show that the ECM scaffolds retain a diverse variety of growth factors including transforming growth factor β1, insulin-like growth factor, epidermal growth factor, bone morphogenetic protein 4, keratinocyte growth factor and etc [21,35]. In addition, the ultrastructural characteristics of the matrix appear to play important roles in modulating the behavior of cells that contact the scaffold by influencing tissue-specific phenotypic differentiation [36,37]. Nevertheless, the “the differentiation efficiency [7]” (the ratio of albumin-positive cells to the input number of MSCs) of the cells in the DCS was relatively low (approximately 5.2%), which was only one-third of the standard differentiation in the TCF culture in the presence of GF.

To achieve maximal hepatic induction, hepatic GF and cytokines were adopted in the differentiation protocol of the seeded cell in the DCS. The analysis of the gene and protein files revealed that the cells in the DCS in the presence of GF expressed higher levels of liver-specific genes over a longer period, and more cells expressed proteins of hepatic markers. The differentiation efficiency of the cells in the DCS with the GF was increased by approximately 24.5%, which was 1.6-fold more than in the TCF with the GF. When compared with the cells cultured in the DCS alone, the extensive analyses of synthetic and metabolic functions demonstrated that the MSCs from the DCS in the presence of GF exhibited more
Fig. 7. Therapeutic potential of transplanted MSCs and MSC-derived hepatocyte-like cells for recovery in the mouse CCl₄ injury model. (A) Survival curve of the injured mice that underwent intravenous cell transplantation. Representative photomicrographs of Sirius Red-stained (B) and H&E-stained (D) mouse livers from the different groups (i, normal mice; ii, sham group; iii, CCl₄ group; iv, MSC group; v, MSC-GF group; and vi, MSC-DCS group). (C) Analysis of the percentage fibrosis using Image J software. (E) Serum ALB and AST levels in the experimental groups. *P < 0.05. Scale bars B, D = 100 μm.
abundant and stable function, which was consistent with the ultrastructural characteristics of the cells. These results suggested that the mature degree of hepatic differentiation of MSCs in the DCS was higher than TCF, irrespective of the present of GF; the combination of the liver-derived biomatrix scaffold and hepatic GF had a synergistic effect on the differentiation of the MSCs into functional hepatocytes in vitro.

To date, only a few studies have investigated the therapeutic effect of the pre-differentiated MSCs on the damaged liver in vivo, and these studies have produced conflicting results, particularly for chronic liver injury [10–12,38–43]. The results from most of the transplantation studies using MSCs that were partially and/or fully pre-differentiated in vitro were encouraging. However, a recent study showed that when MSCs pre-treated with HGF in vitro were injected into a rat model of thioacetamide-induced liver cirrhosis, the transplanted cells engrafted the liver but resulted in a decrease in anti-fibrotic activity [42]. Our findings in the CCl₄-injured livers indicated that the intravenous transplantation of the pre-differentiated MSCs, especially the cells cultured in the DCS in the presence of GF, significantly restored the liver function, which confirmed data from other studies [11,40].

Cell homing and engraftment into the host liver is an integral step in cell-based therapies. The mechanism that governs the recruitment of MSCs is complicated, and to date, several signaling pathways [44,45], growth factors and matrix metalloproteinases [46] have been shown to contribute to the recruitment of bone marrow cells in the injured liver. Although we do not know the exact signals or factors that play a role in the migration of the pre-differentiated MSCs, we have demonstrated that engraftment was significantly improved using the pre-differentiated MSCs by the DCS compared to cells induced in the TCF and the undifferentiated MSCs, confirming the hypothesis that hepatic differentiation was effective in improving the engraftment of the MSCs to the injured liver [11,40,43].

In the mice, a higher proportion of the transplanted cells that were pre-differentiated in the DCS in the presence of GF continued...
to exhibit hepatic phenotypes, such as the expression of ALB and CK19, although these cells were limited to a rare population of the total liver mass and in theory, were not sufficient to reverse the pathological conditions (a percentage of 2.5–5% is necessary for this process [41]). It is unknown whether the paracrine effect of the pre-differentiated MSCs plays an important role in the correction of liver disorders. In this study, the expression of α-SMA in the liver, recognized as an indicator of hepatic stellate cell (HSC) activation, was reduced significantly after the administration of the pretreated MSCs by the DCS than other cells, which was consistent with the morphological changes of liver fibrosis. The transdifferentiation into myofibroblasts or HSC after liver injury, which has been demonstrated in hematopoietic stem cells (another type of bone marrow stem cell) [47], was not detected in our study using MSCs. The robust expression of ALB and HGF in the host liver suggested that the repopulation and functional assembly of the resident hepatocytes were promoted by the transplanted MSCs that were pre-differentiated in vitro.

Although this study is firstly to use the liver biomatrix scaffold to successfully generate functional hepatocyte-like cells from MSCs, a number of questions remain unanswered. For instance, the maturation degree of hepatocyte-like cells from MSCs still fell short of primary hepatocytes. Besides, the decellularized liver and dynamic culture system used in present study made the induction procedure more complex and increased the risk of cell contamination. Herein, we used the biomatrix scaffold from rat for the differentiation of murine MSCs to explore the potential of xenogenic organs as the source of decellularized grafts. The use of xenogenic biomatrix scaffold has been reported in several recent papers, however, the potential immune response between the xenogenic ECM and seeded cells in vitro and especially in vivo has not been fully investigated. Recently, a variety of synthetic fibrous scaffolds has been used for the in vivo delivery of differentiated MSCs [48]. The xenogenic liver biomatrix scaffold is worth exploring as a carrier for cell transplantation or constructing organized liver-tissue equivalents.

5. Conclusions

The decellularized liver matrix is an attractive 3D bioscaffold for cell biology and tissue regeneration studies, particularly because the natural ECM framework allows the spatially defined differentiation of stem cells into mature cell types. We demonstrated that MSCs could be converted in vitro into functional hepatocyte-like cells through induction using a tissue-specific biomatrix scaffold in the presence or absence of hepatic GF. As compared with the conventional induction in TCF, these hepatocyte-like cells exhibited higher level and more stable functions that are potentially useful for the treatment of chronic liver damage. The present study successfully developed a suitable strategy for the production of functional hepatocytes, indicated that the 3D liver biomatrix might have considerable potential for cell-based therapy and tissue engineering. Further investigation of the advantage of dynamic cultured bioscaffold over conventional 2D methods is needed.

Conflict of interest statement

Authors have no conflict of interest to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2012.08.058.

References