Hepatic differentiation capability of rat bone marrow-derived mesenchymal stem cells and hematopoietic stem cells

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AIM: To investigate the different effects of mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs) on hepatic differentiation.

METHODS: MSCs from rat bone marrow were isolated and cultured by standard methods. HSCs from rat bone marrow were isolated and purified by magnetic activated cell sorting. Both cell subsets were induced. Morphology, RT-PCR and immunocytochemistry were used to identify the hepatic differentiation grade.

RESULTS: MSCs exhibited round in shape after differentiation, instead of fibroblast-like morphology before differentiation. Albumin mRNA and protein were expressed positively in MSCs, without detection of alpha-fetoprotein (AFP). HSCs were polygonal in shape after differentiation. The expression of albumin signal decreased and AFP signal increased. The expression of CK18 was continuous in MSCs and HSCs both before and after induction.

CONCLUSION: Both MSCs and HSCs have hepatic differentiation capabilities. However, their capabilities are not the same. MSCs can differentiate into mature hepatocyte-like cells, never expressing early hepatic specific genes, while Thy-1.1+ cells are inclined to differentiate into hepatic stem cell-like cells, with an increasing AFP expression and a decreasing albumin signal. CK18 mRNA is positive in Thy-1.1+ cells and MSCs, negative in Thy-1.1- cells. It seems that CK18 has some relationship with Thy-1.1 antigen, and CK18 may be a predictive marker of hepatic differentiation capability.

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INTRODUCTION
In recent years, liver related stem cells have become a hot spot of research. One of the important findings is that liver stem cells might be derived from bone marrow. Petersen et al. first identified this phenomenon in rat model of liver injury. Later many researchers have reported similar in vivo and in vitro findings. Bone marrow cells have been hypothesized as the third recruitment source in liver regeneration besides hepatocytes and endogenous liver stem cells.

Among bone marrow cells, there are two subsets, hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). HSCs can express CD34, c-kit, Thy-1 and flt-3 receptor mRNA, all of which are markers of HSCs. So HSCs are regarded as the exact source of hepatocytes. However, some studies have found that non-hematopoietic subsets (CD45- subsets) also play such a role. In this paper, we isolated two subsets of stem cells from rat bone marrow. One was MSCs, isolated and cultured by standard methods, the other was Thy-1.1+ cells, isolated and purified by magnetic activated cell sorting (MACS), which were recognized as HSCs of rats. We imitated the circumstances of liver development to define the different capacities of hepatic differentiation of the two subsets in vitro.

MATERIALS AND METHODS
Animals
Male Sprague-Dawley (SD) rats at 6-7 wk of age were obtained from Charles River China. All animal experiments were performed in accordance with the animal guidelines of Peking University Health Science Center.

Isolation and culture of MSCs from rat bone marrow
Rat bone marrow was harvested from rat femurs and tibias, and collected into Dulbecco’s modified Eagle’s medium (DMEM), filtered (40 µm), and centrifuged at 300 r/min to pellet cells. After rinsed in PBS, bone marrow cells were loaded onto 5 mL 57% Percoll (1.073 g/mL in 0.01 mol/L PBS) and centrifuged at 400 r/min for 30 min. Mononuclear cells were collected at the Percoll interface, rinsed twice in PBS, and seeded at 2×10^5/cm^2 in 600 g/L low glucose DMEM (DMEM-LG, Sigma-Aldrich), 400 g/L MCDB-201 (Sigma-Aldrich), with 100 mL fetal bovine serum (FBS; Hyclone, USA) and 100 U/mL rat leukemia inhibitory factor (rLIF; Chemicon, USA). Non-adherent cells were removed after 24 h and culture media were replaced every 3 d. After about 7 d, isolated colonies of MSCs were apparent. Then the cells were trypsinized and replated at 8 000/cm^2, and the cells from passage 3 were used for differentiation protocol.

Differentiation protocols
MSCs from passage 3 were seeded at 2×10^5/cm^2 on 10 ng/mL fibronectin (FN, Sigma-Aldrich, USA) in culture medium as above. After 12 h, media was removed and cells were cultured in 600 g/L DMEM-LG, 400 g/L MCDB-201 supplemented with 50 mL/L FBS, 10^-4 mol/L dexamethasone (Dex, Sigma-Aldrich, USA), 10 ng/mL epidermal growth factor (EGF, R&D, USA), 1×insulin/transferrin/selenium (ITS, Sigma-Aldrich, USA). In some experiments, inducing growth factors were added into culture medium (20 ng/mL acid fibroblast growth factors, aFGF; 10 ng/mL basic fibroblast growth factors, bFGF; and 20 ng/mL hepatocyte growth factor, HGF). aFGF, bFGF, and HGF were all from R&D System Inc, USA.

BASIC RESEARCH
Isolation, culture and differentiation of Thy-1.1* stem cells from rat marrow

After Percoll gradient centrifugation, bone marrow mononuclear cells were collected, then incubated with mouse anti-rat Thy-1.1 monoclonal antibody (Serotech, UK) at 4°C for 30 min. Unbound antibodies were removed by washing twice. Secondary marking was done by incubation with rat-anti-mouse IgG bound magnetic microbeads (MiltenyiBiotec, Germany). The positive cells were absorbed in magnetic field by Mini-MACS columns (MiltenyiBiotec, Germany). The column was removed from the magnetic field and the Thy-1.1+ cells were washed out.

Thy-1.1+ cells were cultured in differentiation media, 600 g/L DMEM-LG, 400 g/L MCDB-201 supplemented with 150 mL/L FBS, 10^-4 mol/L Dex, 10 ng/mL EGF, 1×ITS, 20 ng/mL aFGF, 10 ng/mL bFGF, and 20 ng/mL HGF. After about 7-10 d, apparent colonies of Thy-1.1+ cells appeared.

Flow cytometry determination of cell-surface antigen

The cells were resuspended in washing buffer (10 mL/L bovine serum, 2 g/L sodium azide in PBS), and stained on ice according to the manufacturer’s recommendations with the monoclonal antibodies as follows: R-PE conjugated mouse anti-rat-Thy1.1 (Pharmingen, USA), FITC conjugated mouse anti-rat-CD45 (HarlanSera-Lab, UK), PE conjugated mouse anti-rat-CD34 (Santa Cruz, USA). The cells were resuspended in 300 μL fixation buffer (20 g/L formaldehyde in PBS) and run on a flow cytometer (FACS Calibur, BectonDi-ckinson, USA). The results were analyzed by CellQuest software (BD, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted by using TRIzol (Invitrogen, USA) according to the manufacturer’s instructions and quantified by UV spectroscopy. To prepare RNA for PCR analysis, 2 μg total RNA was converted to the cDNA using SuperScript II reverse transcriptase (Invitrogen, USA) with oligo (dT) (Promega, USA) and random hexamer primers (Promega, USA). PCR was performed by using Taq DNA polymerase (Gibco BRL, USA). For each gene, the DNA primers were originated from different exons to ensure that the PCR products represented the specific mRNA species and not genomic DNA. All PCR experiments were performed using a GeneAmp PCR system 2 400 (Perkin-Elmer, USA).

The following specific oligonucleotide primers were used: albumin (5'-AAGGCACCCCGATTACTCGG-3' (sense), 5'-TGCGAAGTCACCCATCACC-3' (antisense)), AFP (5'-AGGCCTGACTCATCATTAACT-3' (sense), 5'-ATATGTCC TGGCATTTCG-3' (antisense)), CK18 (5'-GGAAGCCAGCAAG ATCATGGC-3' (sense), 5'-CCACGATCTTACGGTGATTTG-3' (antisense)), β-actin (5'-AGAGGGAATGCCTGCGTGAC-3' (sense), 5'-AGGAGCCGGCCAGTAATC-3' (anti-sense)).

Amplification reactions were carried out for 35 cycles (25 cycles for β-actin) at 94°C for 1 min, 58°C for 1 min, and at 72°C for 1 min for albumin; at 94°C for 45 s, at 51°C for 45 s and at 72°C for 45 s for AFP; at 94°C for 1 min, 60°C for 1 min and at 72°C for 1 min for CK18 and at 94°C for 30 s, at 55°C for 45 s, and at 72°C for 45 s for β-actin. The reaction products were subjected to 12 g/L agarose gel electrophoresis and visualized by ethidium bromide staining. The reaction products were 649 bp (albumin), 484 bp (AFP), 515 bp (CK18), and 353 bp (β-actin), respectively. Housekeeping gene β-actin was used as an internal control. Adult liver tissue or newborn rat liver tissue was used as a positive control.

Immunocytochemistry

Cell cultures were washed with PBS twice and fixed with 95% alcohol/acetic acid (99:1) for 10 min at room temperature, then permeabilized with 2 g/L Triton X-100 for 10 min. After washed, cells were incubated overnight at 4°C with primary antibodies, including mouse anti-human CK18 (Sigma, USA), mouse anti-human albumin (Dako, Denmark) and goat anti-human AFP (SantaCruz, USA). Subsequently, the cells were washed with PBS three times and incubated with fluorescence labeled secondary antibody, FITC labeled goat anti-mouse IgG1 and rhodamine labeled donkey anti-goat IgG at 37°C for 1 h. After washed with PBS, cells were mounted with glycerol-PBS (9:1). The cells were visualized and photomicrographed by a fluorescence microscope (Olympus Provis AX80, Japan).

RESULTS

Cell yield and shape of MSC and Thy-1.1* cells before and after differentiation

The number of mononuclear cells by 57% Percoll gradient centrifugation was about 6.44×10^7 per rat. After MACS...
purification, the number of Thy1.1+ cells was about 2.36×10⁷. The recovery rate of Thy1.1+ cells from mononuclear bone marrow cells was 64.65%. Before differentiation, MSCs exhibited a fibroblastic morphology with spindle cell bodies (Figure 1A), while after differentiation, the cells contracted obviously and were inclined to be round in shape (Figure 1B). This change occurred early on d 4 after MSC differentiation protocol. Thy1.1+ cells were small and round in shape when freshly isolated (Figure 1C), and became typical polygonal, much bigger in size after cultured in differentiation media (Figure 1D).

**Quantitative analysis of cell-surface antigen expression**
The percentage of Thy-1.1+ of rat bone marrow mononuclear cells was 56.65%. After MACS, this percentage was 93.45% in positive subset (Figure 2A, B). Thy-1.1+ subset was CD34-, and partial CD45+ (Figure 2C, D). The percentage of CD45 positivity in Thy-1.1+ subset was 36.68%. As displayed in Figure 2E-2G, MSC was Thy-1.1+, CD45-, CD34-.

**RT-PCR analysis of hepatic gene expression of CK18, albumin, and AFP**
We analyzed hepatic gene expression of the cells before and after differentiation protocol. Adult rat liver or newborn rat liver tissue

**Figure 2** Phenotype analysis of MSCs and Thy-1.1+ cells. A: Thy-1.1 of mononuclear bone marrow cells before MACS; B, C, D: Thy-1.1, CD45, CD34 after MACS purification; E, F, G: Thy-1.1, CD45, CD34 of MSCs.

**Figure 3** RT-PCR analysis of hepatic gene expression before and after differentiation. A: Analysis of CK18 mRNA before differentiation, lane 1: Adult liver, lane 2: Mononuclear BM cells.

**Figure 4** Immunocytofluorescence. A: MSCs-albumin, B: Thy1.1+ cells-CK18, C: Thy1.1+ cells-AFP.
was used as positive control. Before differentiation, Thy-1.1+ cells showed a CK18 signal, so did MSCs. However, Thy-1.1+ cells did not express CK18 (Figure 3A). After differentiation, Thy-1.1+ cells were AFP mRNA positive and had decreased signals of albumin, while MSCs expressed albumin only and the signals of albumin increased with the extension of differentiation time (Figure 3B, C). Both cells had a continuous CK18 expression during the process of induction (Figure 3D).

**Immunocytochemical analysis**

MSCs showed positive staining for albumin (Figure 4A), and Thy1.1+ cells showed CK18 and AFP positive staining (Figure 4B, C).

**DISCUSSION**

As stromal cells of bone marrow, MSCs could differentiate into cells of all mesodermal origin, including adipocytes, osteocytes, chondrocytes and myocytes etc. Besides these, MSCs are also capable of “transdifferentiation” into ectodermal cells, such as neural cells[28-31]. So MSCs are multipotential adult stem cells. Regarding hepatic differentiation of MSCs, there has been no specific report yet. Schwartz et al[31] isolated a non-hematopoietic stem cell subset (CD45 GlyA- in humans or CD45 Ter119+ in mice) from bone marrow, termed multipotent adult progenitor cells (MAPCs). MAPCs should be ascribed to MSCs, since they were copurified with MSCs at the same time[22]. Under serial conditions, Schwartz et al[31] induced MAPCs into cells with morphological, phenotypic, and functional characteristics of hepatocytes in vitro. In our study, we tried to induce standard MSCs into hepatocytes, and found hepatic specific gene CK18 was already expressed by MSCs without differentiation protocol. After induction, MSCs also expressed albumin besides CK18. Albumin mRNA appeared early on d 4 after differentiation (data not shown), coinciding with the change of morphology. Albumin signals had a gradual increase with the extension of differentiation time. Immunocytochemical staining confirmed the results of the PCR analysis at protein-level. There were no early markers of hepatocytes before or during differentiation protocol. Morphologic change of MSCs was apparent. Cell shape changed from spindle and fibroblast-like to round and epithelia-like.

HSCs are another subset of stem cells from bone marrow. A large number of papers demonstrated that HSCs could differentiate into hepatocytes or hepatocyte-like cells. Among the papers, CD34, the most general marker of HSCs in humans, demonstrated that HSCs could differentiate into hepatocyte-like cells, not expressing early hepatic specific genes. Under a similar induction condition, Thy-1.1+ cells were inclined to become hepatic stem cell-like cells, with a continuous AFP expression and weak albumin signal. To our interest, CK18 mRNA was positive in Thy-1.1+ cells and MSCs, negative in Thy-1.1+ cells. It seemed that CK18 had some relationship with Thy-1.1 antigen, and it would be an indicative marker of hepatic differentiation ability.

Hepatic stem cells, especially bone marrow-derived hepatic stem cells may be therapeutically useful for treating a variety of diseases that affect the liver. This has been proved in some animal models[25]. Compared with other liver-related stem cells, such as embryonic stem cells[25], pancreatic stem cells[29], and neural stem cells[30], bone marrow-derived stem cells provide several advantages: (1) Bone marrow can be obtained from living donors or recipients themselves using a moderately invasive procedure. There is no problem of limited donors, which restrict liver transplantation and hepatocyte transplantation greatly. (2) Based on its ability of self-renewal, the amplification of bone marrow stem cells could be obtained. (3) Utilizing patients’ own bone marrow or repopulation of both bone marrow and hepatic system from the same donor could avoid or reduce immunological rejection, which usually affects recipients for a life-long time.

**REFERENCES**

1. Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP. Bone marrow as a potential source of hepatic oval cells. *Science* 1999; **284**: 1168-1170
2. Theise ND, Badve S, Saxena R, Henegarui O, Sell S, Crawford JM, Krause DS. Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. *Hepatology* 2000; **31**: 235-240
3. Lagasse L, Connors H, Al-Dhalimy M, Reitsma M, Doehse M, Osborne L, Wang X, Finegold M, Weissman IL, Groome P. Purified hematopoietic stem cells can differentiate into hepatocytes in *in vitro* experiments. *Nature Med* 2000; **6**: 1229-1234
4. Alison MR, Poulsom R, Jeffery R, Dhillon AP, Quaglia A, Jacob J, Novelli M, Prentice G, Williamson J, Wright NA. Hepatocytes from non-hepatic adult stem cells. *Nature* 2000; **406**: 257
5. Theise ND, Nimmakayalu M, Gardner R, Illei PB, Morgan G, Teperman L, Henegarui O, Krause DS. Liver from bone marrow in humans. *Hepatology* 2000; **32**: 11-16
6. Oh SH, Miyazaki M, Kouchi H, Inoue Y, Sakaguchi M, Tsuji T, Shima N, Higashio K, Namba M. Hepatocyte growth factor induces differentiation of adult rat bone marrow cells into a hepatic lineage in *in vitro*. *Biochem Biophys Res Commun* 2000; **279**: 500-504
7. Schwartz RE, Reyes M, Koolde L, Jiang Y, Blackstad M, Lund T, Lenvik T, Johnson S, Hu WS, Verfaillie CM. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. *J Clin Invest* 2002; **109**: 1291-1302
8. Fiegel HC, Lioznov MV, Cortes-Dericks L, Lange C, Kluth D, Fehse B, Zander AR. Liver-specific gene expression in cultured human hematopoietic stem cells. *Stem Cells* 2003; **21**: 98-104
9. Zhang Y, Bai XF, Huang CX. Hepatic stem cells: existence and origin. *World J Gastroenterol* 2003; **9**: 201-204
10. Theise ND, Saxena R, Portmann BC, Thung SN, Yee H, Chiriboga L, Kumar A, Crawford JM. The canals of Hering and hepatic stem cells in humans. *Hepatology* 1999; **30**: 1425-1433
11. Petersen BE, Goff JP, Greenberger JS, Michalopoulos GK. Hepatic oval cells express the hematopoietic stem cell marker Thy-1 in the rat. *Hepatology* 1998; **27**: 433-435
Expression of the stem cell factor receptor c-kit in normal and diseased pediatric liver: identification of a human hepatic progenitor cell? *Hepatology* 1999; 30: 112-117

Ma X, Qiu DK, Peng YS. Immunohistochemical study of hepatic oval cells in human chronic viral hepatitis. *World J Gastroenterol* 2001; 7: 238-242

Omori N, Omori M, Evarts RP, Teramoto T, Hoang TN, Thorgeirsson SS. Partial cloning of rat CD34 cDNA and expression during stem cell-dependent liver regeneration in the adult rat. *Hepatology* 1997; 26: 720-727

Omori M, Omori N, Evarts RP, Teramoto T, Miller MJ, Hoang TN, Thorgeirsson SS. Coexpression of flt-3 ligand/flt-3 and SCF/c-kit signal transduction system in bile-duct-ligated SI and W mice. *Am J Pathol* 1997; 150: 1179-1187

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; 284: 143-147

Gerson SL. Mesenchymal stem cells: no longer second class marrow citizens. *Nat Med* 1999; 5: 262-264

Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells *in vitro*. *J Cell Biochem* 1997; 64: 295-312

Wakitani S, Saito T, Caplan AI. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. *Muscle Nerve* 1995; 18: 1417-1426

Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* 2000; 61: 364-370

Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR. Adult bone marrow stromal cells differentiate into neural cells *in vitro*. *Exp Neurol* 2000; 164: 247-256

Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie CM. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. *Blood* 2001; 98: 2615-2625

Korbling M, Katz RL, Khanna A, Ruifrok AC, Rondon G, Albiter M, Champlin RE, Estrov Z. Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. *N Engl J Med* 2002; 346: 738-746

Goldschneider I, Gordon LK, Morris RJ. Demonstration of Thy-1 antigen on pluripotent hemopoietic stem cells in the rat. *J Exp Med* 1978; 148: 1351-1366

Avital I, Inderbitzin D, Aoki T, Tyan DB, Cohen AH, Ferrarelli C, Rozga J, Arnaout WS, Demetriou AA. Isolation, characterization, and transplantation of bone marrow-derived hepatocyte stem cells. *Biochem Biophys Res Commun* 2001; 288: 156-164

Petersen BE, Goff JP, Greenberger JS, Michalopoulos GK. Hepatic oval cells express the hematopoietic stem cell marker Thy-1 in the rat. *Hepatology* 1998; 27: 433-445

Zhan YT, Wei L, Chen HS, Cong X, Fei R, Wang Y. Differentiation of bone marrow stem cells in rat hepatic fibrogenesis environment. *Zhonghua Ganzangbing Za Zhi* 2003; 11: 673-675

Hamazaki T, Iiboshi Y, Oka M, Papst PJ, Meacham AM, Zon LI, Terada N. Hepatic maturation in differentiating embryonic stem cells *in vitro*. *FEBS Lett* 2001, 497: 15-19

Wang X, Al-Dhalimy M, Lagasse E, Finegold M, Grompe M. Liver repopulation and correction of metabolic liver disease by transplanted adult mouse pancreatic cells. *Am J Pathol* 2001; 158: 571-579

Clarke DL, Johansson CB, Wilbertz J, Veress B, Nilsson E, Karlstrom H, Lendahl U, Frisen J. Generalized potential of adult neural stem cells. *Science* 2000; 288: 1660-1663

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