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An *In Vivo* Model for Monitoring *Trans*-Differentiation of Bone Marrow Cells into Functional Hepatocytes

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The plasticity of bone marrow cells (BMCs) remains controversial. The present study found that persistent injury induces efficient trans-differentiation of BMCs into functional hepatocytes. Mice with liver cirrhosis induced by carbon tetrachloride were injected with 1×10^5 non-treated green fluorescent protein (GFP)-positive BMCs via the tail vein. In these mice, transplanted GFP-positive BMCs efficiently migrated into the peri-portal area of liver lobules after one day, repopulating 25% of the recipient liver by 4 weeks. In contrast, no GFP-positive BMCs were detected following transplantation into control mice with undamaged livers. BMCs trans-differentiated into functional mature hepatocytes via immature hepatoblasts. Serum albumin levels were significantly elevated to compensate for chronic liver failure in BMC transplantation. These results reveal that recipient conditions and microenvironments represent key factors for successful cell therapy using BMCs.

Key words: bone marrow cell, hepatic stem cell, niche, oval cell, *trans*-differentiation.

Abbreviations: BMC, bone marrow cell; CCl_4, carbon tetrachloride; EGFP, enhanced GFP; ES cell, embryonic stem cell; FAH, fumarylacetoacetate hydrolase; GFP, green fluorescent protein; HNF4, hepatocyte nuclear factor 4; MAPCs, multipotent adult progenitor cells; NGS, normal goat serum; NRS, normal rabbit serum; PAP, peroxidase-antiperoxidase.

The use of stem cells in regenerative medicine is attractive as a potential approach to curing patients with severe disease. Embryonic stem (ES) cells are pluripotent cells that differentiate into numerous cell types *in vivo* and *in vitro* (*1*). Although ES cells can be isolated from humans, ethical considerations and problems with the production of teratoma complicate their use in human therapies. Conversely, adult stem cells exist in various tissues (*2–7*). The use of tissue-specific stem cells is attractive, but isolating these cells can sometimes prove difficult, and their capacity for self-renewal is limited. Several groups have recently reported the capacity of bone marrow cells (BMCs) to differentiate into various non-hematopoietic cell lineages (*8–12*). These results suggest BMCs as an attractive cell source for regenerative medicine, as the cells are more easily obtained than other tissue-specific stem cells (*13*). The capacity of BMCs to differentiate into hepatocytes and intestinal cells has been demonstrated using Y chromosome detection in autopsy analyses of human female recipients of BMCs from male donors (*9*, *14*, *15*). However, following reports that spontaneous cell fusion is an important mechanism for trans-differentiation of BMCs and tissue stem cells, many researchers cannot accept the existence of adult BMCs that display a multipotent capacity for differentiation (*16*, *17*). Lagasse *et al*. reported that purified hematopoietic stem cells differentiated into hepatocytes in a fumarylacetoacetate hydrolase (FAH)-deficient model (*12*). Wang and Vassllopoulos *et al*. recently reported that cell fusion is an important mechanism for explaining the differentiation of BMCs into hepatocytes in the FAH-deficient model (*18*, *19*). Conversely, other groups have reported finding no evidence of cell fusion during trans-differentiation of BMCs into pancreatic endocrine cells and buccal epithelial cells in vivo (*20*, *21*). Newsome *et al*. reported that human umbilical cord blood-derived cells can differentiate into hepatocytes in mouse liver without evidence of cell fusion (*22*). Jiang *et al*. established multipotent adult progenitor cells (MAPCs) in bone marrow using a culturing system derived from mesenchymal stem cells (*23*, *24*). The plasticity and trans-differentiation of BMCs thus remain controversial. To develop effective cell therapies using BMCs, a better understanding of the regulatory mechanisms controlling BMC plasticity and differentiation into non-hematopoietic cell lineages is required. To address these issues, we developed a new *in vivo* model to monitor the differentiation of BMCs into functional hepatocytes. In this model, BMCs are transplanted without prior culture. The development of this new model should greatly advance our understanding of

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BMC plasticity. We selected a liver cirrhosis model in mice subjected to injections of carbon tetrachloride (CCl_4) for 4 weeks, due to its similarities to liver cirrhosis in humans. This study used transgenic mice expressing Green Fluorescence Protein (GFP) as a source of BMCs to explore the process of BMC differentiation into hepatocytes (*25*). GFP-positive BMCs were isolated and transplanted into liver cirrhosis mice without the addition of culture step. If BMC transplantation successfully compensates for liver failure, new cell therapies for patients with chronic liver damage can be developed. This model was also used to analyze the differentiation process of BMCs into hepatocytes.

MATERIAL AND METHODS

Mice—C57BL6/Tg14 (act-EGFP) OsbY01 mice were kindly provided by Masaru Okabe (Genome Research Center, Osaka University, Osaka). All tissues from the transgenic mice appeared green under excitation light (*25*). Female C57BL/6 mice were purchased from Japan SLC (Shizuoka). Mice were properly anesthetized during experiments. All procedures including surgical steps were performed in accordance with Yamaguchi University guidelines for experiments involving animals and recombinant DNA.

BMC Preparation and Transplantation—For the isolation of BMCs, C57BL6/Tg 14 (act-EGFP) OsbY01 mice (6-weeks-old) were killed by cervical dislocation, and their limbs were removed. GFP-positive BMCs were flushed from the medullary cavities of the tibias and femurs using a 25G needle and Dulbecco's modified Eagle's culture medium with 10% fetal bovine serum. Marrow cell preparation took approximately 1.5 h.

In the liver damage group, 0.5 ml/kg of CCl_4 was injected into the peritoneum of 6-weeks-old C57BL/6 females twice a week for 4 weeks. Liver cirrhosis resulting from the continuous injections of CCl_4 was confirmed. One day after the eighth injection, 1×10^5 GFP-positive BMCs were injected slowly into the tail vein using a 31G needle and a Hamilton syringe. After transplantation, CCl_4 injections were continued at the same dose twice a week. As a control, 1×10^5 GFP-positive BMCs were injected into female C57BL/6 mice that had not been treated with CCl_4.

Tissue Preparation—The livers were thoroughly perfused *via* the heart with 4% paraformaldehyde (Muto, Tokyo). This step was crucial for washing out contaminating blood cells. For fixation, the perfused livers were incubated with 4% paraformaldehyde (Muto) overnight, then soaked in 30% sucrose for a few more 3 days. Tissues were frozen in dry ice and then sectioned into 18-μm slices using a cryostat (Moriyasu Kounetsu, Osaka) in preparation for dyeing.

Immunohistochemistry and Double Immunofluorescence for GFP—Cells expressing GFP were analyzed by both fluorescent microscopy and conventional immunohistochemistry with anti-GFP antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA). Immunohistochemical analysis was performed in accordance with previously reported methods (*26*). Tissues were soaked overnight in 0.3% Triton X-100/0.05% normal goat serum (NGS; Chemicon, Temecula, CA, USA) or normal rabbit serum (NRS; Chemicon) and phosphate-buffered saline (PBS). The next day, the tissues were immersed in 500 μl of 10% NGS or NRS/0.3% TritonX-100/PBS for 3 h, then washed with 0.3% Triton X-100/PBS/0.05% NGS or NRS for 10 min. Tissues were soaked in 1.5% H_2O_2/50% methanol/DDW for 2 h, then washed with 0.3% Triton X-100/PBS/0.05% NGS or NRS. Sections were incubated overnight with anti-GFP antibody (1:5,000 FL, sc-8334; Santa Cruz Biotechnology), anti–HNF-4 (1:5000 H-171; Santa Cruz Biotechnology), anti-albumin (1:5,000, 55462; ICN Pharmaceuticals, Costa Mesa, CA, USA), anti-Liv2 antibody (1:5,000) (*27*) or A6 monoclonal antibody (1:5,000) for common surface-exposed antigens of mouse on oval cells and biliary epithelial cells (*28*). Tissues were then washed three times for 10 minutes each with 0.3% Triton X-100/PBS/0.05% NGS or NRS. Tissues were incubated with biotin-conjugated IgG anti-rat, -rabbit, -mouse, -goat IgG (Dako, Kyoto) as secondary antibodies for 2 h at 37°C, then washed three times for 20 min each with PBS. After incubation with both peroxidase-antiperoxidase (PAP)-goat, mouse, rabbit (Dako), PAP-rat IgG complex (Chemicon) and streptavidin (Dako) as the third antibody for 3 h at 37°C, the tissues were washed with 0.05 M Tris-HCl buffer (pH 7.4). Samples were then treated with 0.05 M Tris-HCl buffer (pH 7.4) containing 0.02% diaminobenzine and 0.6% nickel ammnonium sulfate (DAB-nickel solution) in the presence of 0.005% H_2O_2 (nickel-enhanced DAB reaction) for 10–15 min at room temperature. For fluorescence immunohistochemistry, tissues were incubated with Alexa Fluor R 488 and 568 donkey anti-goat IgG(H+L) conjugate, Alexa Fluor R 488 goat anti-rabbit IgG(H+L) conjugate and Alexa Fluor R 568 goat anti-rat IgG(H+L) conjugate (Molecular Probes, Eugene, OR) as secondary antibodies for 3 h, then washed with PBS. Sections were mounted on glass slides in 1% gelatin/saline. Positive cells in the liver were quantified using a Provis microscope (Olympus, Tokyo) equipped with a charge coupled devise (CCD) camera, and subjected to computer-assisted image analysis with MetaMorph software (Universal Imaging Corporation, Downingtown, PA). A total of 10 different areas per liver section were analyzed independently, and the areas of positive cells were calculated using the MetaMorph software.

Serum Albumin Levels—Serum albumin levels were measured in experimental mice for 4 weeks after BMC transplantation and in control mice using a SPOTCHEM EZ SP-4430 dry chemical system (Arkray, Kyoto).

Statistical Analysis—Values are shown as mean ± SE. Data were analyzed by analysis of variance with Fisher's projected least significant difference test.

RESULTS

GFP Positive BMCs Migrated into the Peri-Portal Region of Liver Lobules then Distributed into Lobules with Chronic Liver Damage—We injected 1×10^5 GFP-positive BMCs into both liver-damaged and control (undamaged) mice. After transplantation, CCl_4 treatment at the same dose was continued twice each week. Transplanted cells were visualized immunohistochemically using anti-GFP antibody (Fig. 1, a–f). No GFP-positive cells were detected in liver sections of undamaged

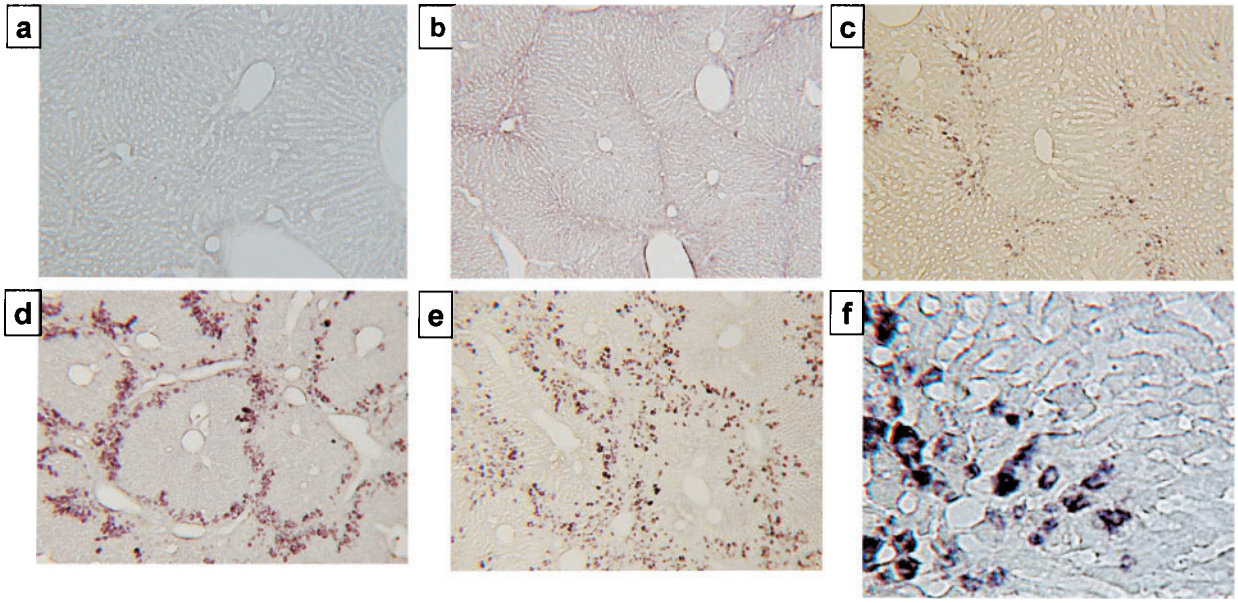

Fig. 1. **Expression of GFP.** a: Anti-GFP staining of a liver section 1 week after BMC transplantation in the control group (no CCl_4 treatment). Magnification ×200. b: Anti-GFP staining of a liver section after 4 weeks of CCl_4-induced liver damage without BMC transplantation. Magnification ×200. c–f: Liver sections of BMC transplant recipients in the CCl_4-induced liver damage group. Magnification ×200. The brown color of the liver sections resulted from a pH difference in the detection buffer, but sensitivity was unaffected. c: 1 d after BMC transplantation. d: 1 week after transplantation. e: 4 weeks. f: Higher magnification at 4 weeks, ×400.

controls (Fig. 1a) or in mice with CCl_4 damage but no BMC transplantation (Fig. 1b). In contrast, the edges of peri-portal regions of liver lobules from BMC recipients treated with CCl_4 contained 0.1–1% GFP-positive BMCs one day after BMC transplantation (Fig. 1c). The number of GFP-positive cells gradually increased, spreading into the liver lobules 1–4 weeks after transplantation (Fig. 1, d and e) and forming liver cell cords (Fig. 1f). The areas occupied by GFP-positive cells increased each week up to 26 ± 1% at 4 weeks (Table 1). These results indicate that GFP-positive BMCs migrate into the peri-portal areas of damaged liver and proliferate to form hepatic cords at 4 weeks only under conditions of persistent liver damage.

BMCs Differentiated into Hepatocytes Expressing Different Differentiation Markers—To monitor the differentiation of transplanted BMCs into hepatocytes, cells were tested for the concommitant expression of GFP and various differentiation markers: hepatoblast markers Liv2; hepatocyte nuclear factor 4 (HNF4) (*29–32*), and hepatocyte marker albumin. Liv2 represents a marker for hepatoblasts in the hepatic bud (*27*). Liv2-positive cells were not detected in bone marrow (data not shown) or in persistently damaged livers without BMC transplantation (Fig. 2a). Liv2-positive cells appeared in the peri-portal regions of the liver 1 week after BMC transplantation (Fig. 2b), and then increased and spread throughout the liver lobules (Fig. 2, b and c). Liv2-positive cells occupied up to 21 ± 1% of affected lobules (Table 1) and were detected at liver cell cords at 4 weeks (Fig. 2d). The co-expression of Liv2 and GFP (yellow color cell) is shown in Fig. 2 (e and f).

HNF4 expression was also analyzed. HNF4 is a transcription factor associated with hepatocyte differentiation (*29–32*). In the livers of BMC-recipient mice, HNF4 expression and HNF4-occupied areas increased over time (Fig. 3, a and b, and Table 1). Co-expression of HNF4 and GFP is shown in Fig. 3c. Staining with an A6 oval cell-specific monoclonal antibody (*28*) detected positive cells in the peri-portal region 1 week after BMC transplantation (Fig. 3d). The area occupied by A6-positive cells was about 5–6% by 4 weeks after BMC transplantaion (Fig. 3e and Table 1). However, A6-positive cells that also expressed GFP were not found in liver after BMC transplantation (data not shown). A6-positive cells were not detected in CCl_4-damaged livers without BMC transplantation (Fig. 3f) or in normal livers (Fig. 3g).

Table 1. **Areas occupied by various differentiation markers after BMC transplantation into the persistent liver damage group.**

	1 week (n = 4)	2 weeks (n = 4)	3 weeks (n = 4)	4 weeks (n = 4)
GFP	12 ± 1	15 ± 1	21 ± 2*,**	26 ± 1*,**,***
Liv2	11 ± 1	14 ± 1*	18 ± 1 *,**	21 ± 1*,**,***
HNF4	16 ± 1	19 ± 1*	16 ± 1**	20 ± 1*,***
A6	5 ± 1	6 ± 1	6 ± 1	6 ± 1
Albumin	9 ± 2	12 ± 2	27 ± 1*,**	42 ± 1*,**,***

Value are shown as mean ± SE. *Significant difference compared with the value at 1 week ($p < 0.05$). **Significant difference compared with the value at 2 weeks ($p < 0.05$). ***Significant difference compared with the value at 3 weeks ($p < 0.05$) (n = 4).

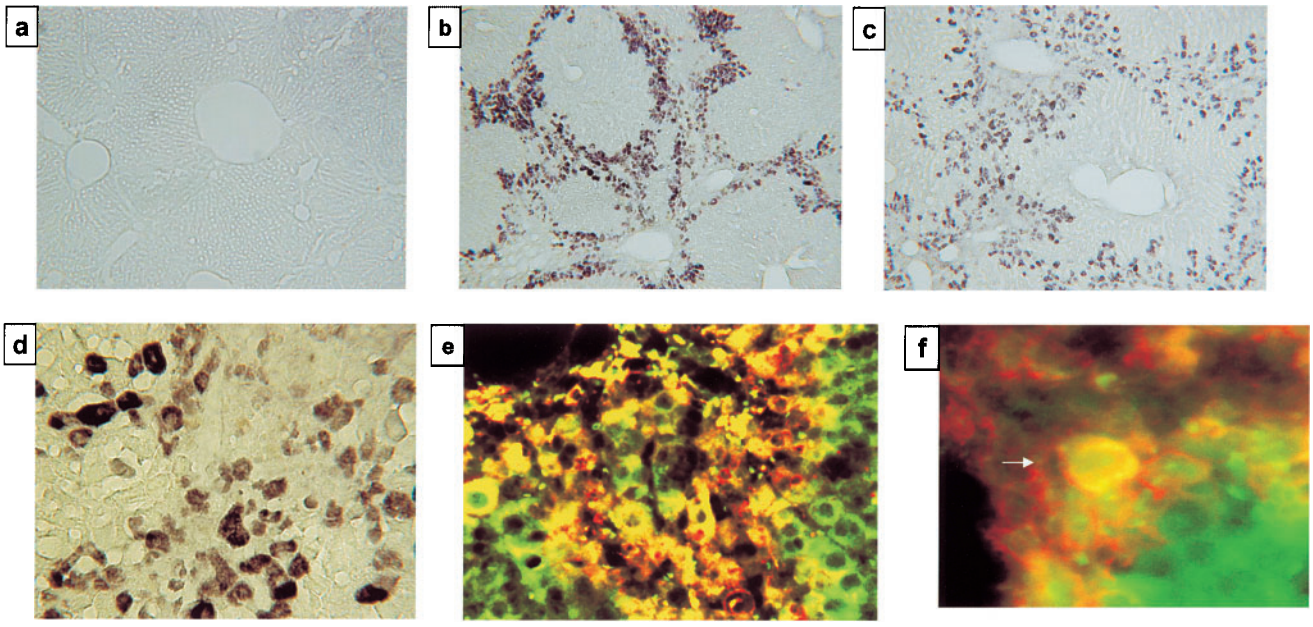

Fig. 2. **Expression of Liv2 antigen.** Immunohistochemistry of liver sections from CCl_4-treated mice. a: Absence of Liv2-positive cells after 4 weeks of CCl_4 treatment in mice without BMC transplantation. Magnification ×200. b, c: Liv2 expression at (b) 1 week and (c) 4 weeks after BMC transplantation. Magnification ×200. d: Magnified image at 4 weeks, ×400. e: Double fluorescent images (red, Liv2; green, GFP; yellow, Liv2 and GFP) at 4 weeks. A GFP-positive cell expressing Liv2 appears yellow, ×400. f: Higher magnification of the cell co-expressing GFP and Liv2, ×400.

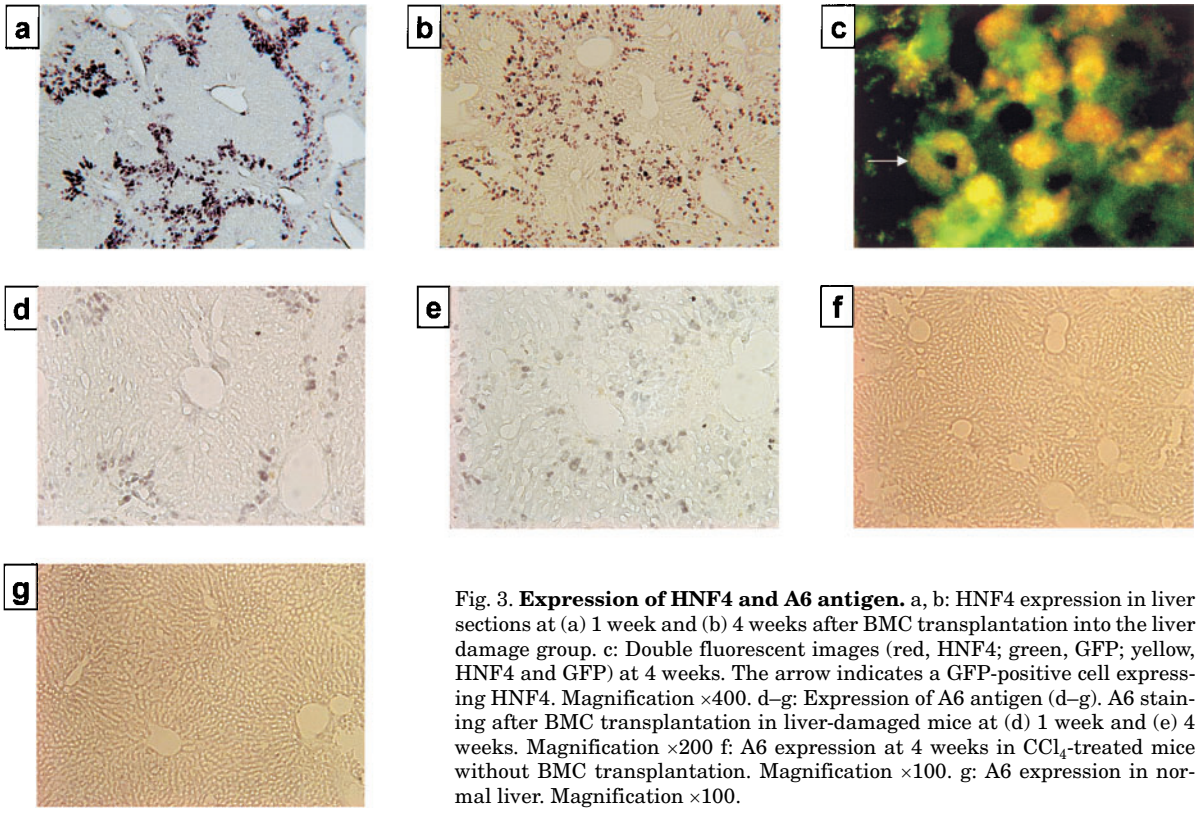

Fig. 3. **Expression of HNF4 and A6 antigen.** a, b: HNF4 expression in liver sections at (a) 1 week and (b) 4 weeks after BMC transplantation into the liver damage group. c: Double fluorescent images (red, HNF4; green, GFP; yellow, HNF4 and GFP) at 4 weeks. The arrow indicates a GFP-positive cell expressing HNF4. Magnification ×400. d–g: Expression of A6 antigen (d–g). A6 staining after BMC transplantation in liver-damaged mice at (d) 1 week and (e) 4 weeks. Magnification ×200 f: A6 expression at 4 weeks in CCl_4-treated mice without BMC transplantation. Magnification ×100. g: A6 expression in normal liver. Magnification ×100.

BMCs Differentiate into Functionally Mature Hepatocytes and Compensate for Chromic Liver Failure Induced by Persistent Liver Damage—Whether transplanted BMCs can differentiate into functional hepatocytes was evaluated by analyzing albumin expression. Albumin was detected as dark staining in hepatocytes of normal mouse liver sections (Fig. 4a). The expression of albumin decreased in the livers of mice treated with CCl_4 for 4 weeks without

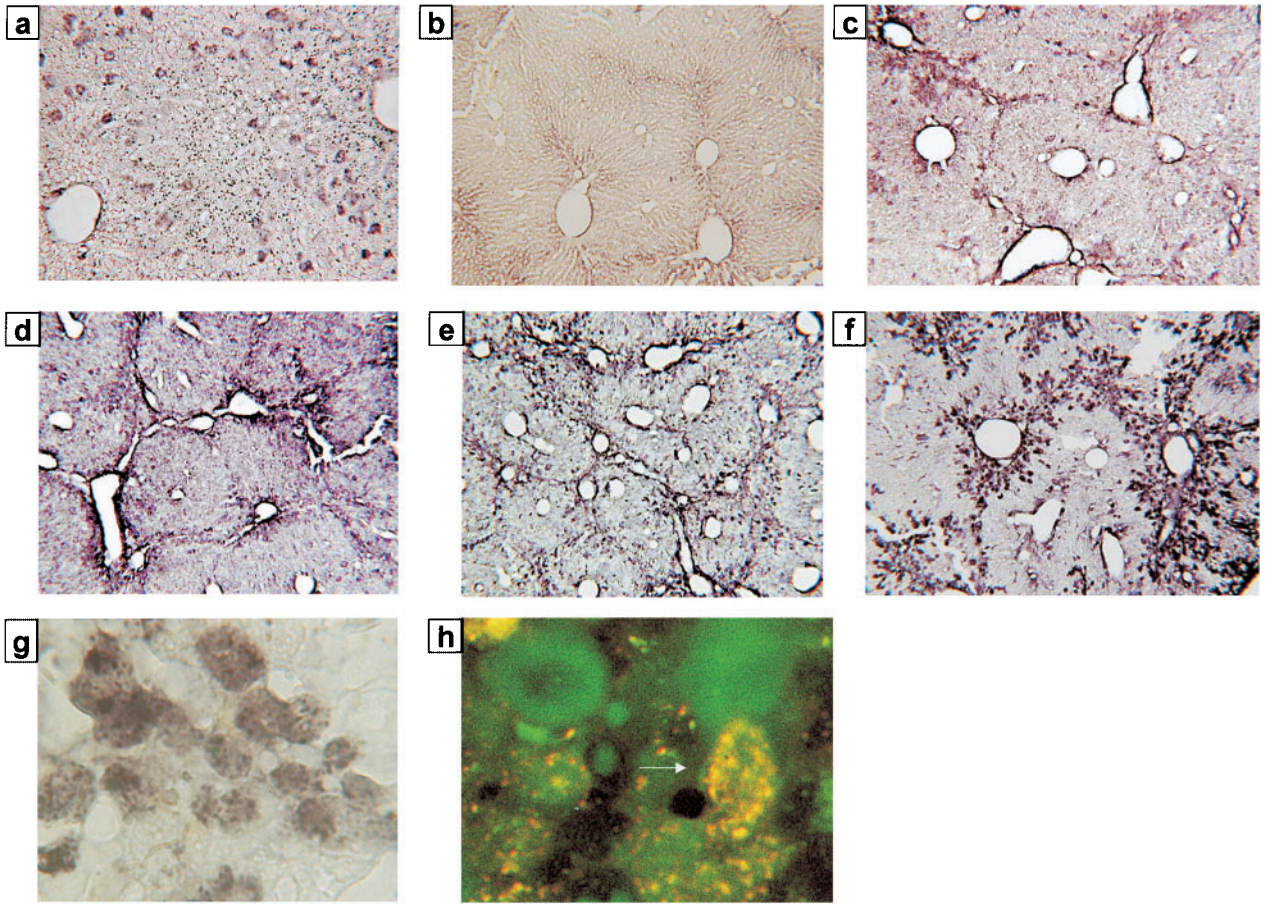

Fig. 4. **Albumin expression.** a: Albumin expression in a liver section from a normal mouse. Magnification ×200. b: Liver section of a mouse after 4 weeks of CCl_4 treatement. Magnification ×200. c–f: Albumin expression after BMC transplantation in the liver damage group at (c) 1 week, (d) 2 weeks, (e) 3 weeks, and (f) 4 weeks. Magnification ×200. g: Higher magnification at 4 weeks. h: Double fluorescent image (red, albumin; green, GFP; yellow, albumin and GFP) at 4 weeks after BMC transplantation in the liver damage group. Magnification ×400. The arrow indicates the co-expression of albumin and GFP in one cell.

BMC transplantation (Fig. 4b). At 1 week after BMC transplantation, a small number of albumin-positive cells were apparent in the peri-portal regions of damaged livers (Fig. 4c). The mean area occupied by albumin-positive cells was 9 ± 2% at 1 week (Table 1). Cells expressing high levels of albumin increased in number and spread from the peri-portal regions into lobules over time (Fig. 4, c–f, and Table 1). Cells expressing high levels of albumin were also detected in liver cell cords (Fig. 4g). Co-expression of GFP (green) and albumin (red) was observed; the albumin in co-expressing cells appeared as fine yellow particles (Fig. 4h). Areas displaying albumin increased to 42 ± 1% at 4 weeks, although the GFP-occupied areas comprised only 26 ± 1% (Table 1). To determine whether BMC transplantation compensates for liver damage, serum albumin levels were analyzed in the persistent liver damage groups with and without BMC transplantation (Fig. 5). The serum albumin level of CCl_4-damaged mice not undergoing BMC transplantation decreased over time, to 1.62 ± 0.15 g/dl at 8 weeks (n = 5). In contrast, the serum albumin levels in CCl_4-damaged mice subjected to BMC transplantation increased to 2.08 ± 0.10 g/dl by 4 weeks after BMC transplantation, representing a significant difference ($p < 0.05$). These results demonstrate that transplanted BMCs differentiate into functional hepatocytes that can compensate for liver damage.

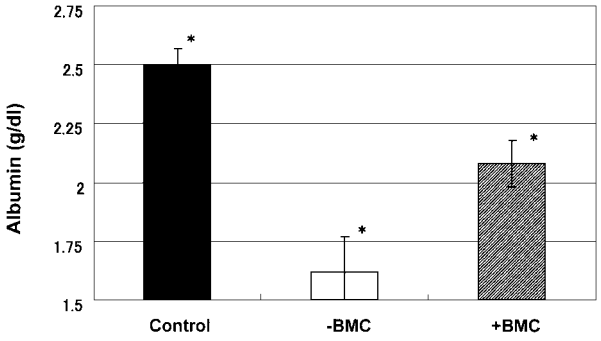

Fig. 5. **Serum albumin levels of CCl_4-treated mice with or without BMC transplantation.** Control, normal mice. Values indicate albumin levels of normal control mice. –BMC, CCl_4-treated mice without BMC transplantation. Values show albumin levels after 8 weeks CCl_4 injection without BMC transplantaion. +BMC, CCl_4-treated mice who underwent BMC transplantation. Values show albumin levels of recipient mice at 4 weeks after BMC transplantation. *Differences between groups were significant (n = 5; $p < 0.05$).

DISCUSSION

The present study described a new *in vivo* model for monitorng the *trans*-differentiation of BMCs into hepatocytes using GFP as a marker protein. In these experiments, the areas occupied by GFP-positive cells comprised around 0.1–1% by 1 day after BMC transplantaion (Fig. 1c). Transplanted GFP-positive BMCs migrated into the peri-portal regions of cirrhotic livers (Fig. 1d). Gangandeep *et al*. reported that hepatocytes transplanted *via* the spleen distributed to the peri-portal region immediately in dipeptidyl peptidase IV–deficient F344 rats with CCl_4-induced cirrhosis (*33*). Although the BMCs in our model were transplanted *via* the tail vein, the same efficient migration of transplanted cells to the peri-portal regions of cirrhotic livers occurred. The areas of liver occupied by GFP-positive cells increased to 26 ± 1% by 4 weeks after BMC transplantation, despite continued induction of liver damage by the injection of CCl_4 (Table 1). Some previous studies have reported that CCl_4 injection enhances the repopulation of hepatocytes following hepatocyte tranplantation *via* the spleen (*34*, *35*). The same situation might have occurred in the present model. In FAH-deficient mice, transplanted hematopoietic stem cells were found to form foci of hematopoietic stem cell-derived hepatocytes to compensate for liver failure (*12*). Conversely, in our experiments, transplanted GFP-positive BMCs formed hepatic cords in the liver in a different manner (Fig. 1f), representing a major difference between the FAH model and this CCl_4 model.

Recently, cell fusion has been reported as an important mechanism for the plasticity of BMCs and tissue stem cells (*16*, *17*). The differentiation of BMCs into hepatocytes in the FAH model seems to indicate cell fusion as an important mechanism of differentiation (*18*, *19*). However, other groups have reported finding no evidence of cell fusion during the *trans*-differentiation of BMCs into other cell lineages *in vivo* (*20*, *21*). We analyzed the rate of cell fusion using cultured Neo-resistant ES cells and GFP-positive BMCs under the same culture conditions as Terada *et al*. (*17*), who reported a cell fusion rate of $1/10^5$–10^6. Our in vitro assay yielded similar results (data not shown). Next we analyzed the differentiation step from transplanted BMC into hepatocyte. To monitor the differentiation of transplanted BMCs into hepatocytes, the expressions of hepatoblast markers Liv2 (*27*), HNF4, and hepatocyte marker albumin, and the hepatic oval cell marker A6 were analyzed. During mouse liver development, Liv2-positive cells appear in the hepatic bud at E 9.5 d. In our study, Liv2-positive cells were not detected in bone marrow (data not shown) or in persistently damaged liver without BMC transplantation (Fig. 2a). Liv2-positive cells appeared in the peri-portal regions of the liver 1 week after BMC transplantation and had spread throughout the damaged liver lobules (Fig. 2, b–d), to occupy 21 ± 1% of liver area at 4 weeks (Table 1). Liv2-expressing cells were also detected in liver cell cords at 4 week (Fig. 2d). The co-expression of Liv2 and GFP (yellow colored cells) is shown in Fig. 2 (e and f), but cells co-expressing Liv2 and albumin were not observed (data not shown). These results suggest that transplanted BMCs change to an immature phenotype that expresses Liv2 antigen before maturing into albumin-expressing hepatocytes. HNF4 is a transcription factor associated with hepatocyte differentiation (*29–32*). In the livers of BMC-recipient mice, HNF4 expression and the HNF4-occupied area increased over time (Fig. 3, a and b, and Table 1). The co-expression of HNF4 and GFP is shown in Fig. 3c. These results suggest that differentiating BMCs take on the hepatocyte phenotype. Oval cells are considered to represent a type of hepatic stem cell derived from the Canal of Hering following severe liver damage (*6*, *37*). Petersen et al. also reported that under certain conditions, oval cells can be derived from BMCs (*8*). Therefore, the activation of oval cells was also analyzed using an A6 antibody. A6-positive cells were detected in the peri-portal regions 1 week after BMC transplantation (Fig. 3d). The A6-positive oval cells were activated, but the area occupied by A6-positive cells remained at around 5% even after 4 weeks (Fig. 3, d and e, and Table 1). However, A6-positive cells also expressing GFP were not detected in liver following BMC transplantation (data not shown). Furthermore, A6-positive cells were not detected in CCl_4-damaged livers without BMC transplantation (Fig. 3f), or in normal livers (Fig. 3g). These results suggest that while some signals activating oval cells are induced by BMC transplantation, oval cells might not be derived from transplanted BMCs. Although the possibility of cell fusion occurring in our model can not be excluded, BMCs were found to *trans*-differentiate into Liv2-positive hepatoblasts that subsequently differentiated into functional hepatocytes. The differentiation process resembles that observed during hepatogenesis (*38*).

Finally, we analyzed whether transplanted BMCs could differentiate into functional hepatocytes by examining albumin expression. Albumin was detected as a dark staining of hepatocytes in normal mouse liver sections (Fig. 4a), and its expression was decreased by persistent liver damage (Fig. 4b). At 1 week after BMC transplantation, a small number of albumin-positive cells appeared in the peri-portal regions of damaged livers (Fig. 4c), with the mean area occupied by albumin-positive cells being 9 ± 2% at 1 week (Table 1). Cells expressing high levels of albumin increased in number and spread from the peri-portal regions into damaged lobules over time (Fig. 4, c–f, and Table 1). Cells expressing high levels of albumin were also detected in liver cell cords (Fig. 4g). Co-expression of GFP (green) and albumin (red) was observed, with the albumin in co-expressing cells appearing as fine yellow particles (Fig. 4h). The area occupied by albumin-expressing cells increased to 42 ± 1% at 4 weeks, compared with the GFP-occupied area of only 26 ± 1% (Table 1). During the *trans*-differentiation of BMCs into hepatocytes, A6-positive oval cells are activated (Fig. 3, d and e and Table 1). These results suggest that the *trans*-differentiation of BMCs might affect albumin expression in surrounding cells *via* oval cell activation. Serum albumin levels decreased in persistent CCl_4-damaged mice without BMC transplantation, but increased significantly in CCl_4-damaged mice who underwent BMC transplantation at 4 weeks after transplantation (Fig. 5). These results demonstrate that transplanted BMCs differentiate into functional hepatocytes that can compensate for chronic liver failure.

Previously, several authors have reported the existence of hepatic stem/progenitor cells in bone marrow (*8*, *12*,

39). Jiang *et al*. derived MAPCs from bone marrow mesenchymal cells (*23*, *24*) and engrafted 1.0×10^6 MAPCs into NOD/SCID mice. The MAPCs were found to occupy 5–8% of the liver area after 4–24 weeks. While the MAPCs demonstrated multipotency, their efficiency of differentiation into hepatocytes was lower than that observed in the present system, in which 1×10^5 BMC efficiently differentiated and occupied $26 \pm 1\%$ of the liver area by 4 weeks after transplantation. A significant increase in serum albumin levels demonstrated that the BMC-derived cells restored liver function. Exactly why the BMCs in our model were able to differentiate efficiently into functional hepatocytes remains an intriguing question. Only isolated BMCs were transplanted in the present system, without the addition of a culture period, representing one key difference from the methods described by Jiang *et al*. (*24*). Although exactly which cells trans-differentiate into hepatocytes remains unknown, we believe that the persistent liver damage induced by CCl_4 represents a key factor in our method, creating a special "differentiation niche" able to induce the trans-differentiation of BMCs into functional hepatocytes. Stress-induced signaling pathways are known to play crucial roles in hepatogenesis, and knockout mice for various inflammation signal molecules display massive liver degeneration (*40–42*). Based on these results, we postulate that the *trans*-differentiation of BMCs might be enhanced by unknown signals related to stress and inflammation.

In conclusion, much about the trans-differentiation of transplanted BMCs into hepatocytes in our model remains yet to be explained. However, our model shows that recipient condition and the timing of BMC transplantation are important. This system should facilitate the development of cell therapies for liver damage by providing a model for testing factors critical to BMC differentiation into hepatocytes.

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