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A subpopulation of bone marrow cells depleted by a novel antibody, anti-Liv8, is useful for cell therapy to repair damaged liver☆,☆☆

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Abstract

We previously reported a new in vivo model named as "GFP/CCl_4 model" for monitoring the transdifferentiation of green fluorescent protein (GFP) positive bone marrow cell (BMC) into albumin-positive hepatocyte under the specific "niche" made by CCl_4 induced persistent liver damage, but the subpopulation which BMCs transdifferentiate into hepatocytes remains unknown. Here we developed a new monoclonal antibody, anti-Liv8, using mouse E 11.5 fetal liver as an antigen. Anti-Liv8 recognized both hematopoietic progenitor cells in fetal liver at E 11.5 and CD45-positive hematopoietic cells in adult bone marrow. We separated Liv8-positive and Liv8-negative cells and then transplanted these cells into a continuous liver damaged model. At 4 weeks after BMC transplantation, more efficient repopulation and transdifferentiation of BMC into hepatocytes were seen with Liv8-negative cells. These findings suggest that the subpopulation of Liv8-negative cells includes useful cells to perform cell therapy on repair damaged liver.

Keywords: Bone marrow cell; Cell therapy; Regenerative medicine; Hepatic stem cell; Migration; Transdifferentiation; Mesenchymal stem cell; Hematopoietic stem cell; Liver regeneration; Niche

Recently, several groups have reported the possible plasticity of bone marrow cells (BMCs) to transdifferentiate into a variety of non-hematopoietic cell lineages [1–4]. Ever since the transdifferentiation of BMC into hepatocytes was documented following a bone marrow transplant from a man donor to a woman recipient [5,6],

☆ *Abbreviations:* BMC, bone marrow cell; CCl_4, carbon tetrachloride; FAH, fumarylacetoacetate hydrolase; GFP, green fluorescent protein; EGFP, enhanced GFP; GFP-Tg mice, C57BL6/Tg14 (act-EGFP) OsbY01 mice; HSC, hematopoietic stem cell; E, embryonic day; MSC, mesenchymal stem cells; MAPC, multipotent adult progenitor cell.

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BMC has been an attractive cell source in regenerative medicine because getting BMC is easier than obtaining other tissue-specific stem cells [7].

However, the results of recent studies have been mixed in that some studies found that BMC was hardly transdifferentiated while others documented high levels of transdifferentiation [8,9]. Successful transdifferentiation in cell therapy involves various cell and recipient factors, and these factors interact in a complex manner. Therefore, it is difficult to identify the conditions necessary for transdifferentiation, contributing to the varied results among past studies. A past study using a fumarylacetoacetate hydrolase (FAH) knockout mice (metabolic tyrosinemia model) showed that hepatic functions could be compensated by transplanting Lin-Kit + Sca + Thy1low (KTLS) marrow cells [10]. In the FAH model, KTLS cells form foci and transdifferentiate into hepatocytes. Results of recent studies suggest that KTLS cells transdifferentiate into hepatocytes due to fusion with hepatocytes [11,12]. The FAH model is a specialized model of metabolic liver damage, making it possible to analyze the transdifferentiation of BMC into hepatocytes and functional compensation. However, a model with which the transdifferentiation of BMC can be analyzed under conditions of more general liver damage is needed. Using autologous transplantation in GFP transgenic mice [13], we established an isogenic transplantation model to assess the transdifferentiation of BMC into hepatocytes. This model is unique in that uncultured BMCs efficiently migrate into the peri-portal area of the liver and transdifferentiate into immature hepatoblasts and differentiate into mature hepatocytes under the specific "niche" of persistent liver damage induced by persistent intraperitoneal administration of carbon tetrachloride (CCl_4) [14]. In this model, liver cirrhosis was induced by 4 weeks CCl_4 injection, and BMCs isolated from GFP transgenic mice were transplanted through the caudal vein. It is possible to chronologically observe colonization and transdifferentiation of BMC in the liver by continuous administration of CCl_4, and we have named this model as the "GFP/CCl_4 model." Furthermore, in this model, as in the natural development of the liver, BMCs appear to be transdifferentiated into hepatoblasts and then into hepatocytes. In our GFP/CCl_4 model, the timing of cell transplantation and the state of recipients appear to be suitable for the transdifferentiation of BMC into hepatocytes. Cell transplantation and continuous liver damage made efficient transdifferentiation of BMC into hepatocytes. In a system similar to ours, human hematopoietic stem cells (HSCs) were transplanted into the bone marrow of immunologically tolerant NOD/SCID mice before administration of CCl_4, and these cells differentiated into albumin-positive hepatocyte-like cells after the CCl_4 administration [15]. These findings suggest that a special "niche" created by CCl_4-induced liver damage is important for the migration of BMC to the liver and transdifferentiation into hepatocytes. Also, it has been reported recently that CCl_4 administration is effective for improving the colonization of HSC to liver of NOD/SCID [16].

The liver functions as a metabolic organ, but during the fetal period, from embryonic day (E) 12 to 16 (E12–E16), the liver functions as a hematopoietic organ [17]. Several studies have reported that mesenchymal cells affect hepatic hematopoiesis during the fetal period [18,19]. After this hematopoietic period, hepatoblasts are involved in a complex manner to develop the liver as a metabolic organ. However, documentation of the existence of HSC in the adult liver suggests that, even in the adult liver, blood cells and hepatocytes still play some role in the maintenance of hepatic function [20]. To further analyze this aspect, we prepared new rat monoclonal antibodies using the fetal liver on E 11.5 as an antigen. One of these antibodies, anti-Liv2, specifically recognizes hepatoblasts in the fetal liver from E 9.5 to 12.5. The results of past studies using the anti-Liv2 antibody have shown that SEK1, a stress-signaling kinase, plays an important role in the proliferation of hepatoblasts, thus suggesting that inflammatory signals are involved in the proliferation of hepatoblasts [21].

Although various theories explain the existence of pluripotent stem cells in BMC, the exact composition of stem cells in BMC is not clear at this time; the following cell types are known to exist in bone marrow: HSC [4,10], side population cells [22], and mesenchymal stem cells (MSC) [23]. Although past studies used the existing antibodies and techniques, there have not been any studies based on the findings associated with natural liver development. Using fetal liver as an antigen, we prepared a new monoclonal antibody, anti-Liv8 antibody, to analyze which subpopulation of BMC could differentiate into hepatocytes under CCl_4-induced continuous liver damage in the GFP/CCl_4 model [14]. This anti-Liv8 antibody recognizes hematopoietic cells using a specific cell surface marker and it can be used to separate cells. In the present study, we used this new antibody to separate BMC of adult mice and then transplanted the different types into mice under identical conditions of the GFP/CCl_4 model to ascertain which types of BMCs transdifferentiate into hepatocytes.

Materials and methods

Mice. C57BL6/Tg14 (act-EGFP) OsbY01 mice (GFP-Tg mice) showed GFP expression in multiple tissue and cells and were kindly provided by Masaru Okabe (Genome Research Center, Osaka University, Osaka, Japan) [13]. C57BL/6 female mice were purchased from Japan SLC (Shizuoka, Japan). AML1 knockout mice were generated

as described previously [24]. The genetic background of these mice used in this study was C57BL/6 mice. Male and female mice were mated overnight and female mice were scored based on vaginal plaques taken to represent E 0.5. Mice were anesthetized at the completion of experiments. All processes, including surgical steps, were undertaken with the guidance of the committee for animal and recombinant DNA experimentation at Yamaguchi University.

Production of rat monoclonal antibody, Liv8. Eight-week-old WKY/NCrj female rats were immunized in the hind footpads with 100 μg E 11.5 murine fetal liver lysate in complete Freund's adjuvant (0.2 ml). Anti-Liv8 antibodies were raised according to a previously described protocol [21].

Immunohistochemical staining for fetal liver. Fetal liver at E11.5 was obtained from c57/BL/6 mice and AML1 knockout mice. Tissue preparation and immunohistochemical analysis were performed according to a previously described protocol [21]. We analyzed anti-Liv2- and anti-Liv8-positive cells in fetal liver.

Preparation of GFP-positive BMC. For isolation of BMC, GFP-Tg mice were sacrificed by cervical dislocation and the limbs were removed. GFP-positive BMCs were flushed from the medullary cavities of tibias and femurs with PBS culture solution using a 25 G needle. The cell solution was filtered through a cell strainer (16 μm) to remove particular matter and centrifuged at 500*g* for 5 min. After centrifugation, the supernatant was removed and cells were resuspended to prepare 1.0×10^6 cells/ml GFP-positive BMC solutions. Preparation of BMC takes approximately 1.5 h.

FACS analysis of BMC using Liv8 antibody. Prepared GFP-positive BMCs were reacted with rat biotin anti-Liv8 IgG antibody, R-Phycoerythrin (R-PE)-conjugated rat anti-CD45 (leukocyte common antigen) monoclonal antibody (PharMingen, San Diego, USA) at the rate of 1 μg per 10^6 total cells, mixed well, and incubated in the gobos for 30–40 min at 4 °C. Following the incubation with the first antibody, the cells were washed twice by 0.02 M PBS and centrifuged at 500*g* for 5 min. Labeled cells were then reacted to streptavidin–fluorescein isothiocyanate (FITC) conjugate (PharMingen) at the rate of 1 μg per 10^6 total cells, mixed well, and incubated in the gobos for 30–40 min at 4 °C. After that, these were washed out once with 0.02 M PBS and centrifuged at 500*g* for 5 min. The labeled cells were analyzed using FACS Calibur (Becton–Dickinson).

Sort GFP positive BMC by Liv8 antibody. Prepared BMCs were reacted to rat anti-Liv8 IgG antibody at the rate of 1 μg per 10^6 total cells, mixed well, and incubated in the gobos for 30–40 min at 4 °C. Then cells were washed two times by 0.02 M PBS and centrifuged at 500*g* for 5 min. Cells were labeled with rat anti-Liv8 IgG antibody by reacting with Goat Anti-Rat IgG MicroBeads (Miltenvi Biotec GmbH, Bergisch Gladbach, Germany) at the rate of 20 μl per 10^7 total cells, mixed well, and incubated for 20–30 min at 4 °C. Labeled cells were washed once by 0.02 M PBS and centrifuged at 500*g* for 5 min. These cells were separated into Liv8-positive cells or negative cells by the Auto Magnetic Cell Sorting system (Auto MACS) (Miltenvi Biotec GmbH) for 10 min per tube.

Transplantation of Liv8-positive or negative BMC into persistent liver damaged mice. We developed a new in vivo model "GFP/CCl_4 model" for monitoring differentiation of BMCs into hepatocytes [14]. To generate a liver damage group, 0.5 ml/kg of CCl_4 was injected into the peritoneum of 6-week-old C57BL/6 females twice a week for 4 weeks. Liver cirrhosis resulting from the continuous injections of CCl_4 was confirmed. A control group of C57BL/6 mice that had not been treated with CCl_4 was also used. One day after the eighth injection, sorted Liv8-positive or Liv8-negative BMC (1×10^5 cells) was slowly injected into the caudal tail vein of mice using a 31 G needle and a Hamilton syringe. After transplantation, CCl_4 injections (0.5 ml/kg) were continued twice a week. Mice were sacrificed weekly up to 4 weeks.

Tissue preparation. The livers were thoroughly perfused via the heart with 4% paraformaldehyde (Muto, Tokyo, Japan). This step was crucial for washing out contaminating blood cells. For fixation, the perfused livers were incubated with 4% paraformaldehyde (Muto) overnight and 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, Japan) in preparation for dyeing.

Immunohistochemistry and double immunofluorescence for GFP. To avoid autofluorescence, we used immunostaining to assess the expression of 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 according to a previously described protocol [14,25]. Sectioned tissues were incubated with anti-GFP antibody (1:5000 FL, sc-8334; Santa Cruz Biotechnology), anti-albumin (1:5000, 55462; ICN Pharmaceuticals, Costa Mesa, CA, USA), and anti-Liv2 antibody (1:5000) [21]. 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. Positive cells in the liver were quantified using a Provis microscope (Olympus, Tokyo, Japan) equipped with a charge coupled devise (CCD) camera and subjected to computer-assisted image analysis with MetaMorph software (Universal Imaging, 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 level analysis. Serum albumin levels during the 4 weeks after Liv8-positive or Liv8-negative BMC transplantation were analyzed using the SPOTCHEM EZ SP-4430 dry chemical system (Arkray, Kyoto, Japan).

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

Results

Anti-Liv8 antibody detected hematopoietic progenitor cell in fetal liver at E 11.5

Previously we had raised a rat monoclonal antibody, anti-Liv2, which recognized hepatoblasts at E 9.5 [21]. As shown in Fig. 1A, Liv2-positive cells were also detected in fetal liver at E 11.5. Using the antibody developed in this study, Liv8-positive cells were seen in the fetal liver on E 11.5 (Fig. 1B). Fetal liver at E 11.5 functions as a secondary hematopoietic organ [17]. We analyzed whether anti-Liv8 positive cell is associated with hepatoblast or hematopoietic cell. We found Liv2-positive cells (Fig. 1C), but no Liv8-positive cells (Fig. 1D), in the fetal liver of AML1$^{-/-}$ embryos which do not undergo definitive hematopoiesis [24]. These results suggested that anti-Liv-8 recognizes hematopoietic progenitor cell in fetal liver.

Liv8-positive cells exist in adult bone marrow and express CD45

Next, we investigated Liv8-positive cells in the BMC of adult GFP Tg mice. Liv8-positive cells were found to be present among adult BMCs in adult bone marrow when analyzed in GFP-Tg mice. We found around 32%

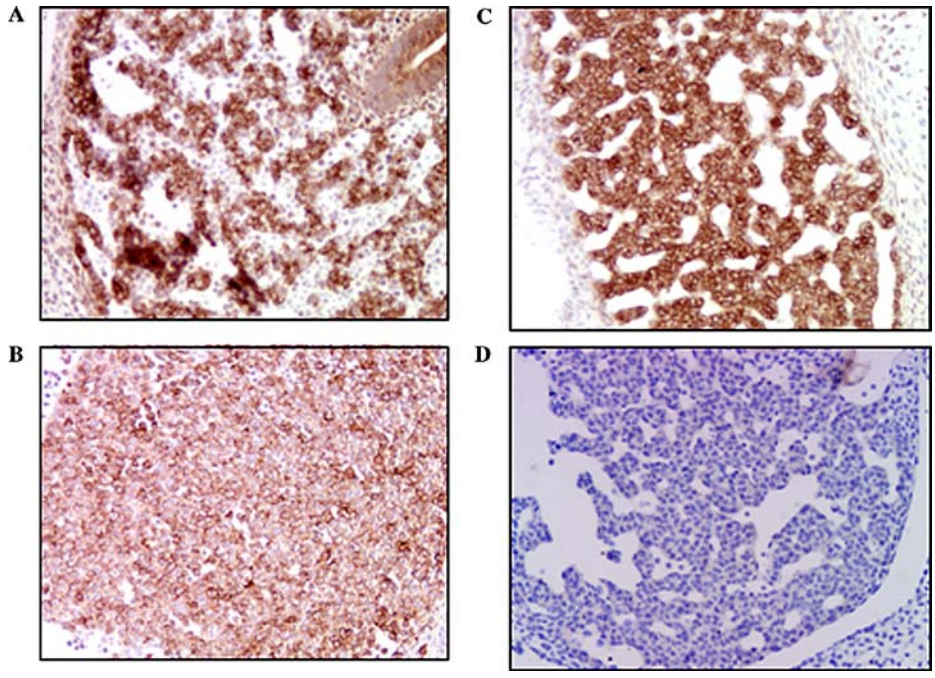

Fig. 1. (A–D) Liv2 and Liv8 expression at E 11.5 in normal and AML1$^{-/-}$ mice. Liv2 (A,C) and Liv8 (B,D) expression at E 11.5 in normal fetal liver (A,B) and AML1$^{-/-}$ mice (C,D). Magnification: (A–D) at 200×.

of Liv8-positive cells in adult GFP-Tg mice (Fig. 2A). We also analyzed the relationship between Liv8 and CD45, and found that 54% of Liv8-positive cells also expressed CD45 (Fig. 2B). These results showed that anti-Liv8 is useful to separate hematopoietic cell and non-hematopoietic cell.

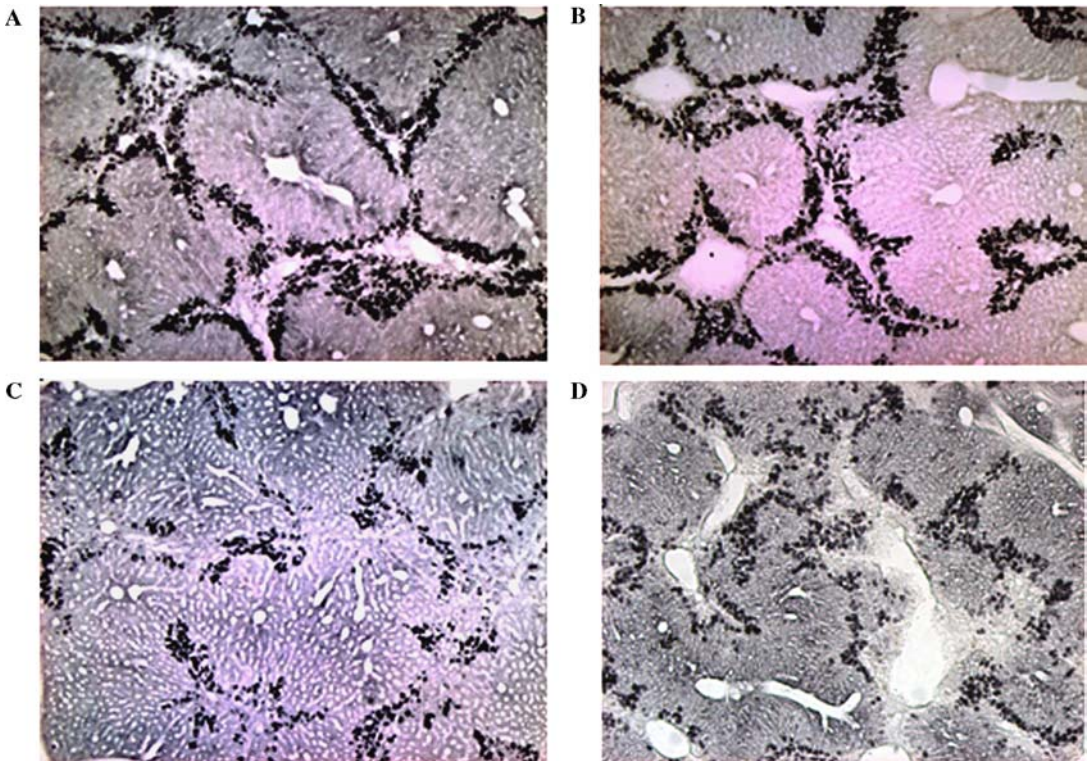

Fig. 3. (A–D) Expression of GFP in liver after transplantation of Liv8-positive and Liv8-negative cells. GFP expression in the liver after transplantation of Liv8-positive BMCs at 1 week (A) and 4 weeks (C), GFP expression at the liver after Liv8-negative BMC transplantation at 1 week (B) and 4 weeks (D) after cell injection. Magnification 200×.

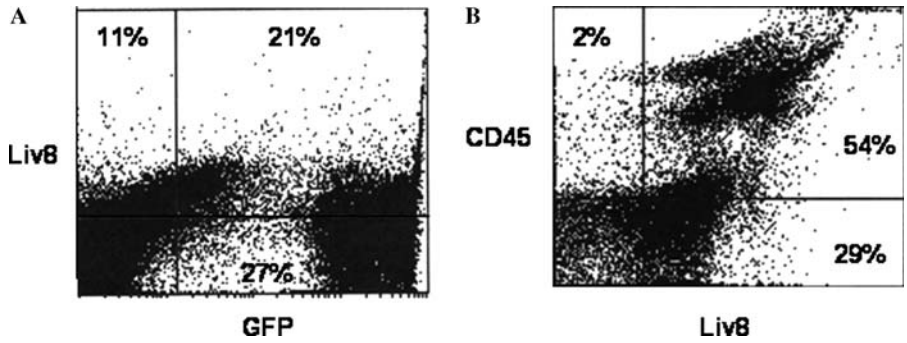

Fig. 2. Expression of CD45, Liv8 in bone marrow cell. FACS analysis of all BMCs of GFP-Tg mice. (A) Staining with Liv8 and GFP. (B) Staining with CD45 and Liv8.

Liv8-negative cells repopulated at the liver more than Liv8-positive cells

After separating Liv8-positive cells from Liv8-negative cells using AutoMACS, these cells were transplanted to recipient mice with CCl_4-induced liver cirrhosis. At one week after transplantation, both Liv8-positive (Fig. 3A) and Liv8-negative cells (Fig. 3B) colonized around the portal vein, with no marked differences in the rate of colonization (Table 1). In the Liv8-positive cell transplanted group, the number of GFP-positive cells in the liver increased transiently, but at four weeks after transplantation, the number of GFP-positive cells was significantly lower in the Liv8-positive cell group (Fig. 3C) than in the Liv8-negative cell group (Fig. 3D). Furthermore, GFP-positive cells were colonized inside the hepatic lobes in the Liv8-negative cell group at four weeks after transplantation. These results showed that Liv8-negative cell repopulated more than Liv8-positive cell.

The Liv8-negative cells transdifferentiate into hepatoblast phenotype

We showed in previous studies that transplanted BMCs transdifferentiate into Liv2-positive hepatoblasts and then further differentiate into hepatocytes [14,21]. In the present study, we also investigated the presence of cells expressing Liv2. Liv2-positive cells were identified by immunostaining, and the results showed that Liv2-positive cells were seen around the portal region one week after transplantation, but that there was no significant difference in the number of Liv2-positive cells between Liv8-positive and Liv8-negative cell groups (Figs. 4A and B, and Table 1). With time, the number of Liv2-positive cells in the liver decreased significantly for the Liv8-positive cell group (Figs. 4C and D and Table 1). The transdifferentiation of myelogenic GFP cells into Liv2 cells was investigated. Cells that expressed both Liv2 and GFP were detected at four weeks after transplantation, and fluorescent staining showed that the expression of Liv2 by myelogenic cells was higher for the Liv8-negative cell group (Figs. 4E and F). These results indicated that Liv8-negative cell could be transdifferentiated into hepatoblast phenotype.

Albumin expression in the liver and serum albumin level following transplantation of Liv8-positive and Liv8-negative BMCs

At one week after cell transplantation, there was no marked change in the expression of albumin for both Liv8-positive and Liv8-negative cell groups (Figs. 5A and B). However, at four weeks after transplantation, the expression of albumin decreased with time for the Liv8-positive cell group (Fig. 5C), but remained the same for the Liv8-negative cell group (Fig. 5D). Furthermore, at four weeks after cell transplantation, the number of yellow cells expressing both albumin and GFP was higher for the Liv8-negative cell group

Table 1
Percent of area for each differentiation marker after Liv8(+) and Liv8(−) cell transplantation under the persistent liver damage

		1 week ($n = 5$)	2 weeks ($n = 5$)	3 weeks ($n = 5$)	4 weeks ($n = 5$)
GFP	Liv8(+)	11.1 ± 1.7	15.1 ± 2.1	9.4 ± 0.8	$5.1 \pm 0.6^*$
	Liv8(−)	11.7 ± 1.0	13.2 ± 0.8	12.4 ± 2.6	$9.5 \pm 3.6^*$
Liv2	Liv8(+)	6.0 ± 1.1	7.3 ± 3.5	8.2 ± 1.8	3.3 ± 0.9
	Liv8(−)	5.5 ± 1.3	5.8 ± 0.8	9.2 ± 0.6	7.7 ± 0.9
Albumin	Liv8(+)	15.0 ± 1.9	14.9 ± 2.5	$6.8 \pm 2.6^*$	$3.7 \pm 1.4^*$
	Liv8(−)	12.7 ± 3.2	12.5 ± 3.2	$14.8 \pm 1.3^*$	$10.6 \pm 2.1^*$

Values shown are percent of the area occupied.
*showed significant differences at each sampling point ($n = 5$) at $p < 0.05$ between Liv8(+) and Liv8(−) cell transplantation groups.

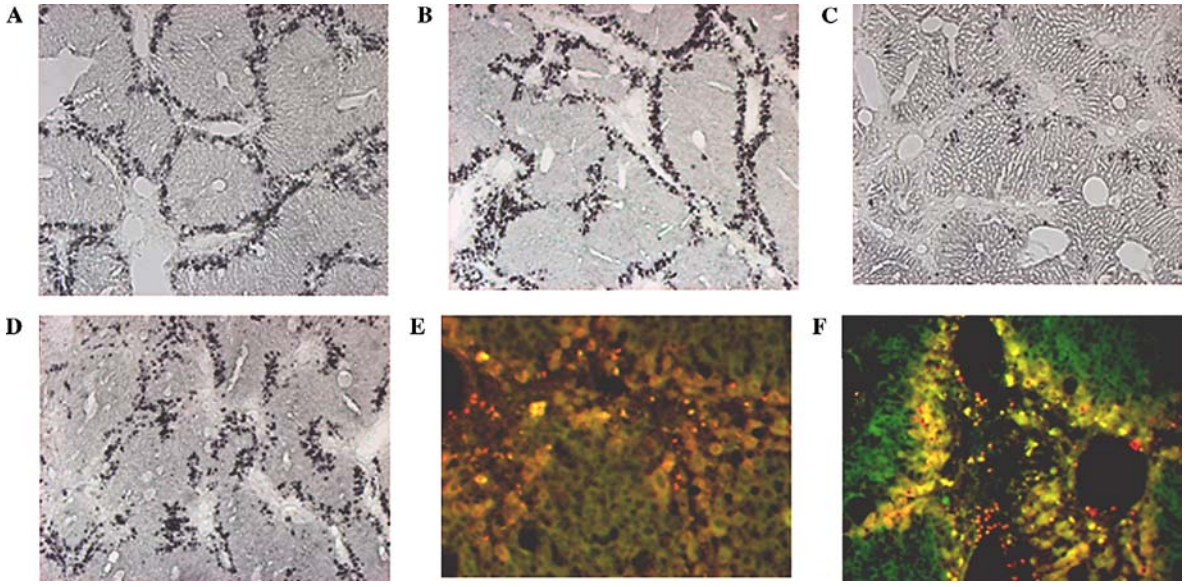

Fig. 4. (A–F) Expression of Liv2 antigen in liver after transplantation of Liv8-positive and Liv8-negative cells. Liv2 antigen expression at 1 week (A) and 4 weeks (C) after Liv8-positive BMC transplantation. Magnification at 200×. Liv2 antigen expression at liver at 1 week (B) and 4 weeks (D) after Liv8-negative BMC transplantation. Double fluorescent staining (red, Liv2; green, GFP; and yellow, Liv2 & GFP) of the liver at 4 weeks after Liv8-positive cell transplantation (E) and Liv8 negative cell transplantation (F) Magnification: (A–D) 200×, (E,F) 400×.

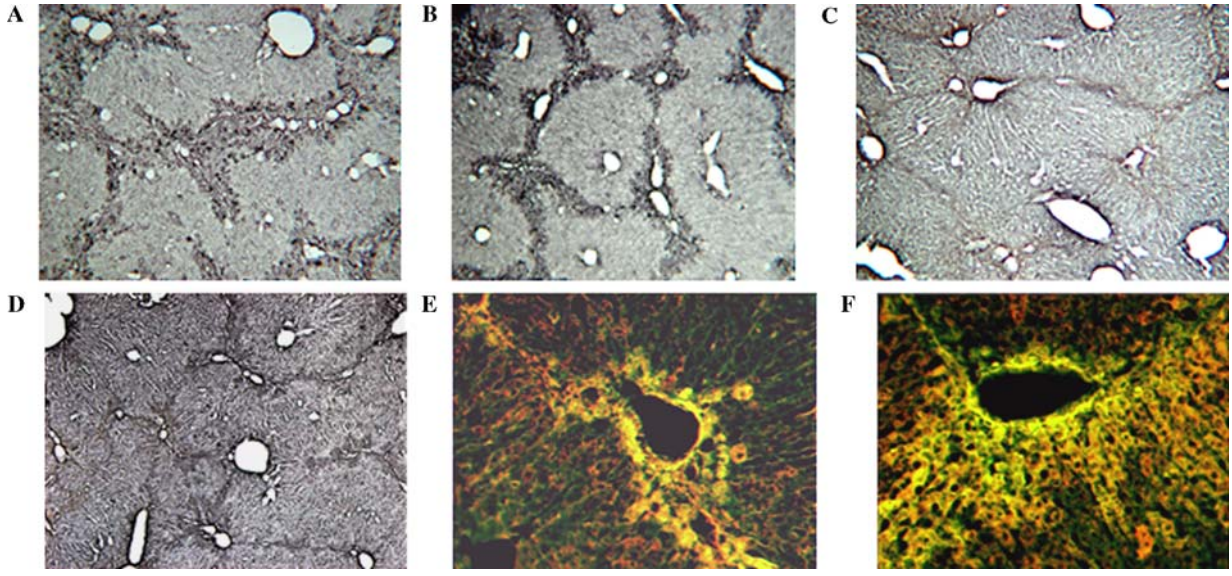

Fig. 5. (A–L) Expression Albumin after transplantation of Liv8-positive and Liv8-negative cells. Albumin expression at 1 week after transplantation of Liv8-positive cells (A) and Liv8-negative cells (B). Albumin expression at 4 weeks after transplantation of Liv8-positive cells (C) and Liv8-negative cells (D). Double fluorescent staining (red, albumin; green, GFP; and yellow, albumin & GFP) of liver at 4 weeks after transplantation of Liv8-positive cells (E) and Liv8-negative cells (F). Magnification: (A–D) 200×, (E,F) 400×.

(Figs. 5E and F). To ascertain whether transplanted cells were functioning as hepatocytes, serum albumin levels were measured. Serum albumin levels increased for both groups and were higher for the Liv8-negative cell group than the Liv8-positive cell group. The serum albumin levels at 4 weeks after Liv8-negative BMC transplantation showed the significantly higher levels for Liv8-negative cell group compared to the Liv8-positive BMC group ($n = 5$, $p < 0.05$) (Fig. 6). These results also showed that Liv8-negative cell could transdifferentiate into albumin-positive hepatocyte.

Discussion

The anti-Liv8 antibody is a useful antibody to separate hematopoietic cells and non-hematopoietic cells in adult bone marrow. We found Liv8-positive cells in fetal

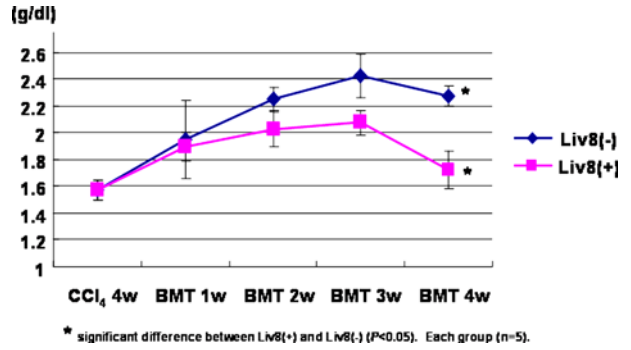

Fig. 6. The level of serum albumin. Serum albumin levels after Liv8-positive or Liv8-negative cell transplantation. CCl_4 4w, 4 weeks CCl_4 injection group. BMT 1w, 1 week after BMC transplantation. BMT 2w, 2 weeks after BMC transplantation. BMT 3w, 3 weeks after BMC transplantation. BMT 4w, 4 weeks after BMC transplantation. * showed significant differences at each sampling point ($n = 5$) at $p < 0.05$.

liver at E11.5, but could not detect no-positive cells in fetal liver of AML1 knockout mice (Fig. 1C) at E 11.5. This result suggested that anti-Liv8-positive cell might be associated with the generation of HSC. We used FACS analysis to understand more about the characterization of Liv8-positive cells in the bone marrow. Around 32% of all BMCs, which were positive for Liv8, also expressed CD45 (Figs. 2A and B). CD45 is the pantrophic marker for hematopoietic cell marker [26,27]. These results suggest that anti-Liv8 recognizes most hematopoietic cells. We separated BMCs into Liv8-positive cells and Liv8-negative cells using AutoMACS, and the repopulation and transdifferentiation of these cells into liver was analyzed in the GFP/CCl_4 model [14].

First we analyzed the colonization of transplanted Liv8-positive or negative cell. There was no change in the ratio of GFP-positive cells one week after transplantation between the Liv8-positive and Liv8-negative cell groups (Figs. 3A and B). In both groups, GFP-positive cells were found around the portal vein. The expression of GFP decreased with time for the Liv8-positive cell group (Fig. 3C), but in the Liv8-negative cell group, GFP-positive cells entered the hepatic lobes (Fig. 3D). At four weeks after transplantation, the rate of colonization for the Liv8-positive cell group was significantly lower than that for the Liv8-negative cell group (Table 1). Previously we found that colonization was not observed when BMCs were transplanted to normal recipients, but colonization was observed when BMCs were transplanted to recipients with liver cirrhosis caused by administration of CCl_4 [14]. Some previous studies also have reported that CCl_4 injection enhances the repopulation of hepatocytes following hepatocyte transplantation via the spleen [28,29]. It has been documented that elevated levels of SDF1 and matrix metalloprotease 9 (MMP9) might have an important role for the migration of BMCs to the liver at liver damage by CCl_4 administration [16,30]. In the GFP/CCl_4, the expression of MMP9 was also increased by the transplantation of BMCs (I. Sakaida, unpublished data). At 1 week after transplantation, there was no marked difference in colonization between the Liv8-positive and negative transplantation groups. These results suggest that the early migration of BMC into liver was determined by the recipient condition. Next we analyzed the transdifferentiation of BMC into functional hepatocyte in the "niche" where transdifferentiation of BMC into hepatocyte is favorable [14]. The results of our past analyses have shown that transplanted BMCs transdifferentiate into Liv2-positive hepatoblasts and then differentiate into hepatocytes only under continuous inflammation. The persistent liver damage made by injection of persistent CCl_4 injection is important for the transdifferentiation of BMC [14]. When human HSCs were transplanted to immunologically tolerant NOD/SCID mice and followed up with administration of CCl_4, it was found that transplanted human HSC was differentiated into albumin express hepatocyte-like cell [15]. Albumin/promoter-Alb-DsRed2 Tg rat was established to monitor the transdifferentiation into albumin positive cell. Albumin-producing DsReds cell was increased by repeated administration of CCl_4 [31]. A study reported recently that the transdifferentiation of BMCs was low when inducing liver damage by CCl_4 administration before or after transplantation [32]. Different results were obtained with these systems because chronic liver damage before and after transplantation was not evident. The persistent liver damage might be the key factor to induce the transdifferentiation of BMC into hepatocyte. We investigated the transdifferentiation of Liv8 positive and negative BMCs into hepatoblast and hepatocytes by Liv2 and albumin expression. Like GFP, Liv2-positive cells were seen around the portal vein one week after transplantation for both Liv8-positive and Liv8-negative cell groups, and there was no marked difference between the two groups (Figs. 4A and B). On the other hand, at four weeks after transplantation, the expression of Liv2 for the Liv8-positive cell group was significantly lower than that for the Liv8-negative cell group (Figs. 4C and D). The results of double staining at four weeks after transplantation also showed that the number of myelogenic Liv2-positive cells was greater for the Liv8-negative cell group (Figs. 4E and F). Figs. 5C and D show the expression of albumin four weeks after transplantation and the expression of albumin for the Liv8-negative cell group was higher (Fig. 5D). The expression of albumin and GFP in myelogenic cells was significantly higher for the Liv8-negative cell group (Fig. 5F). Furthermore, we investigated functional recovery by comparing improvement in hepatic failure between the Liv8-positive and Liv8-negative cell groups.

As shown in Fig. 6, when CCl_4 was administered in the same manner to the Liv8-positive and Liv8-negative cell groups, and the level of serum albumin increased in both groups, but a significant finding in this analysis was significant improvements in the serum albumin levels at four weeks after transplantation in the Liv8-negative cell group compared to the Liv8-positive cell group ($p < 0.05$). These findings support those of immunostaining. These results can be summarized that Liv8-negative cells are more likely to transdifferentiate into hepatocytes with time passed. The subpopulation which was deleted by anti-Liv8 will be useful cells to use cell therapy using BMC to repair damaged liver. The Liv8 negative cell was thought to be non-hematopoietic cells. For example, multi-potent adult progenitor cells (MAPCs) from BMCs differentiate into functional hepatocyte like cells [33,34]. Our results might support that mesenchymal cells may differentiate into pluripotent cells under certain conditions.

Still the precise mechanisms to regulate repopulation and transdifferentiation BMC into hepatocyte are uncertain. To develop a cell therapy using BMC to repair damaged liver, we are planning to further analyze these mechanisms.

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