

(Shizuoka, Japan). Mice were properly anesthetized during experiments.

Experimental Protocol. Six-week-old female C57BL6 mice were treated with 1 mL/kg CCl₄ dissolved in olive oil (1:1) twice a week for 4 weeks. One day (24 hours) after the eighth injection of CCl₄, 1 × 10⁵ green fluorescent protein (GFP)-positive BMCs or sorted Liv8-positive or Liv8-negative BMCs (1 × 10⁵ cells) or same volume of saline as a control (described also as mice treated with CCl₄ alone) were injected into the tail vein as described previously.^{11,12} Mice continued to be treated with CCl₄. After 1, 2, 3, or 4 weeks, mice were then sacrificed to assess the extent of liver fibrosis. For examination of the survival rate, mice were treated with CCl₄ for 4 weeks and divided into 2 groups (15 mice each) with bone marrow transplantation or the same volume of saline injection. All mice were then treated with CCl₄ for a further 25 weeks.

BMC Preparation. For BMC isolation, GFP-transgenic mice (TgN(β-act-EGFP)Osb) (6 weeks old) were killed by cervical dislocation and the limbs removed. GFP-positive BMCs were flushed with Dulbecco's Modified Eagle medium (DMEM) culture medium with 10% fetal bovine serum (FBS) from the medullary cavities of tibias and femurs using a 25-G needle.

Production of Rat Monoclonal Antibody, Liv8. Eight-week old WKY/NCrj female rats were immunized in the hind footpads with 100 μg of E11.5 murine fetal liver lysate in complete Freund's adjuvant (0.2 mL). Anti-Liv8 antibodies were raised according to a previously described protocol.¹³

Fluorescence-Activated Cell Sorter Analysis of Fetal Liver Cells and BMCs Using Anti-Liv8 Antibody. Prepared mouse fetal liver cells (E11.5) and adult BMCs were reacted with biotin-conjugated anti-Liv8 antibody,¹² phycoerythrin-conjugated rat anti-CD45 (Becton Dickinson Bioscience, San Jose, CA), fluorescein isothiocyanate-conjugated anti-c-kit (Becton Dickinson Bioscience), phycoerythrin-conjugated anti-Thy 1 (Becton Dickinson Bioscience), and fluorescein isothiocyanate-conjugated anti-B220 antibodies (Becton Dickinson Bioscience) at the rate of 1 μg per 10⁶ total cells, mixed well, and incubated in the tube for 30 to 40 minutes at 4°C. Following the incubation with the first antibody, the cells were washed twice by 0.02 mol/L phosphate-buffered saline (PBS) and centrifuged at 500g for 5 minutes. Labeled cells were then reacted to allophycocyanin-conjugated streptavidin (Becton Dickinson Bioscience) at the rate of 1 μg per 10⁶ total cells, mixed well, and incubated in the tube for 30 to 40 minutes at 4°C. After that, these were washed out once with 0.02 mol/L PBS and centrifuged at 500g for 5 minutes. The labeled cells were analyzed using FACS Calibur (Becton Dickinson Bioscience).

Preparation of Liv8-positive and Liv8-negative BMCs Liv8-positive and Liv8-negative BMCs were prepared as described previously.¹² Briefly, prepared BMCs were reacted to rat anti-Liv8 immunoglobulin G (IgG) antibody at the rate of 1 μg per 10⁶ total cells, mixed well, and incubated in the tube for 30 minutes at 4°C. Cells were then washed twice by 0.02 mol/L PBS and centrifuged at 500g for 5 minutes. Cells were labeled with rat anti-Liv8 IgG antibody by reacting with goat anti-rat IgG MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) at the rate of 20 μL per 10⁷ total cells, mixed well, and incubated for 20 minutes at 4°C. Labeled cells were washed once by 0.02 mol/L PBS and centrifuged at 500g for 5 minutes. These cells were separated into Liv8-positive cells or Liv8-negative cells by the autoMACS magnetic cell sorting system (Miltenyi Biotec GmbH) for 10 minutes per tube.

Tissue Preparation and Immunohistochemistry. The liver was perfused via the heart with 4% paraformaldehyde to flush out blood cells and incubated with 4% paraformaldehyde overnight. Tissues were then soaked in 30% sucrose for 3 days. Tissues were frozen with liquid nitrogen to prepare for sectioning with a cryostat for immunohistochemistry.

Cells expressing GFP and matrix metalloproteinase (MMP)-9 (or α-smooth muscle actin) were analyzed by both fluorescent microscopy and conventional immunohistochemistry using anti-GFP, anti-MMP-9 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-α-smooth muscle actin antibodies (Sigma-Aldrich, St. Louis, MO). Tissues were soaked in 0.3% Triton X-100 with 0.05% normal goat serum (NGS) (Chemicon, Temecula, CA) or normal rabbit serum (NRS) (Chemicon) in PBS overnight. The next day, the tissues were put in 500 mL of 10% NGS or NRS in 0.3% Triton X-100 of PBS for 2 hours, then washed with 0.3% Triton X-100 with 0.05% NGS or NRS in PBS for 10 minutes. We soaked the tissues in 1.5% H₂O₂ in 50% methanol with distilled water for 2 hours. The tissues were then washed in 0.3% Triton X-100 with 0.05% NGS or NRS in PBS. Sections were incubated with anti-GFP and anti-MMP-9 (ICN Pharmaceuticals Inc., Kanagawa, Japan) antibodies. Anti-biotin-conjugated anti-goat IgG, anti-rabbit IgG, biotin-conjugated rabbit anti-goat IgG, and biotin-conjugated rabbit anti-mouse IgG were purchased from Dako Japan (Kyoto, Japan) and used as the secondary antibodies. PAP-goat (B0157), PAP-mouse (B0650), and PAP-rabbit (Z0113) polyclonal antibodies (Dako Japan) were used as third antibodies.

For fluorescent immunohistochemistry, we used Alexa Fluor R 488 and 568 donkey anti-goat- or anti-rabbit-

or anti-mouse-IgG (H + L)-conjugated antibodies (Molecular Probe Inc., Eugene, OR) as second antibodies.

For the evaluation of fibrosis, picro-sirius red staining was performed using 0.1% picro-sirius red solution as previously described.¹⁴

Quantitative Analysis of Liver Fibrosis. We quantified the liver fibrosis area with picro-sirius red staining using an Olympus Provis microscope equipped with a CCD camera (Tokyo, Japan), as described previously.¹⁵ Briefly, the red area, considered the fibrotic area, was assessed by computer-assisted image analysis with MetaMorph software (Universal Imaging Corporation, Downingtown, PA) at a magnification of $\times 40$. The mean value of 6 randomly selected areas per sample was used as the expressed percent area of fibrosis.

Microarray Analysis. Microarray analysis was performed as described previously.¹⁶

Briefly, total RNA of liver was isolated using the Atlas Pure Total RNA labeling system (Clontech Laboratories, Inc.) from mice 1 week after BMC transplantation ($n = 3$) or from mice treated with CCl_4 for 5 weeks ($n = 3$) according to the manufacturer's recommendations. Differential hybridization analysis was done using an Atlas Mouse complementary DNA expression array (BD Bioscience Clontech, Tokyo, Japan). Complementary DNA probe preparation and hybridization were done according to the manufacturer's recommendations. The array results were scanned with a Strom 840 PhosphoImager (Molecular Dynamics, Sunnyvale, CA) and analyzed with Atlas Image software (BD Bioscience Clontech). The results show the mean values of 3 mice in each group.

Hydroxyproline Content. Hydroxyproline content was determined by a modification of Kivirikko's method, as previously reported.¹⁷ Briefly, liver specimens were weighed, and 20 mg of the freeze-dried sample was hydrolyzed in 6 mol/L HCl at 110°C in an autoclave at a pressure of 1.2 kg force/cm² for 24 hours. After centrifugation at 2,000 rpm at a temperature of 4°C for 5 minutes, 2 mL of supernatant was mixed with 50 mL of 1% phenolphthalein and 8 N KOH to obtain a total volume of 5 mL of liquid at pH of 7 to 8. Absorbance was measured at 560 nm. The hydroxyproline content of the liver was expressed as micrograms per gram of wet weight.

In Situ Hybridization. *In situ* hybridization was performed essentially as described previously.¹⁸ Briefly, digoxigenin (DIG)-11-UTP-labeled single-stranded RNA probes were prepared with DIG RNA labeling mix and the corresponding T3 or T7 RNA polymerase (Boehringer Mannheim Japan, Tokyo, Japan) according to the manufacturer's instructions. The mouse MMP-9 probe was a 150-base pair fragment from the 3' untranslated region cloned in the pBluescript (Stratagene, Tokyo,

Japan) vector. *In situ* hybridization was performed on tissue sections placed on Superfrost Plus slides postfixed in 4% paraformaldehyde in PBS, rinsed in PBS containing 0.1% active diethyl pyrocarbonate, and prehybridized for 2 hours at 58°C in 50% formamide, $5 \times \text{SSC}$ (standard saline citrate), and 40 μg of salmon-sperm DNA per milliliter. Hybridization was carried out at 58°C for 16 hours in a humid chamber with 400 ng of DIG-labeled probe per milliliter diluted in the same solution used for prehybridization. After hybridization, the sections were successively washed in $2 \times \text{SSC}$ at room temperature for 30 minutes, $2 \times \text{SSC}$ for 1 hour at 65°C , and $0.1 \times \text{SSC}$ at 65°C for 1 hour. For the reaction of anti-DIG antibodies, slides were preincubated in buffer A (100 mmol/L Tris, 150 mmol/L NaCl [pH 7.5]), and then with an alkaline phosphatase-coupled anti-DIG antibody (Boehringer Mannheim Japan) diluted 1:5,000 in buffer A containing 0.5% Boehringer blocking reagent for 2 hours at room temperature. The slides were washed in buffer A and then preincubated in buffer C (100 mmol/L Tris, 50 mmol/L MgCl_2 [pH 9.5]). Alkaline phosphatase was then revealed as described for 16 to 24 hours at room temperature. The enzymatic reaction was stopped with Tris-ethylenediaminetetraacetic acid (EDTA) for 15 minutes. The slides were rinsed in water for several hours and then dried, cleared in xylene, and mounted directly.

In Situ Zymography. *In situ* zymography was performed as described.¹⁹

The fresh specimens of CCl_4 treated with BMC-transplanted liver tissues (1 week after BMC transplantation) were embedded without fixation in Tissue-Tek optimal cutting temperature compound (Miles, Elkhart, IN). Serial frozen sections were made using a cryostat (MicroM, Walldorf, Germany) and mounted on gelatin films that were coated with 7% gelatin solution (Fuji Photo Film, Tokyo, Japan). The films with sections were incubated for 24 hours at 37°C in a moisture chamber and stained with 1.0% amido black 10B. The gelatin in contact with the proteolytic areas of the sections was digested, and thus zones of enzymic activity were indicated by negative staining. The digested areas in the sections were compared with serial sections stained with hematoxylin-eosin. As a control, liver tissues treated with CCl_4 alone (5 weeks) were used, and the frozen sections were treated in a manner similar to that already described.

Statistical Analysis. Results are presented as the mean \pm SD. Differences between groups were analyzed by 1-way ANOVA.

The survival rate was examined using the Breslow-Gehan-Wilcoxon test.

Ethical Considerations. This experiment was reviewed by the Committee of Animal Experiment Ethics at

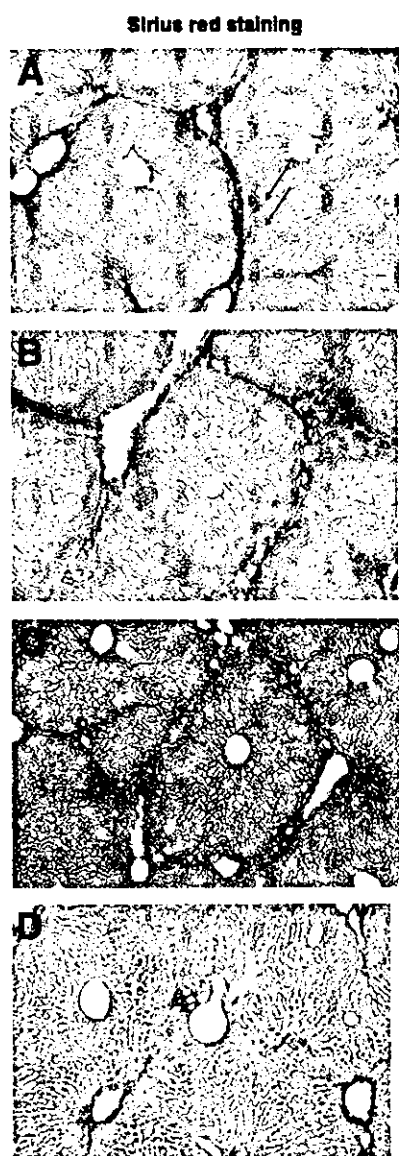


Fig. 1. Photomicrographs of liver sections stained with sirius red from mice after BMC transplantation with continuous CCl_4 treatment. Six-week-old C57BL6 mice were treated with CCl_4 twice a week for 4 weeks. Then, 1×10^5 GFP-positive BMCs were injected through the tail vein. Mice continued to be treated with CCl_4 . After (A) 1, (B) 2, (C) 3, and (D) 4 weeks, mice were killed to assess the extent of liver fibrosis. (Original magnification, $\times 100$.)

the Yamaguchi University School of Medicine and was carried out under the guidelines for animal experiments at Yamaguchi University School of Medicine (no. 105).

Results

Five weeks after CCl_4 injection, liver fibrosis was already seen (Supplementary Fig. 1A). One week after BMC transplantation (5 weeks after CCl_4 injection), BMCs were seen along with the fibers recognized by light red staining (black arrows), different from hepa-

toocytes (Fig. 1A) with sirius red staining. More BMCs were seen after 2 weeks (Figs. 1B and 2A,C) and 3 weeks (Figs. 1C and 2B,D), and large spheroid-shaped cells (blue arrows) and small cells (green arrows) (Fig. 2B) were found in the area presumably occupied by fibers (Fig. 2D), shown by sirius red and GFP staining.

Surprisingly, 4 weeks later, the BMC-transplanted liver clearly showed reduction of liver fibrosis (Fig. 1D) compared with the liver treated with CCl_4 alone at 8 weeks (Supplementary Fig. 1D), although CCl_4 was injected throughout the experimental period. Quantitative image analysis of liver fibrosis indicated that the percent area of liver fibrosis at 1 week after BMC transplantation was $5.36\% \pm 0.90\%$, but at 4 weeks after transplantation it was significantly decreased to $4.16\% \pm 0.53\%$ ($P < .001$, $n = 8$ each; Fig. 3).

Treatment with CCl_4 alone for 8 weeks showed an increased hydroxyproline content of $630 \pm 93 \mu\text{g/wet g liver}$ (Table 1). BMC transplantation significantly reduced this to $392 \pm 59 \mu\text{g/wet g liver}$ 4 weeks later ($P < .01$, $n = 8$ each). This hydroxyproline content was significantly reduced even compared with that of 1 week after BMC transplantation ($494 \pm 74 \mu\text{g/wet g liver}$, $P < .05$).

The mouse fetal liver at E11.5 functions as a definitive hematopoietic organ, and Liv8-positive cells of the fetal liver at E11.5 include c-kit-positive immature hematopoietic cells and CD45-positive lymphoid cells. These results indicate that almost all Liv8-positive cells are of hematopoietic origin (Supplementary Fig. 2).

In addition, all c-kit-positive mouse adult BMCs belong to the Liv8-positive fraction, and Liv8-positive BMCs include almost all of the CD45- and Thy-1-positive BMCs, in addition to B220, a marker of B lymphocytes (Fig. 4).

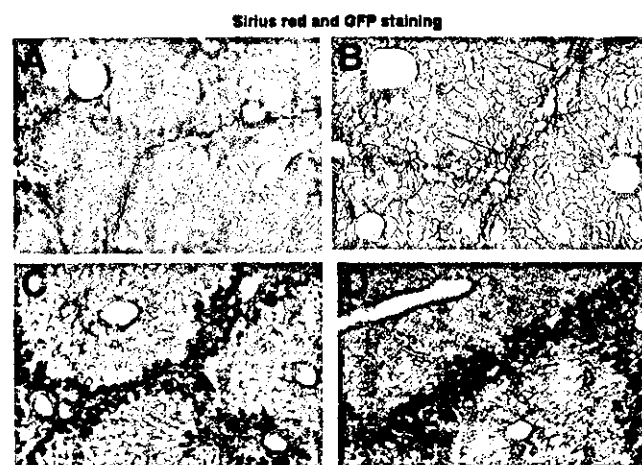


Fig. 2. Photomicrographs of liver sections stained with sirius red or sirius red and GFP from a mouse (A, C) 2 weeks and (B, D) 3 weeks after BMC transplantation. (Original magnification, [A] $\times 200$; [B] $\times 200$; [C] $\times 200$; [D] $\times 200$.)

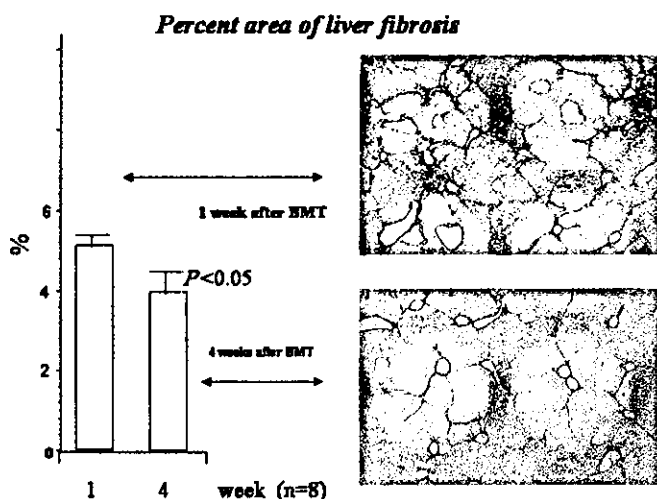


Fig. 3. Quantitative analysis of liver fibrosis after bone marrow cell transplantation (BMT). Percent area of liver fibrosis was calculated using sirius red staining as described in Materials and Methods. Results are expressed as mean \pm SD of 8 samples. (Original magnification, $\times 40$.)

These results strongly suggest that Liv8-positive cells include both immature and mature hematopoietic cells.

Liv8-negative BMCs significantly reduced liver fibrosis compared with that of the liver treated with CCl₄ alone for 8 weeks, although Liv8-positive BMCs had no effect on liver fibrosis (Table 2).

Microarray analysis of the liver 1 week after BMC transplantation indicated increased expression of MMP-2, MMP-9 and MMP-14 with decreased expression of tissue inhibitor of metalloproteinase-3 (TIMP-3) compared with that of the liver treated with CCl₄ alone for 5 weeks (Table 3). Because the expression of MMP-9 was marked, we investigated it in this model.

Immunohistochemistry of MMP-9 showed localization of these cells similar to that of transplanted BMCs (Fig. 5A). However, the liver treated with CCl₄ alone showed only a few MMP-9-positive nonparenchymal cells (black arrows, Fig. 5B).

The expression of MMP-9 with *in situ* hybridization coincided with the immunohistochemical staining of MMP-9 (Fig. 5C).

Table 1. Hydroxyproline Content

Treatment (No. of Mice)	Hydroxyproline (μ g/g Liver)
CCl ₄ , 5 wk (8)	464 \pm 93
CCl ₄ , 8 wk (8)	630 \pm 93
CCl ₄ /BMT, 5 wk (8)	494 \pm 74
CCl ₄ /BMT, 8 wk (8)	392 \pm 59*†

NOTE. Mice were treated with CCl₄ for 4 weeks; then, 1 week or 4 weeks after bone marrow cell transplantation (BMT) or saline injection with CCl₄ treatment, they were killed to measure liver hydroxyproline content. Results are typical of 1 of 3 independent experiments.

*P < .01 vs. CCl₄ (8 weeks).

†P < .05 vs. CCl₄/BMT (5 weeks).

FACS Analysis of adult bone marrow cells

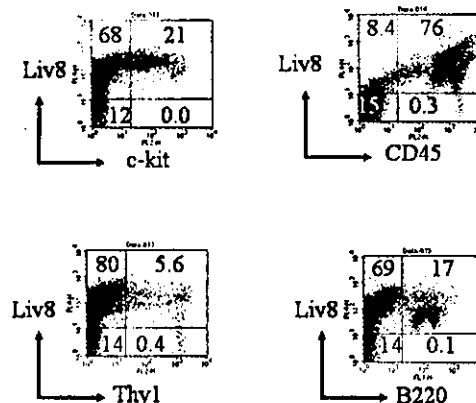


Fig. 4. Expression of Liv8, c-kit, CD45, Thy 1, and B220 in adult BMCs.

Although double fluorescent-positive cells were not seen in the liver treated with CCl₄ alone (Fig. 6A), double-positive yellow-colored cells (black arrows) were seen in the BMC-transplanted liver (Fig. 6B). With high magnification, double fluorescent immunohistochemistry showed the expression of MMP-9 (red) on the GFP-positive (green) cell surface (Fig. 6C).

A double fluorescent (anti-GFP with green color and anti- α -smooth muscle actin with red color) study indicated that a fine network pattern of stellate cells (red) existed in the liver treated with CCl₄ alone for 5 weeks (Fig. 7A). Conversely, GFP-positive green-colored cells (green arrow) were seen with a reduced fine network pattern (red arrow) in the liver 1 week after BMC transplantation (Fig. 7B). Double fluorescent-positive yellow-colored cells (yellow arrow), presumably stellate cells, were then seen without the fine network pattern (red arrow) in the liver 2 weeks after BMC transplantation (Fig. 7C). The shape of these cells was different from other GFP-positive cells, and the number of the double-positive cells was very small.

Next, we examined the direct activity of MMP-9 using *in situ* zymography. Film *in situ* zymographic analysis revealed strong gelatinolytic activity in the periportal area

Table 2. Hydroxyproline Content

Treatment (No. of mice)	Hydroxyproline (μ g/g Liver)
CCl ₄ (8)	687 \pm 102
CCl ₄ /Liv8-positive (8)	638 \pm 94
CCl ₄ /Liv8-negative (8)	415 \pm 77*

NOTE. Mice were treated with CCl₄ for 4 weeks followed by transplantation with Liv8-positive or Liv8-negative BMCs or saline. After 4 weeks of CCl₄ treatment (total 8 weeks), mice were killed to measure liver hydroxyproline content. Results are typical of 1 of 3 independent experiments.

*P < .01 vs. CCl₄.

Table 3. Microarray Analysis

	CCl ₄ + BMC vs. CCl ₄ Alone	
	Increased	Decreased
MMP-2	1.7	
MMP-9	3.9	
MMP-14	2.1	
TIMP-3		0.67

NOTE. Mice were treated with CCl₄ for 4 weeks and were killed 1 week after BMC transplantation. Microarray analysis was performed using 3 livers from each group. The expression ratios (CCl₄ + BMC vs. CCl₄ alone) larger than 1.5 are shown. Values indicated are the difference between the mean values of 3 mice. Results are typical of 1 of 2 independent experiments.

coinciding with the location of MMP-9-positive BMCs compared with the liver treated with CCl₄ alone (Fig. 8).

This gelatinolytic activity was completely blocked by the addition of 1,10-phenanthroline, an MMP inhibitor (data not shown).

Finally, the mice that underwent BMC transplantation with continuous CCl₄ injection showed a gradually increased serum albumin level (Supplementary Fig. 3) resulting in a significantly improved survival rate after BMC transplantation compared with mice treated with CCl₄ alone (Supplementary Fig. 4).

Discussion

In this report, transplanted BMCs can degrade collagen fibers and clearly reduce liver fibrosis with strong expression of MMPs, especially MMP-9, as indicated by both *in situ* zymography and the double staining of GFP and MMP-9 using fluorescent microscopy. The reason for the strong expression of MMP-9 is still unknown.

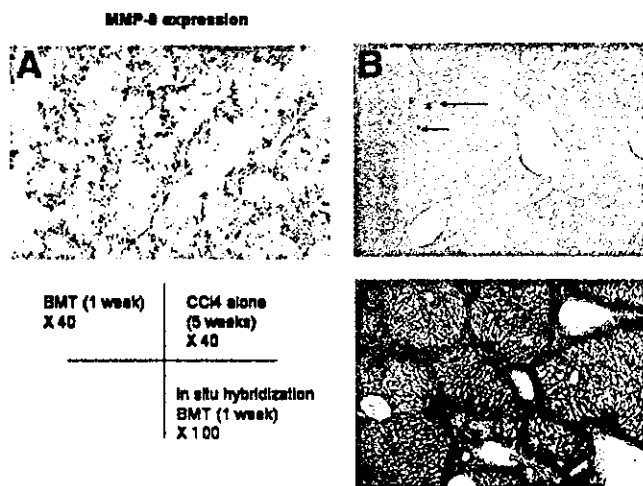


Fig. 5. Photomicrograph of a liver section stained with anti-MMP-9 antibody (A) from a mouse 1 week after BMC transplantation (BMT) and (B) from a mouse treated with CCl₄ alone for 5 weeks. (Original magnification, ×40.) (C) *In situ* hybridization of a liver section from a mouse 1 week after BMC transplantation. (Original magnification, ×100.)

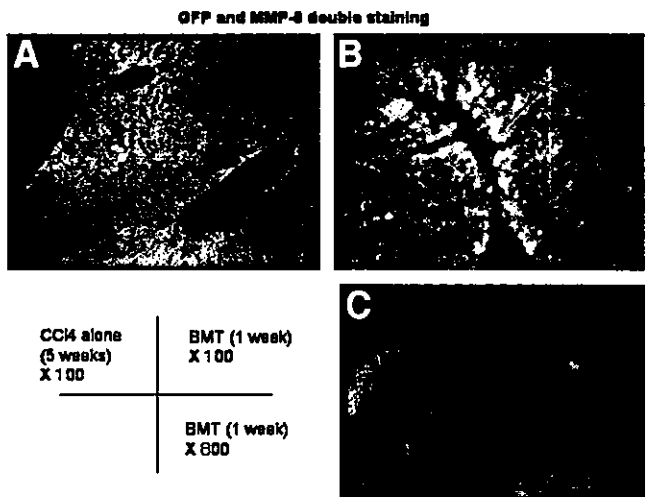


Fig. 6. Double fluorescent immunohistochemistry of a mouse liver after (A) 5-week treatment with CCl₄ alone and (B, C) 1 week after BMC transplantation (BMT) with CCl₄ treatment for 5 weeks. (Original magnification, [A and B] ×100; [C] ×800.)

However, Heissing et al.^{20,21} recently reported that MMP-9 induced in BMCs released soluble Kit-ligand, which might be related to the transfer of stem cells in BMCs to the proliferative niche. Therefore, MMP-9 in our model could play an important role in the degradation of extracellular matrix and also by releasing some factors, *e.g.*, soluble Kit-ligand, related to the differentiation and proliferation of transplanted BMCs in liver inflammation induced by continuous injection of CCl₄. It has also been shown that MMP-9 plays an important role in the migration of mast progenitor cells to inflammatory

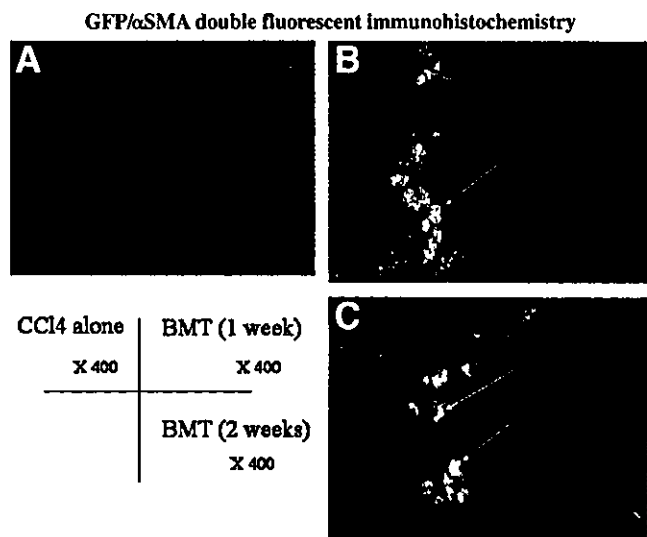


Fig. 7. Double fluorescent immunohistochemistry for α-smooth muscle actin (αSMA) and GFP of a mouse liver (A) treated with CCl₄ alone for 5 weeks and (B) 1 week, or (C) 2 weeks after BMC transplantation (BMT) with CCl₄ treatment. (Original magnification, ×400.)

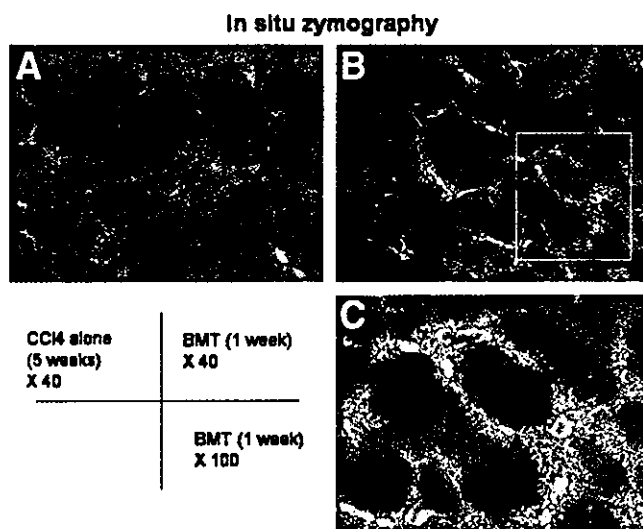


Fig. 8. *In situ* zymography of a mouse liver after (A) 5-week treatment with CCl₄ alone and (B, C) 1 week after BMC transplantation (BMT) with CCl₄ treatment for 5 weeks. (Original magnification, [A] \times 40; [B] \times 40, [C] \times 100.)

tissue.^{22,23} Therefore, the increased expression of MMP-9 in this study was somehow related to the migration of BMCs to the inflammatory liver.

Film *in situ* zymography clearly showed that these MMP-9-positive cells possessed high gelatinolytic activity compared with the liver treated with CCl₄ alone. Thus, the BMCs that migrated acted in the degradation of liver fibrosis (fibrolysis).

According to our present data, increased expression of MMP-14 (MT1-MMP [membrane-type 1 matrix metalloproteinase]) will contribute to degrading interstitial collagens²⁴ to gelatin that MMP-9 can degrade, resulting in the regression of fibrosis (fibrolysis).

Recently, Kollet et al.²⁵ reported that the expression of MMP-9 was increased with the migration of human CD34⁺ progenitor cells in CCl₄-treated NOD/SCID mice and that an inhibitor of MMP-9 reduced this migration. Thus, proteolytic activity seems to be necessary for the cell migration in addition to matrix degradation activity.

It seems to be very important how many cells can migrate into the damaged liver to degrade fibers, but a recent paper²⁶ reported little evidence of bone marrow-derived hepatocytes in the CCl₄-treated liver. However, the dose of CCl₄ was only 4% (0.08 mL/kg) of our dose (0.5 mL/kg), and the number of mice used was too small (1 or 2). The reason they did not see the BMCs that migrated is most likely due to the cessation of CCl₄ injection after BMC transplantation. Even in our experimental model,¹¹ the cessation of CCl₄ after BMC transplantation dramatically reduced the number of BMCs migrating into the

damaged liver (I.S., unpublished data, 2003). Thus, the extent of continuing liver damage may limit BMC migration to the liver with matrix degradation activity.

Transplanted BMCs differentiated into albumin-producing hepatocytes with an increased serum albumin level, and the degradation of the extracellular matrix may presumably lead to improved liver function resulting in better survival of mice with BMC transplantation compared to that of treated with CCl₄ alone, although only 1 transplantation of BMCs was performed.

As shown by double fluorescence, our data may also indicate that transplanted BMCs seem to become stellate cells, in agreement with a recent report,²⁷ although the number was very small in our experimental model. This result seems to be contradictory to our result of resolution of liver fibrosis by BMC transplantation because transdifferentiated stellate cells may produce collagens. Our preliminary results indicated reduced messenger RNA expression of type I procollagen, transforming growth factor- β 1 (TGF- β 1) and no change of hepatocyte growth factor messenger RNA expression in the liver 1 week after BMC transplantation compared with the liver treated with CCl₄ alone (I. Sakaida, unpublished data, 2003). As shown in Fig. 7, migrated BMCs seemed to reduce the fine network pattern of activated stellate cells. Thus, transplanted BMCs may affect activated stellate cells by reducing their number—*e.g.*, by leading them to apoptosis. However, further examinations are necessary to determine the exact relationship between BMCs and resident stellate cells.

Our recent data¹² indicated that the subpopulation of BMCs, nonhematopoietic cells in bone marrow, separated using an anti-Liv8 antibody, will transdifferentiate into hepatocytes in the liver damaged by CCl₄ induction. The present study clearly indicates that this subpopulation of BMCs is also responsible for the resolution of liver fibrosis (fibrolysis) induced by CCl₄ treatment.

In conclusion, the present study introduces a new concept for the treatment of liver fibrosis.

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Molecular signature associated with plasticity of bone marrow cell under persistent liver damage by self-organizing-map-based gene expression[☆]

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Abstract The mechanism that regulates the plasticity of bone marrow cells (BMCs) into hepatocytes is poorly understood. We developed a green fluorescent protein/carbon tetrachloride model to find that BMC transplantation recovered liver damage. Serum albumin level and liver fibrosis were recovered by BMC transplantation. To understand the mechanism, we used DNA-chip technology to profile the change of transient gene expression before and after BMC transplantation. On the basis of gene expression with self-organizing map using specific equation, genes were classified into 153 clusters. The information is useful to understand the dramatic gene activation during the process of the plasticity of BMC.

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Keywords: Bone marrow cell; Plasticity; Regenerative Medicine; Gene expression; Microarray analysis; Self-organizing map; Liver regeneration

1. Introduction

Recently, several groups have reported the possible plasticity of bone marrow cells (BMC) to differentiate into a variety of non-hematopoietic cell lineages [1,2]. Ever since, the differentiation of BMC into hepatocytes in human was documented following a bone marrow transplantation from a man to a woman [3]. The mechanism of the plasticity of BMC was discussed whether that was occurred with cell fusion, nuclear reprogramming [4–6] or trans-differentiation [7,8]. We think both cell fusion and trans-differentiation might be important to understand the mechanism of BMC plasticity. On the other hand, in cardiovascular medicine, clinical research has been conducted to evaluate the use of BMCs in regenerating the myocardium and vessels, and some positive results have been obtained [9,10]. These findings suggest the usefulness of BMCs as the source of cells in developing the next-generation of treatment for liver regeneration [11]. We first tried to understand how we could use BMC to repair damaged liver. We have developed a model [named as a green fluorescent protein/carbon tetrachloride (GFP/CCl₄)] to evaluate the usefulness of BMC transplantation for damaged liver [12,13]. In this model, 0.5 ml/kg of carbon tetrachloride (CCl₄) is administered twice weekly to induce liver cirrhosis and then GFP-positive BMCs are transplanted through the causal vein [14]. Under continuous liver injury, immunostaining using anti-GFP antibodies [15] showed that GFP-positive BMCs migrated into the marginal area of the hepatic lobule starting from day 1 after BMC transplantation, and with time, while forming a hepatic cord towards the central vein, the distribution of GFP-positive BMCs expands [12,16]. Also, using Liv2, a hepatoblast-specific antibody that we developed [17], it has been shown that BMCs first trans-differentiate into Liv2-positive hepatoblasts and then differentiated into albumin-positive hepatocytes. Furthermore, the level of serum albumin significantly increases with time in recipient mice. Liver fibrosis induced by CCl₄ injection was recovered by BMC transplantation [18]. These findings suggest that this GFP/CCl₄ model can be used

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Abbreviations: BMC, bone marrow cell; SOM, self-organizing map; CCl₄, carbon tetrachloride; EGFP, enhanced GFP; GFP, green fluorescent protein; RT, reverse transcriptase; HNF4- α , hepatocyte nuclear factor 4 alpha; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; FAH, fumarylacetoacetate hydrolase; TNFR, tumor necrosis factor receptor; FGF, fibroblast growth factor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; NumbL, Numblike; HOX, homeobox; GPI, glucose-6-phosphatase isomerase

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to understand the process of plasticity of BMCs under persistent liver damage condition. It is important to understand what had happened in GFP/CCl₄ model after BMC transplantation in mRNA level. DNA chips are recently developed tools used in genetic analyses [19]. While it is possible to obtain genetic data using DNA chips, the vast amount of information collected makes it difficult to precisely interpret the factors involved in the gene expression. Therefore, in the present study, patterns of global gene expression at different

times were compared between mice with BMC transplantation and those without. Self-organizing map (SOM) is a statistical technique that has been recently used in analyzing microarray data and, via this method, it is possible to visualize a vast amount of complicated and multidimensional data [20]. In this analysis, we made a specific equation to extract genes with expressions that altered in relation to BMC transplantation. Here, we present the results obtained from this study.

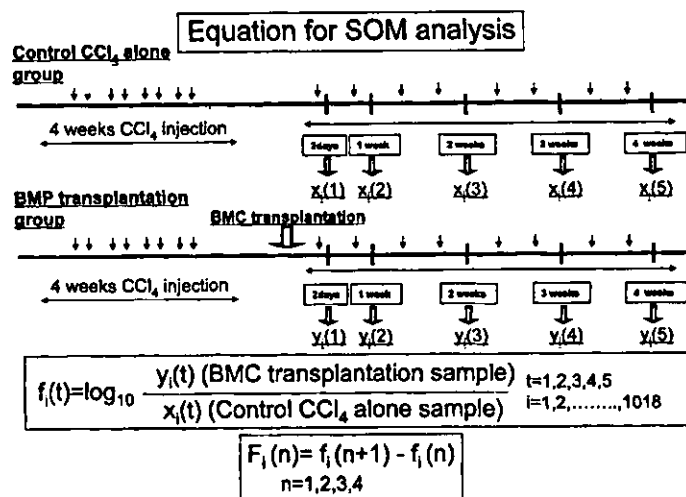


Fig. 1. Defined equation in this analysis. (1) Arrow indicates CCl₄ injection twice a week in GFP/CCl₄ model. We analyzed gene expression in each time point (2 days, 1 week, 2 weeks, 3 weeks, and 4 weeks). $y_i(t)$ showed the gene expression level of the liver after BMC transplantation. $x_i(t)$ showed the gene expression level of the liver CCl₄ alone injection group. We define $f_i(t)$: $f_i(t) = \log_{10} y_i(t)/x_i(t)$. By using this, we succeeded in extracting the change of gene expression by BMC transplantation. We are interested in following the change of the values of $F_i(t) = f_i(t+1) - f_i(t)$ with the increase in t .

Table 1
Lists of primers for selected 13 genes

Cluster	Gene name (Accession No.)	Primer – forward (5'–3') Primer – reverse (5'–3')	Target	Tm
86	c-kit (NM02099)	TCCAACGATGTGGGCAAGAG AATGAGCAGCGGCGTGAA	90	55
86	FGP6 (M92415)	CATGGTCTATACCGGCCACA GGCTGTGACATGAAACCAAAG	88	63
125	MMP2 (NM008610)	CCCTGATGCCAGCAAGTAGATG ATTCCAGGAGTCTGCGATGAG	148	62
125	MMP9 (NM013599)	ACGACATAGACGGCATCCAGTA TCGGCTGTGGTTTCAGTTGTG	90	53
92	TIMP2 (NM011594)	ACACGCTTAGCATCACCAGCA TGTGACCCAGTCCATCCAGAG	137	63
13	HGF (NM010427)	CCCAAACATCCGAGTTGGCTAC TTCCCATTGCCACGATAACA	84	63
1	NumbL (NM010950)	TATGCAGCCTCCGTTTGTG GCGTTGGCTACCATCTGTGAA	102	62
1	HOXD3 (NM010468)	CCATAAATCAGCCGCAAGGA GGATGGGTCGAGGACTTACCCTAG	112	63
152	GPI (NM008155)	TGGACGGCAAAGATGTGATG CGATGTTGATGATGTCCTGGA	129	63
152	VEGF (NM009505)	ATGCGGATCAAACCTCACCA CCGCTCTGAACAAGGCTCAC	129	63
136	TNFR1 (NM011609)	CTGCTCTACGAATCACTCTGCTC ACAGCATACAGAATCGAAGGTC	113	62
151	HNF4 (NM008261)	CCAAGTACATCCGGCCCTTC CTAGGAGCAGCAGTCTTAAAC	132	62
151	FGF2 (NM008006)	GGCTGCTGGCTTCTAAGTGTG ACTGCCAGTTCGTTTCAGTG	129	62

2. Materials and methods

2.1. Experimental protocol (GFP/CCL₄ model)

We developed a new *in vivo* model in which we could monitor the plasticity of BMCs into hepatocytes [12,16]. The mouse line C57BL/6 Tg14 (act-EGFP) OsbY01 was a kind gift from Dr. Masaru Okabe (Genome Research Center, Osaka University, Osaka, Japan) [14]. C57BL/6 female mice were purchased from Japan SLC (Shizuoka, Japan). We injected 0.5 ml/kg body weight of CCL₄ into C57BL/6 mice at 6 weeks of age via the peritoneum twice a week for 4 weeks to induce persistent liver damage. At this time, the condition of recipient mice was liver cirrhosis. One day after 4 weeks of CCL₄ injection, 1×10^5 GFP-positive BMCs were injected slowly using a 31 G needle and Hamilton syringe via the tail vein. The mice that were injected with CCL₄ only were used as the control group. After BMC transplantation, the same dose of CCL₄ was injected twice a week. Individual mice were killed at 18 h after initial CCL₄ injection (2 days after BMC transplantation) and once a week after BMC transplantation for 4 weeks. All processes including surgical steps confirmed to the guidance of Yamaguchi University for animal and recombinant DNA experiments.

2.2. RNA preparation and microarray analysis

In both the BMC transplantation and control groups, the liver was excised 2 days and 1, 2, 3, and 4 weeks after transplantation. The mice were killed by cervical dislocation. The whole liver was removed and immediately frozen in liquid nitrogen. Liver samples were pooled at least two from whole liver of both mice groups (BMC transplantation and control CCL₄ damage at each point). Total RNA was isolated from pooled liver samples using an Atlas Glass Total RNA Isolation Kit (Clontech, Palo Alto, CA) [21]. Single strands of cDNA were synthesized using the primer mix, dNTP, aminoacryl dUTP, and MMLV-RT using an Atlas Glass Fluorescent Labeling Kit (Clontech). The synthesized cDNA probes were coupled to monoreactive Cy3 for fluorescent labeling. Probes were prepared in the same manner for the control group (no BMC transplantation) and BMC transplantation group at the same time. The DNA microarray analysis was conducted using an Atlas Glass Mouse 1.0K Microarray System (Clontech) [22]. The above-mentioned cDNA probes were hybridized to a DNA chip composed of about 1100 DNA fragments by incubating the chip overnight at 50 °C with the probe. After incubation, the chip was washed using GlassHyb Wash Solution, RNase water and 20× SSC, rinsed with distilled water and then air dried. The signal intensity of each gene was measured using a fluorescent scanner (Axon Instruments, CA). The spot intensity of expression of each gene was assessed using the ArrayGauge System (Fuji Film, Tokyo, Japan). The raw data of the spot intensity were used for SOM analysis (All raw data of microarray are available at <http://liver-project.med.yamaguchi-u.ac.jp/research/>). We performed several analyses to obtain representative data.

2.3. SOM analysis for microarray

The microarray analysis showed that, of the 1100 genes on the DNA chips, although the expression of some genes was too small for further analysis, the expression data recorded of the remaining 1018 genes were sufficient for SOM data analysis. At each of five sampling times, i.e., 2 days and each week for 4 weeks, expression levels of 1018 genes for both control CCL₄ damage and BMC transplantation group were measured, respectively. To extract the genes that are differentially expressed before and after BMC transplantation, we defined the following equation (Fig. 1):

$$f_i(t) = \log_{10} \frac{y_i(t)}{x_i(t)}, \quad t = 1, 2, 3, 4, 5, \quad i = 1, 2, \dots, 1018 \quad (1)$$

where $x_i(t)$ is the expression level of the control CCL₄ alone sample (CCL₄ alone without BMC transplantation) and $y_i(t)$ is the expression level of BMC transplantation sample for gene i (1, 2, ..., 1018) at sampling point t , respectively. The term $f_i(t)$ represents the expression level of GFP/CCL₄ group normalized by the control group. If $y_i(t) = x_i(t)$, at that the value of $f_i(t)$, i.e., $\log_{10} 1$, was zero. By using this, we succeeded in extracting the change of gene expression by BMC transplantation. Point t shows time. $t = 2d, 1w, 2w, 3w,$ and $4w$ showed 1, 2, 3, 4, and 5, respectively. To extract the change of gene expression with time, we followed the change of the values of

$f_i(t+1) - f_i(t)$ with the increase in t . Using $f_i(t)$, we defined the 4-dimensional vector $F_i = [F_i(1), F_i(2), F_i(3), F_i(4)]^T$ for gene i , where

$$F_i(1) = f_i(2) - f_i(1)$$

$$F_i(2) = f_i(3) - f_i(2)$$

$$F_i(3) = f_i(4) - f_i(3)$$

$$F_i(4) = f_i(5) - f_i(4)$$

All genes were described and then used as input patterns for SOM analysis. SOM was performed using the SOM toolbox in MATLAB (The Mathworks Inc., Natick, MA; and <http://www.cis.hut.fi/projects/somtoolbox/>).

Each element of these vectors [$F_i(i = 1, 2, 3, \dots, 1018)$] represents a chronological change of gene expression after BMC transplantation in GFP/CCL₄ model.

2.4. Reverse transcriptase (RT)-PCR analysis

Total RNA was isolated from the whole liver of both mice groups (BMC transplantation and control CCL₄ damage ($n = 2$, each group) using Isogen Total-RNA isolation kit (Nippon Gene Co., Ltd., Tokyo, Japan) at each of five sampling times, i.e., 2 days and each week for 4 weeks. These samples were obtained by independent experiments from microarray analysis. RT step was performed using SYBR RT-PCR kit (Takara Co., Tokyo, Japan).

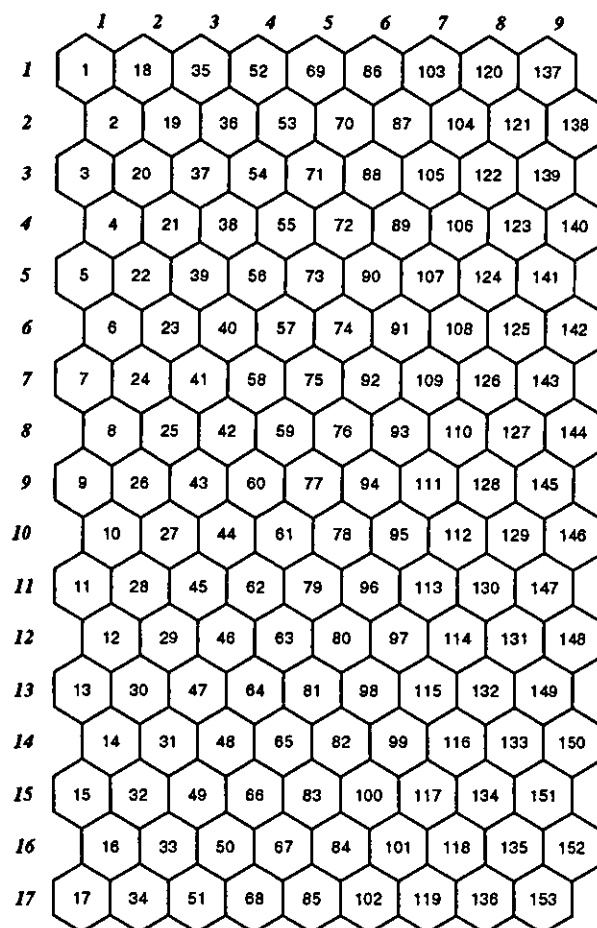


Fig. 2. The 1018 genes analyzed using microarray were divided into 153 clusters and arranged in a 17×9 matrix (height \times width) of 153 hexagons (all raw data of microarray are available at <http://liver-project.med.yamaguchi-u.ac.jp/research/>). The number of genes varied among the clusters. Clusters with similar elements were arranged close to each other in the matrix.

Two μl of cDNA solution (100 ng of initial RNA) was amplified in 20 μl of reaction mixture containing 5 pmol of forward and reverse primer. PCR was performed for a total of 45 cycles, each of 95 °C for 5 s and 60 °C for 20 s [23]. We selected 13 genes to further clarify the difference expression pattern of each gene. c-kit, fibroblast growth factor (FGF)-6, matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitor of metalloproteinase (TIMP)-2, Hepatocyte growth factor (HGF), Numbl like (NumbL) and homeobox (HOX) - D3, Glucose-6-phosphatase isomerase (GPI), vascular endothelial growth factor (VEGF), tumor necrosis factor receptor (TNFR)-1, hepatocyte nuclear factor (HNF)-4 and FGF-2 were selected. The primer used in this study is shown in Table 1. The relative ratio of each gene expression was determined referring with the mean expression level of control house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18 S ribosomal RNA expression.

2.5. SOM analysis compared between RT-PCR and microarray

To validate the results of SOM analysis depend on microarray, we compared SOM analysis between microarray and RT-PCR. We used the same equation and performed SOM analysis (Fig. 1) based on both the data of RT-PCR and microarray.

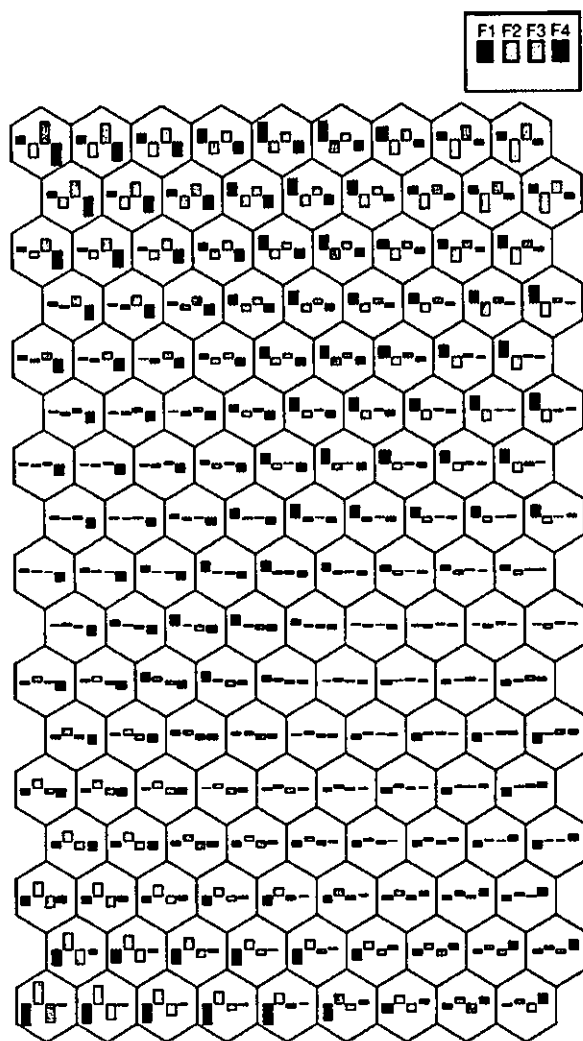


Fig. 3. The median value for gene expression for F_1 , F_2 , F_3 , and F_4 in each cluster is presented as a bar chart. As a reference, in cluster 1, the median value in F_1 and F_3 was increased, while that in F_2 and F_4 was decreased.

3. Results

The 1018 genes that could be analyzed by the DNA chips were classified into 153 clusters by SOM (Fig. 2). Genes in the same cluster showed similar gene expression pattern during the process of BMC trans-differentiation. On the SOM matrix, clusters with similar vector F_i elements (F_1 – F_4) were arranged in close proximity to each other. Therefore, adjacent clusters on the matrix exhibited similar chronological changes in gene expression profiles during the process of plasticity of BMC into hepatocyte. Fig. 3 shows bar charts that represent the median value of gene expression for each cluster in F_1 , F_2 , F_3 , and F_4 . By analyzing each element (F_1 – F_4) of vector F_i , the clusters were color coded to aid visualization of the SOM data (Fig. 4). For example, in the F_1 output, clusters 69, 70, and 86 containing upregulated genes in F_1 were colored dark brown. On the other hand, clusters with downregulated genes in F_1 were colored dark blue. The color bar on the right-hand side of the figure indicates the degree of gene expression from F_1 to F_4 , and a value of 0 indicates that there was little transient change in gene expression between the BMC transplantation and control CCl_4 injection groups. The following clusters exhibited marked changes in transient gene expression: in F_1 , clusters 69, 70, 86, 92, 125, 140, 141, 142, and 143; in F_2 , clusters 13, 14, 15, 16, 17, 32, 33, and 34; in F_3 , clusters 1, 2, 18, and 137; and in F_4 , clusters 118, 119, 133, 134, 135, 136, 148, 149, 150, 151, 152, and 153. To validate the data of SOM analysis based on microarray analysis, we performed SOM

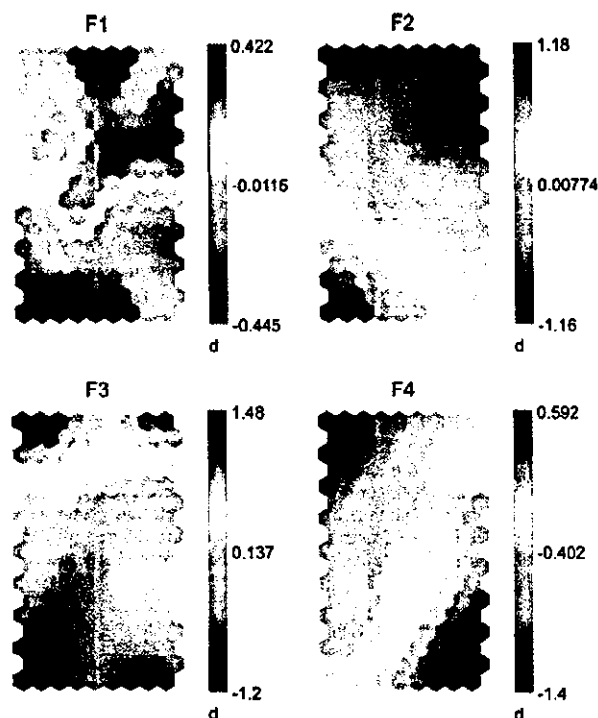


Fig. 4. The clusters were color-coded based on the median values for gene expression at the F_1 , F_2 , F_3 , and F_4 time periods. The color bar on the right hand side indicates the values in each period; dark brown for upregulated and dark blue for downregulated. A value of 0 indicates that there was little chronological change in gene expression between the transplantation and control groups.

analysis based on the data of RT-PCR for selected 13 genes using the same equation (Figs. 5A and B). Table 2 shows the list of genes in each cluster.

4. Discussion

By using specific equation to extract change of gene expression after BMC transplantation, we found that there were dramatic changes for both gene expression and distribution of gene clusters after BMC transplantation in GFP/CCl₄ model (Figs. 3 and 4). 1018 genes were classified into 153 patterns of change of gene expression using SOM analysis. These results might show that many genes had important reciprocal roles during the process of differentiation of BMC into albumin positive hepatocyte. To validate the SOM analysis based on microarray analysis, we performed SOM analysis based on selected 13 gene expressions analyzed by RT-PCR independently. *c-kit*, *FGF6*, *MMP2*, *MMP9*, and *TIMP2* were selected from serious clusters at *F1* periods (clusters 86, 92, and 125). *HGF* was selected from *F2* periods (cluster 13). *NumbL* and *HOXD3* were selected from *F3* (cluster 1). *GPI*, *VEGF*, *TNFR1*, *HNF4*, and *FGF2* were selected from *F4* (clusters

136, 151, and 152). As shown in Fig. 5, we found the similar position of these selected genes. This means that the change of gene expression from microarray analysis is similar to that from RT-PCR analysis. These results showed the consistency of SOM analysis based on microarray using specific equation.

Cluster with color deeper than dark orange (69, 70, 125, 141, 142, and 143) showed the dramatic change in *F1* period (Fig. 4). The *c-kit* gene, which was present in cluster 86, encodes a stem cell factor receptor which is related with rat hepatic stem cell, oval cell, activation [24]. *FGF-6* was also extracted in cluster 86. *FGF* was known to have an important role of hepatocyte proliferation and liver development [25]. To focus on the change of *F1* to *F4* in cluster 86, we found that genes in cluster 86 were upregulated soon after BMC transplantation suggesting that the expression of these genes changes dynamically and might have an important role in the early stage of plasticity of BMC (Fig. 3). In cluster 125, genes involved in the regulation of liver fibrosis such as *MMP2* and *MMP9* were pointed out. These results were consistent with liver fibrosis recovered by BMC transplantation. *MMP9* has been reported to facilitate the induction of hematopoietic cells from the marrow via the kit signal transduction pathway [26]. This result might suggest that ECM might be important for the

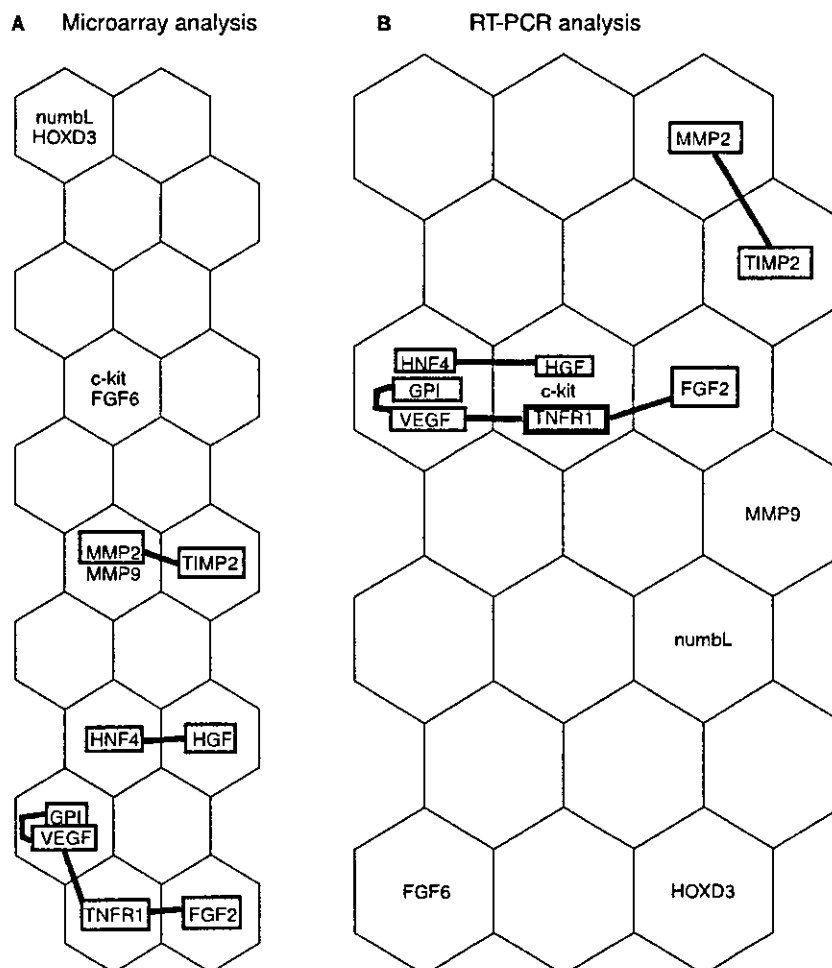


Fig. 5. For the genes, the data of microarray (A) and RT-PCR (B) were classified into clusters by SOM, respectively. Clusters with similar elements were arranged close to each other in the matrix.

Table 2
Selected *gene* grouped to clusters

Cluster No.	GenBank ID	Gene description	
Clusters with remarkable variation of gene expression in F1			
86	U52951	Enhancer of zeste homolog 2 (EZH2); ENX1H	
	X06368 X68932	Macrophage colony-stimulating factor 1 receptor (CSF1R) o-fms proto-oncogene	
	Y00864 NM02099	c-kit proto-oncogene	
	U14173	ski proto-oncogene	
	M92415	Fibroblast growth factor 6 (FGF6)	
69	X07439	Homeobox protein 3.1 (HOX3.1)	
	X57487	Paired box protein 8 (PAX8)	
	U43788	POU domain class 2-associating factor 1 (POU2AF1); OCT-binding factor 1 (OBF1); BOB1; OCA-B	
	M84819	Retinoic acid receptor- γ (RXR- γ ; RXRG)	
	AF015848	E2F transcription factor 3 (E2F3)	
	Z22649	mpl proto-oncogene; thrombopoietin receptor	
	L27105	MOESIN-ezin-radin-like protein (MERLIN); schwannomin (SCH); neurofibromatosis 2(NF2)	
	U51196	EB1 APC-binding protein	
	Y00671	met proto-oncogene	
	Z12604	Matrix metalloproteinase 11 (MMP11); stromelysin 3 (STMY3)	
70	D90156	Myogenin (MYOG); myoD1-related protein	
	U51037	CCCTC-binding factor (CTCF)	
	U06119	Cathepsin H	
142	J04946	Angiotensin-converting enzyme (ACE); dipeptidyl carboxypeptidase I (DCPI); kinase II	
	U10551	Gem induced immediate early protein	
	L34290	Transducin β -5 subunit GTP-binding protein G(t)/G(s)/G(t) β subunit 3	
	M96653	Adenylate cyclase 6	
	Z71173	Inositol 1,4,5-triphosphate receptor 2	
	L76946	Phosphodiesterase 1C	
	U12919	Adenylate cyclase type VII (AIP pyrophosphate-lyase) (adenyl cyclase) (KTAA0037)	
	D31788	BP3 alloantigen	
	AA000715	S100 calcium-binding protein A1; S-100 protein α chain	
	U97327	Calcylin binding protein	
	U73004	Antileukoproteinase 1 (ALP1); secretory leukocyte protease inhibitor	
	140	U58992	Mothers against decapentaplegic homolog 1 (MADH1; mSMAD1); TGF- β signaling protein 1
		U36203	snoN: ski-related oncogene
D17571		NADPH-cytochrome P450 reductase (CPR); POR	
U68058		Secreted frizzled-related protein 3 (SFRP3; mFIZ); frezzled	
U77638		Mothers against dpp homolog 5 (SMAD5; MADH5)	
M33385		Neurotrophic tyrosine kinase receptor type 2 (NTRK); tyrosine kinase receptor B (TRKB)	
X57277		ras-related C3 botulinum substrate 1 (RAC1)	
M21531		Calbindin-28K: calbindin 1 (CALB1)	
U53142		Nitric oxide synthase 3, endothelial cell	
U37775		Tuberin: TSC2 (tuberous sclerosis 2 protein)	
J05261		Lysosomal protective protein; cathepsin A; carboxypeptidase C (CPC); MO54	
M81591		Membrane metallo endopeptidase	
M14222		Cathepsin B (CTSB)	
X15475		Peripherin (PRPH)	
141	U61969	Wingless-related MMTV integration site 10a protein (WNT10A)	
	X76292	Desert hedgehog homolog (DHH); HHG3	
	X67962	Interleukin 7 (IL7)	
	X66196	Recoverin (RCV1; RCVRN); cancer-associated retinopathy protein (CAR protein), 23-kDa photoreceptor cell-specific protein	
	U57311	14-3-3 protein η ; protein kinase C inhibitor protein 1 (KCIP1); tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein η polypeptide (YWHAH)	
	X16490	Macrophage plasminogen activator inhibitor 2 (PAI2; PLANH2)	
	X05211	Laminin γ 1 subunit (LAMC1); laminin B2 subunit	
125	U03421	Interleukin 11 (IL11)	
	M84324	Matrix metalloproteinase 2 (MMP2)	
	NM008610		
	X72795	Matrix metalloproteinase 9 (MMP9)	
	NM013599 M59470	Cystatin C; cystatin 3 (CST3)	

(continued on next page)

Table 2 (continued)

Cluster No.	GenBank ID	Gene description	
143	U94350	Radical fringe homolog (RFNG)	
	U37720	Cell division cycle 42 homolog (CDC42); 25-kDa GTP-binding protein (G25K)	
	X73985	Calbindin 2 (CALB2); calretinin	
	X61432	Calmodulin (CALM; CAM)	
	U48853	ork-associated substrate (CRKAS; CAS)	
	U24680	Dishevelled 2 homolog (DVL2)	
	D00611	Basic immunoglobulin superfamily (BASIGIN; BSG); membrane glycoprotein 42 (GP42), neurothelin; CD147 antigen	
	M28730	Tubulin β 4 (TUBB4)	
	Z21848	DNA polymerase δ catalytic subunit (POLD1)	
	92	U37331	T-box protein 6 (TBX6)
M61909		relA proto-oncogene; NF- κ B transcription factor p65 subunit (NF- κ B p65)	
U18542		Calcitonin receptor 1b	
U17985		Cannabinoid receptor 1 (CNR1; CBR), brain cannabinoid receptor	
U41285		Segment polarity protein dishevelled homolog 3 (DVL3), DSH homolog 3	
X62622		Tissue inhibitor of metalloproteinase 2 (TIMP2)	
NM011594			
L07803		Thrombospondin 2 (THBS2; TSP2)	
X53929		Decorin (DCN); bone proteoglycan II (PG-S2), PG40	
X61435		Neuronal kinesin heavy chain (NKHC); KIF5C	
D10061		DNA topoisomerase I (<i>Top1</i>)	
D28492		Caspase 2 (CASP2); NEDD2 protein; ICH1 cysteine protease	
X66323		X-ray repair-complementing defective repair in Chinese hamster cells 5 (XRCS5)	
Clusters with remarkable variation of gene expression in F2			
17		AF017085	BAP-135 homolog; DIWSIT; general transcription factor II-1 (GTF2I)
		U77969	Neuronal PAS domain protein 2
		X97817	Semaphorin F (SEMAF)
	M84487	Vascular cell adhesion molecule 1 (VCAM1)	
	M59378	Tumor necrosis factor receptor superfamily member 1B2 (TNFRSF1B2); tumor necrosis factor receptor 2 (TNFR2)	
	U25995	Receptor (TNFRSF)-interacting serine-threonine protein kinase 1 (RIPK1; RIP)	
	L22472	BCL2-associated X protein membrane isoform alpha (BAX-alpha)	
	L33406	Uromodulin	
	AF007268	Fibroblast growth factor 15 (FGF15)	
	AF030433	Dickkopf homolog 1 (mDKK1)	
	AF031896	Cerberus-related protein1 (CERR1)	
	X58995	Calcium/calmodulin-dependent protein kinase IV catalytic subunit (CAM kinase-CR; CAMKIV);	
	34	D32132	Hairy and enhancer of split protein 5 (HES5)
		Z93947	Semaphorin H (SEMAH)
U69535		Semaphorin J (SEMAJ)	
X97818		Semaphorin G (SEMAG); SEMA5B	
U25416		Tumor necrosis factor receptor superfamily member 8 (TNFRSF8); CD30L receptor	
U39643		fas-associated factor 1 (FAF1)	
Z22703		Fibroblast growth factor 7 (FGF7)	
X63615		Calcium/calmodulin-dependent protein kinase type II β subunit (CAM-kinase II β ; CAMK-II β)	
U43187		Mitogen-activated protein kinase kinase kinase 3 (MAPKKK3; MAP3K3; MAPK/ERK kinase kinase 3 (MEK kinase 3; MEKK3)	
L35236		Mitogen-activated protein kinase 10 (MAP kinase 10 MAPK10; PRKM10); MAP kinase p49 3F12; Stress-activated o-jun N-terminal kinase 3 (JNK3); SAPK/ERK kinase 2 (SERK2)	
U03856		Receptor-type protein tyrosine phosphatase (PTPRCAP); C polypeptide-associated protein; CD45-Associated protein (C045-AP), LSM	
16	L20331	Adenosine A3 receptor	
	L41145	Bone morphogenetic protein 5 (BMP5)	
	AB006787	Mitogen-activated protein kinase kinase kinase 5 (MAPKKK5; MAP3K5); MAPK/ERK kinase kinase 5 (MEKK5); apoptosis signal-regulating kinase 1 (ASK1)	
	U92456	Serine/alanine-rich protein specific kinase 2 (SRPK2), WW domain binding protein 6 (WBP6)	
15	X81579	Insulin-like growth factor-binding protein 1 (IGF-binding protein 1; K3FBP1)	
	U60530	Mothers against decapentaplegic homolog 2 (MADH2; mSMAD2)	
	AB005141	Klotho protein (KL)	
	U77039	Four and a half LM domains 1 (FLH1); KyoT	
51	Z32675	Hairless protein (HR)	
	L10075	DNA-binding protein SMBP2	
	S56660	Retinoic acid receptor β (RAR- β ; RARB); nuclear receptor subfamily 1 group B member 2	

Table 2 (continued)

Cluster No.	GenBank ID	Gene description
32	X57413	Transforming growth factor β 2 (TGF- β 2; TOFB2)
	M20473	cAMP-dependent protein kinase type I β regulatory chain (PRKAR1B)
	Y00703	Guanine nucleotide-binding protein alpha stimulating activity polypeptide (GNAS)
	Y07836	Stimulated by retinoic acid protein 14 (STRA14); STRA13; E47 interaction protein 1 (EIP1)
	X85993	Collapsin 1; semaphorin IIIA (SEMA3A), SEMAD
	L28177	Growth arrest and DNA damage-inducible protein (GADD45), DNA damage-inducible transcript 1
	L33768	Janus tyrosine-protein kinase 3 (JAK3)
	U07617	Growth factor receptor-bound protein 2 (GRB2); ASH protein
	L02526	Mitogen-activated protein kinase kinase 1 (MAP kinase kinase 1; MAPKK1; MAP2K1; PRKMK1); MAPK/ERK kinase 1 (MEK1)
	U66058	DNA ligase III; polydeoxyribonucleotide synthase (ATP) (DNL3)
33	L28819	Involucrin (IVL)
	X97052	Mitogen-activated protein kinase kinase 6 (MAP kinase kinase 6; MAPKK6; MAP2K6; PRKMK6); MAPK/ERK kinase 6 (MEK6); SAPKK3
	L09562	Protein-tyrosine phosphatase γ (R-PTP- γ ; PTPRG)
	Z32767	DNA damage repair & recombination protein 52 homolog (RAD52)
14	X81466	Ephrin type A receptor 7 (ephrin receptor A7; EPHA7), embryonic brain receptor tyrosine kinase (EBK); developmental kinase 1 (mDK1)
	U37522	Tumor necrosis factor superfamily member 10 (TNFSF10); TNF-related apoptosis inducing ligand
	D38258	Fibroblast growth factor 9
	X77113	Growth differentiation factor 9 (GDF9)
	X06381	Leukemia inhibitory factor (LIF); cholinergic differentiation factor
	Z22532	Syndecan 1 (SYND1)
	M75716	Alpha-1-antitrypsin, 1-2 (AAT2), serine proteinase inhibitor 1-2 (SPII-2); alpha 1 protease inhibitor 2; alpha-1-antitrypsin
	M64292	Anti-proliferative B-cell translocation gene 2 (BTG2); NGF-inducible protein TIS21
	L23971	Fragile X mental retardation syndrome 1 homolog (FMR1; FMRP)
	13	U95610
U05252		Special AT-rich sequence-binding protein 1 (SATB1)
U70017		Cyclin-D binding Myb-like protein (hDMPI)
AF001287		Neural cell adhesion molecule 2 (NCAM2), olfactory axon cell adhesion molecule (OCAM)
J04806		Osteopontin (OP); bone sialoprotein 1; minopontin; early T-lymphocyte activation 1 protein (ETA1); secreted phosphoprotein 1 (SPP1), calcium oxalate crystal growth inhibitor protein
X83930		Cadherin 5 (CDH5); vascular epithelial cadherin (VE-cadherin)
AF003747		Zinc transporter 4 (ZNT4)
M16472		Myelin proteolipid protein (PLP), lipophilin; DM20
XI5830		7B2 neuroendocrine protein; secretogranin V (SGV; SCG5)
X72307		Hepatocyte growth factor (HGF)
D84372	Non-receptor type II protein cytosome, phosphocytosome phosphatase	
M38700	ATP-dependent DNA helicase II 70-kDa subunit 70-kDa thyroid autoantigen; lupus Ku autoantigen protein p70 CTC box-binding factor 75-kDa subunit (CTCBF; CTC75)	
cluster with remarkable variation of gene expression in F3		
1	UB6441	numfaiike (numbL; m-nbl)
	NM010950	CD3 antigen zeta (CD3Z)
	M33158	Low-affinity IgG Fc receptor II β (FCGR2B)
	X04648	LIM-homeodomain protein L3; LHX8
	D49658	Engrailed protein (En-2) homolog
	L12705	SRY-box containing gene 17 (SOX17)
	D49474	Homeobox protein D3 (HOXD3)
	X73573	
	NM010468	
	U62522	Sp4 zinc finger transcription factor
	U25096	Lung Kruppel like factor (LKLf)
	X90329	Lbx 1 transcription factor
	X61753	Heat shock factor 1
	U53925	Transcription factor C1
	U97076	FLICE-like inhibitory protein long form (FLIP-L)
18	M55S12	WT1; Wilms tumor protein; tumor suppressor
	M16449	Myeloblastosis proto-oncogene (MYB)
	X13945	Lung carcinoma myc-related oncogene 1 (L-myc; mycL1)
	M26391	Retinoblastoma-associated protein 1 (RB1); phosphoprotein 105 (PP105)
	U65594	Breast cancer type 2 susceptibility protein (BRCA2)
	U04807	FMS-like tyrosine kinase 3 ligand (FLT3L)
	M34563	T-ceB-specific surface glycoprotein CD28

(continued on next page)

Table 2 (continued)

Cluster No.	GenBank ID	Gene description
2	M32240 M63801 AF013282 U63386 J05154	Peripheral myelin protein 22 (PMP22); CD25 antigen; SR13 myelin protein Gap junction alpha 1 protein (GJA1), connexin 43 (CXN43; CX43) T-box protein 15 (TBX15); TBX14; TBX8 Early development regulator 1 (EDR1); pdyhomeotic 1 homolog (mPHI) Lecithin cholesterol acyltransferase (LCAT); phosphatidylcholine sterot acyltransferase; phospholipid cholesterol acyltransferase
137	M55171 D50311 X14943 U12570 V00727 U36799 S59388 Z31683 S53216 AF039601 X06203	Rhodopsin (RHO), opsin (mOPS) Myocyte enhancer factor 2B (MEF2B) Contactin 1 (CNTN1); F3 neuronal cell adhesion molecule (F3CAM) von Hippel-Lindau syndrome homolog (VHLH) fos proto-oncogene retinoblastoma-like protein 2 (RSL2); retinoblastoma-related protein PRB2/p130 Erythropoietin receptor (EPOR) activin A receptor type 1B Tyrosine-protein kinase ryk; kinase vik; nyk-R TGF-beta receptor type 1B (betaglycan); candidate tumor suppressor gene Interleukin 8 (IL6)
Clusters with remarkable variation of gene expression in F4		
152	L12140 M98502 S79463 X91144 U04294 M14220 NM008155 M95200 NM009505 M30643 U51866 U17112 U67916 U49739	Groucho gene-related protein (GRG); amino enhancer of split protein (AES) Zinc finger protein 46 Semaphorin I (SEMAI) P-selectin glycoprotein ligand 1 (PSGL1; SELPLG; SELP1) Electrocardiographic QT syndrome 2 potassium channel subunit Glucose-6-phosphate isomerase (GPI) Vascular endothelial growth factor (VEGF) Heparin-binding growth factor 5 (HBGF5); fibroblast growth factor 5 (FGF5) Casein kinase II alpha 1 related sequence 4 (CSNK2A1-RS4) Dentin sialophosphoprotein (DSPP) Unconventional myosin VI
153	S663B5 X85994 U28724	CREB-binding protein Semaphorin IIIc (SEMA3C); SEMAE Postmeiotic segregation increased 2 homolog (PMS2)
135	X12875 L24755 X83106 U34960 D86726	Neural cell adhesion molecule LI (N-CAM LI; LtCAM; CAML1) Bone morphogenetic protein 1 (BMP1) MAX dimerization protein (MAD) Transducin beta-2 subunit MCM6 DNA replication licensing factor (P105MCM)
136	D31967 U59496 X85992 X07640 X57796 NM011609 X53798 X78850 U43144 X83536 Y13602	Jumonji protein Hypoxia inducible factor 1 alpha subunit (HIF1-alpha; HIF1A); ARNT-interacting protein Semaphorin C (SEMAC) Cell surface glycoprotein MAC-1 alpha subunit; CR-3 alpha subunit; GD11B antigen; leukocyte adhesion receptor MO1; integrin alpha-M (ITGAM) Tumor necrosis factor receptor 1 (TNFR1) Small inducible cytokine subfamily B member 2 (SCYB2); macrophage inflammatory protein 2 (MIP2) Mitogen activated kinase-activated protein kinase 2 (MAPKAP kinase 2; MAPKAPK2); 60-kDa ribosomal protein S6 kinase polypeptide 1 (RPS6KC1) Phospholipase C beta 3 (PLC-beta 3; PLCB3) Matrix metalloproteinase 14 (MMP14); membrane-type matrix metalloproteinase 1 (MTMMP1) Filensin, beaded filament structural protein in lens 1 (BFSP1)
150	AF033011 D63644 S70632 S70756 S70629 S81932 X75330 U89487 U89489 U36340 U42554 U33626	distal-less homeobox protein 5 (DLX5) Aryl hydrocarbon receptor nuclear translocator 2 (ARNT2) T-cell leukemia homeobox 1 (TLX1); homeobox protein 11 (HOX11) distal-less homeobox protein 3 (DLX3) Drosophila NK5 transcription factor-related locus 1 (NKX-5.1), H6 homeobox protein 3 (HMX3) LIM homeobox protein cofactor 1A (CUM1A); CUM1B; LIM domain-binding protein 3 (LDB3) CACCC-box-binding protein basic Kruppel like factor (BKLf); Kruppel-like factor 3 (KLF3) Single-minded 2 (SIM2) Promyelocytic leukemia gene (PML)

Table 2 (continued)

Cluster No.	GenBank ID	Gene description
	X56135	Prothymosin alpha (PTMA)
	U43512	Dystroglycan 1
	M13071	A-raf proto-oncogene
	M36829	Heat shock 84-kDa protein 1 (HSP84-1); HSP90
	M89802	Wingless-related MMTV integration site 7b protein (WNT7B)
	M30903	B-lymphocyte kinase (BLX)
	U43298	Laminin β 3 subunit (LAMB3); kalinin B1 subunit
151	D29015	Hepatic nuclear factor 4-alpha (HNF4-alpha)
	NM008281	
	M31042	Immediate early response protein 2 (IER2); T-lymphocyte activated protein; cycloheximide-induced Protein 1 (CHX1)
	U43900	STAM; signal transducing adaptor molecule
	M13177	Transforming growth factor β 1 (TGF- β 1; TGFB1)
	M30644	Fibroblast growth factor 2 (FGF2)
	NM008006	
	M76601	Cardiac myosin heavy subunit alpha isoform (MYH6; MYHCA)
148	AB009453	Transcription factor 21 (TCF21); basic helix-loop-helix factor COR1; POD1
	S71659	LIM homeobox protein 4 (LHX4); GSH4
	S79041	Genomic screened homeobox protein 2 (GSH2)
	U58533	est2 repressor factor (ERF)
	J03770	Homeobox protein D4 (HOXD4); HOX4.2; HOX5.1
	X59252	Homeobox protein 8 (HOX8)
	J03168	Interferon regulatory factor 2 (IRF2)
	S68108	Brahma-related protein 1 (BRG1); swi/snf-related matrix-associated actin dependent regulator of chromatin subfamily a member 4 (SMARCA4)
	D78382	Transducer of erbB2 (TROB; TOB)
	X59421	Fli-1 ets-related proto-oncogene
	M62860	Myelin protein zero
	D31942	Oncostatin M (OSM)
	M29464	Platelet-derived growth factor A subunit (PDGFA; POGF1)
	M69042	PKC- δ ; protein kinase C δ type
	U29539	Retinoic acid-inducible E3 protein; stimulated by retinoic acid 13 (STRA13), hematopoietic-specific Protein E3; orfB
	U26967	Cordon-bleu protein (COBL)
149	L38248	LIM homeobox protein 3 (LHX3; UM3)
	X56230	Octamer-binding transcription factor 1 (OCT1; OTF1); NF-A1; POU domain class 2 transcription factor 1 (POU2F1)
	X13721	Homeobox protein 2.4 (HOX2.4)
	L04662	γ -Aminobutyric acid transporter 4 (GABA-A transporter 4; GABT4)
	U59746	B-cell lymphoma protein W (BCLW); BCL2-like protein 2 (BCL2L2)
	M16819	Lymphotoxin alpha (LTA), tumor necrosis factor β (TNF- β ; TNFB)
	U12147	Laminin alpha 2 subunit (LAMA2). dystrophia muscularis protein (DV); merosin heavy chain; laminin M subunit
	U22421	Leptin (LEP); obese factor (OB)
133	D10329	CD7 antigen
	D86603	btb and cnc homolog 1 (BACH1)
	U36384	Dermis expressed 1 protein (DERM01)
	M58633	P58/GTA; galactosyl transferase associated protein kinase (cdc2-related protein kinase)
	D83698	Activator of apoptosis harakiri (HRK); neuronal death protein 5 (DP5); BID3
	L08235	Clusterin (CLU); clustrin; apolipoprotein J (APOJ); sulfated glycoprotein 2 (SGP2; mSGP2)
	X07414	DNA excision repair protein ERCC1
134	U61155	LIM homeobox protein 2 (LIM2); LHX5
	U20553	p57kip2; cdk-inhibitor kip2 (cyclin-dependent kinase inhibitor 1 B); member of the p21CP1 Cdk inhibitor family; candidate tumor suppressor gene
	U05671	Adenosine A ₁ receptor (ADORA1)
	L37663	Acetylcholine receptor alpha 7 neural
	M97017	Bone morphogenetic protein 8A (BMP8A), osteogenic protein 2 (OPS)
	AF027503	guanylate kinase membrane-associated inverted protein 1 (GUKMI1; MAGI-1)
	M84817	Retinoid X receptor alpha (RXR- α Jpha; RXRA)
	J03520	Tissue plasminogen activator (T-plasminogon activator PLAT; TPA)
118	X99063	Zyxin(ZYX)
	X14194	Nidogen (NID); entactin (ENT)
119	Y15001	Iroquois-related homeobox protein 3 (IRX3)
	Z23066	Microphthalmia-associated transcription factor (MITF; MI); microphthalmia-related protein
	X73360	Transducin-like enhancer of split protein 3 (TLE3; ESG)

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Table 2 (continued)

Cluster No.	GenBank ID	Gene description
	U65091	Melanocyte-specific gene 1 (MSG1); Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1 (CITED1)
	X97986	Desmocollin 1A/1B (DSC1)
	U81317	Myelin-associated oligodendrocytic basic protein
	U21050	TNF receptor-associated factor 3 (TRAF3); TRAFAMN; C040 receptor-associated factor 1 (CRAF1)
	D83966	Protein tyrosine phosphatase
	L19622	Tissue inhibitor of metalloproteinase 3 (TIMP3); SUN
	AF021031	DiGeorge syndrome chromosome region 6 protein (DGCR6)

plasticity of BMC. In the F2 time period, clusters 13, 14, 15, 16, 17, 32, 33, and 51 were dramatically changed. In cluster 13, hepatocyte growth factor (HGF) was discovered. HGF is involved in hepatocyte proliferation [27,28]. HGF might also have an important role in GFP/CCl₄ model. In the F3 time period, clusters 1, 2, 18, and 137 were focused. NumbL is involved in asymmetric division of nerve precursor cells [29]. The HOXD3 genes encode information important for determining the positional relationships of the antero-posterior axis in embryogenesis [30]. NumbL and HOXD3 might have an important role in regulating the plasticity of BMC. In F4, clusters 118, 119, 133, 134, 135, 136, 148, 149, 150, 151, 152, and 153 were found. In this period, genes involved in hepatocyte differentiation and homeostasis, such as GPI and HNF4, were focused [31]. This enzyme is essential for the glycolytic metabolism of hepatocytes. In the GFP/CCl₄ model, the level of albumin in bone marrow-derived hepatocytes increased significantly [12]. The fact that an enzyme such as GPI was induced at this period suggests that, at 4 weeks after BMC transplantation, transplanted BMCs begin to possess some of the metabolic functions of hepatocytes. HNF4- α was also upregulated in F4 period. HNF4 plays an important role in the metabolic regulation of hepatocytes [32,33]. These results might be related with BMC differentiated into functional hepatocyte at this period in GFP/CCl₄ model. VEGF was also upregulated. VEGF promotes vasculogenesis and liver regeneration [34]. VEGF might also have an important role in accelerating the liver regeneration in GFP/CCl₄ model. Gene involved in inflammation such as TNF-R1 was also pointed out in GFP/CCl₄ model. TNF- α related inflammation signal is important in the generation of hepatoblast [17]. These results also showed that TNF- α related signal might be important for plasticity of BMC in GFP/CCl₄ model.

Here, we analyzed the change of molecular signature after BMC transplantation in GFP/CCl₄ model in mRNA level. Still many precise things are unconfirmed, but we think the information is useful to understand the mechanism of the plasticity of BMC in GFP/CCl₄ model. In the future, we are planning to further analyze the mechanisms.

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肝細胞癌に対する新しいバルーンマイクロカテーテルを用いた
リピオドール併用肝動脈バルーン閉塞下ラジオ波凝固療法

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<短 報>

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リピオドール併用肝動脈バルーン閉塞下ラジオ波凝固療法

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緒言: 肝細胞癌に対して経皮的ラジオ波凝固療法(以下 RFA)が導入されて、約5年が経過している。その間にも腫瘍の凝固範囲を拡大させる工夫がさまざま報告されており¹⁻³⁾、われわれも肝動脈を遮断することで血流によるラジオーター効果を低下させ、凝固範囲を拡大させる肝動脈バルーン閉塞下ラジオ波凝固療法(balloon-occluded RFA; BoRFA)を開発し、有用性について報告した^{4,5)}。しかし、血管歩行等により、すべての症例で固有肝動脈に通常のバルーンカテーテルを留置するのは不可能であり、そのような症例に対してわれわれはバルーンマイクロカテーテルを用いた肝動脈バルーン閉塞下ラジオ波凝固療法(balloon-microcatheter-occluded RFA; BMoRFA)を開発した⁶⁾。その際用いたバルーンマイクロカテーテルは、Commodore™ temporary occlusion balloon catheter (Cordis, Johnson and Johnson Co., USA)であったが、5 Fr カテーテルへの挿入が不可能であり、5.1 Fr カテーテルへの交換が必要で手技が煩雑な点、およびその後の検討で予想以上の凝固範囲拡大が得られなかった点⁷⁾が、問題点であり、改良の余地が必要であった。今回、上記の問題点を解決すべく、新しいバルーンマイクロカテーテルを用い、同療法にリピオドールを併用したリピオドール併用肝動脈バルーン閉塞下ラジオ波凝固療法を考案したので報告する。

対象と方法: 各種画像診断にて肝細胞癌と診断した4例(4結節)を対象とした。3例が女性、平均年齢69歳、腫瘍径31.3±11.9 mm(24-49 mm)、全例Child-Pugh Aであった。使用装置は、Radionics社のCool-tip RF Systemで、全例3.0 cm針を使用し、1回穿刺で12分間を基本凝固時間としたが、1例のみ腫瘍径が大きいため、引き抜いて12分間追加凝固を行った。使用したバルーンマイクロカテーテルは、Equinox™ occlusion balloon catheter (Micro Therapeutics, Inc. USA)の4×15 mmのバルーン径を用いた。今回の治療としては、既報⁶⁾と異なりリピオドール併用で行った。方法としては、通常の腹部血管造影の手技にて、5 Fr カテーテル(セレンカテーテル:クリニカルサブライ)を用い、精査を行い、マイクロカテーテルにてTranscatheter arterial chemoembolization(TACE: epirubicin hydrochloride, Mitomycin C, lipiodol 使用)を施行し、腫瘍内にlipiodolをpoolingさせた後、Equinox™ occlusion balloon catheterを担癌領域の肝動脈に留置し、バルーン閉塞後にRFAを施行した。同バルーンマイクロカテーテルは5 Fr カテーテルへの挿入が容易であり、バルーンは造影剤入り生理食塩水を0.2 cc注入することで、

4×15 mmのバルーン径が得られた。治療効果判定は、1週間後の造影CTにて施行し、凝固範囲を検討した。

結果: 表1のごとく、12分間1回での凝固範囲が検討できたのは、症例2(図1)と4であり、それぞれ、50×47 mm、55×44 mmであった(症例3は、肝梗塞合併のため、計測していない)。同治療1週間後の造影CTでは、全例で治療部位の早期濃染像は見られなかったが、症例4では、脈管近傍のsafety marginが不足していたため、経皮的エタノール注入療法(PEI)を追加した。合併症では、症例1で発熱の遷延、症例3に肝梗塞を認めたが、保存的に改善した。その他は全例に発熱および疼痛を認めたが、対処療法で改善している。また、一時的に肝機能悪化は認めたが、約1週間後にはほぼ前値に復している。

考察: 今回のRFAの工夫としては、新しいバルーンマイクロカテーテルを用いた点およびリピオドール併用肝動脈バルーン閉塞下ラジオ波凝固療法を考案した点の2つである。使用した新しいバルーンマイクロカテーテルは、以前使用したCommodore™ temporary occlusion balloon catheterとは違い、5 Fr カテーテルでの挿入が可能であり、手技の煩雑さが緩和された。今回の治療法は、肝動脈バルーン閉塞下ラジオ波凝固療法の変法として、ラジオーター効果をより低下させる目的で、リピオドールを併用した。リピオドールは腫瘍内だけでなく、門脈枝にも流入し、腫瘍周囲の門脈血流を低下させること⁸⁾から、動脈および門脈血流の同時遮断効果⁹⁾を狙い、同療法を考案した。通常のバルーンカテーテル(バルーン径9 mm)での検討では、リピオドール併用肝動脈バルーン閉塞下ラジオ波凝固療法において非併用群と比較して、長径では有意差はないものの、短径で有意差を認めている(データ未発表)。リピオドール併用ラジオ波凝固療法での成績は、われわれは持ち合わせていないが、以前の報告¹⁰⁾よりも凝固範囲は広く、肝動脈閉塞を併用する意義は十分あるものと考える。以前のCommodoreを用いたBMoRFAの1回凝固(12分間)の成績では長径38.3±5.7 mm、短径31.5±4.6 mmと、通常のRFAでの凝固範囲(長径35.3±4.7 mm 短径25.9±3.7 mm)と比較して、短径のみの有意差であった⁷⁾。今回は1回凝固範囲の成績が2例のみであり、なおかつリピオドール併用であるが、50×47 mm、55×44 mmと十分な凝固範囲を認めている。また以前のバルーン径は4×10 mmであったが、新しいバルーンマイクロカテーテルは4×15 mmと短径は4 mmのままであるが、長径が15 mmと長くなっていることが、血流遮断に寄与し、凝固範囲の拡大につながったのかもしれない。今後症例を重ねた検討が必要であるが、新しいバルーンマイクロカテーテルは、通常のBoRFA不可能例の適応に応用可能と考える。また、リピオドール併用肝動脈バルーン閉塞下ラ

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