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Lesson from the GFP/CCl4 model — Translational Research Project: the development of cell therapy using autologous bone marrow cells in patients with liver cirrhosis

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Abstract
The plasticity of bone marrow has been confirmed by the analysis of autopsy findings in female recipients of bone marrow cells transplanted from male donors. To establish new clinical cell therapies using autologous bone marrow cells for patients with liver failure, we developed a new in vivo model, the "green fluorescent protein (GFP)/carbon tetrachloride (CCl_4) model". Using the GFP/CCl_4 model, we found that transplanted Liv8-negative cells efficiently repopulated into cirrhotic liver tissue and trans-differentiated into albumin-producing hepatocytes under conditions of persistent liver damage induced by CCl_4. Moreover, one marrow cell transplantation into liver cirrhosis mice improved their liver function, ameliorated liver fibrosis, and improved their survival rate. Results from the GFP/CCl_4 model showed that cell therapy using autologous bone marrow cells has the potential to become an effective treatment for patients with liver failure. Here we describe the findings from the GFP/CCl_4 model and the scope of the translational research project.

Key words Bone marrow cells · Bone marrow cell transplantation · Liver cirrhosis · GFP (green fluorescent protein)/carbon tetrachloride (CCl_4) model · Liver fibrosis · Stem cell · Translational research · Trans-differentiation · Niche · Liver fibrosis

Introduction

Liver failure in patients with liver cirrhosis with endstage chronic liver disease is very difficult to cure. At present, liver transplantation is one effective therapy for curing these patients; however, this treatment faces serious problems, such as lack of donors, operative damage, rejection, and high expense. On the other hand, cell transplantation therapy is a minimally invasive procedure with fewer potential complications. Regenerative medicine using stem cells is an attractive therapy for the cure of patients with severe liver disease. The capacity of bone marrow cells (BMCs) to differentiate into hepatocytes and intestinal cells was confirmed through the detection of the Y chromosome in the analysis of autopsy findings in human female recipients of BMCs from male donors.[1–4] BMCs are an attractive cell source for regenerative medicine, because it is easier to obtain BMCs than it is to obtain other tissue-specific stem cells.[5,6] BMC transplantation is also an established treatment for hematological diseases. Clinical studies have evaluated the use of BMCs in regenerating the myocardium and vessels in patients with heart failure and those with limb ischemia.[7–10] Based on these findings, we began to focus on BMCs as a new cell source for liver regenerative therapy. The mechanism of BMC plasticity was previously examined with respect to cell fusion[11,12] and trans-differentiation.[13,14] We think that the most important aspect of developing a new clinical therapy using BMCs is evaluating its effectiveness for liver disease. We think both cell fusion and trans-differentiation could be important for furthering the understanding of the mechanisms of BMC plasticity. Indeed, the development of an effective cell therapy using BMCs requires better understanding of events in the recipient mouse liver after BMC transplantation. We developed a new in vivo model, named the "green fluorescent protein (GFP)/carbon tetrachloride (CCl_4) model",[15] in which to monitor the differentiation of BMCs into functional hepatocytes. Here, we describe the newest findings from the GFP/CCl_4 model. These lessons will be important for proceeding with the translational project of cell therapy using autologous BMCs to treat patients with liver cirrhosis.

The GFP/CCl_4 model

First, we sought to understand how to use BMCs to repair liver damage, using our GFP/CCl_4 model.[15] In the

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model, 0.5 ml/kg of CCl_4 is administered twice weekly to C57BL/6 female mice to induce liver cirrhosis, and then GFP-positive BMCs obtained from GFP-Tg mice (C57BL6/Tg14 (act-EGFP) OsbY01 mice)[16] are transplanted through the caudal vein (donor and recipient mice are of the same strain). In this model, we transplanted 1×10^5 GFP-positive BMCs that had not been cultured. By analyzing GFP-positive BMCs in the recipient mice, we evaluated the repopulation and differentiation of BMCs under conditions of continuous liver injury. Immunostaining, using anti-GFP antibodies,[17] showed that GFP-positive BMCs migrated into the marginal area of hepatic lobules, starting 1 day after BMC transplantation; with time, the distribution of GFP-positive BMCs expanded,[15,18] with they formed a hepatic cord towards the central vein. The use of Liv2, a hepatoblast-specific antibody that we developed,[19] also showed that BMCs first trans-differentiated into Liv2-positive hepatoblasts and then differentiated into albumin-positive hepatocytes. Furthermore, the level of serum albumin significantly increased with time in the recipient mice. These findings suggest that the GFP/CCl_4 model can be used to understand the process of BMC differentiation into hepatocytes. On the other hand, GFP-positive cells were not detected in the liver tissues of control mice (those with no liver damage) following BMC transplantation. The persistent liver damage induced by CCl_4 injection is important for producing a specific differentiation "niche" in order to activate the plasticity of BMCs and their subsequent differentiation into hepatocytes. Oval cells were thought to be one type of hepatic stem cells derived from the Canal of Hering following severe liver damage.[20,21] Based on the finding of Petersen et al.,[22] that, under some conditions, oval cells are derived from bone marrow cells, we also analyzed the activation of oval cells, using a specific oval-cell marker, A6 antibody. A6-Positive cells were detected in the periportal region 1 week after BMC transplantation in the GFP/CCl_4 model, but these A6-positive oval cells did not increase in the 4 weeks after BMC transplantation in this model. We could not detect A6-positive cells that also expressed GFP in the liver after BMC transplantation. These results suggest that, while some signals that activate oval cells are induced by BMC transplantation into CCl_4-induced cirrhotic liver, the oval cells may not be derived from transplanted BMCs. In summary, BMCs transplanted into the GFP/CCl_4 model trans-differentiated into hepatoblast phenotypes and differentiated into albumin-producing hepatocytes in the "differentiation niche" created by the persistent cirrhosis induced by CCl_4 injection.

Characteristics of candidate BMCs for cell therapy of liver disease

Although various theories explain the existence of pluripotent stem cells in BMCs, the exact composition of the stem cells in BMCs is not clear at present. The following cell types are known to exist in bone marrow: hematopoietic stem cells (HSCs),[13,23] side population cells (SPCs),[24] and mesenchymal stem cells (MSCs).[25] Although past studies used existing antibodies and techniques, there have not been any studies based on the findings of natural liver development studies. The liver functions as a metabolic organ, with the exception of a short period during the fetal stage from embryonic day (E) 12 to 16 (E12–E16), when the liver functions as a hematopoietic organ.[26] We decided to analyze the usefulness of various cell populations of BMCs, based on the analysis of fetal liver development. We prepared a new monoclonal antibody, anti-Liv8 antibody, which recognizes hematopoietic cells using a specific cell-surface marker, in order to identify and separate subpopulations of BMCs capable of differentiating into hepatocytes under CCl_4-induced continuous liver damage in the GFP/CCl_4 model.[15] Next, we investigated Liv8-positive cells in the BMCs of adult GFP Tg mice. Liv8-positive cells were present in bone marrow in adult GFP-Tg mice; around 32% of BMCs were Liv8-positive. With regard to the relationship between Liv8 and CD45, we found, by flow cytometry, that CD45-positive cells expressed Liv8. These results show that anti-Liv8 is a useful antibody for separating hematopoietic and non-hematopoietic cells. Liv8-positive cells are thought to be hematopoietic cells and Liv8-negative cells are thought to be non-hematopoietic cells. The separated cells were then transplanted into a CCl_4-induced liver damage recipient model. At 4 weeks after BMC transplantation, more efficient repopulation and trans-differentiation of BMCs into hepatocytes was seen with Liv8-negative cells. These findings suggest that the subpopulation of Liv8-negative cells includes cells useful for performing cell therapy in damaged livers.[18]

Improvements in liver function and survival rate, and amelioration of liver fibrosis by BMC transplantation

Using the GFP/CCl_4 model, we also evaluated the recovery of liver function and the effect on liver fibrosis and survival rate. Transplanted BMCs trans-differentiated into albumin-producing hepatocytes, leading to an increase in the serum albumin level. Interestingly, we found an amelioration of liver fibrosis after BMC transplantation.[27] Although the exact mechanism of the fibrolysis remains unclear, transplanted BMCs migrated along fibers that had strong expression of matrix metalloproteinase (MMP)-9, resulting in the resolu-

tion of fibrosis. The degradation of the extracellular matrix presumably led to improved liver function, resulting in the better survival of mice following BMC transplantation. We also analyzed differences in liver fibrosis following the transplantation of Liv8-positive or Liv8-negative BMCs. Our results showed that Liv8-negative BMC transplantation ameliorated liver fibrosis to a greater extent than Liv8-positive BMC transplantation. These results show that subpopulations of Liv-8-negative cells will be useful for treating liver cirrhosis. We think that BMC transplantation into liver cirrhosis mice has two effects: BMC trans-differentiation into albumin-producing hepatocytes and the recovery of liver fibrosis.[18,27] These effects of BMC transplantation accelerate the improvement of liver function and the survival rate.

Molecular mechanisms that regulate the trans-differentiation of BMCs into hepatocytes in the GFP/CCl4 model (microarray-self-organizing map [SOM] analysis)

Recently, cell fusion has been reported to be an important mechanism for the trans-differentiation of BMCs and tissue stem cells.[11,12] The differentiation of BMCs into hepatocytes in the fumarylacetoacetate hydrolase (FAH) model was thought to show the importance of cell fusion in the differentiation of HSCs into hepatocytes.[28,29] However, other groups have reported little evidence of in vivo cell fusion during the trans-differentiation of BMCs into other cell lineages.[14,30] We analyzed the cell fusion rate, using cultured Neo-resistant Embryonic stem (ES) cells and GFP-positive BMCs under the same culture conditions as those used by Terada et al.[11] (cell fusion rate of $1/10^5$–10^6), and found similar cell fusion rates in our in vitro assay (data not shown). Mouse hepatocytes have ploidy values of 2N, 4N, 8N, or 16N. The cell fusion of diploid (2N) BMCs with hepatocytes produces cells with ploidy values of 4N, 6N, 10N, or 18N.[28,29] We were afraid that the variety in ploidy values would make it very difficult to analyze cell fusion. We analyzed the DNA ploidy patterns of primary hepatocytes isolated from mice with livers with persistent CCl_4 damage, with and without BMC transplantation, at 4 weeks. We were able to isolate around 1.2×10^8 hepatocytes from recipient mice at 4 weeks by a two-step collagenase method, and we analyzed DNA ploidy patterns by fluorescence activated cell sorting (FACS), We found 2N, 4N, 8N, and 16N DNA bands. Comparisons of these DNA ploidy patterns showed that the 2N and 4N bands were similar, but the peaks, representing 8N and 16N bands, were slightly different (data not shown). These results show that cell fusion may have occurred in the GFP/CCl_4 model. Next, we analyzed the differentiation of transplanted BMCs into hepatocytes. Although we could not neglect the possibility that cell fusion had occurred in our model, the BMCs trans-differentiated into Liv2-positive hepatoblasts and functional hepatocytes. We think that the trans-differentiation of BMCs actually occurred in the GFP/CCl_4 model. We analyzed the mechanism of this plasticity using DNA chips, which are recently developed tools of genetic analysis. While it is possible to obtain vast amounts of genetic data with DNA chips,[31] interpretation of the factors involved in gene expression requires the application of a statistical technique such as the SOM to visualize the vast amounts of complicated and multidimensional data.[32] In this analysis, we made a specific equation to identify genes that regulate the trans-differentation of BMCs into hepatocytes. In the GFP/CCl_4 model, genes related to morphology were dramatically activated at an early stage, while genes associated with hepatocyte differentiation were upregulated at a later state. In the early stage after BMC transplantation, we found that genes such as *FGF* and c-*kit*, as well as *HOX* and *HLH* transcription factors, might be important. In later stages, genes associated with metabolic function, such as hepatocyte nuclear factor 4 (*HNF4*) and glucose-6-phosphatase (*G6Pase*) isomerase were induced, suggesting that, at 4 weeks after BMC transplantation, the transplanted BMCs had begun to assume some of the metabolic functions of hepatocytes.[33] Although many details remain unconfirmed, we think that the microarray-SOM analysis for the GFP/CCl_4 model confirms the idea that the BMCs trans-differentiated into immature cells and then differentiated into mature hepatocytes. This information will be useful for understanding the mechanism of BMC plasticity in the GFP/CCl_4 model.

Summary of the lessons learned from the GFP/CCl_4 model, and brief report of a clinical study of autologous BMC transplantation into patients with liver cirrhosis

We summarized the findings from the GFP/CCl_4 model. As shown in Fig. 1, transplanted GFP-positive BMCs (especially the Liv8-negative cell population, without culturing) migrated into the periportal regions of the cirrhotic liver. With time, the transplanted GFP-positive BMCs trans-differentiated into Liv2-positve hepatoblasts and then differentiated into albumin-producing hepatocytes. The transplanted BMCs formed a hepatic cord. The differentiation "niche" created by the persistent liver damage caused by continuous CCl_4 injection is a key factor. Microarry-SOM analysis showed that, at an early stage after BMC transplanta-

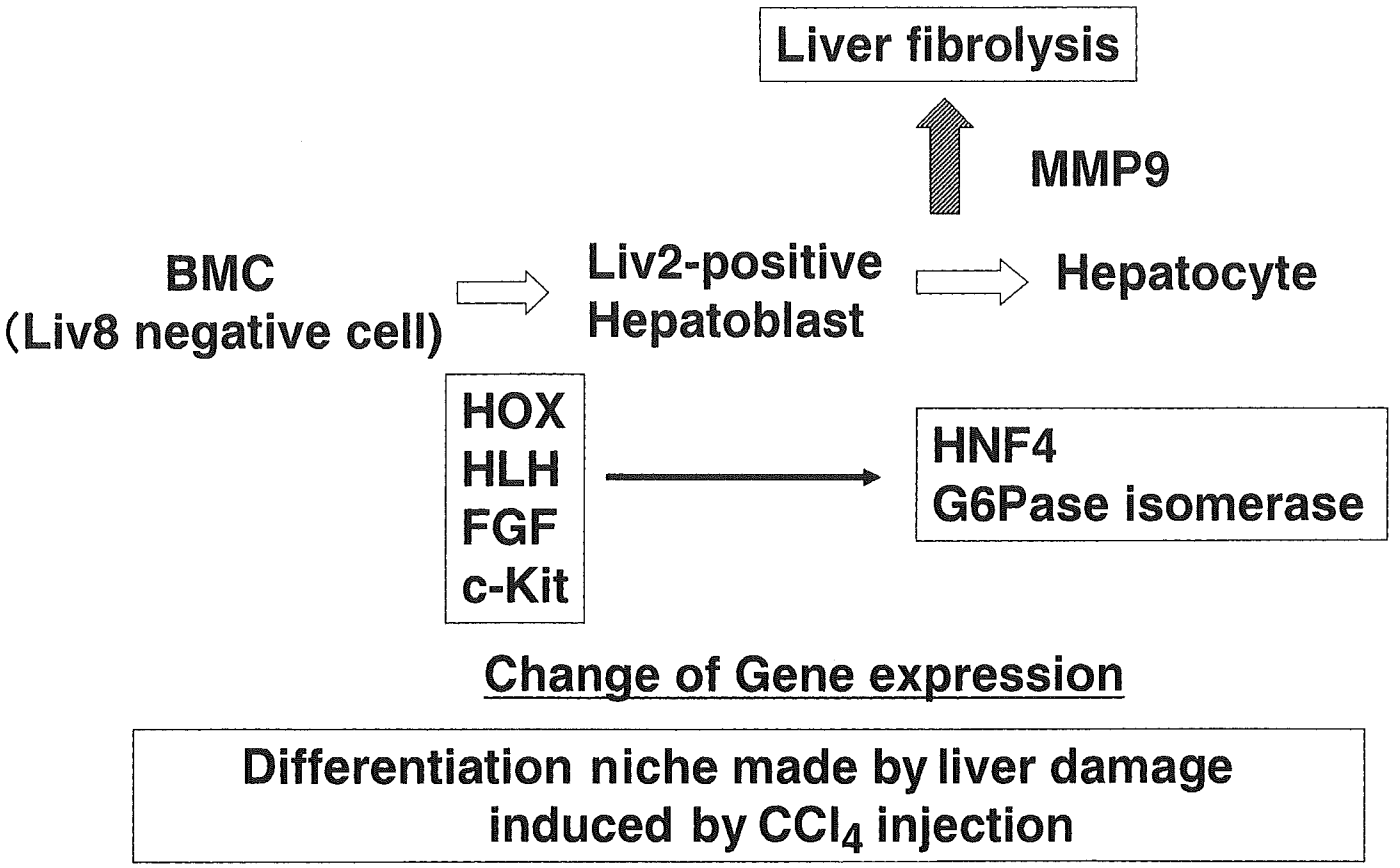

Fig. 1. Summary of the green fluorescent protein/carbon tetrachloride (*GFP/CCl_4*) model. *BMC*, bone marrow cell; *MMP-9*, matrix metalloproteinase-9; *G6Pase*, glucose-6-phosphatase; *HNF4*, hepatocyte nuclear factor 4

tion, the genes related to morphology were activated, and later, genes associated with liver metabolism were activated. Finally, BMC transplantation improved liver function, ameliorated liver fibrosis, and improved the survival rate. These findings strongly support the development of a new cell therapy, using BMCs, to cure liver cirrhosis, especially because autologous BMC transplantation has few ethical problems and BMC transplantation itself is already an established treatment for hematological diseases. Based on the results obtained in basic research using the GFP/CCl_4 model, we prepared a clinical study. We started a phase I clinical study called "Autologous BMC transplantation for liver cirrhosis patients", on November 14, 2003. The study is a first and important step toward the development of a new cell therapy to cure liver failure. We will report the outcome of the phase I clinical study at a later time, and we will combine the basic and clinical studies as part of our translational research, in order to develop a new therapy to cure liver cirrhosis.

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Editorial

Use of bone marrow cells for the development of cellular therapy in liver diseases

Currently, liver transplantation is one of the most effective therapies for advanced liver diseases. However, transplantation has many problems such as "lack of donor", "operative damage", "rejection", and "high expense". Cell transplantation therapy will be a minimally invasive procedure with potentially fewer complications.

A somatic human stem cell that could be propagated in large quantities while retaining its ability to differentiate into different cell types could serve as a highly valuable resource for the development of cellular therapy in liver diseases. If we limit the definition of stem cell to its ability to self renew and reconstitute a given tissue in vivo, hepatocytes fulfill both criteria.

But hepatocyte transplantation has very rarely produced therapeutic effects in human clinical trials mainly because their numbers are too low to achieve a biological effect [1,2]. Under certain conditions when hepatocyte replication is blocked, bipotent oval cells proliferate and participate in liver regeneration. However, the fact that they have been shown to generate hepatocellular carcinoma and cholangio carcinoma in rodents is a concern for their use for cell therapy.

As a result bone marrow cells are now being considered. The capacity of bone marrow cells (BMCs) to differentiate into hepatocytes and intestinal cells was found using Y chromosome detection in autopsy analysis of human female recipients of BMCs from male donors [3–6]. BMC transplantation itself is an established treatment for hematological diseases. These results suggest that bone marrow is an attractive cell source for regenerative medicine, because obtaining BMC is easier than for other tissue specific stem cells [7,8]. In the field of cardiovascular diseases, clinical studies have been performed to evaluate the use of BMCs in regenerating the myocardium and vessels of limb ischemia [9–12]. Although various theories explain the existence of pluripotent stem cells in BMCs the exact composition of stem cells in BMCs remains unclear. The following cell types are known to exist in bone marrow: hematopoietic stem cell (HSC) [13,14], side population cells (SP) [15], and mesenchymal stem cells (MSC) [16]. Although past studies used the existing antibodies and techniques, there have been no studies based on the findings associated with natural liver development. Hematopoietic stem cells (HSCs) have been shown to differentiate into hepatocytes under selective pressure like fumarylacetylacetate (FAH)-deficient mice simply fused with resident hepatocyte [14]. This fusion event has been demonstrated to occur between resident hepatocytes and myelomonocytes [17,18], and also in normal mice using the Cre-cox system [19]. However, using the same approach Harris et al. recently demonstrated that epithelial cells can develop from bone marrow cell without cell fusion [20]. Also, many recent publications have suggested that bone marrow-derived hepatocytes could have originated from the mesenchymal compartment rather than the hematopoietic compartment [21–24]. Moreover, it has been demonstrated that BMC transplantation in a CCl_4-injured liver reduced liver fibrosis resulting in an improved survival rate [25,26]. This effect was brought by the subpopulation of non-hematopoietic cells in the bone marrow.

From 14 November 2004, we initiated a Phase I clinical study (autologous BMC transplantation for liver cirrhosis patients). This study is the first attempt to develop a new cell therapy to cure patients with liver failure. In our next report, we will detail the outcome of our Phase I clinical study. The combination of a basic and clinical study is translational research. We will proceed to translational research to develop a new therapy to cure liver cirrhosis patients.

Thus, there are still many questions to be answered, e.g. is fusion necessary? Shi et al. [27] in the issue of Hepatology Research, clearly showed the potential of bone marrow cells in cellular therapy for liver disease.

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REGULAR ARTICLE

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Fibroblast growth factor 2 facilitates the differentiation of transplanted bone marrow cells into hepatocytes

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Abstract We have developed an in vivo mouse model, the green fluorescent protein (GFP)/carbon tetrachloride (CCl_4) model, and have previously reported that transplanted GFP-positive bone marrow cells (BMCs) differentiate into hepatocytes via hepatoblast intermediates. Here, we have investigated the growth factors that are closely related to the differentiation of transplanted BMCs into hepatocytes, and the way that a specific growth factor affects the differentiation process in the GFP/CCl_4 model. We performed immunohistochemical analysis to identify an important growth factor in our model, viz., fibroblast growth factor (FGF). In liver samples, the expression of FGF1 and FGF2 and of FGF receptors (FGFRs; FGFR1, FGFR2) was significantly elevated with time after bone marrow transplantation (BMT) compared with other factors, and co-expression of GFP and FGFs or FGFRs could be detected. We then analyzed the effect and molecular mechanism of FGF signaling on the enhancement of BMC differentiation into hepatocytes by immunohistochemistry, immunoblotting, and microarray analysis. Treatment with recombinant FGF (rFGF), especially rFGF2, elevated the repopulation rate of GFP-positive cells in the liver and significantly increased the expression of both Liv2 (hepatoblast marker) and albumin (hepatocyte marker). Administration of rFGF2 at BMT also raised serum albumin levels and improved the survival rate. Transplantation of BMCs with rFGF2 specifically activated tumor necrosis factor-alpha (TNF-α) signaling. Thus, FGF2 facilitates the differentiation of transplanted BMCs into albumin-producing hepatocytes via Liv2-positive hepatoblast intermediates through the activation of TNF-α signaling. Administration of FGF2 in combination with BMT improves the liver function and prognosis of mice with CCl_4-induced liver damage.

Keywords Bone marrow cell · Stem cell · Hepatocyte · Differentiation · Liver · Fibroblast growth factor · Tumor necrosis factor · Mouse

Abbreviations BMC: bone marrow cell · BMT: bone marrow transplantation · GFP: green fluorescent protein · CCl_4: carbon tetrachloride · SOM: self-organizing map · FGF: fibroblast growth factor · EGF: epidermal growth factor · EGFR: epidermal growth factor receptor · FGFR: fibroblast growth factor receptor · HGF: hepatocyte growth factor · VEGF: vascular endothelial growth factor · VEGFR: vascular endothelial growth factor receptor ·

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PDGF: platelet-derived growth factor · PDGFR: platelet-derived growth factor receptor · TGFβ: transforming growth factor β · TGFβR: transforming growth factor β receptor · rFGF: recombinant fibroblast growth factor · TNF-α: tumor necrosis factor-α · TNFIP3: tumor necrosis factor-α induced protein 3 · NF-κB: nuclear factor-κB

Introduction

Liver cirrhosis is the end-stage of chronic liver disease and is extremely difficult to treat. Currently, liver transplantation is one of the effective therapies available to patients who face this life-threatening condition. However, this treatment presents serious problems, such as the lack of a donor, operative damage, rejection, and high cost. Regenerative therapy by stem cell transplantation is a promising approach for the treatment of patients with severe liver disease. The capacity of bone marrow cells (BMCs) to differentiate into hepatocytes and intestinal cells was first identified through the detection of Y-chromosome-containing cells in postmortem samples from female recipients of BMCs from male donors (Alison et al. 2000; Theise et al. 2000). Bone marrow transplantation (BMT) is now an established treatment for hematological diseases, and several clinical studies have evaluated the potential of BMCs in regeneration of the myocardium and blood vessels (Orlic et al. 2001; Stamm et al. 2003; Wexler et al. 2003). Together, these findings suggest that BMCs will be an effective cell source for regenerative therapy in the liver.

To realize the potential for cell therapy by BMCs, we have developed an in vivo mouse model to monitor the differentiation of BMCs into hepatocytes, viz., the green fluorescent protein (GFP)/carbon tetrachloride (CCl_4) model (Terai et al. 2003). We have shown that transplanted GFP-positive BMCs populate the damaged liver and differentiate into albumin-producing hepatocytes via hepatoblast intermediates under CCl_4-induced persistent liver-damage conditions (Terai et al. 2003; Yamamoto et al. 2004). Furthermore, BMT elevates serum albumin levels, reduces liver fibrosis, and improves the survival rate of CCl_4-treated mice (Sakaida et al. 2004). These results suggest that BMT could become an effective treatment for patients with liver failure.

Several lines of evidence support the idea that chronic liver injury by CCl_4 is required to induce the differentiation of transplanted stem cells into hepatocyte-like cells (Wang et al. 2003; Kollet et al. 2003). In our model, similarly, some feature associated with continuous intraperitoneal administration of CCl_4, which causes persistent liver damage, actually appears to facilitate or induce migration of BMCs to the liver and differentiation of BMCs into hepatocytes. In addition, we have used microarray analysis, together with a self-organizing map (SOM), to show that dramatic gene activation occurs after BMT into mice with CCl_4-induced liver damage (Omori et al. 2004). Genes associated with morphology are activated at an early stage, whereas genes that regulate differentiation of hepatocytes are up-regulated at a later stage.

Growth factors are known to affect cell proliferation and differentiation and reportedly participate in repair processes of many organs. Moreover, clinical trials have been carried out with several growth factors for the therapy of peripheral vascular disorders, ischemic heart diseases, and cutaneous chronic wounds through neoangiogenesis (Laham et al. 2000; Lederman et al. 2002; Fu et al. 2002). Here, we report the identification of a growth factor that induces cellular repopulation of damaged liver and the differentiation of transplanted BMCs into hepatocytes. Furthermore, we report the results of our investigation into the way that the identified growth factor, namely fibroblast growth factor (FGF), affects these processes in the GFP/CCl_4 mouse model of liver damage and regenerative treatment.

Materials and methods

Experimental protocol: GFP/CCl_4 model

C57 BL/6 Tg14 (act-EGFP) OsbY01 mice (GFP transgenic mice) were kindly provided by Dr. Masaru Okabe (Genome Research Center, Osaka University, Osaka, Japan), and C57 BL/6 female mice were purchased from Japan SLC (Shizuoka, Japan). In this study, injection of 1.0 ml/kg body weight of CCl_4 into recipient mice was performed at 6 weeks of age via the peritoneum twice a week for 4 weeks to induce persistent liver damage. On one day after 4 weeks of CCl_4 treatment, 1×10^5 GFP-positive BMCs were transplanted slowly, by using a 31-gauge needle and Hamilton syringe, via the tail vein as previously described (Terai et al. 2003). After BMT, the same dose of CCl_4 was continuously injected twice a week to maintain persistent liver damage. Mice treated with CCl_4 without BMT were used as a control group. Individual mice were killed at 48 h and every week after BMT. All processes, including surgical steps, conformed to the guidelines of Yamaguchi University for animal and recombinant DNA experiments.

Immunohistochemical staining

We obtained liver samples from six independent mice in each group, and immunohistochemical staining was performed as previously described (Shinoda et al. 1992). Immunohistochemical samples were quantified by using a Provis microscope (Olympus, Tokyo, Japan) equipped with a charge-coupled device camera; the obtained images were subjected to computer-assisted analysis with MetaMorph software (Universal Imaging, Downingtown, Pa.). A total of 30 random fields per group were analyzed independently, and the percentage of stained area was calculated by using MetaMorph software. For screening, we examined the expression of several growth factor receptors by using commercial antibodies against the following: epidermal growth factor (EGF) receptor (EGFR), FGF receptor 1 (FGFR1), and FGFR2, all from Santa Cruz Biotechnology (Calif., USA); hepatocyte growth factor (HGF) receptor (c-Met) from R&D Systems (Minn., USA); vascular

endothelial growth factor (VEGF) receptor 1 (VEGFR1), VEGFR2, platelet-derived growth factor (PDGF) receptor α (PDGFRα), PDGFRβ, transforming growth factor β (TGFβ) receptor 1 (TGFβR1), and TGFβR2, all from Santa Cruz Biotechnology. In addition, we analyzed the expression of FGFRs and FGFs by using antibodies against FGFR1, FGFR2, FGF1, and FGF2 (Santa Cruz Biotechnology) in two groups: the BMT(+) group that received both CCl_4 treatment and BMT, and the BMT(−) control group that received CCl_4-only treatment without BMT. Immunofluorescent detection of GFP and FGFRs or FGFs was performed with the following secondary antibodies: Alexa Fluor R-488 and R−568 donkey anti-goat IgG (H+L) conjugates and Alexa Fluor R-488 goat anti-rabbit IgG (H+L) conjugate (Molecular Probes, Eugene, Ore., USA). The analysis was performed as previously described (Terai et al. 2003; Sakaida et al. 2004).

Analysis for the effect of recombinant FGF

Recombinant FGF1 (rFGF1) or rFGF2 (R&D Systems) was administered as follows. A treatment dose of 30 μg/kg was deemed appropriate based on previous reports of clinical trials for ischemic heart diseases and peripheral vascular disorders (Laham et al. 2000; Lederman et al. 2002). C57 BL/6 female mice with CCl_4-induced chronic liver damage were divided into six groups: (1) CCl_4 group (treatment with neither BMCs nor rFGFs); (2) rFGF1 group (rFGF1-only treatment without BMT); (3) rFGF2 group (rFGF2-only treatment without BMT); (4) BMC group (BMC-only transplantation without rFGFs); (5) BMC+rFGF1 group (treatment with both BMCs and rFGF1); and (6) BMC+rFGF2 group (treatment with both BMCs and rFGF2). Isolated GFP-positive BMCs were incubated with rFGF1 or rFGF2 for approximately 30 s before transplantation and then injected into recipient mice with a 31-gauge needle and Hamilton syringe via the tail vein. After transplantation, CCl_4 injections were continued at the same dose twice a week. Mice were killed at 48 h after BMT and every week thereafter for up to 4 weeks. Immunohistochemical analysis with anti-GFP (Santa Cruz Biotechnology), anti-Liv2 (hepatoblast marker; Watanabe et al. 2002), anti-albumin (Bethyl Laboratories, Tex., USA), and anti-tumor necrosis factor-α (TNF-α; TECHNE, Min., USA) antibodies was performed on these samples (n=6 in each group). The proportion of stained area was calculated by using MetaMorph software at a total of 30 random fields per group. Moreover, serum albumin level was measured (n=6 in each group) by using the SPOTCHEM EZ SP-4430 dry chemical system (Arkray, Kyoto, Japan; Terai et al. 2003; Sakaida et al. 2004). We calculated the survival rate with follow-up for 150 days after transplantation (n=15 in each group) by using the Kaplan-Meier method.

DNA-microarray analysis: first screen and second screen

We excised an equal amount of liver sample from three independent mice in each group at 48 h and 1 week after transplantation. Total RNA was isolated from the samples by using an Atlas Glass Total RNA Isolation Kit (Clontech, Palo Alto, Calif.), and single strands of cDNA were synthesized by using an Atlas Glass Fluorescent Labeling Kit (Clontech). DNA-microarray analysis was subsequently conducted with the Atlas Glass Mouse 1.0K Microarray System (Clontech; Omori et al. 2004; Ishigaki et al. 2002). The signal intensity of each gene was measured by a fluorescent scanner (Axon Instruments, Calif., USA), and the transient differences in gene expression between two groups were assessed with the Array Gauge System (Fuji Film, Tokyo, Japan). These methods were identical to those previously performed (Omori et al. 2004). In this study, a total of 1,108 genes on the DNA-microarray were analyzed, and two screens were carried out.

We did the first screen to select genes that were significantly up-regulated by BMT with rFGF2 treatment as compared with BMT-alone. For each gene, the gene expression was analyzed by the specific equations as follows:

x_{i1}; expression level of gene i at 48 h after transplantation, in the BMC group
y_{i1}; expression level of gene i at 48 h after transplantation, in BMC+rFGF2 group
x_{i2}; expression level of gene i at 1 week after transplantation, in BMC group
y_{i2}; expression level of gene i at 1 week after the transplantation in BMC+rFGF2 group

$(i = 1, 2, 3, , , , , 1108)$

The chronological change of the expression level of gene i in each group was expressed as follows:

$$\text{BMC group; } f_{i1} = \log_{10}(x_{i2}/x_{i1})$$

$$\text{BMC + rFGF2 group; } f_{i2} = \log_{10}(y_{i2}/y_{i1})$$

The difference in the chronological change of the gene expression between two groups was defined by

$$F_i = f_{i2} - f_{i1} = \log_{10}\left[(y_{i2}/y_{i1})/(x_{i2}/x_{i1})\right]$$

If $(x_{i2}\text{-}x_{i1}) = (y_{i2}/y_{i1})$, the value of F_i, i.e., $\log_{10}1$ is zero. We focused on the genes with a large value of F.

For each gene, we computed the value of F and selected genes that fulfilled the conditions, $f_{i1}>0$, $f_{i2}>0$, and $F_i>0.477(=\log_{10}3)$. An $F_i>0.477$ means that the ratio of y_{i2}/y_{i1} to x_{i2}/x_{i1} is more than 3. These genes were considered to be significantly up-regulated by rFGF2 treatment in addition to BMT.

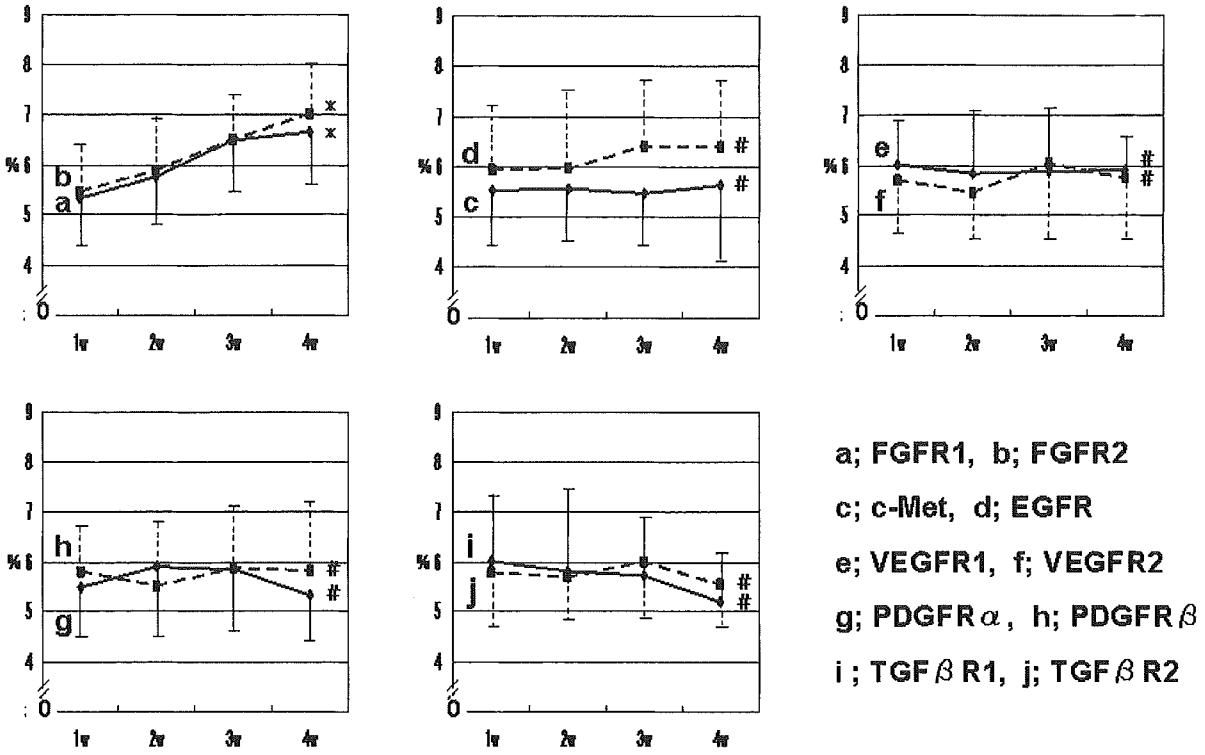

Fig. 1 Time-course of distribution of several growth factor receptors in the GFP/CCl_4 model system. Expression of FGFR1 and FGFR2 significantly increases with time after BMT (*x-axis* time after BMT (in weeks), *y-axis* percentage of stained area in immunohistochemistry). *Significant difference compared with the value at 1 week ($P<0.05$). #No significant difference compared with the value at 1 week ($P>0.05$)

We carried out the second screen to separate the gene that was most specifically activated by rFGF2 treatment with BMT out of the genes selected from the screen I. This analysis was performed by using the additional equations as follows:

$x_i'1$; expression level of gene i at 48 h in CCl_4 group
$y_i'1$; the expression level of gene i at 48 h in rFGF2 group
$x_i'2$; the expression level of gene i at 1 week in CCl_4 group
$y_i'2$; the expression level of gene i at 1 week in rFGF2 group

CCl_4 group; $f_i'1 = \log_{10}(x_i'2/x_i'1)$

rFGF2 group; $f_i'2 = \log_{10}(y_i'2/y_i'1)$

We compared the f value (chronological change of the gene expression) of each gene selected from screen I in two

Table 1 Percent immunohistochemically stained area in areas treated with anti-FGFR1 or anti-FGFR 2 and anti-FGF1 or anti-FGF2

Receptor or factor	Treatment	One week[a]	Four weeks[b]	Percentage change[c]
FGFR1	BMT(+)[d]	5.3%±1.0[e]	6.7%±1.1[e, f]	26.4%
	BMT(−)[g]	3.7%±0.7	4.2%±0.9	13.5%
FGFR2	BMT(+)	5.4%±0.9[e]	7.0%±1.0[e, f]	29.6%
	BMT(−)	3.8%±0.9	4.4%±1.0	15.8%
FGF1	BMT(+)	5.5%±0.9[e]	7.1%±1.0[e, f]	29.1%
	BMT(−)	3.8%±0.7	4.5%±0.5	18.4%
FGF2	BMT(+)	5.7%±1.1[e]	7.5%±1.1[e, f]	31.6%
	BMT(−)	3.9%±1.0	4.6%±1.0	17.9%

[a]One week in the BMT(−) group represents 5 weeks of CCl_4 injection
[b]Four weeks in the BMT(−) group represents 8 weeks of CCl_4 injection
[c]Percentage change over time (from 1 week to 4 weeks) in each group
[d]BMT(+): BMC transplantation group with CCl_4-induced persistent liver damage
[e]Significant difference compared with the value at same period in the BMT(−) group ($P<0.05$)
[f]Significant difference compared with the value at 1 week ($P<0.05$)
[g]BMT(−): control CCl_4-induced persistent liver damage group without BMT
BMT significantly elevates the expression of FGFRs and FGFs with time compared with the BMT (−) control group
Values are shown as means±SD

groups: the CCl_4 group and rFGF2 group. Those for the BMC group and BMC+rFGF2 group had been computed in screen I.

Western blot analysis of TNF-α

We isolated cell lysate from the liver sample at 1 week after transplantation in four groups: CCl_4 group, rFGF2 group, BMC group, and BMC+rFGF2 group. Protein was obtained by homogenization with lysis buffer (20 mM TRIS-HCl pH 7.5, 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycol-bis-N, N'-tetraacetic acid, 1% Triton X, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) and then centrifuged. A total of 100 μg protein were analyzed by sodium dodecylsulfate/polyacrylamide gel electrophoresis and immunoblot. Protein was electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad, CA, USA), which was incubated with a 1:500 dilution of goat anti-mouse TNF-α antibody, washed, and then incubated with a 1:5,000 dilution of anti-goat IgG conjugated to horseradish peroxidase (Amersham Biosciences, N.J., USA). Bands were visualized with an enhanced chemiluminescence Western blotting detection system (Amersham Biosciences, NJ, USA). All experiments were repeated independently at least three times with reproducible results.

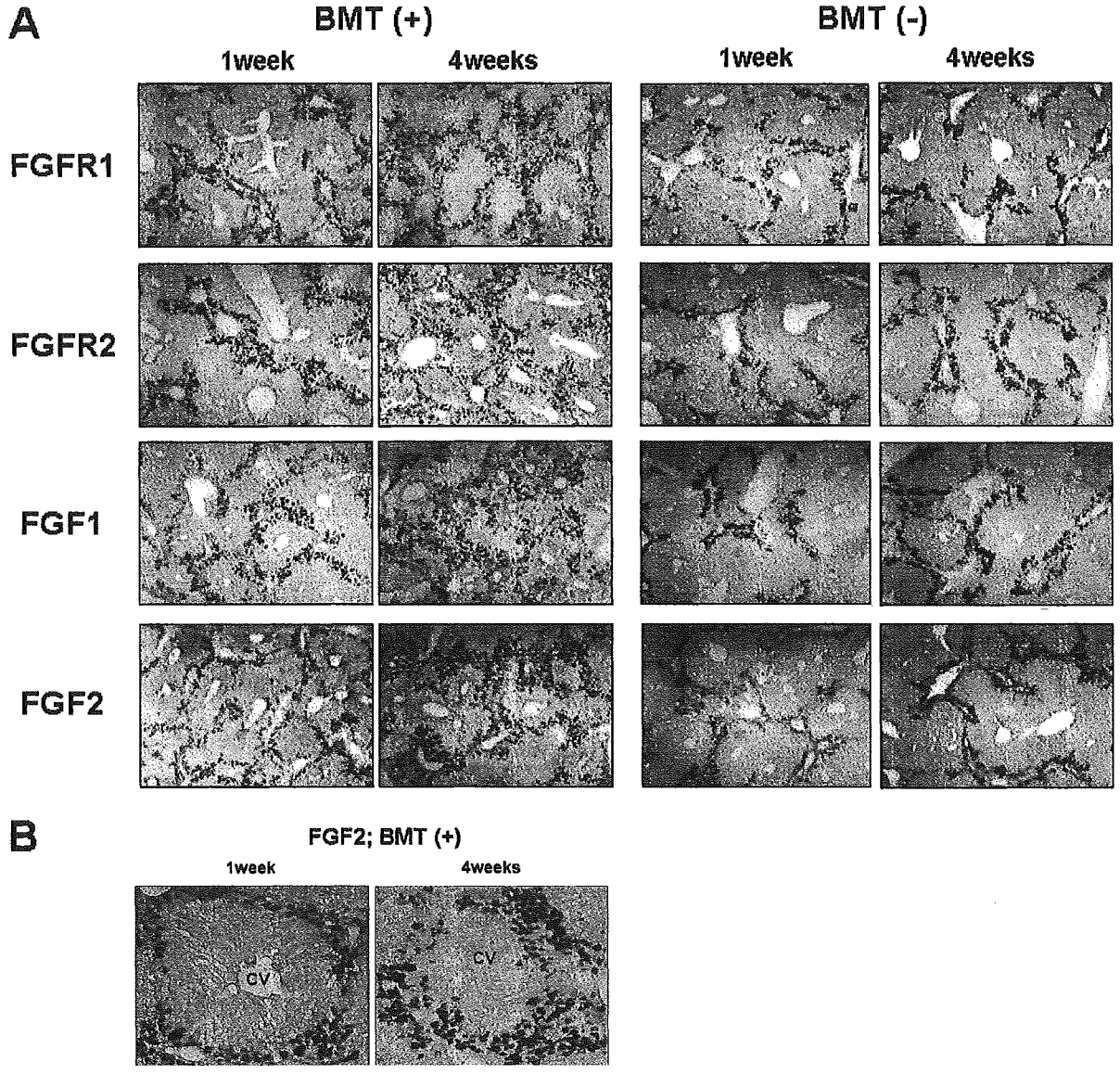

Fig. 2 Expression of FGFRs and FGFs (*BMT(+)* BMC transplantation group with CCl_4-induced persistent liver damage, *BMT(−)* control CCl_4-induced persistent liver damage group without BMT). One week (*1week*) in BMT(−) group represents 5 weeks of CCl_4 injection, and four weeks (*4weeks*) in BMT(−) group represents 8 weeks of CCl_4 injection. **A** Immunohistochemistry of FGFR1, FGFR2 and FGF1, FGF2 in BMT(+) and BMT(−) groups. In the BMT(+) group, the expression of FGFRs and FGFs significantly increases with time as compared with the BMT(−) control group, and the distribution of the proteins eventually extends from the peri-portal area into the intra-lobule. ×40 **B** Typical higher magnification image of FGF2 immunohistochemical staining in BMT(+) group. FGF2-positive cells proliferate and spread from the peri-portal area into the central area with time after BMT (*CV* central vein). ×200

Statistical analysis

Values are shown as means±SD. Data were analyzed with Fisher's exact test. A P-value of <0.05 was considered statistically significant.

Results

Expression of FGFRs significantly increases with time after BMT

For screening, we performed immunohistochemical staining with ten different antibodies recognizing growth factor receptors in the GFP/CCl_4 model. As shown in Fig. 1a,b, the percentage of stained area for FGFR1 and FGFR2 gradually and significantly increased each week after BMT (P<0.05). On the other hand, the expression of other growth factor receptors (c-Met, EGFR, VEGFR1/2, PDGFR α/β, and TGFβR 1/2) could be detected, but no significant chronological change in the expression level after BMT was found (Fig. 1c–j). These results suggest that FGF-FGFR signaling is most closely related to the process of BMC differentiation into hepatocytes in our model.

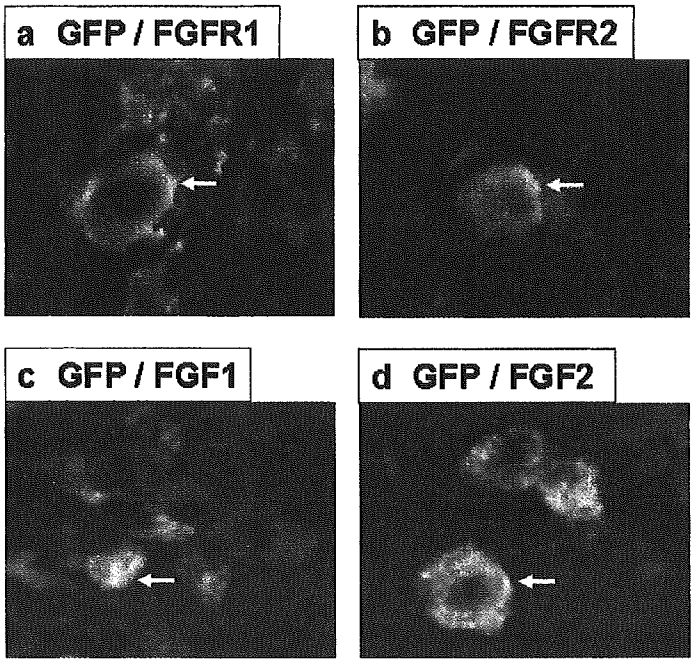

Fig. 3 Co-expression of GFP and FGFRs or FGFs in GFP/CCl_4 model. FGFRs are detectable at the cell surface of GFP-positive cells, and FGFs are detected in the cytoplasm of GFP-positive cells. Double-fluorescent merged images (*arrows* co-expression regions). **a** GFP (*green*), FGFR1 (*red*), co-expression of both GFP and FGFR1 (*yellow*). **b** GFP (*green*), FGFR2 (*red*), co-expression of both GFP and FGFR2 (*yellow*). **c** GFP (*green*), FGF1 (*red*), co-expression of both GFP and FGF1 (*yellow*). **d** GFP (*green*), FGF2 (*red*), co-expression of both GFP and FGF2 (*yellow*). ×400

Transplanted BMCs express FGFs and FGFRs during differentiation into hepatocytes

We further analyzed the expression of FGF1 and FGF2 and their receptors, FGFR1 and FGFR2, in the BMT(+) and BMT(−) groups. As shown in Table 1, BMT significantly elevated the percentage of the stained area of FGFRs and FGFs with time as compared with the BMT(−) control group (P<0.05). Furthermore, for each protein, the "percentage change" over time in the BMT(+) group was higher than that in the controls. Another obvious difference between the two groups was the distribution of the stained regions. Expression of FGFs and FGFRs was not detectable in the livers of normal mice (data not shown). In the BMT(−) group, expression of FGFs and FGFRs could be detected but consistently remained localized only to the peri-portal area. In contrast, in the BMT(+) group, expression of FGFs and FGFRs appeared to be up-regulated relative to control group, and the distribution of the proteins eventually extended from the peri-portal area into the intra-lobule (Fig. 2A). For example, the typical enlarged image of FGF2 immunohistochemical staining is shown in Fig. 2B. FGF2-positive cells proliferated and spread from the peri-portal area into the central area with time after BMT. To determine whether FGFs and FGFRs were detectable in the transplanted BMCs themselves, we analyzed the co-expression of GFP and FGFs or FGFRs. As shown in Fig. 3, co-expression of GFP and FGFRs was detectable at the cell surface. In addition, GFP and FGFs were both detected in the cytoplasm. These results indicate that transplanted BMCs express both FGFs and FGFRs during differentiation into hepatocytes.

FGF2 significantly elevates the repopulation rate of GFP-positive cells in the liver and increases the expression of both Liv2 and albumin

We investigated the effect of rFGF treatment on the process of the differentiation of BMCs into hepatocytes. In each group (BMC group, BMC+rFGF1 group, and BMC+rFGF2 group), the number of GFP-positive cells in the liver gradually increased after transplantation. GFP-positive cells were first detected in the peri-portal area and then in liver lobules with actively forming hepatic cords, suggesting a gradual spread into that region of the liver (Fig. 4A). In the BMC+rFGF1 group, the percentage of GFP-positive cells in the liver was 7.7±1.1% (1.4-fold more than in the BMC group) at 1 week, and 9.7±1.1% (1.2-fold more than in the BMC group) at 4 weeks after transplantation. The BMC+rFGF2 group showed the highest rate of liver repopulation among the three groups with a significant difference (P<0.05): 8.7±1.2% (1.6-fold more than in the BMC group, 1.1-fold more than in the BMC+rFGF1 group) at 1 week, and 10.7±1.2% (1.3-fold more than in the BMC group, 1.1-fold more than in the BMC+rFGF1 group) at 4 weeks after transplantation (Fig. 4B). The percentages of Liv2- and albumin-stained areas are summarized in Table 2. Treatment with rFGF, especially rFGF2, in combination with BMT

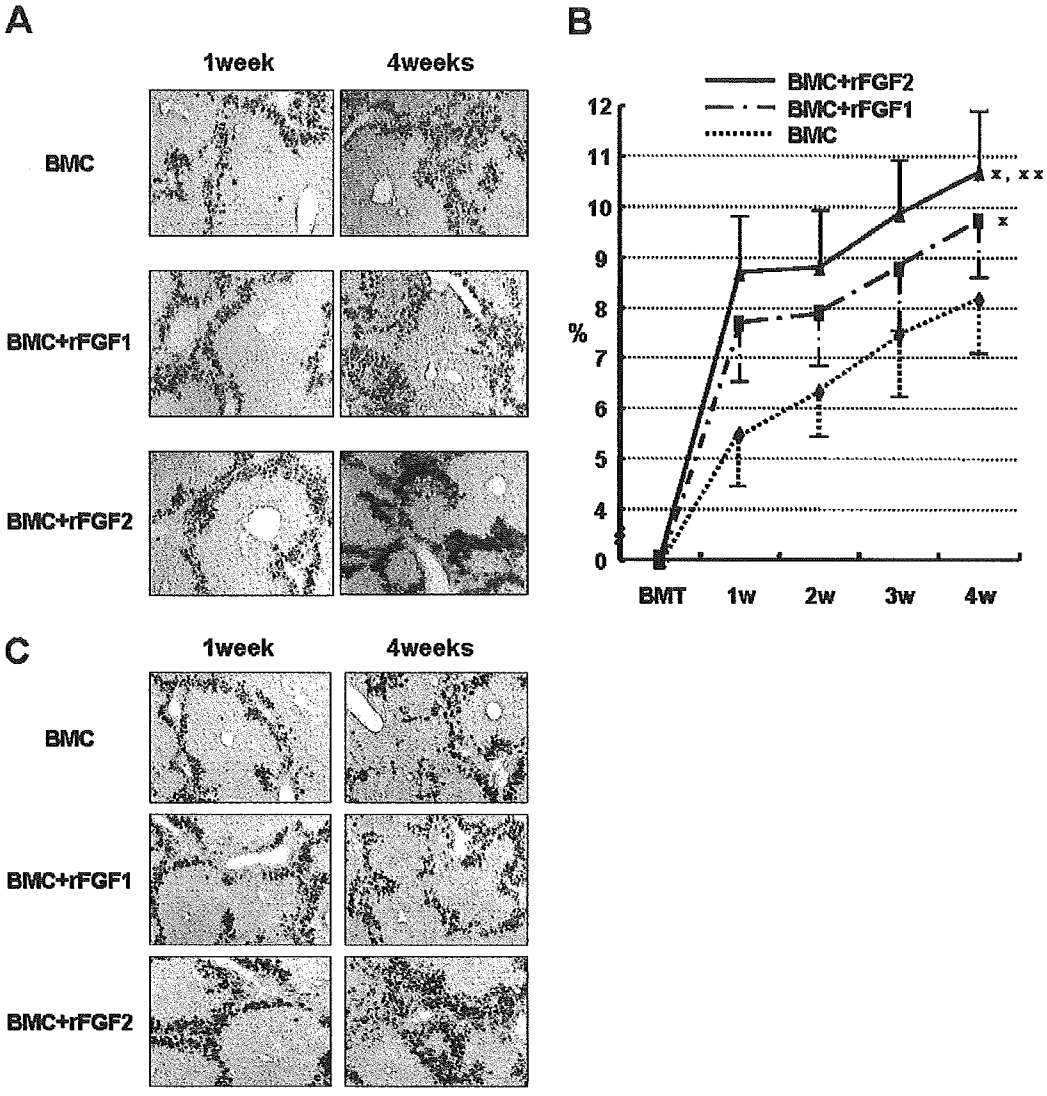

Fig. 4 Expression of GFP and Liv2 in BMC, BMC+rFGF1, and BMC+rFGF2 groups (*BMC* BMC-only transplantation without rFGF treatment *BMC+rFGF1* treatment with both BMCs and rFGF1, *BMC+rFGF2* treatment with both BMCs and rFGF2. **A** Immunohistochemistry for GFP. GFP-positive cells in the liver gradually increase and spread from the peri-portal area into the intra-lobule with actively forming hepatic cords in each group. The level of GFP expression in the BMC+rFGF2 group is the highest amongst the three groups. ×200. **B** Time-course of the percentage of GFP-positive cells in the liver. The BMC+rFGF2 group shows the highest percentage of stained area of GFP among three groups. *Significant difference compared with the value at same period in BMC group ($P<0.05$). **Significant difference compared with the value at same period in BMC+rFGF1 group ($P<0.05$). **C** Immunohistochemistry for Liv2. The distribution of Liv2-positive cells in the liver is similar to that of GFP-positive cells, and the level of Liv2 expression in the BMC+rFGF2 group is the highest amongst the three groups. ×200

Table 2 Percentage area stained immunohistochemically for Liv2 and albumin

Protein	Treatment	One week	Four weeks
Liv2 (hepatoblast marker)	BMC[a]	5.4%±0.9	6.0%±0.7
	BMC+rFGF1[b]	6.0%±0.6[c]	6.8%±0.9[c]
	BMC+rFGF2[d]	6.3%±0.8[c]	7.2%±0.9[c, e]
Albumin (hepatocyte marker)	BMC	11.1%±1.0	12.1%±1.0
	BMC+rFGF1	11.9%±0.9[c]	13.1%±1.0[c]
	BMC+rFGF2	12.1%±0.8[c]	13.6%±0.9[c, e]

[a]*BMC* BMC-only transplantation without rFGFs
[b]*BMC+rFGF1* Treatment with both BMCs and rFGF1
[c]Significant difference compared with the value at the same period in BMC group ($P<0.05$)
[d]*BMC+rFGF2* Treatment with both BMCs and rFGF2
[e]Significant difference compared with the value at the same period in BMC+rFGF1 group ($P<0.05$)

significantly increased the expression of both Liv2 and albumin compared with the BMC-only group ($P<0.05$). Moreover, the distribution of Liv2-positive cells in the liver was similar to that of GFP-positive cells (Fig. 4C). On the other hand, we could not detect Liv2 expression in either the rFGF1 group or the rFGF2 group without BMT (data not shown). Thus, FGFs, especially FGF2, are likely, directly or indirectly, to facilitate the ability of transplanted BMCs to populate the liver and to differentiate into hepatocytes via hepatoblast intermediates.

Administration of rFGF2 in combination with BMT significantly elevates serum albumin level and survival rate of mice with damaged liver

Next, we focused on FGF2 and further analyzed the effect of rFGF2 treatment with BMT. We compared serum albumin levels and survival rates for four groups: CCl_4 group, rFGF2 group, BMC group, and BMC+rFGF2 group (Fig. 5A,B). BMT itself significantly elevated both values compared with those of the control CCl_4 group ($P<0.05$), and BMT and rFGF2 treatment together resulted in serum albumin levels and survival rates that were significantly the

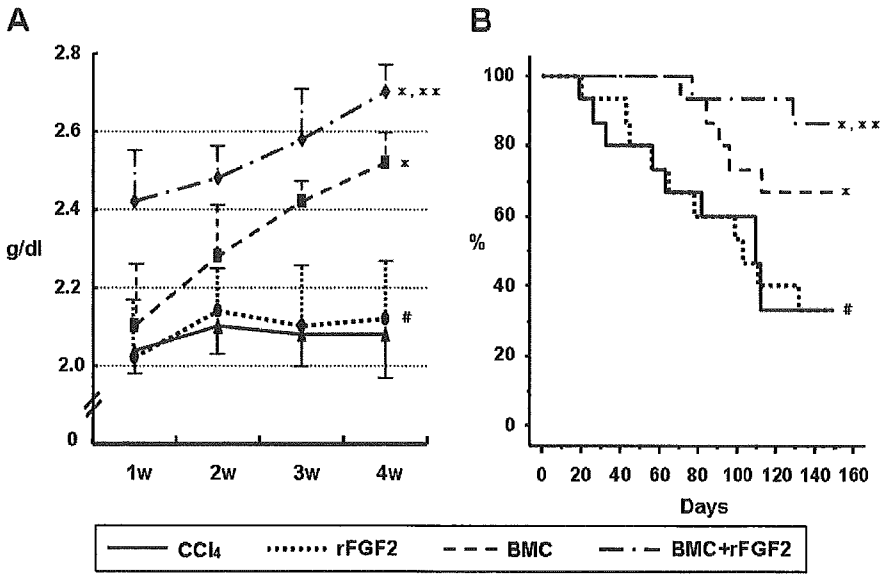

Fig. 5 Effect of rFGF2 treatment with BMT (*CCl_4* treatment with neither BMCs nor rFGFs, *rFGF2* rFGF2-only treatment without BMT, *BMC* BMC-only transplantation without rFGFs, *BMC+ rFGF2* treatment with both BMCs and rFGF2). **A** Time-course of serum albumin level. Serum albumin level is most significantly elevated by rFGF2 treatment in combination with BMT among four groups. *Significant difference compared with the value at the same period in the CCl_4 group ($P<0.05$). **Significant difference compared with the value at the same period in the BMC group ($P<0.05$). #No significant difference compared with the value at the same period in the CCl_4 group ($P>0.05$). **B** Cumulative survival analysis evaluated by the Kaplan-Meier method. Administration of rFGF2 with BMT improves the survival rate, which is significantly the highest among the four groups. *Significant difference compared with CCl_4 group ($P<0.05$). **Significant difference compared with BMC group ($P<0.05$). #No significant difference compared with CCl_4 group ($P>0.05$)

highest among the four groups ($P<0.05$). On the other hand, there was no significant difference between the CCl_4 group and rFGF2 group. These data suggest that administration of rFGF2 in combination with BMT is significantly the most effective for mice with damaged liver.

TNF-α signaling is activated by BMT with rFGF2 treatment

To improve our understanding of the molecular mechanism of the effect of FGF2 on the enhancement of the repop-

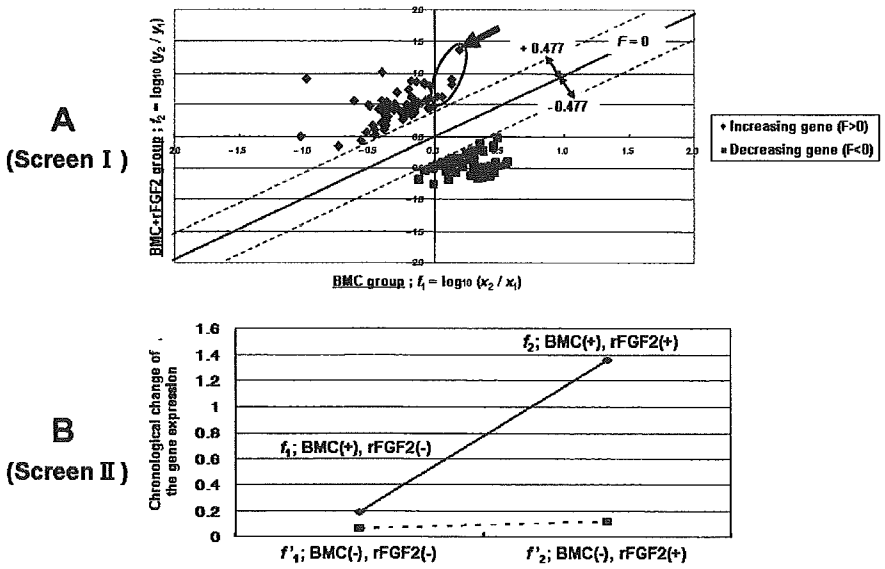

Fig. 6 Identification of the specific gene involved in repopulation and differentiation by using DNA-microarray analysis and two screens. **A** Plot of genes in the f_1–f_2 space (*Screen I*). The five specific genes out of 1108 genes are selected in screen I (*encircled plots* the five selected genes, *large arrow* TNFIP3). **B** Chronological change of the gene expression of TNFIP3 (*Screen II*). TNFIP3 is most specifically activated by treatment with both BMCs and rFGF2 compared with others in Screen II. The *f* value represents the chronological change of the expression level of a gene in each group (*f1';BMC(−),rFGF2(−)* CCl_4 group treated with neither BMCs nor rFGFs, *f2';BMC(−),rFGF2 (+)* rFGF2-only treatment without BMT, *f1;BMC(+),rFGF2(−)* BMC-only transplantation without rFGFs, *f2;BMC(+),rFGF2(+)* treatment with both BMCs and rFGF2)

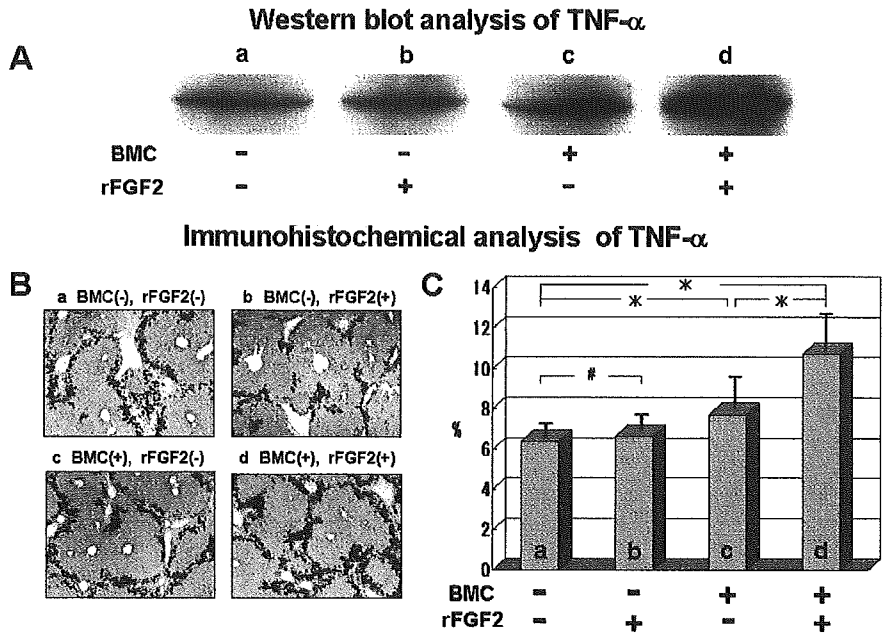

Fig. 7 Expression of TNF-α in CCl_4, rFGF2, BMC, and BMC+rFGF2 groups (*a* CCl_4 group: treatment with neither BMCs nor rFGFs, *b* rFGF2 group: rFGF2-only treatment without BMT, *c* BMC group: BMC-only transplantation without rFGFs, *d* BMC+rFGF2 group: treatment with both BMCs and rFGF2). **A** Western blot analysis of TNF-α. The level of TNF-α expression in BMC+rFGF2 group is the highest amongst the four groups in immunoblot analysis. **B** Immunohistochemical analysis of TNF-α. Expression of TNF-α slightly increases with BMT-alone and is significantly elevated by rFGF2 treatment in combination with BMT compared with other treatments. ×40 **C** Percentage of stained area of TNF-α. The BMC+rFGF2 group shows the highest percentage of stained area of TNF-α amongst the four groups. *Significant difference between two groups ($P<0.05$). #No significant difference between two groups ($P>0.05$)

ulation and differentiation of BMCs in the GFP/CCl_4 model, we used microarray analysis to profile the chronological change in gene expression before and after rFGF2 treatment. We carried out two screens to identify the specific gene that was activated by rFGF2 treatment in combination with BMT in mice with CCl_4-induced liver damage. Figure 6A shows the plot of genes in the 2-dimensional space spanned by f_1 and f_2 in screen I. We could select five genes that fulfilled the conditions $f_1>0$, $f_2>0$ and $F>0.477$ ($=\log_{10}3$), out of 1,108 genes. These specific genes are TNF-α induced protein 3 (TNFIP3), hypoxia-inducible factor 1α, neural plakophilin-related arm-repeat protein, semaphorin B, and zinc-finger protein 46. Screen II was performed to separate the gene that was most specifically up-regulated by rFGF2 treatment with BMT out of five genes selected from screen I. In this analysis, TNFIP3 was most significantly activated when BMT and treatment with rFGF2 were combined compared with others (Fig. 6B). We next analyzed the expression of TNF-α protein in the liver from four groups at 1 week after transplantation: the CCl_4 group, rFGF2 group, BMC group, and BMC+rFGF2 group. Immunoblot analysis (Fig. 7A) revealed that BMT itself slightly elevated TNF-α expression, and the level of TNF-α expression in the BMC+rFGF2 group was the highest amongst the four groups. Immunohistochemical analysis confirmed the results of the immunoblot (Fig. 7B,C). Treatment of rFGF2 without BMT, by contrast, did not activate TNF-α signaling in our system. Together, these results suggest that FGF2 significantly activates TNF-α signaling during the process of the differentiation of BMCs into hepatocytes.

Discussion

A number of reports have demonstrated the potential of BMCs to differentiate into a variety of cell types, including hepatocytes (Theise et al. 2000; Orlic et al. 2001; Ferrari et al. 1998; Lagasse et al. 2000; Krause et al. 2001; Kotton et al. 2001; Petersen et al. 1999; Okamoto et al. 2002). We have previously developed an in vivo model, the GFP/CCl_4 mouse model, and have reported that transplanted BMCs differentiate into albumin-producing functional mature hepatocytes via Liv2-positive immature hepatoblast intermediates (Terai et al. 2003). Furthermore, BMT elevates the serum albumin level, reduces liver fibrosis, and improves the survival rate in our model (Sakaida et al. 2004). The mechanism behind the BMC plasticity that we have observed in the model is the subject of much debate. For example, the mechanism could reportedly involve cell fusion, nuclear reprogramming, or trans-differentiation (Terada et al. 2002; Ying et al. 2002; Ianus et al. 2003; Hochedlinger et al. 2004; Harris et al. 2004; Jang et al. 2004), and several lines of evidence have led us to believe that both cell fusion and trans-differentiation might be important to BMC plasticity. We have previously performed microarray analysis (using specific equations with SOM) to analyze the molecular cues that controlled the differentiation of BMCs into hepatocytes in GFP/CCl_4

model and found a dramatic change of gene expression during BMC differentiation (Omori et al. 2004). In the early stage after BMT, genes known to regulate morphology, such as homeobox, helix-loop-helix transcription factors, and FGFs are up-regulated. In later stages, however, the genes that show relatively increased levels can be categorized as those associated with hepatocyte differentiation, such as hepatocyte nuclear factor-4 and glucose-6-phosphatase isomerase (Omori et al. 2004).

Several growth factors have previously been reported to play important roles in the repair processes in myocardium, vessel, nerve, skin, and bone (Detillieux et al. 2003; Kowalczyk and Pasyk 2002; Werner and Grose 2003; Goodman et al. 2003). Indeed, clinical trials of various growth factors (e.g., FGF, VEGF, HGF) for the treatment of peripheral vascular disorders, ischemic heart diseases, and cutaneous chronic wounds have been performed (Laham et al. 2000; Lederman et al. 2002; Fu et al. 2002). In this study, we have analyzed the expression of several growth factors and their receptors during the process of the repopulation and differentiation of BMCs into hepatocytes. We have found that the expression of FGFs and FGFRs significantly increases with time after BMT compared with other factors, and that the expression levels of FGFs and FGFRs are significantly higher in the BMT(+) group than in the BMT(−) control group ($P<0.05$; Figs. 1, 2, Table 1). These results suggest that the FGF-FGFR system is important in our GFP/CCl_4 model. Furthermore, transplanted GFP-positive BMCs express both FGFs and FGFRs (Fig. 3). Our data indicate that transplanted BMCs differentiate into hepatocytes under the control of autocrine regulation by FGF signaling in the model system. These findings agree well with previous studies showing that FGF is crucial for the initial process of liver development (Kinoshita and Miyajima 2002; Jung et al. 1999; Deutsch et al. 2001). Indeed, a previous microarray-SOM analysis in our laboratory has demonstrated that FGF is an important factor at an early stage in the GFP/CCl_4 model (Omori et al. 2004). Taken together, these reports support our current finding that FGF-FGFR signaling plays important roles, especially during the early differentiation of BMCs into hepatocytes.

We have also investigated the way that the administration of rFGF affects the repopulation and differentiation of BMCs into hepatocytes in the GFP/CCl_4 model. Treatment with rFGF, especially rFGF2, significantly elevates the repopulation rate of GFP-positive cells in the liver and increases the expression of both Liv2 and albumin ($P<0.05$; Fig. 4, Table 2). Furthermore, the serum albumin level is significantly elevated ($P<0.05$; Fig. 5A), and the survival rate is significantly improved ($P<0.05$; Fig. 5B) by treatment with rFGF2 in combination with BMT. In our present microarray analysis and screen I, we have been able to identify specific five genes that are significantly activated by BMT together with rFGF2 treatment compared with BMT-alone (Fig. 6A). In addition, as shown in Fig. 6B, screen II has revealed that TNFIP3 is most specifically up-regulated by rFGF2 treatment in combination with BMT amongst the five genes identified by screen I. Interestingly, the induction of TNFIP3 (also called as A20) has been reported to be caused by TNF-α stimulation, and A20 is involved in feedback suppression of nuclear factor-κB (NF-κB) activation induced by TNF-α (Idel et al. 2003). We have not been able to obtain information regarding TNF-α expression itself, because our microarray system does not include it. We have lastly confirmed the expression of TNF-α protein during the differentiation of BMCs in GFP/CCl_4 model mice. BMT-alone slightly elevates TNF-α expression, and moreover, the level of TNF-α expression is significantly the highest when BMT and treatment with rFGF2 are combined, as shown by immunoblot and immunohistochemical analysis (Fig. 7). TNF-α is a pluripotent mediator that affects several cellular processes, including adhesion, migration, angiogenesis, and apoptosis. TNF-α-regulated inflammation signals, such as stress-activated protein kinase/extracellular signal regulated-kinase kinase 1/mitogen-activated protein-kinase kinase 4-mediated c-jun NH_2-terminal kinase signaling, are important for the generation of hepatoblasts (Watanabe et al. 2002). Yamada and Fausto (1998) have shown that the TNF receptor 1 signaling pathway involving NF-κB, interleukin-6, and the signal transducer and activator of transcription 3, is an important component of the hepatocyte mitogenic response induced by CCl_4 injury in mouse liver. From these reports and our present studies, we speculate that the transplantation of BMCs and concurrent treatment with rFGF2 has a synergistic effect that facilitates or potentiates the differentiation of transplanted BMCs into hepatocytes through the activation of TNF-α signaling.

In conclusion, we have found that FGF2 is the most important growth factor in the GFP/CCl_4 model of liver damage and regenerative treatment. Our present studies suggest that FGF2 facilitates the differentiation of transplanted BMCs into albumin-producing hepatocytes via Liv2-positive hepatoblast intermediates through the activation of TNF-α signaling. Additionally, administration of FGF2 in combination with BMT improves the liver function and prognosis of mice with CCl_4-induced liver damage and might thus have the potential to become an effective and efficient therapy for patients with severe liver disease.

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RESEARCH ARTICLE

Proteomic analysis of serum marker proteins in recipient mice with liver cirrhosis after bone marrow cell transplantation

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We previously found that transplantation with bone marrow cells (BMCs) improves liver function and liver fibrosis in cirrhotic mice. In the presence of liver damage induced by carbon tetrachloride (CCl_4), transplanted BMC migrated into the peri-portal region and trans-differentiated into hepatocytes that produce albumin. Thus under these conditions, BMC transplantation induces liver regeneration. Detecting serum marker proteins is important to monitor the recovery of liver function of cirrhotic mice after BMC transplantation. We therefore initially resolved proteins extracted from serum samples at 48 h after BMC transplantation by 2-DE and compared spot intensity between control and BMC groups of mice. Six protein spots increased in the BMC group compared with the control group. MS revealed that these spots comprised apolipoprotein A1 (apoA1), apolipoprotein C3 (apoC3), vitamin D-binding protein, alpha-1-antitrypsin and proteasome subunit alpha type 1. We subsequently confirmed the levels of apoA1 in serum and liver samples by immunoblotting. ApoA1 increased at early stage (48 h and 1 wk) after BMC transplantation in this mouse model of liver cirrhosis. The early elevation of apoA1 might be useful to predict liver regeneration in cirrhotic mice after BMC transplantation.

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Abbreviations: apoA1, apolipoprotein A1; **apoC3**, apolipoprotein C3; **AT**, alpha-1-antitrypsin; **BMC**, bone marrow cell; **CCl_4**, carbon tetrachloride; **DBP**, vitamin D binding protein; **GFP**, green fluorescence protein; **PCNA**, proliferating cell nuclear antigen

1 Introduction

The plasticity of bone marrow cell (BMC) differentiation into hepatocytes has been revealed by detecting Y chromosome-positive hepatocytes in female recipients after BMC transplantation from a male donor [1–5]. To analyze the effectiveness of cell therapy using BMC on regeneration of the cirrhotic liver, we developed an *in vivo* model to monitor the trans-differentiation of BMC into hepatocytes. Liver cirrhosis was established in mice by continuous injections of carbon