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Blockade of CD26 Augments the Therapeutic Potential of Marrow Stem Cells in the Liver

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Key words：CD26, stromal cell-derived factor-1 (SDF-1), bone marrow stem cells, liver cirrhosis

Abstract　***Background & Aims:*** The therapeutic potential of marrow stem cells for liver disease is currently unclear. The aim of this study was to investigate whether potentiating the chemotaxis ability of stromal cell-derived factor 1 could augment the engraftment of marrow stem cells and ameliorate tissue damage. ***Methods:*** Female mice reconstructed with β-galactosidase-positive male marrow cells were repeatedly injected with carbon tetrachloride to induce liver cirrhosis. The statuses of stromal cell-derived factor 1 and its inhibitor CD26 were examined by immunohistochemical staining, enzyme-linked immunosorbent assays and colorimetric assays. Recipients were treated with an inhibitory peptide for CD26 (Diprotin A) together with granulocyte colony-stimulating factor, and the marrow-derived cells were tracked through the β-galactosidase activity or sex-determining region on the Y chromosome. ***Results:*** Stromal cell-derived factor 1 expression and CD26 activity were both significantly increased and marrow-derived hepatocytes were not observed in liver cirrhosis, suggesting that the potential of stem cell homing is impaired by liver damage. Granulocyte colony-stimulating factor only induced a few cell clusters comprising 2-6 marrow-derived hepatocytes at a late stage of liver cirrhosis. In contrast, when animals with liver cirrhosis were treated with Diprotin A after granulocyte colony-stimulating factor-induced stem cell mobilization, marrow-derived hepatocytes increased to 2-4% of the total hepatocytes and both the serum albumin and hepatic hydroxyproline contents were ameliorated ($p < .05$). ***Conclusions:*** Maintaining the chemotaxis ability of stromal cell-derived factor 1 may represent an effective strategy for validating stem cell therapy.

Introduction

Recent progress in stem cell biology has revealed the plastic ability of bone marrow stem cells (BMSCs) to trans-differentiate into non-hematological lineages, thereby raising high expectations for stem cell therapies toward various types of refractory diseases. However, in the case of liver diseases, a critical question regarding the therapeutic potential of BMSCs has been raised by experimental animal model studies, especially under conditions that do not provide a selective growth advantage for BMSCs.[1)] Since the reported engraftment rates of BMSCs in the liver have been controversial, the substantial question of whether BMSCs can contribute functionally to the liver remains unsolved.

Taking the mouse model of carbon tetrachloride (CCl_4)-induced liver injury as an example, the ratio of marrow-derived hepatocytes has been reported to vary from 0-25%.[2)-4)] Several studies have reported that granulocyte colony-stimulating factor (G-CSF) administration augments the

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formation of BMSC-derived hepatocytes, although the observed engraftment rates were rather low.[5),6)] Since BMSC chimerism in the liver has been suggested to be influenced by the nature of the tissue injury,[7),8)] the different experimental conditions used in the previous studies may explain the controversy over the therapeutic potential of BMSCs. Unfortunately, however, the molecular mechanism that links the severity of liver injury with BMSC chimerism is unclear and an effective strategy for augmenting the formation of BMSC-derived hepatocytes remains to be determined.

Recently, the chemokine stromal cell-derived factor 1 (SDF-1), the ligand of the G-protein-coupled receptor CXCR4 expressed on various types of cells, was found to play an essential role in recruiting BMSCs to injured organs, such as the liver, heart and subcutaneous tissue.[9)-11)] Since SDF-1 is induced by hypoxia inducible factor-1 (HIF-1) or vascular endothelial growth factor (VEGF) in accordance with tissue damage,[11),12)] the regulatory mechanism for SDF-1 expression may be one of the plausible determinants of BMSC chemotaxis to injured sites. Of note, SDF-1 is naturally inactivated via specific processing of the first two amino acids in the N-terminal domain by enzymes such as elastase, cathepsin G and the membrane-bound extracellular peptidase CD26/dipeptidyl peptidase IV.[13)-16)] Several studies have recently suggested that cleavage of SDF-1 is an important event involved in BMSC mobilization, since G-CSF-induced BMSC mobilization was shown to require cleavage of marrow SDF-1 in order to induce efflux of hematopoietic cells into the bloodstream.[13),14)] More recently, inhibition of the enzymatic activity of CD26 was reported to restore the function of SDF-1 in the marrow, leading to a reduction in G-CSF-induced BMSC mobilization[15)] or enhanced engraftment rate of donor cells in the recipient marrow.[16)] These lines of evidence strongly suggest that, among several types of SDF-1 inhibitors, CD26 may play a critical role in the chemotaxis activity of SDF-1 during G-CSF-induced stem cell mobilization, thereby leading to the hypothesis that manipulation of CD26 activity may lead to effective BMSC engraftment in injured livers.

To assess the validity of stem cell therapy toward liver disease, we examined the statuses of SDF-1 and CD26 in a mouse model of CCl_4-induced liver injury. We further investigated whether inhibition of CD26 activity *in vivo* could enable increased engraftment of BMSC-derived hepatocytes via restoration of functional SDF-1 in the injured liver.

Materials and Methods

Animals

In the present study, Balb/c strain mice were used to investigate the prevailing effect of G-CSF *in vivo*.[17)] All studies were carried out under the guidelines of the Committee of Animal Experiment Ethics at Niigata University Hospital of Medicine and Dental Science. To generate bone marrow chimeric mice, 6-week-old female mice (CLEA Japan Inc., Tokyo, Japan) with preparative irradiation (700 cGy) were transplanted with 1×10^7 marrow mononuclear cells from 6-week-old male Balb/c background β-galactosidase (β-gal) transgenic ROSA26 (Jackson Laboratories, Rochester, NY). After 4 weeks, the reconstitution of donor cells in the blood was 60-80% and the recipients were processed for the following four experiments. In the first experiment, mice (n = 5) were subcutaneously (s.c.) injected with recombinant human G-CSF (Kyowa Hakko Chemical Co. Ltd., Tokyo, Japan) dissolved in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin at a dose of 250 μg/kg body weight every day for 5 days, while controls (n = 5) were injected with vehicle in the same manner. In the second experiment, mice (n = 5) were intra-peritoneally (i.p.) injected with CCl_4 (Wako Pure Chemical Industries Ltd., Osaka, Japan) at 1 mL/kg biweekly and s.c. injected with G-CSF (250 μg/kg) daily for 4 weeks, while controls (n = 5) were injected with the vehicle for G-CSF. In the third experiment, mice (n = 5) were i.p. injected with CCl_4 at 1 mL/kg biweekly and s.c. injected with G-CSF (250 μg/kg) daily for 12 weeks, while controls (n = 5) were injected with the vehicle for G-CSF. In the fourth experiment, three groups of mice were used. In group 1, mice (n = 5) were i.p. injected with CCl_4 at 1 mL/kg biweekly for 12 weeks and s.c. injected with G-CSF (250 μg/kg) for 5 days from week 5 of the experiment, at which time the histological features of liver cirrhosis were established. In group 2, mice (n = 5) were injected with CCl_4 and G-CSF as described for group 1, and an inhibitory peptide of CD26 (Diprotin A; lle-Pro-lle; Bachem AG, Bubendorf, Switzerland) was s.c. injected 4 times a day at a dose of 5 μM/body during the same period as the G-CSF treatment. In group 3, mice (n = 5) were injected with

CCl_4 and G-CSF as described for group 1 and Diprotin A (5 μM/body) was s.c. injected 4 times a day for 5 days from the last day of the G-CSF treatment. Controls (n = 5) for all three groups were injected with CCl_4 and the vehicle for G-CSF in the manner described for group 1.

Tissue Preparation and Biochemical Analysis

Mice were properly anesthetized and blood was collected via the heart. Liver tissue samples were processed according to one of the following five procedures: snap-frozen in liquid nitrogen; fixed in 10% formalin and embedded in paraffin; fixed in 4% paraformaldehyde and embedded in a cryo-embedding compound (OCT); reacted with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal;Wako); or processed for hydroxyproline content analysis as previously described.[18] The hepatic SDF-1 content was analyzed by enzyme-linked immunosorbent assay (ELISA) sing a Quantikine Mouse SDF-1α Immunoassay Kit (R&D Systems Inc., Minneapolis, MN). CD26 activity was measured by a colorimetric assay as previously described.[19]

Immunohistochemical and Immunofluorescence Analyses

For immunohistochemical analysis, deparaffinized tissue sections were microwaved in citrate buffer and then incubated for 2 hours at room temperature with one of the following primary antibodies: anti-SDF-1 monoclonal antibody (1:50 dilution; R&D Systems Inc.) using a Mouse-on-Mouse Immunodetection Kit (Vector Laboratories, Burlingame, CA); anti-CD26 goat polyclonal antibody (1:100 dilution; R&D Systems Inc.); or anti-mouse collagen type 1 rabbit polyclonal body (1:100 dilution; Cedarlane Laboratories, Ontario, Canada). Some tissue sections processed for X-gal assays were reacted with an anti-E-cadherin monoclonal antibody (1:100 dilution; R&D Systems Inc.). To detect male donor-derived cells, serial deparaffinized tissue sections (4 μm in thickness) were washed twice with 0.005% Triton X-100 and incubated with an anti-mouse sex-determining region on the Y chromosome (SRY) goat polyclonal antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours at room temperature. The reacted sections were stained using an Elite ABC kit (Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride (DAB). Substitution of each primary antibody with the corresponding normal immunoglobulin (Ig) was used for negative controls. For immunofluorescence analysis, an Axio Imager.A1 microscope (Carl Zeiss, Göttingen, Germany) was used. Thin slices (6 μm) of OCT-embedded tissue samples were reacted with the anti-SRY goat polyclonal antibody (1:100 dilution) and an anti-mouse albumin rabbit polyclonal antibody (1:100 dilution; Bethyl Laboratories, Montgomery, TX) for 2 hours at room temperature. The secondary antibodies used were indocarbocyanine (Cy3) -conjugated donkey anti-goat IgG (Chemicon, Temecula, CA) and fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (GeneTex, San Antonio, TX). The sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and the average percentages of donor-origin hepatocytes were calculated by examining at least 10 tissue sections.

Statistical Analysis

The results are presented as the mean ± SD. Student's *t*-test was used for statistical evaluation, and values of $p < .05$ were considered significant.

Results

SDF-1 is Increased in the Injured Liver and Augmented by G-CSF

In healthy livers, immunohistochemical staining revealed that SDF-1 was expressed on biliary epithelial cells (Figure 1*A*), consistent with a previous report.[9] G-CSF treatment induced faint SDF-1 expression on sinusoidal endothelial cells and 1-2% of the hepatocytes (Figure 1*B*). In the livers of mice injected with CCl_4 for 4 weeks, SDF-1 was diffusely expressed on sinusoidal endothelial cells (Figure 1*C*) and G-CSF treatment resulted in the appearance of SDF-1-positive hepatocytes (1-2% of the total cells) (Figure 1*D*). When CCl_4 was injected into mice for 12 weeks, severe liver injury showing hepatic lobular reconstitution and marked fiber accumulation was established, resembling human liver cirrhosis. In this setting, SDF-1-positive histiocyte-like cells that were multinucleate and oval were found around the fibrosis area (Figure 1*E*) and G-CSF treatment resulted in the formation of clusters comprising 4-50 SDF-1-positive hepatocytes (Figure 1*F*). ELISA analyses revealed that the hepatic SDF-1 contents were increased by 3-4-fold and 8-10-fold in mice injected with CCl_4 for 4 and 12 weeks, respectively, compared to healthy mice without tissue injury. G-CSF augmented the levels of

hepatic SDF-1 by 1.3-1.5-fold compared to the levels in mice administered CCl_4 alone ($p < .05$) (Figure 1*G*).

G-CSF Induces a Few BMSC-derived Hepatocytes in Liver Cirrhosis

X-gal assays did not detect any β-gal-positive cells in the livers of healthy mice with and without G-CSF treatment (Figure 2A and *B*). No β-gal-positive cells were observed in mice injected with CCl_4 for 4 weeks (Figure 2*D*), while G-CSF treatment of these mice induced a few β-gal-positive spindle cells (0.2-1.0% of the total number of hepatic cells) in the sinusoid space and fibrotic area, which could be readily distinguished from the large polygonal hepatocytes located in the hepatic lobular architecture (Figure 2*E-G*). No β-gal-positive hepatocytes were detected in mice treated with CCl_4 for 12 weeks, whereas the number of β-gal-positive non-parenchymal cells was increased by 2-4-fold around the portal veins of mice injected with CCl_4 for 4 weeks (Figure 2*H*) and almost all their bile duct epithelial cells were β gal-positive (Figure 2*H*, *inset*). G-CSF treatment resulted in scattered clusters comprising 2-6 β-gal-positive hepatocytes (Figure 2*I*), indicating that G-CSF only induced the formation of marrow-derived hepatocytes in cases of severe liver injury.

Enzymatic Activity of CD26 is Increased in the Injured Liver

Immunohistochemical staining revealed that CD26 was preferentially expressed at the plasma membrane of hepatocytes (Figure 3*A*), consistent with a previous report,[20] and significantly increased according to the progress of liver injury (Figure 3*B* and *C*). The CD26 activity was 3-4-fold and 8-10-fold higher in the livers of mice injected with CCl_4 for 4 and 12 weeks, respectively, compared to that in healthy controls. G-CSF treatment tended to augment the CD26 activity in the liver, although the difference did not reach statistical significance (Figure 3*D*).

Blockade of CD26 after G-CSF Treatment Augments the Chimerism of BMSC-derived Hepatocytes in Liver Cirrhosis

To address whether temporary administration of G-CSF exerts an effect on BMSC chimerism in liver cirrhosis, mice repeatedly injected with CCl_4 for 12 weeks were administered G-CSF for 5 days during the middle of the experimental period. Subsequent X-gal assays showed that the number of β-gal-positive non-parenchymal cells was increased by 2-4-fold in the liver of G-CSF-treated mice (Figure 4*A*) compared to mice injected with CCl_4 alone (Figure 2*H*). Double-immunofluorescence staining for SRY/albumin revealed that 0.01-0.50% of the hepatocytes were of donor origin in the liver of G-CSF-treated recipients (Figure 4*B*). When mice were simultaneously administered Diprotin A and G-CSF for 5 days, the total number of β-gal-positive cells (Figure 4*C*) did not differ significantly from that in mice injected with CCl_4 alone (Figure 2*H*) and less than 0.01% of the total hepatocytes were SRY-positive (Figure 4*D*). In contrast, when Diprotin A was administered for 5 days immediately after the cessation of G-CSF treatment, the number of BMSC-derived hepatocytes was significantly increased. Both X-gal assays and immunofluorescence staining for SRY/albumin detected clusters comprising 4-30 donor-derived hepatocytes, predominantly in the peri-portal region (2-4% of the total hepatocytes) (Figure 4*E* and *F*). Of these, approximate 80% of the binuclear SRY-positive hepatocytes expressed SRY in the odd nucleus and the remaining 20% expressed SRY in both nuclei (Figure 4*G*), indicating that most of the cells had originated through cell fusion between donor-derived marrow cells and recipient hepatocytes.

Blockade of CD26 after G-CSF Treatment Ameliorates Liver Cirrhosis

Biochemical analyses revealed that increased chimerism of BMSC-derived hepatocytes appeared to contribute to hepatic function in liver cirrhosis. Among mice repeatedly injected with CCl_4 for 12 weeks, the hepatic hydroxyproline content was decreased in the group treated with Diprotin A after G-CSF compared with that in control mice injected with CCl_4 alone (618 ± 89 vs. 894 ± 150 μg/wet g tissue, $p < .05$) (Figure 4H). Moreover, the serum albumin levels were increased by Diprotin A treatment after G-CSF administration compared to that in control mice injected with CCl_4 alone (2.8 ± 0.4 mg/mL vs. 2.0 ± 0.3 mg/mL, $p < .05$) (Figure 4*I*).

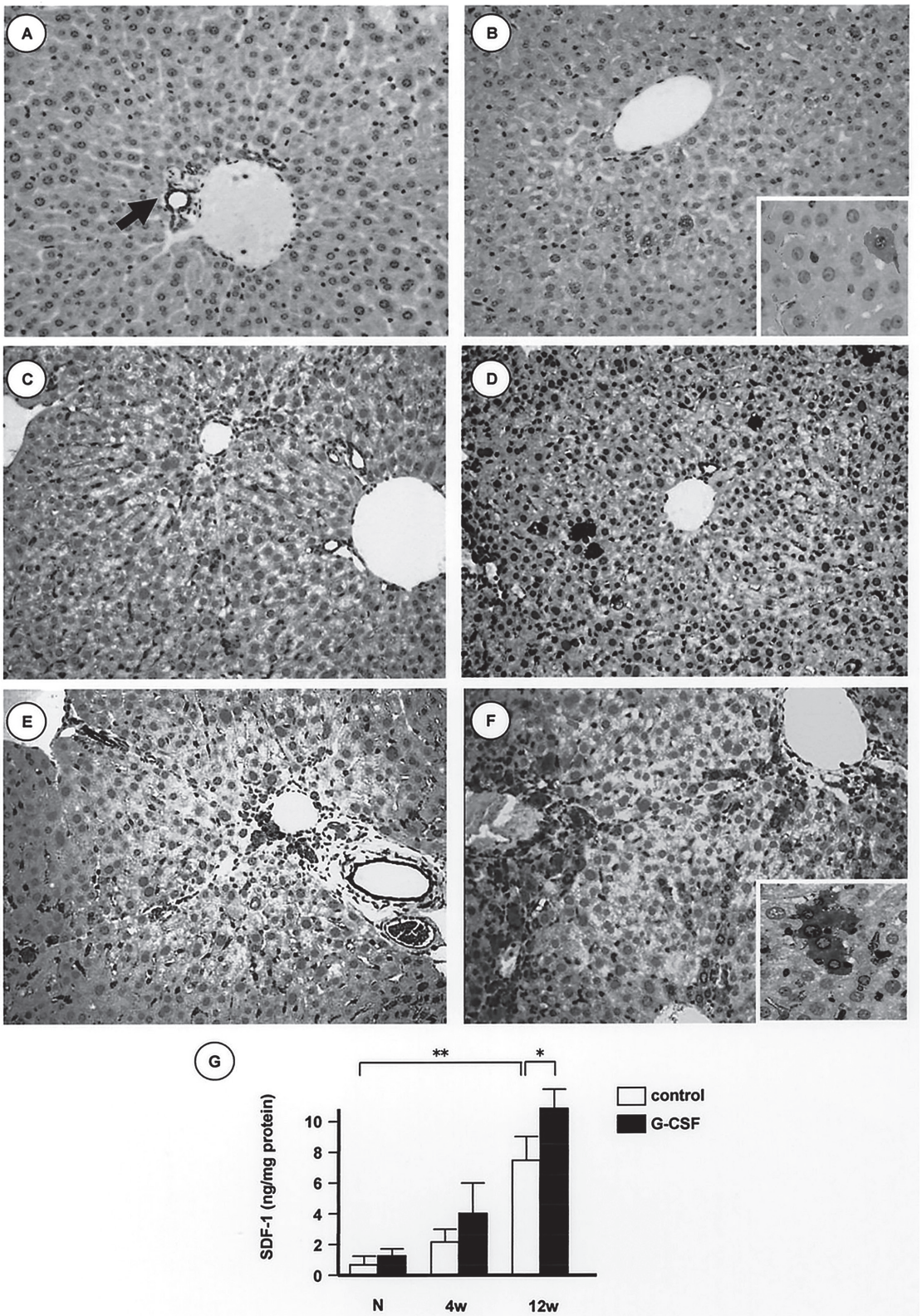


Figure 1. SDF-1 expression in the liver. (*A-F*) Immunohistochemical staining for SDF-1. (*A*) Healthy mice. (*B*) G-CSF-treated healthy mice. (*C*) Mice treated with CCl_4 for 4 weeks. (*D*) Mice treated with CCl_4 plus G-CSF for 4 weeks. (*E*) Mice treated with CCl_4 for 12 weeks. (*F*) Mice treated with CCl_4 plus G-CSF for 12 weeks. *Arrow*, bile duct (magnificati ons: x20; inset, x40). (*G*) SDF-1 levels in the livers of untreated (*white bars*) and G-CSF-treated (*black bars*) mice. *N*, healthy mice; *4W*, mice treated with CCl_4 for 4 weeks; *12W*, mice treated with CCl_4 for 12 weeks (*: $p < .05$, **: $p < .01$).

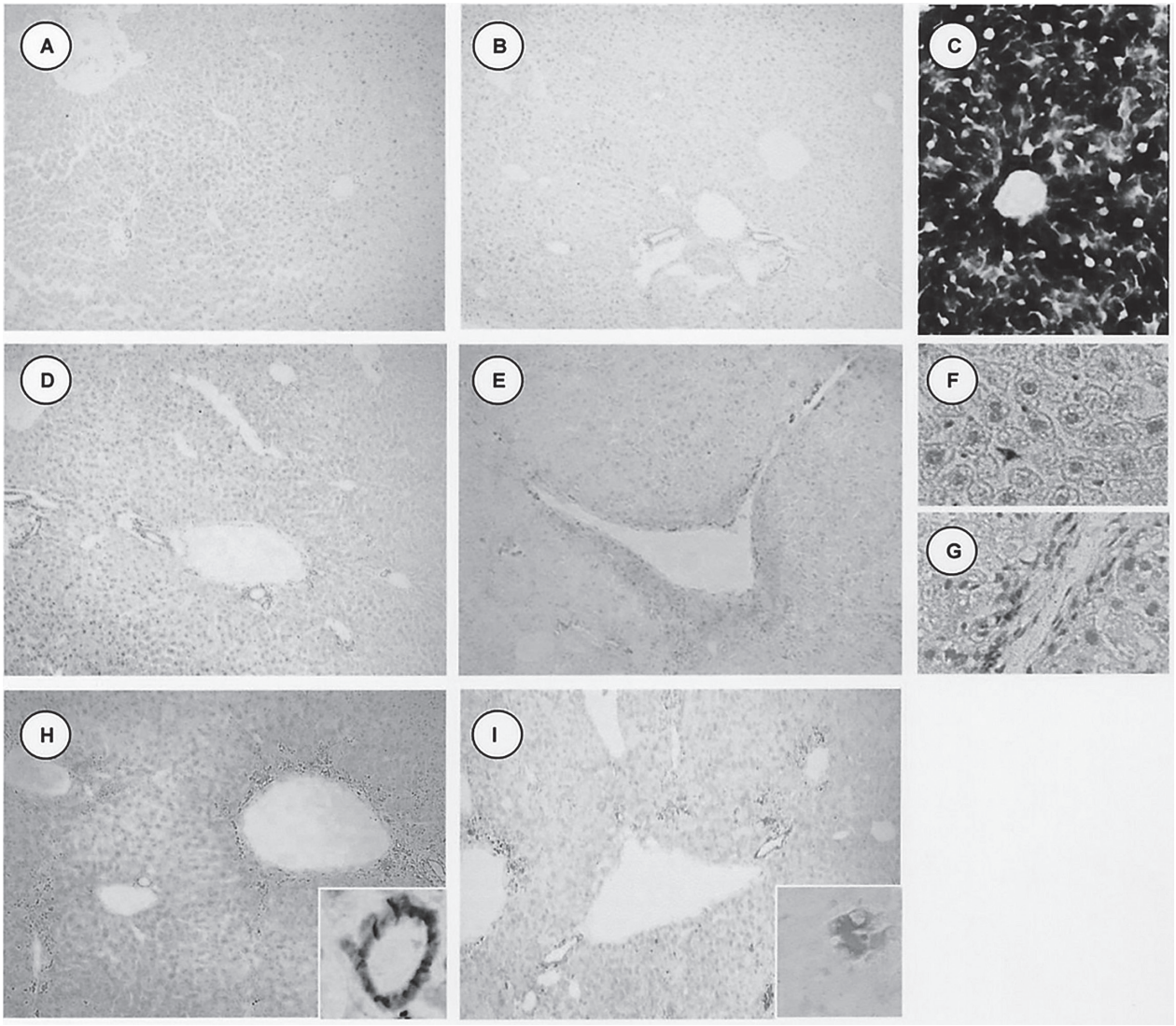


Figure 2. X-gal assays of recipient livers. (*A*) Healthy mouse. (*B*) G-CSF-treated mouse. (*C*) ROSA26 mouse. (*D*) Mouse treated with CCl_4 for 4 weeks. (*E-G*) Mouse treated with CCl_4 plus G-CSF for 4 weeks. (*H*) Mouse treated with CCl_4 for 4 weeks (inset: staining for E-cadherin). (*I*) Mouse treated with CCl_4 plus G-CSF for 12 weeks (magnifications: *A, B, D, E, H* and *I*, x4; *C*, x20; *F, G* and insets in *H* and *I*, x40).

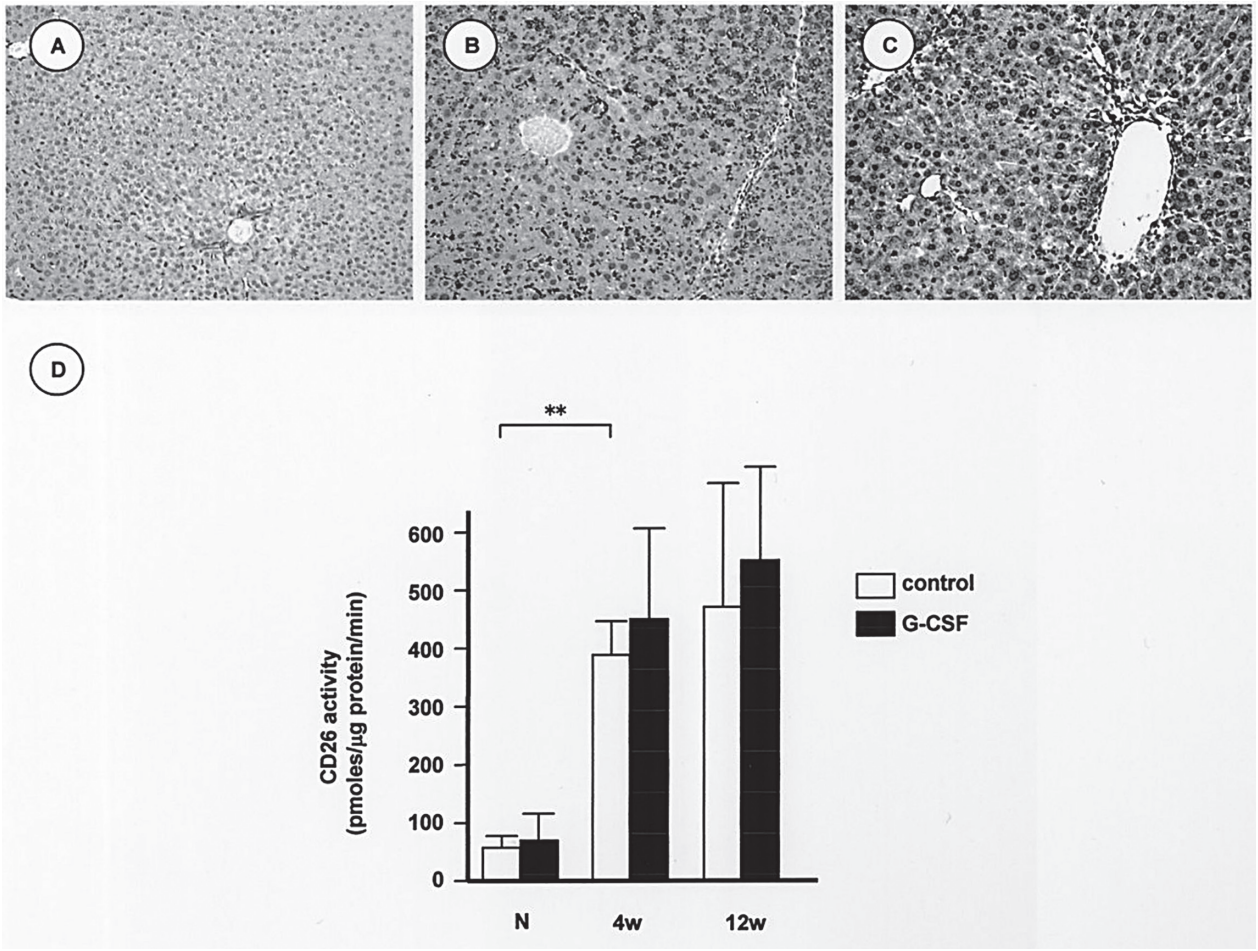
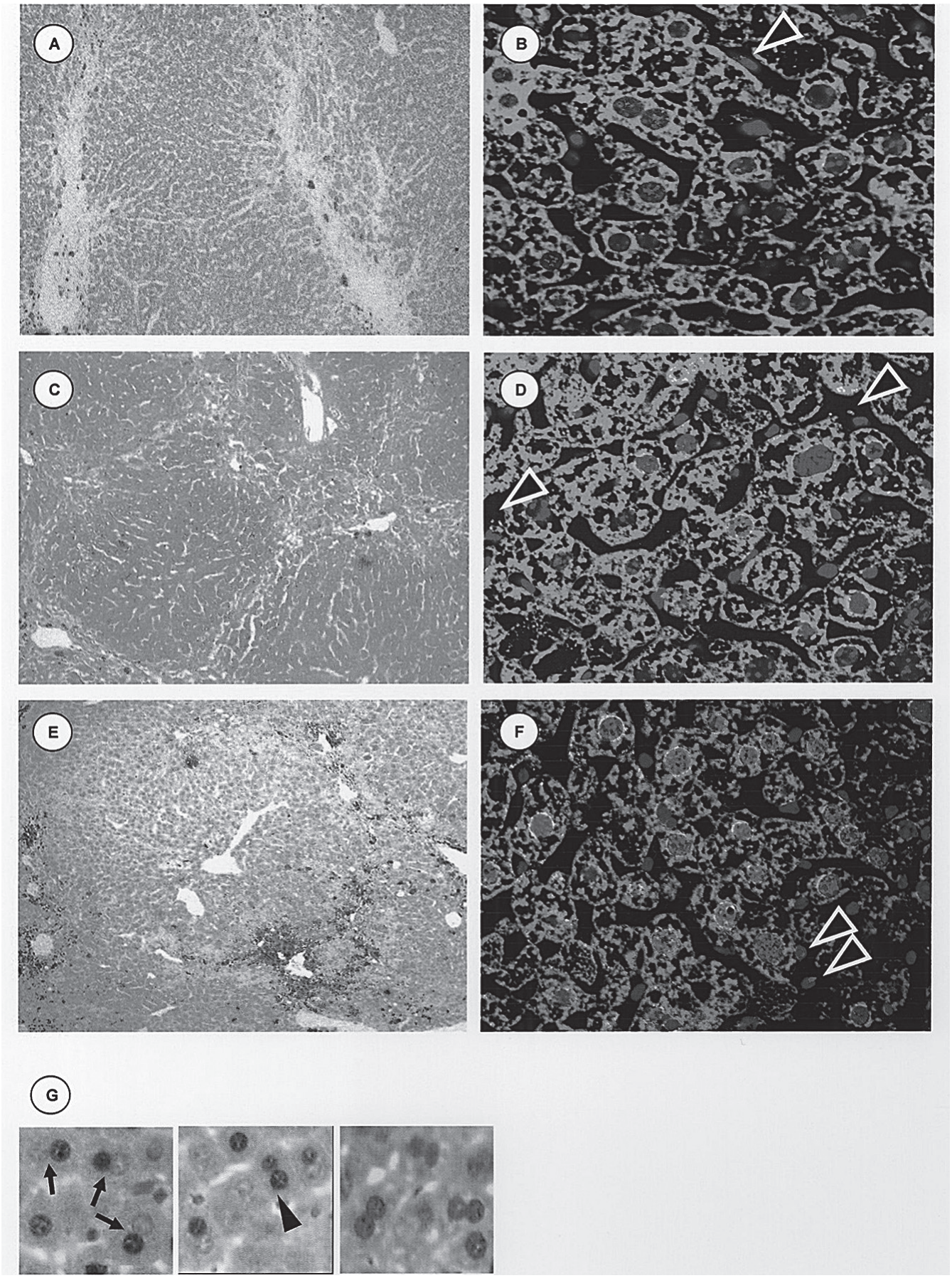


Figure 3. Status of CD26 in the liver. (*A-C*) Immunohistochemical staining for CD26. (*A*) Healthy mouse. (*B*) Mouse tre ated with CCl_4 for 4 weeks. (*C*) Mouse treated with CCl_4 for 12 weeks (magnifications: x20). (*D*) CD26 activities in the livers of untreated (*white bars*) and G-CSF-treated (*black bars*) mice. *N*, healthy mice; *4W*, mice treated with CCl_4 for 4 weeks; *12W*, mice treated with CCl_4 for 12 weeks (**: $p < .01$).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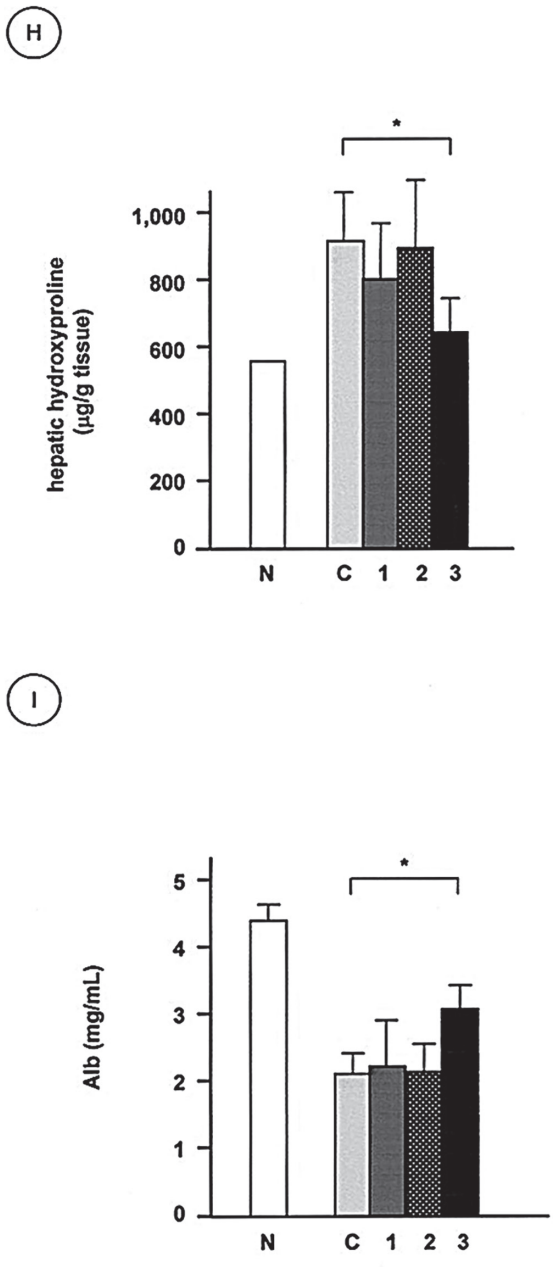

Figure 4. Marrow stem cell chimerism in the liver of mice treated with CCl_4 for 12 weeks. (*A* and *B*) Mice treated with G-CSF for 5 days. (*C* and *D*) Mice simultaneously treated with G-CSF and Diprotin A for 5 days. (*E* and *F*) Mice treated with Diprotin A for 5 days from the last day of the G-CSF treatment. (*A, C* and *E*) X-gal assays (magnifications: x4). (*B, D* and *F*) Immunofluorescence staining for SRY (*red*) and albumin (*green*). *Arrowhead*, SRY-positive non-parenchymal cells (magnifications: x40). (*G*) Immunohistochemical staining for SRY in the liver. *Left* and *middle*, mice treated with Di protin A after G-CSF treatment; *right*, mouse treated with CCl_4 alone. *Arrowhead*, binuclear hepatocyte expressing SRY in both nuclei; *Arrow*, binuclear hepatocytes expressing SRY in the odd nucleus (magnification: x40). (*H*) Hepatic hydroxyp roline contents in mice treated with CCl_4 for 12 weeks. (*I*) Serum albumin levels treated with CCl_4 for 12 weeks. *N*, healt hy mice; *C*, control mice with CCl_4-induced liver injury; *1*, mice treated with G-CSF alone for 5 days from week 5 of the ex periment; *2*, mice treated with Diprotin A during the same period as the G-CSF treatment; *3*, mice treated with Diprotin A for 5 days after G-CSF treatment (*: $p < .05$).

Discussion

The liver has a remarkable ability to regenerate after partial removal, because virtually all the hepatocytes have the ability to replicate and restore the hepatic mass. However, when excessive tissue damage continues for a long time period, liver regeneration is impaired due to phenomena such as hepatocyte-specific telomere shortening.[21] Many studies have addressed whether BMSC transplantation could compensate for liver cirrhosis, but the observed results have been disappointing.[1-8]

In the present study we investigated whether the functional status of SDF-1, a chemokine that plays a critical role in the homing of marrow stem cells,[9)10)13)14)] may affect the therapeutic potential of BMSCs. By generating a mouse model of CCl_4-induced liver injury, we found that hepatic SDF-1 was significantly increased along with the progress of tissue damage. Interestingly, G-CSF treatment augmented the number of SDF-1-positive hepatocytes. Since G-CSF receptors are not expressed on hepatocytes,[22] some sort of paracrine action, such as VEGF release from G-CSF-stimulated stem cells,[23] may induce SDF-1 expression on hepatocytes. However, similar to a previous report,[6] we found that the chimerism of BMSC-derived hepatocytes was rather low. Even when G-CSF was administered daily, X-gal assays did not detect any BMSC-derived hepatocytes in healthy mice or mice treated with CCl_4 for 4 weeks. Only mice treated with CCl_4 for 12 weeks showed a small number of cell clusters comprising 2-6 BMSC-derived hepatocytes after G-CSF treatment.

One possible reason for the functional impairment of SDF-1 in our animal model may be physiological inactivation via specific processing of the N-terminal domain by enzymes such as elastase, cathepsin G and CD26.[13)-16)] Among several types of SDF-1 inhibitors, we focused on CD26 because previous clinical studies have reported increased levels of CD26 in the serum or liver of individuals with liver cirrhosis.[24),25)] As expected, both the expression levels and the enzymatic activity of CD26 were significantly increased in the liver of CCl_4-treated mice. When mice were treated with CCl_4 for 4 and 12 weeks, the CD26 activity was increased 3-4-fold and 8-10-fold, respectively, compared to that in the liver of healthy mice. We surmise that the increased CD26 activity in the liver may prevent excessive infiltration of CXCR4-positive lymphocytes into the injured sites with SDF-1 overexpression, and would also dampen stem cell homing to the liver and result in the rare engraftment of BMSC-derived hepatocytes.

To investigate whether manipulation of the CD26 activity *in vivo* could augment BMSC engraftment in the liver, we administered the CD26 inhibitor Diprotin A to mice with CCl_4-induced liver cirrhosis. When mice were treated with Diprotin A after G-CSF-induced marrow stem cell mobilization, the number of BMSC-derived hepatocytes increased to 2-4% of the total hepatocytes. In contrast, when mice were administered Diprotin A at the same time as the G-CSF treatment, the engraftment rate of BMSC-derived hepatocytes was less than 0.01%, and almost the same as that in mice injected with CCl_4 alone. We also found that daily administration of Diprotin A alone resulted in rare engraftment of BMSC-derived hepatocytes in the liver of mice treated with CCl_4 for 12 weeks (not shown). Therefore, the increased emergence of BMSC-derived hepatocytes may be caused by restoration of SDF-1 function through inhibition of CD26, rather than by a pharmacological effect of Diprotin A.

To date, the principal mechanism of the marrow cell contribution to hepatocytes has remained unclear. Several studies have identified BMSC-derived hepatocytes following cell fusion of marrow-derived stem cells or myelomonocytic cells with resident hepatocytes,[1),6),26)-28)] whereas other studies have reported a lack of cell fusion.[8),29)] As many studies have suggested, analytical methods using donor-specific markers (e.g., the Y chromosome, green fluorescent protein or β-gal) in marrow-transplanted animal models can sometimes be insensitive or non-specific, leading to difficulties in detecting the frequency of cell fusion *in vivo*. In the present study, we focused on the diploid hepatocytes that are frequently observed in the liver. Diploid hepatocytes are useful for assessing cell fusion, because they return to their diploid status by a reduction division after fusion with diploid blood cells.[28)] Immunostaining for SRY, which is specifically expressed on male donor cells, revealed that approximately 80% of the binuclear SRY-positive hepatocytes expressed SRY in the odd nucleus, indicating that most of them had emerged through cell fusion between donor marrow cells and recipient hepatocytes. To further investigate this cell fusion in the liver, additional studies using techniques such as cytogenetic analyses are required. In any case, even if most

of the BMSC-derived hepatocytes do emerge through cell fusion, the marrow cells may contribute to the liver function since we found that the serum albumin and hepatic hydroxyproline contents were ameliorated in mice with increased engraftment of BMSC-derived hepatocytes.

Finally, we have shown that potentiating SDF-1 through blockade of CD26 activity could increase the emergence of BMSC-derived hepatocytes in liver cirrhosis. Even though the engraftment rate of BMSC may be less significant in the liver, we suggest that marrow stem cells could exert a therapeutic potential under conditions of increased chemotaxis ability of the injured tissue.

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