# Hematopoietic Mobilization in Mice Increases the Presence of Bone Marrow–Derived Hepatocytes Via *In Vivo* Cell Fusion

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**The mechanisms for *in vivo* production of bone marrow–derived hepatocytes (BMDHs) remain largely unclear. We investigated whether granulocyte colony–stimulating factor (G-CSF)–mediated mobilization of hematopoietic cells increases the phenomenon. Recurrent liver injury in mice expressing green fluorescent protein (EGFP) in all hematopoietic-derived cells was produced by 3 months of carbon tetrachloride ($CCL_4$) injections. Histologically, there were necrotic foci with histiocyte-rich infiltrates, but little oval cell proliferation. Subsequently, some animals were mobilized with G-CSF for 1, 2, or 3 weeks. Animals were sacrificed 1 month after growth factor treatment. BMDH percentages were lower than previously reported, though G-CSF mobilization significantly augmented BMDH production in injured livers. BMDHs originating from *in vivo* fusion were evaluated by transplanting female $EGFP^+$ cells into male mice. Binucleated, $EGFP^+$ hepatocytes with one Y chromosome, indicating fusion, were identified. In conclusion, (1) mobilization of hematopoietic cells increases BMDH production and (2) as with the FAH-null model, the first model demonstrating hematopoietic/hepatocyte fusion, recurring $CCl_4$-induced injury has macrophage-rich infiltrates, a blunted oval cell response, and a predominantly *in vivo* fusion process for circulating cell engraftment into the liver. These findings open the possibility of using hematopoietic growth factors to treat nonhematopoietic degenerative diseases. *Supplementary material for this article can be found on the* HEPATOLOGY *website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).* (HEPATOLOGY 2006;43:108-116.)**

During recent years, the central tenet of developmental biology—that origin in the three embryonic germ layers rigidly determines subsequent differentiation—has been questioned as the result of a newly demonstrated potential of adult cells commonly referred to as *cellular plasticity*. Plasticity describes the conversion of a cell from one tissue lineage to a completely different one, with a loss of specific markers and of previous functionality in the original tissue, and with the acquisition of markers and functions related to the new lineage.[1,2] Bone marrow (BM) cells have extensively demonstrated this capability. This has been studied in lethally irradiated and transplanted hosts that were damaged in the target tissues.[2,3] Expression of specific markers from BM donors has been described for nonhematopoietic cells in a broad range of tissues.[1,4]

The incidence of plasticity has been shown to be very variable, from extremely rare events to between 20% and 40% of the tissue.[2] In the liver, different models of hepatic damage have been applied to demonstrate plasticity of hematopoietic cells to hepatocytes, with heterogeneous results.[5,6] It is also clear that BM cells may help restore hepatic degenerative diseases.[7,8] Hepatic[9] and hematopoietic[10,11] growth factors have been proved to increase the regenerative process after carbon tetrachloride ($CCl_4$) hepatic injury. Although the treatment can be beneficial, the contribu-

*Abbreviations: BMDH, bone marrow–derived hepatocyte; G-CSF, granulocyte colony–stimulating factor; EGFP, expressing green fluorescent protein; BM, bone marrow; PBS, phosphate-buffered saline; DAPI, 4,6-diamidino-2-phenylindole; Y-ch, Y chromosome.*

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tion of bone marrow–derived hepatocytes (BMDHs) to the injury repair is not completely understood.

Two primary mechanisms for the generation of nonhematopoietic tissues from circulating marrow–derived cells have been demonstrated: direct differentiation into mature cells of different tissues following engraftment and fusion between hematopoietic cells and differentiated cells of the target nonhematopoietic tissue. In either case, modulation of the hepatic potential of some BM-derived populations might be a useful means of treating liver disease and is therefore worth exploring for the development of alternative treatments of hepatic disorders in human patients.

Many different soluble factors regulate the proliferation and differentiation of the hematopoietic cells.[12] Some of these factors have the ability to mobilize hematopoietic progenitors and hematopoietic stem cells from the BM to the periphery, increasing the total number of circulating hematopoietic cells. In particular, granulocyte colony–stimulating factor (G-CSF) was the first factor described with this property.[13-15] G-CSF mobilization of hematopoietic progenitors is widely used for hematopoietic transplantation.[16] Very recently, it has been described that G-CSF treatment can speed the recovery after liver damage with $CCl_4$ in mice via endogenous hepatic mechanisms without contribution of BMDH.[11,17]

In the present study, we show that treatment of $CCl_4$-hepatic damaged animals with G-CSF significantly increases the presence of hepatocytes derived from BM cells. Moreover, we demonstrate that in our model, the generation of these BMDHs occurs via *in vivo* cell fusion events between endogenous hepatocytes and hematopoietic cells.

## Materials and Methods

***Animals and Bone Marrow Transplants.*** Eight- to ten-week-old C57BL/6JxDBA/2 F1 mice (B6D2F1; CD45.2 phenotype) or B6.SJL-*Ptprc*$^{a/b}$*Pep*$^{3b}$/BoyJx-DBA/2 female mice (P3D2F1; CD45.1 phenotype) were used as recipients of BM cells. Breeding pairs were bred at the CIEMAT animal facility. The F1 male mice of C57BL/6J-*βactinEGFP* (kindly provided by Dr. M. Okabe, Osaka, Japan) xDBA/2 (BDGF1; CD45.2 EGFP$^+$ phenotype) were used as donors of BM cells. Recipients of BM transplants were irradiated with Philips MG324 X ray equipment (Philips, Hamburg, Germany). For *in vivo* fusion studies, the sex of donors and recipients were switched to facilitate the identification of the BMDH origin.

***Recurring Hepatic Damage and Hematopoietic Cell Mobilization.*** Chimeric mice were treated with 10 mL of 10% $CCl_4$ (Fluka, Buchs, Switzerland) in olive oil per kilogram of body weight or with phosphate-buffered saline (PBS) (Sigma-Aldrich, Steinheim, Germany) via intraperitoneal injection once a week over 3 months. Hepatic damage was confirmed via analysis of serum hepatic parameters with a Konelab 20 equipment (Termo Clinical Labsystems, Vataa, Finland).

For G-CSF treatment, 50 μg/mouse of pegylated G-CSF (Neulasta, Amgen, Breda, The Netherlands) in PBS/0.1% bovine serum albumin was administered weekly via subcutaneous injection. One weekly injection of pegylated G-CSF induces the same effect of a 12-hour injection for 5 days' protocol using nonpegylated G-CSF.[18,19]

***Tissue Collection.*** Animals were transcardially perfused with 10 mL cold PBS/20 mmol/L EDTA, followed by 25 mL cold 4% paraformaldehyde (Merck, Darmstadt, Germany)/PBS. Livers were excised and one part of each liver lobe was additionally fixed in 4% paraformaldehyde/PBS at 4°C. Samples were then incubated in 30% sucrose/PBS at 4°C overnight and kept in Optimal Cutting Temperature (OCT) solution (Sakura Finetek, Zoeterwoude, Netherlands) at −70°C. The rest of each liver lobe was fixed in 4% formaline and paraffin-embedded.

***Histological and Immunohistological Analysis.*** For liver histological studies, 5 μm paraffin-embedded sections were stained with hemotoxylin-eosin. For immunohistochemistry in frozen tissue, 12-15 μm frozen sections were fixed in acetone, blocked with 5% fetal calf serum (Sigma Chemicals, St. Louis, MO) in PBS, incubated with rabbit anti-GFP antibody (Molecular Probes, Leiden, The Netherlands) and a biotinylated rat anti-mouse CD45 (clone 30-F11; BD Bioscience Pharmingen, San Jose, CA), and washed and incubated with goat anti-rabbit labeled with FITC (Jackson Immunoresearch Laboratories, Cambridgeshire, UK) and avidin Texas Red (Vector Laboratories, Burlingame, CA). Albumin and carcinoembrionic antigen were detected as indicated in supplemental material. Finally, sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and mounted in Mowiol solution.

To detect the presence of hepatocellular glycogen, periodic acid-Schiff staining (Merck, Darmstadt, Germany) was performed according to manufacturer's recommendations.

To identify oval cell reaction, staining for biliary-type cytokeratins was performed using broad spectrum rabbit anti-cow cytokeratin antibodies (panK; DakoCytomation, Carpinteria, CA) on paraffin-embedded, fixed tissue using standard techniques, and colorizing with diaminobenzidine and Mayer's hematoxylin. The number of oval cells per portal tract were counted around the 25 smallest

portal tracts in each tissue section examined according to established protocol.[20]

***Fluorescence In Situ Hybridization.*** Five-micrometer slides were hybridized for Y chromosome identification according to the STARFISH manufacturer's protocol (Cambio, Cambridge, UK) (see supplemental material for details).

***Quantification of BMDHs.*** Tissue sections were analyzed with an Axioplan 2 imaging fluorescent microscope (Zeiss, Jena, Germany) or with a Bio-Rad Radiance 2100 confocal microscope system (Zeiss). Quantification of BMDHs was performed by counting all the cells accomplishing the required characteristics (see Results) in each tissue section. At least four different tissue sections from two different lobes of each analyzed liver were evaluated. To estimate the total number of hepatocytes and infiltrating hematopoietic cells in each liver section, 5 to 8 random ×100 images were counted and extrapolated to the total tissue section by multiplying by the total number of ×100 images obtained per slide. The percentage of BMDHs was then obtained using the following formula: total number of BMDHs per tissue section/estimated number of total hepatocytes per tissue section.

***Statistical Analysis.*** Data are expressed as the mean ± SEM. Significance of differences was determined via nonparametric Wilcoxon Mann-Whitney W test and Kruskal Wallis test using Statgraphics software (Manugistics Inc., Rockville, MD).

## Results

***Evaluation of Damage With Continued $CCl_4$ Administration to Study the Contribution of BM Cells to the Regeneration of the Liver.*** For the development of a mouse model of hepatic damage (Fig. 1A), CD45.1 female mice were lethally irradiated and transplanted with $10^7$ total BM cells from male CD45.2 congenic mice, which also express EGFP under the control of the β-actin ubiquitous promoter. Exogenous hematopoietic engraftment was analyzed 1 and 3 months after BM transplantation. The percentage of blood donor cells ($CD45.2^+$ and $GFP^+$) was analyzed by flow cytometry. A mean percentage of 96.15 ± 0.73% $CD45.2^+$ was observed in the recipient mice, indicating almost complete regeneration of the hematopoiesis from the exogenous cells. When the percentage of EGFP cells was analyzed, 79.36 ± 0.72% of total cells were $EGFP^+$. Variations of EGFP expression were also confirmed in the liver of the donor animals, where 10% to 15% of hepatocytes did not express the transgene.

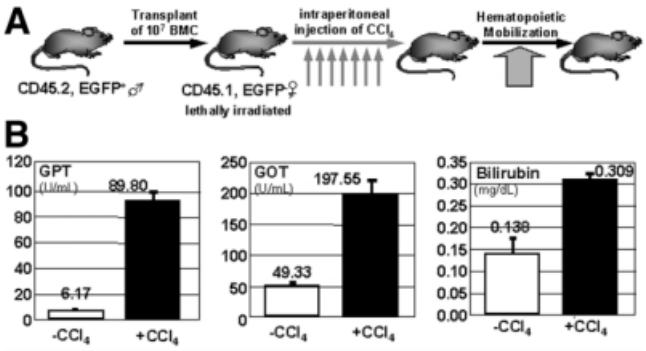


Fig. 1. Liver damage induced by continuous treatment with $CCl_4$. (A) Scheme of the experimental protocol used to induce chronic liver failure. (B) Hepatic parameters in plasma of untreated (white bars) and $CCl_4$-treated (black bars) at the end of chronic liver damage. BMC, bone marrow cells; EGFP, enhanced green fluorescent protein; GPT, glutamic-pyruvic aminotransferase; GOT, glutamic-oxaloacetic aminotransferase.

To induce an ongoing liver damage, 10 mL of 10% (v:v) $CCl_4$/kg of body weight was injected intraperitoneally every week for 3 months. Different plasma parameters such as glutamic-pyruvic aminotransferase, glutamic-oxaloacetic aminotransferase, and bilirubin were analyzed. As shown in Fig. 1B, glutamic-pyruvic aminotransferase values in $CCl_4$-treated mice were approximately 15 times higher than in nontreated mice, glutamic-oxaloacetic aminotransferase values increased nearly four times, and bilirubin values increased two times. Histological evaluation of hepatic slides (Fig. 2) showed that in contrast to nontreated mice, in which all hepatocytes had a similar size and there were few hematopoietic cells infiltrating the liver parenchyma (Fig. 2A), the livers of $CCl_4$-treated mice showed hepatocellular regeneration with prominent anisonucleosis and focal, scattered necrotic foci within the parenchyma without obvious zonal distribution (Fig. 2B). Cellular infiltrates in these foci were predominantly clustered histiocytes expressing the EGFP protein (Fig. 2C-D). No significant scarring or nodularity was present, thus a transition to cirrhosis was not observed.

Prominent oval cell reactions as are seen in many acute injury models like acetaminophen toxicity,[20] were not identified in response to $CCl_4$. Here, the highest number of oval cells per portal tract was twice normal, though not statistically significant (data not shown).

***G-CSF Mobilization Increases the Number of Hematopoietic Cells Infiltrating the Liver Structure.*** To study whether the increase of total hematopoietic cells and hematopoietic stem cells in the circulation could increase the number of exogenous hematopoietic cells in the liver, groups of nontreated or $CCl_4$-treated animals were additionally treated with G-CSF for 1, 2, or 3 weeks. Between 3 and 6 animals/group were studied. In all mobilized animals, blood cell numbers increased 4 to 10

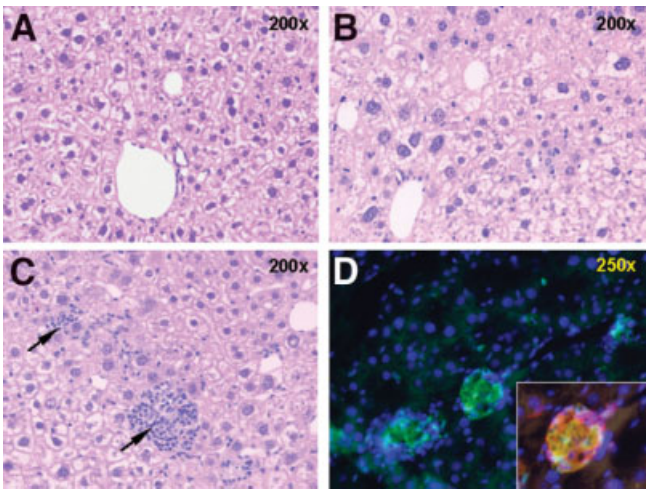


Fig. 2. Histological features induced by $CCl_4$ treatment. The micrographs show hematoxylin-eosin–stained liver sections from (A) untreated and (B-C) $CCl_4$-treated animals. Large cells with (B) high nuclear variability and (C) hematopoietic infiltrations (arrows) can be seen. (D) EGFP (green) and DAPI nuclei staining (blue). Inset: CD45 (red) and EGFP plus CD45 expression (yellow) detected in the histiocytic nodules. Original magnification is shown in each panel.

times depending on the number of weeks with G-CSF treatment. One week after the last injection, animals were sacrificed, and the presence of hematopoietic cells in the liver was detected via immunofluorescence using anti-CD45 and anti-EGFP antibodies. To minimize the contamination with hematopoietic cells present in liver sinusoids, animals were extensively perfused with saline solution before fixation. In non–G-CSF–treated, as well as in mobilized animals, we observed the presence of $EGFP^+$ cells (Figs. 2D, 3A, green). The majority of these cells were also $CD45^+$ (Fig. 2A, yellow indicating colocalization of EGFP and CD45) demonstrating that they were hematopoietic cells. Clusters of these cells, probably macrophages by morphology, were also observed in the injured liver of $CCl_4$-treated mice (Fig. 2D).

When we compared nontreated versus $CCl_4$-treated animals, the level of hematopoietic cells (evaluated as $CD45^+$ [red] cells/total liver cells) in damaged livers was increased (Fig. 3B). This hematopoietic infiltration was distributed mainly as cell groups, predominantly macrophages positive for the F4/80 monoclonal antibody (not shown), in necrotic areas in the $CCl_4$-treated group, probably recruited from circulating monocytic precursors as part of the inflammatory response. In the nonmobilized group, the cells were distributed regularly, primarily in the liver sinusoids. When the animals were treated with G-CSF for 1, 2, or 3 weeks, a progressive increase of hematopoietic cells in the liver was observed that directly correlated with the number of weeks of G-CSF treatment (Fig. 3A-B).

***Functional Hepatocytes Derived From the BM Are Detected in the Liver of $CCl_4$-Treated Mice.*** Due to the reported plasticity phenomena of hematopoietic cells, we next looked for BMDHs in the different groups. In the livers of $CCl_4$-treated animals, we detected $EGFP^+$ cells, which were large, polygonal, single, or multiple round nuclei often with prominent nucleoli and a moderate to low nucleus/cytoplasm ratio (*i.e.*, the typical morphology of hepatocytes). These cells appeared integrated into hepatocellular trabeculae and had an appearance identical to surrounding hepatocytes. They did not express the CD45 hematopoietic antigen (Fig. 4A). To confirm the complete absence of hematopoietic marker expression on these cells, we took confocal image sequences of these cells along the z-axis all the way through the tissue section

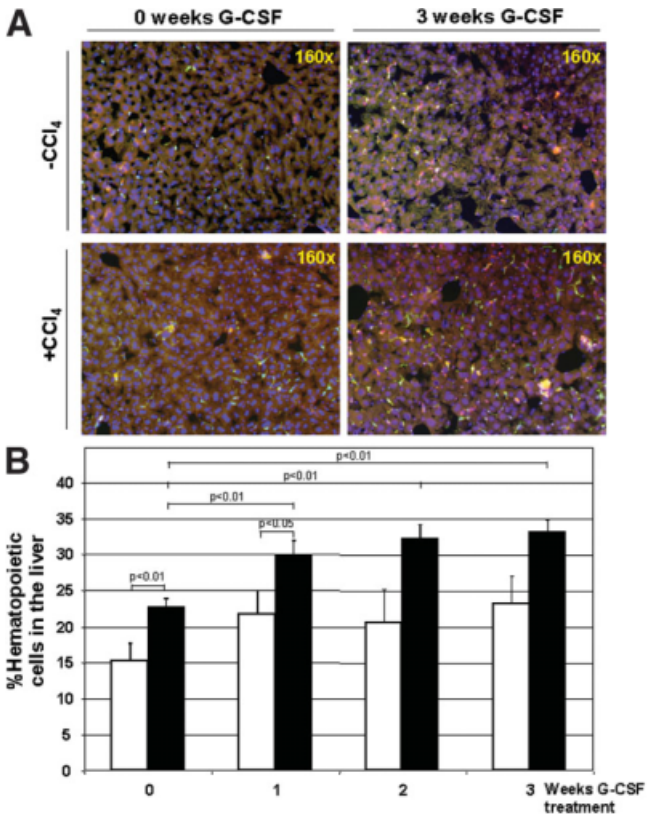


Fig. 3. G-CSF mobilization induces hematopoietic liver infiltration in untreated and $CCl_4$-treated mice. (A) Triple-staining for EGFP (green), CD45 (red), and nuclei (blue) of liver cryosections from untreated and $CCl_4$-treated animals mobilized with G-CSF or not for 3 weeks. Livers were extracted 1 week after the last dose of pegylated G-CSF; nuclei were stained with DAPI (blue); colocalization is represented in yellow. Negative and positive controls of the immunostaining are provided in the supplemental material. (B) Quantification of the presence of hematopoietic cells in livers after 0, 1, 2, and 3 weeks of pegylated G-CSF treatment. See Materials and Methods for details of quantification. Statistically significant differences between untreated (white bars) and $CCl_4$-treated (black bars) animals and between $CCl_4$-treated and nonmobilized ($n = 9$) or mobilized for 2 ($n = 4$) or 3 ($n = 10$) weeks with G-CSF are represented (Mann-Whitney test, $P < .05$ and $P < .01$ as indicated). G-CSF, granulocyte colony–stimulating factor.

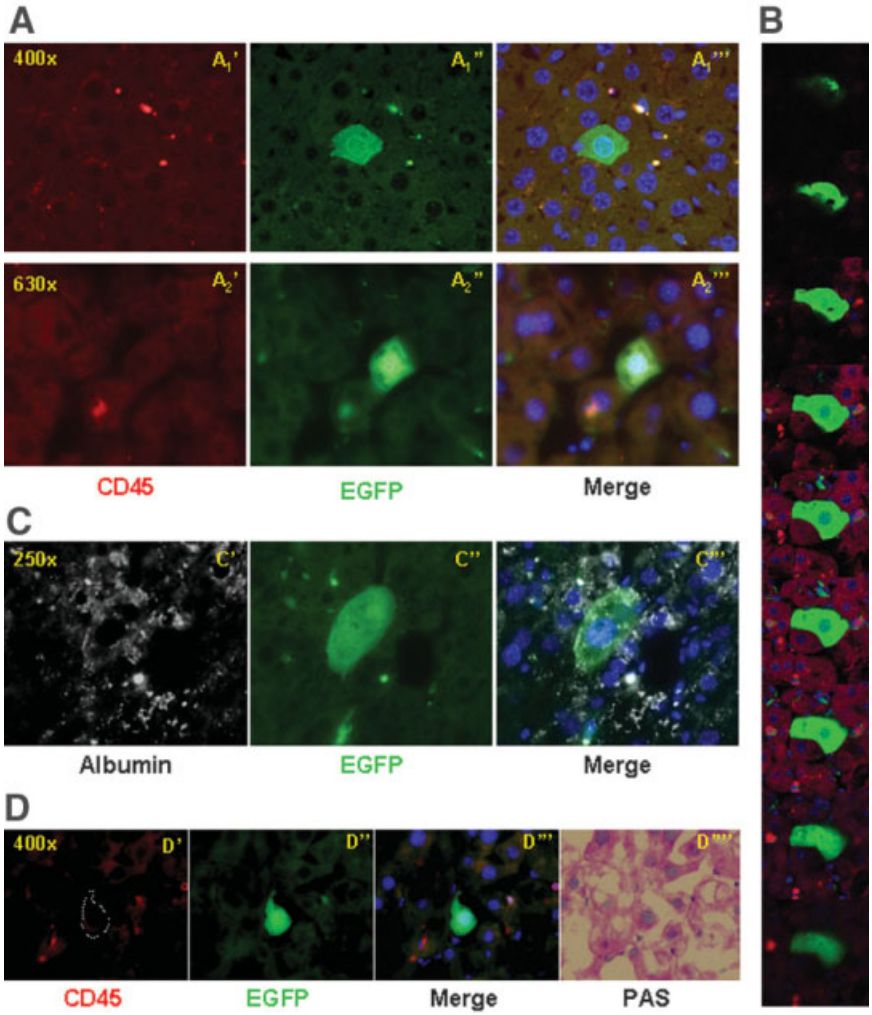


Fig. 4. Identification of BMDHs. (A) Two different examples ($A_1$, $A_2$) of cells accomplishing the conditions defining a BMDH. A', $CD45^-$ (red negative); A'', $EGFP^+$ (green), hepatocyte-like nucleus, and cytoplasm; A''', merged images. (B) Confocal microscopy Z-axis analysis of a BMDH to demonstrate the nonexpression of CD45 antigen. Images correspond to successive 0.25-$\mu$m thick sections separated by 1 $\mu$m; (C) Expression of specific hepatocyte proteins by BMDHs. C', albumin expression (white); C'', EGFP expression (green); C''', merged images together with DAPI staining (blue). (D) Periodic acid-Schiff staining to demonstrate the capacity to accumulate glycogen of BMDHs. D', $CD45^-$; D'', $EGFP^+$; D''', merged image of D', D'', and DAPI; D'''', periodic acid-Schiff $^+$ staining of the selected cell. EGFP, enhanced green fluorescent protein; PAS, periodic acid-Schiff.

(12-15 $\mu$m thick). CD45 expression was never seen in any confocal section of these polygonal cells (Fig. 4B). However, when we observed other small cells, identified as hematopoietic cells, EGFP and CD45 expressions were colocalized in the same section or alternated in consecutive sections. Thus, we assumed that there were BM-derived cells that were negative for hematopoietic markers (CD45) and had hepatocyte morphology (Fig. 4A-B). The majority of these BMDHs were located as isolated cells, although pairs of cells were also observed.

To determine if BMDHs had hepatocyte functionality, we analyzed the expression of specific hepatic proteins. The expression of albumin, biliary glycoprotein 1 (as specifically identified via its cross-reaction with rabbit polyclonal anti-carcinoembryonic antigen[21]) and hepatocyte-type cytokeratins was analyzed via immunofluorescence on paraffin-embebbed sections. Albumin expression, which is heterogeneously expressed in endogenous hepatocytes and is detected as cytoplasmatic granules, was clearly observed in $EGFP^+$ cells (Fig. 4C). The hepatocyte-specific CEA antigen was also positive in BMDHs (not shown). Hepatocytes accumulate glycogen in their cytoplasm due to their gluconeogenesis metabolic function. We also analyzed glycogen storage using periodic acid-Schiff analysis and confirmed that BMDHs accumulate glycogen (Fig. 4D). Other characteristics of hepatocytes, such as positive cytokeratin staining or characteristic high red autofluorescence due to the presence of porphyrins,[22] were also observed in these cells (data not shown). In conclusion, we demonstrated that BMDHs are fully functional by morphological and functional parameters. Interestingly, BMDHs ($GFP^+/CD45^-$) were only detected in damaged livers. Their frequency was very low (1 BMDH per 250,000 hepatocytes), suggesting that $CCl_4$ damage was required to induce the presence of BMDHs in the liver, though at a low level.

***G-CSF Mobilization Significantly Increases the Appearance of BMDH in the Livers of $CCl_4$-Treated Animals.*** Next, we investigated if G-CSF treatments would also increase the number of BMDHs. The analysis of the damaged and mobilized mice revealed a direct cor-

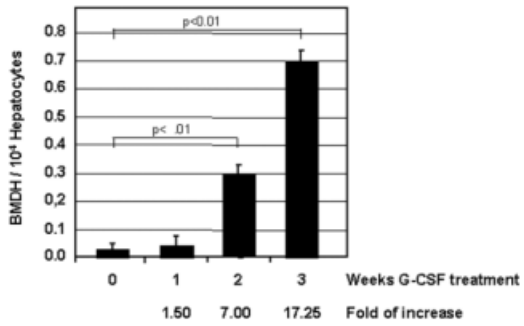


Fig. 5. G-CSF increases the percentage of BMDHs in a dose-dependent manner. The number of BMDHs present in the livers of animals treated with $CCl_4$ for 3 months and then with pegylated G-CSF for 0, 1, 2, or 3 weeks are shown. See Material and Methods for details of counting. The fold of increase with respect to no G-CSF treatment is shown. Statistically significant differences between $CCl_4$-treated and nonmobilized (n = 6) mice or mice mobilized for 1 (n = 3), 2 (n = 4), or 3 (n = 5) weeks with G-CSF are represented (Mann-Whitney test, $P < .01$). BMDH, bone marrow–derived hepatocytes; G-CSF, granulocyte colony–stimulating factor.

relation between the number of weeks with G-CSF treatment and the number of BMDHs present in the livers. Increments of 1.5-, 7-, and 17-fold were observed after 1, 2, and 3 weeks of G-CSF treatment, respectively (Fig. 5).

Although strongly significant, the number of BMDHs in the liver was very low, even after 3 weeks of G-CSF (1 BMDH versus 18 BMDHs per 250,000 hepatocytes; average of 5 and 6 animals analyzed, respectively; $P < .05$). Thus, a significant contribution of the BMDH in the recovery of the liver function would not be expected. In fact, we could not observe any shortening in the recovery to normal levels of the serum hepatic parameters after G-CSF treatment (data not shown).

***BM-Derived Hepatocytes Were Originated by Cellular Fusion.*** Finally, we investigated whether BM hepatocytes originated from a direct differentiation process in which cells from BM directly change their hematopoietic fates to a hepatic program upon entry into the liver and engraftment, or whether they were generated by an *in vivo* fusion process between endogenous hepatocytes and hematopoietic cells infiltrating the liver. We transplanted BM cells from female BDGF1 mice (constitutively expressing EGFP) into irradiated male P3D2F1 animals and then applied the $CCl_4$+3w-G-CSF protocol. In this design, if an $EGFP^+$ hepatocyte originated from a direct differentiation process, it would express the EGFP marker but would not have any male marker (Y chromosome [Y-ch]). On the contrary, if BMDHs resulted from an *in vivo* fusion event, they would also have the recipient's Y-ch (Fig. 6A). Thus, we analyzed the colocalization of Y-ch signal, EGFP, and cytokeratin expression using simultaneous fluorescence *in situ* hybridization, immunofluorescence, and immunohistochemistry in liver sections of $CCl_4$-treated and G-CSF–mobilized animals (n = 6; two sections from two different lobes per animal). We found that 74 ± 7% BMDHs were simultaneously $EFGP^+$ and $Y\text{-}ch^+$. Moreover, BMDHs expressed hepatic cytokeratins (inner box in Fig. $6B_2$'''), and the majority of them contained two nuclei: one large, round, and positive for the Y-ch, and the other one smaller, more condensed, and Y-ch–negative (Fig. 6B). As an internal control, we also analyzed the presence of Y-ch in $CD45^+$ cells and endogenous hepatocytes. No Y-ch signal was detected in the nuclei of hematopoietic cells infiltrating the liver tissue, and 81 ± 4% (n = 4) of the endogenous hepatocytes had a Y-ch signal. Because no statistical significance was observed between $Y\text{-}ch^+$ BMDHs and $Y\text{-}ch^+$ endogenous hepatocytes, we assumed that BMDHs originated through cell fusion. Interestingly, the observation of these two nuclei with very different structure suggested that cytoplasmic but not nuclear fusion had occurred. These data strongly suggest that the emergence of $EGFP^+$ BMDHs induced by $CCl_4$ treatment and aug-

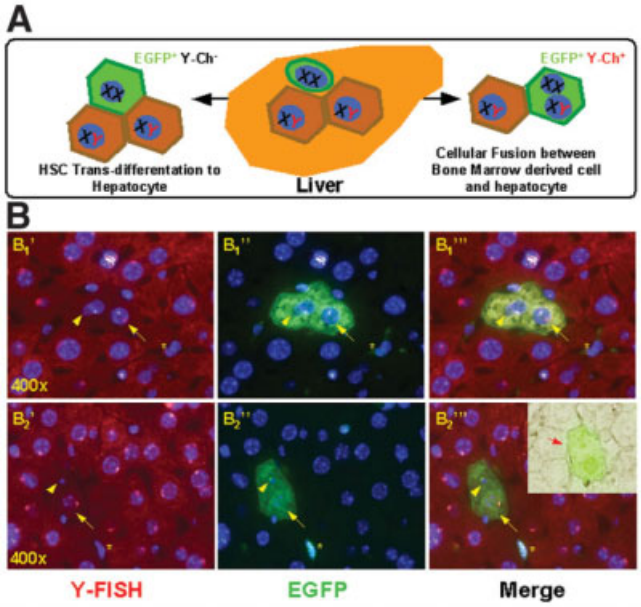


Fig. 6. BMDHs originate via *in vivo* fusion events. (A) Diagram showing the read-out of the presented experiment to distinguish the two possibilities of BMDH generation. (B) Two different examples of male hepatocytes ($Y\text{-}ch^+$) expressing the EGFP protein are shown ($B_1$, $B_2$). B', fluorescence *in situ* hybridization (red) and DAPI staining (blue); B'', EGFP stain (green) and DAPI (blue); B''', merged images showing the presence of the $Y\text{-}ch^+$ in one nucleus of the binucleated cells. Typical hepatocyte nuclei positive for the $Y\text{-}ch^+$ are shown indicating the receptor origin (arrows), smaller nuclei from donor BM (arrowheads) in BMDHs, and hematopoietic cells (asterisks). Inset: cytokeratin staining using panK polyclonal antibody visualized with a peroxidase conjugated secondary antibody merged with the EGFP staining. The characteristic pericytoplasmic staining of this antigen (red arrow) is observed in $Y\text{-}ch^+/EGFP^+$ cells. EGFP, enhanced green fluorescent protein; Y-ch, Y chromosome; FISH, fluorescence *in situ* hybridization.

mented by G-CSF mobilization is mainly due to *in vivo* fusion events between BM-derived cells and endogenous hepatocytes.

## Discussion

In the present study, we show that G-CSF treatment of mice with chronic liver damage increases the number of hematopoietic cells infiltrating the liver as well as the number of BMDHs. Moreover, we demonstrate that the principal mechanism accounting for the production of BMDHs occurs via *in vivo* cell fusion between resident hepatocytes and hematopoietic cells.

To study the plastic properties of BM cells, we used an established protocol of ongoing liver damage through the periodic injection of a chemical compound, $CCl_4$, over 3 months.[6,11,23-25] The data clearly demonstrate liver injury in the treated animals with necrotic areas, regenerative hepatocytes, and increase of infiltrated hematopoietic cells.

G-CSF treatment has been described to increase the number of peripheral blood cells and to mobilize progenitors and stem cells from the BM to the periphery.[13-15,18,26] Increase in liver infiltration of primitive hematopoietic cells has been described after G-CSF treatment,[27] and it has been observed that G-CSF treatment accelerates liver recovery by at least 1 week after acute $CCl_4$ damage.[11] Our results indicate that the continuous treatment of recipients with G-CSF increases the number of hematopoietic cells infiltrating the liver in normal as well as liver-injured animals. This increase is directly related to the number of weeks of G-CSF treatment and is strongly increased in $CCl_4$-treated animals. However, we have not detected any amelioration in the recovery of liver parameters (data not shown). Considering that we studied all the animals more than 1 month—instead of 2 to 5 days—after the last $CCl_4$ injection,[11] the endogenous liver repair potential would have restored normal liver functions in all groups.

To identify BMDHs, we looked for cells that showed the following features: (1) hepatocyte morphology (large polygonal cells with a regular cytoplasm, single or multiple large nuclei with prominent nucleoli, and a moderate to low nucleus/cytoplasm ratio); (2) integration into the normal trabecular architecture; (3) expression of the EGFP donor reporter protein; (4) no expression of hematopoietic markers (*e.g.*, CD45 antigen) on their surfaces; (5) expression of markers of hepatocyte-specific function (*e.g.*, production of albumin, canalicular staining with pCEA, hepatocyte-type cytokeratin expression). Observing these characteristics, we found that the presence of BMDH was clearly detectable, although very low, in the $CCl_4$-damaged liver (1 BMDH/250,000 total hepatocytes). However, no BMDHs were identified in nontreated animals.

The presence of BMDHs has been extensively discussed, and data indicating from very low to very high BM contribution to the liver parenchyma have been reported (reviewed in Masson et al.[28]). It seems clear that if the liver has been injured, the incidence of BMDHs is increased.[26,29-31] However, this phenomenon does not appear to occur after partial hepatectomy.[32] Our data support the idea that liver damage is needed for the contribution of BM cells to the formation of hepatocytes. However, the contribution of BMDHs is very low, in agreement with data previously reported[5,6] and in contrast with reports that describe up to 20% BM contribution.[26] Differences in liver damage protocols and in the way BMDHs are defined probably account for these discrepancies. Another explanation for our lower incidence of BMDHs could be the differences in the timing selected in our protocol set-up. We analyzed animals between 2 and 4 weeks after the last liver insult. Similarly, Kanazawa and Verma[6] analyzed $CCl_4$-damaged animals and found very similar data to those reported here. Others have analyzed animals earlier and have found much higher numbers of BMDHs.[11,29] A "short-term engraftment" of BMDH, analogous to that recognized in marrow transplantation of hematopoietic progenitors, that could function as a transient population would explain these differences. This could perhaps relate to a limited life span in BMDHs or a reduced ability to replicate (though the presence of occasional doublets suggests otherwise).

We report that treatment of the damaged animals with G-CSF increases the presence of BMDHs in the injured liver. This effect is observed only in $CCl_4$-damaged animals and not in non–$CCl_4$-treated mice, suggesting a specific effect of G-CSF treatment in the $CCl_4$-damaged liver. Interestingly, $CCl_4$ treatment alone induces a transient leukocytosis (data not shown). Other authors have also described an increase in the presence of BMDHs after treatment with G-CSF.[11] Furthermore, we have observed a clear relationship between the levels of BMDHs and the duration of G-CSF treatment (up to 3 weeks), indicating that this factor potentiates the contribution of BMDHs to the hepatic structure.

Finally, we demonstrate that in our murine model of $CCl_4$ damage and G-CSF mobilization, BMDHs originate through *in vivo* fusion mechanisms. *In vivo* fusion has been extensively described in FAH-null mice,[7-9,33] in which the selective advantage of fused cells strongly amplifies its contribution to the liver. Stadtfeld and Graf,[34] using a very elegant transgenic approach, have suggested—although not formerly demonstrated—that BMDHs could derive from fusion events. We have

demonstrated that endogenous hepatocytes and cells derived from BM fused together in a murine liver damage model in which no selective advantage has been shown.

Interestingly, this form of $CCl_4$-induced injury is known to be endothelial-toxic,[35] has shown a predominantly macrophage infiltration in areas of hepatic necrosis with local expansion of the infiltrated sinusoids, and has failed to show a prominent oval-cell proliferative response. These features are also seen in the FAH-null model.[36] Thus, we may speculate that this constellation of injuries favors engraftment by fusion, by increasing the number of fusogenic macrophages in the liver, obliterating the endothelial barrier between hepatocytes and macrophages. Moreover, irradiation has also been described to increase engraftment via cell fusion.[37] Thus, irradiation could also be involved in the process. Additionally, in a model of exogenous BM engraftment into muscle cells, it has been demonstrated that exogenous-fused nuclei, though integrated, do not express muscle-specific proteins.[37] In the present study, although BMDHs functioned as normal hepatocytes and expressed donor-derived proteins (EGFP), we did not address the reprogramming of the donor-fused nuclei (experiments in that direction are now underway). Finally, it has been described that one of the characteristics of the FAH model is nuclear instability with high rates of aneuploidy even before the animals are taken off of NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione). This fact, which was not addressed in our model, should facilitate nucleus/nucleus fusion in that model. Although we cannot exclude the possibility of nuclear fusion followed by reduction division, though no mononucleated GFP positive hepatocytes were found, this would result in 100% resolution of tetraploid-fused nuclei into two separate diploid nuclei, which would be possible but not expected.

In conclusion, using a hematopoietic growth factor (G-CSF), we have been able to modulate generation of BMDHs in a growth factor dose-dependent manner and in the absence of any exogenous selective cellular advantage. In this model, the plasticity observed was very limited and was lower than previously described in other models of injury or with other analytic approaches in the same model. In particular, this protocol involved analysis months after the final insult, perhaps representing a true end point of the postinjury repair process. Additionally, we have demonstrated that the main source of plasticity in our experimental conditions is the *in vivo* cellular fusion, presumably related to injury/repair mechanisms that invoke prominent infiltration of the liver by macrophages, endothelial injury, and a blunted, endogenous oval-cell response. Furthermore, fusion itself can be seen in low level injury as well as in the more prominent effects demonstrated previously in the FAH-null model; failure to expand is likely due to the absence of the strong selective advantage of that model.

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