Hematopoietic myelomonocytic cells are the major source of hepatocyte fusion partners

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Several recent reports have demonstrated that transplantation of bone marrow cells can result in the generation of functional hepatocytes. Cellular fusion between bone marrow–derived cells and host hepatocytes has been shown to be the mechanism of this phenomenon. However, the exact identity of the bone marrow cells that mediate cellular fusion has remained undetermined. Here we demonstrate that the hematopoietic progeny of a single hematopoietic stem cell (HSC) is sufficient to produce functional hepatic repopulation. Furthermore, transplantation of lymphocyte-deficient bone marrow cells and in vivo fate mapping of the myeloid lineage revealed that HSC-derived hepatocytes are primarily derived from mature myelomonocytic cells. In addition, using a Cre/lox–based strategy, we directly demonstrate that myeloid cells spontaneously fuse with host hepatocytes. Our findings raise the possibility that differentiated myeloid cells may be useful for future therapeutic applications of in vivo cellular fusion.

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Introduction
Several recent studies have demonstrated the ability of bone marrow (BM) cells to produce cells of multiple tissues, including skeletal and cardiac muscle (1–3), endothelium (2, 4), neurons (5), and epithelial cells of the lung (6), gut (6, 7), skin (6, 8), and liver (9–11). Collectively, these reports led many investigators to question the view that germ layer and lineage commitment are temporally restricted to embryonic development. These data bear singular importance not only because of their biological interest, but also due to their enormous therapeutic implications. However, the concept of so-called stem cell “plasticity” has been challenged by recent findings. Failure to reproduce initial experiments (12–14), the very low levels of transdifferentiation events in several animal models, and recent findings demonstrating cell fusion as the mechanism of transdifferentiation (15–17) argue against the physiological significance of stem cell plasticity. A further understanding of the cellular mechanisms involved in the regeneration of nonhematopoietic tissues by BM cells is required before we can apply any of these observations in the clinical setting.

In the case of BM transdifferentiation into liver, it was originally demonstrated that cells derived from the BM could generate functional hepatocytes and rescue a liver metabolic disease (10). In the report by Lagasse et al., transplantation of as few as 50 highly purified hematopoietic stem cells (HSCs) was sufficient to generate functional hepatocytic nodules (10). However, formal and direct demonstration that a single cell could serve as a progenitor for both hematopoietic and hepatic lineages was never provided. In addition, recent data have demonstrated that cellular fusion between BM-derived cells and host hepatocytes accounts for the principal mechanism of blood-to-liver regeneration (15–17). However, the identity of the BM-derived cells that act as hepatocyte fusion partners has remained undetermined. In the present work, by transplanting single isolated HSCs, we demonstrate, for the first time to our knowledge, that one hematopoietic cell can serve as progenitor for both blood and functional hepatocytes. Furthermore, we establish that BM-derived hepatocytes are primarily derived from hematopoietic cells of the myeloid, but not of the lymphoid, lineage. In addition, using a Cre/lox DNA recombination–based strategy, we directly show that mature myeloid cells spontaneously fuse with host hepatocytes. Our findings raise the possibility that localized administration of fusogenic cells such as myeloid cells could be a new strategy for cellular therapy of multiple tissues.

Results
In order to unambiguously demonstrate that HSCs could give rise to both blood and hepatocytes, we decided to follow the progeny of a single prospectively isolated HSC after transplantation into lethally irradiated host mice. Single side population (+) CD45+ cells from CD45.2 Rosa26 mice were transplanted into irradiated CD45.1 congenic recipients; hematopoietic and hepatic engraftment were selected for liver-engraftment analysis. Two of these mice were treated with the hepatotoxin 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) (18). Fourteen months after transplantation, liver samples were examined by X-gal staining. We detected donor cells displaying a characteristic hepatocyte-like morphology at frequencies of 1 in 300,000 and 1 in 150,000 for the noninjured and DDC-treated mice, respectively (Figure 1A). Donor hepatocyte-like cells were found primarily as isolated cells, and, in a few cases in the DDC-treated group, as clusters of two and, in one case, three cells (data not shown).
In order to more rigorously assess the hepatic potential of HSCs, BM cells derived from the single HSC were isolated by flow cytometry on the basis of their expression of the CD45.2 allele and transplanted into lethally irradiated recipients that were deficient in expression of fumarylacetoacetate hydrolase (FAH), an essential liver enzyme (19). Fab<sup>−/−</sup> mice can be maintained on the drug 2-(2-nitro-4-trifluoromethylbenzol)-1,3-cyclohexadione (NTBC), and transplantation of whole BM (WBM) cells has previously been shown to rescue this deficiency (10, 20). In the absence of NTBC, normal hepatocytes possess a remarkable selective growth advantage and can repopulate a mutant liver. In order to maximize survival of BM-recipient mice, NTBC therapy was intermittently reinstituted and withdrawn. After verification of hematopoietic engraftment, 4–7 months after transplantation, the Fab<sup>−/−</sup> transplant recipients were sacrificed and their livers analyzed histologically. Whole-mount X-gal staining of liver lobes revealed extensive blue nodular repopulation (Figure 1B). Hepatic engraftment, in agreement with previous reports (10, 20), appeared oligoclonal in nature, with approximately 200–400 β-gal<sup>−</sup> nodules of repopulation per mouse liver, similar to the prevalence of repopulation clusters observed after WBM transplantation. Histochemical analysis of livers of transplanted mice revealed extensive areas of cells with hepatocyte morphology expressing both the hepatocyte-specific FAH enzyme and β-gal (Figure 1, C–E). Furthermore, serum biochemical measurements of liver function were taken. Transplanted mutant mice showed nearly wild-type levels of all functional indicators analyzed (Table 1). These results strongly indicate that cells derived from a single HSC can produce, in addition to hematopoietic cells, fully functional hepatocytes and correct a metabolic disease.

Although these results indicate that cells derived from a single HSC can produce functional hepatocytes, it was unclear whether the HSC itself or its differentiated hematopoietic progeny fused with the host hepatocytes. In order to test whether mature hematopoietic cells of the lymphoid lineage could act as fusogenic partners for hepatocytes, we transplanted 1 × 10<sup>6</sup> WBM cells from mice deficient in both recombinase-activating gene 2 (Rag-2) and the common cytokine receptor γ chain (γc) into Fab<sup>−/−</sup> hosts. Absence of both Rag-2 and γc in mice results in the complete absence of circulating B, T, and NK cells, but normal numbers of myeloid and erythroid progeny (21). Four weeks after BM transplantation, NTBC withdrawal was initiated in order to select for FAH<sup>−/−</sup> hepatocytes. Transplanted mice were able to survive in the absence of NTBC and were maintained without the drug for more than 5 months. Immunohistochemical staining of liver sections from these mice revealed typical FAH<sup>−/−</sup> nodules throughout the liver parenchyma (data not shown). The kinetics of repopulation and the number of FAH<sup>−/−</sup> nodules per section (Table 2) in these mice were no different from those in mice transplanted with normal BM. These data suggest that cells of the lymphocyte lineage are not required as hepatocyte fusion partners.

Next we wanted to evaluate the hepatic contribution of mature hematopoietic cells of the myeloid lineage. In order to trace myeloid cells in vivo, we used Cre/lox recombination, wherein Cre recombinase, specifically expressed in myeloid cells under the endogenous regulatory sequences of the lysozyme-M (LysM) gene (22), irreversibly unblocks expression of a β-gal reporter (R26R) (23), thus marking all myeloid cells and their downstream progeny. The LysM locus in mice is exclusively active in hematopoietic cells of the myelomonocytic lineage, being expressed moderately in committed myeloid progenitors and expressed highly in mature macrophages and neutrophils. We transplanted 1 × 10<sup>6</sup> BM cells from mice that were transgenic for both the LysM-Cre gene and the R26R gene (LysM-Cre/R26R), and that also carried the CD45.2 allele, into lethally irradiated CD45.1 Fab<sup>−/−</sup> recipients. The degree

Figure 1
Hepatic differentiation after transplantation of a single HSC. Fourteen months after transplantation of a single β-gal<sup>−</sup> HSC, random liver sections were analyzed by X-gal staining. (A) Cells with hepatocyte morphology (large cells with abundant cytoplasm) that clearly expressed β-gal and were integrated into the liver parenchyma were detected in primary single-cell recipients. The micrographs were taken at ×20 magnification, with the inset at ×100. Clonally derived hematopoietic BM cells were harvested from primary recipients and transplanted into Fah<sup>−/−</sup> mice. (B) Five months after transplantation, whole-mount X-gal staining on liver lobes revealed extensive blue nodular repopulation (arrowheads). (C) FAH immunohistochemistry (dark brown staining) shown on a liver section confirms widespread oligoclonal repopulation throughout the host parenchyma (arrowheads). (D) Increased magnification of a repopulated nodule reveals typical hepatocyte morphology of the brown FAH<sup>−/−</sup> cells. (E) Frozen serial sections stained with X-gal and anti-FAH show colocalization of staining, confirming that the FAH expression is derived from the original Rosa26 (lacZ<sup>−</sup>) HSC.
of donor-derived hematopoietic chimerism in these hosts averaged 85% ± 2.9% in their peripheral blood. In order to verify the myeloid specificity of LysM-Cre expression, donor CD45.2 blood cells were analyzed for β-gal expression. Whereas only 2–8% of circulating B cells (B220+) and 1–6% of T cells (CD3+) expressed β-gal, more than 70% of granulocytic (Gr-1 high) and 80% of monocytic (Mac-1 high) cells were β-gal+ (Figure 2A).

Six months after transplantation, all Fah−/− recipient mice were NTBC independent; thus, they were sacrificed and analyzed for donor-derived hepatic contribution by X-gal whole-mount staining of liver lobes. As a positive control, staining of livers of Fah−/− mice transplanted with WBM from a ubiquitously expressed β-gal reporter (Rosa26 mice; ref. 24) revealed widespread engraftment with transplanted WBM recipients also demonstrating extensive hepatic repopulation. As a positive control, staining of livers of NTBC independent; thus, they were sacrificed and analyzed for non-myelomonocytic progenitors. As expected, histochemical analysis of two livers of Fah−/−/R26R recipients 5 months after transplantation revealed widespread repopulation by fused β-gal+ hepatic nodules (Figure 2B) that were also positive for FAH expression (Figure 2D). The number of nodules in these livers also correlated very well with that in mice transplanted with Rosa26 BM (Table 2), indicating that the great majority of hepatic nodules in the FAH model arise from random fusion of myeloid cells.

**Discussion**

Our work here significantly extends the recent observations of fusion as a mechanism for BM-to-liver transdifferentiation (15, 16) by defining the specific cell lineage involved in these phenomena. Although our studies do not directly rule out a potential hepatic contribution by granulocytic cells, we hypothesize that within the myeloid lineage, differentiated macrophages are the cell entities that act as hepatic fusion partners. The fusogenic ability of macrophages has been described in the literature for more than a century. Langhans first described the presence of multinucleated giant cells in tuberculosis granulomas in 1869 (26). Langhans’s giant cells, osteoclasts, and foreign-body giant cells were later shown to originate from the fusion of macrophage-lineage cells (26). Thus, it is very likely that the resident liver macrophages, the Kupffer cells, because of their abundance in the liver and proximity to hepatocytes, are the direct hepatic fusion partners.

Thus, although our experiments do not directly rule out that a few HSCs might be contributing to hepatocyte regeneration, they directly demonstrate that the great majority of this contribution is through a myeloid cell intermediate.

We designed an additional experiment to confirm the myeloid origin of the repopulating nodules. For this experiment we transplanted LysM BM into Fab−/− recipients that also carried an allele for the R26R reporter. In this context, only hematopoietic cells actively expressing high levels of Cre at the time of spontaneous fusion with hepatocytes would activate expression of β-gal in Fab−/−/R26R host nuclei. Since expression of Cre in HSCs seems to be oscillatory and transient and at much lower levels than in myeloid cells (25), this experiment would more effectively rule out contribution by non-myelomonocytic progenitors. As expected, histochemical analysis of two livers of Fab−/−/R26R recipients 5 months after transplantation revealed widespread repopulation by fused β-gal+ hepatic nodules (Figure 2B) that were also positive for FAH expression (Figure 2D). The number of nodules in these livers also correlated very well with that in mice transplanted with Rosa26 BM (Table 2), indicating that the great majority of hepatic nodules in the FAH model arise from random fusion of myeloid cells.

**Table 1**

Functional parameters of single-HSC–derived hepatocytes

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<th>Biochemical parameter</th>
<th>Units</th>
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<th>FAH (single cell transplanted), n = 3</th>
<th>FAH (untransplanted), n = 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>U/l</td>
<td>45 ± 10</td>
<td>112 ± 50</td>
<td>407 ± 30</td>
</tr>
<tr>
<td>Direct bilirubin</td>
<td>mg/dl</td>
<td>0</td>
<td>0</td>
<td>5.88 ± 2.5</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>μM</td>
<td>54 ± 15.1</td>
<td>69.3 ± 14</td>
<td>617 ± 18</td>
</tr>
<tr>
<td>Methionine</td>
<td>μM</td>
<td>41 ± 4</td>
<td>71.3 ± 27</td>
<td>170 ± 6.7</td>
</tr>
<tr>
<td>Alanine</td>
<td>μM</td>
<td>257 ± 41</td>
<td>443 ± 170</td>
<td>1,858 ± 186</td>
</tr>
<tr>
<td>Glycine</td>
<td>μM</td>
<td>163 ± 14</td>
<td>212 ± 27</td>
<td>466 ± 49</td>
</tr>
<tr>
<td>Glutamine</td>
<td>μM</td>
<td>501 ± 94</td>
<td>521 ± 123</td>
<td>2,680 ± 221</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>μM</td>
<td>58 ± 6.5</td>
<td>69 ± 14</td>
<td>89.7 ± 10.5</td>
</tr>
<tr>
<td>Ornithine</td>
<td>μM</td>
<td>74 ± 11</td>
<td>108 ± 34</td>
<td>672 ± 86</td>
</tr>
</tbody>
</table>

Data from *fah−/−* recipient mice represent analysis done at 4 months (one mouse) and 6 months (two mice) after transplantation. Untransplanted *Fah−/−* mice were kept off NTBC for 4 weeks before analysis was performed. ALT, Alanine aminotransferase.

**Table 2**

Contribution of different hematopoietic lineages to hepatocytes in the FAH model

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Months after transplantation</th>
<th>Number of lacZ+ nodules</th>
<th>Number of FAH+ nodules per section</th>
</tr>
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<tbody>
<tr>
<td>Rosa26 (n = 5)</td>
<td>Fab−/−</td>
<td>6</td>
<td>279 ± 38</td>
<td>36 ± 4</td>
</tr>
<tr>
<td>Rag2−/−/Cre−/− (n = 3)</td>
<td>Fab−/−</td>
<td>6</td>
<td>ND</td>
<td>39 ± 5</td>
</tr>
<tr>
<td>LysM-Cre/R26R (n = 4)</td>
<td>Fab−/−</td>
<td>6</td>
<td>247 ± 45</td>
<td>38 ± 3</td>
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<tr>
<td>LysM-Cre (n = 2)</td>
<td>Fab−/−/R26R</td>
<td>4.5</td>
<td>229 ± 43</td>
<td>32 ± 5</td>
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Donor cells were 1 × 10^6 freshly isolated BM cells. The number of lacZ+ nodules was determined by whole-mount X-gal staining of liver lobes. The number of FAH+ nodules was determined by analysis of 4-μm paraffin-embedded sections stained with anti-FAH antibody. At least three liver sections from each mouse were analyzed. ND, not determined.
Our results have important implications for the design of potential therapies based on the concept of stem cell plasticity. First, our data here and elsewhere (12) and the data of others (17) question the rationale behind clinical studies based on the idea that transdifferentiation of HSCs can lead to the de novo generation of heart or brain cells. Second, our observations that myeloid cells can act as fusogenic partners for hepatocytes and skeletal myofibers (12) raise the possibility that these cells or their derivatives could be used as vehicles for cellular therapy. Direct and localized administration of fusogenic cells could be an effective approach to deliver therapeutic DNA, bypassing the toxicity associated with traditional BM transplantation. Third, the fact that, after BM transplantation, Kupffer cells in the recipient livers are entirely donor-derived after 21 days (27) argues that recruitment of the fusogenic cell type is not the rate-limiting step in the generation of HSC-derived hepatocytes. Therefore, the extremely low incidence of initial BM-derived hepatocytes is due either to the low efficiency of fusion events in the liver or to the low probability of obtaining a productive nuclear reprogramming.

Finally, although our results argue against an unexpected developmental plasticity of HSCs, our work here and elsewhere (12) and the work of others (15–17) show that at least partial nuclear reprogramming of a differentiated adult cell can occur after cellular fusion. Activation of tissue-specific genes normally not expressed in hematopoietic cells has been reported in skeletal muscle (1, 3, 12), brain (28), and liver (10, 16). In the FAH model, hematopoietic myeloid nuclei are probably reprogrammed by transcription factors provided in trans by adjacent liver nuclei, which confer the function of FAH expression onto the donor nucleus, as well as a complete metabolic rescue. It will be interesting to see whether the donor nucleus undergoes an entire epigenetic reprogramming or whether only selected genes, necessary for selection and survival, are activated. Further investigation of the molecular mechanisms driving hepatocyte-specific expression in hematopoietic cells could facilitate identification of the factors necessary for targeted reprogramming.

Methods

Single SP sorting. Single SP sorting and transplantation were done largely as described previously (12). Briefly, BM was flushed from the tibiae and femurs of 2-month-old Rosa26 transgenic mice backcrossed onto the C57BL/6 background that also expressed the CD45.2 allele. Hoechst 33342 staining (Sigma-Aldrich, St. Louis, Missouri, USA) and antibody incubations were performed as described previously (29). Sorting gates for single cells were set on the lowest part of the SP tail, shown to contain the most primitive HSCs (30), so that only 10% of the SP cells were selected. SP cells that were double positive for Sca-1 and CD45 were then sorted on a triple-laser instrument (MooFlo; Cytomation Inc., Fort Collins, Colorado, USA). Single cells were sorted directly into individual wells of a flat-bottom 96-well plate containing 150 μl of StemPro buffer (Invitrogen, Carlsbad, California, USA) by a single-cell deposition unit.

HSC and WBM transplantation and analysis. Five- to six-week-old CD45.1 C57BL/6 mice were lethally irradiated with 10 Gy of γ-irradiation in a split dose, with 3 hours between doses. The reconstituting cells were injected retro-orbitally within 24 hours of irradiation. In order to maximize the recovery of the single cells from wells, we first aspirated the contents of each well and then washed the same well with 150 μl of buffer containing the corresponding number of nontransgenic CD45.1 C57BL/6–carrier cells — either 1 × 10^6 WBM cells depleted of cKit+Sca-1^ double-positive cells (in two experiments) or 600 Lin Sca-1^-cKit+ CD34^high cells (in one experiment). The volume in the well was then re-aspirated and injected. Carrier cells, isolated using the two parameters described above, are highly devoid of long-term HSC activity, and their in vivo lifespan ranges between 4 and 8 weeks (31). Mice were tested for hematopoietic engraftment at different time points beginning at 2 months after transplantation. For transplantation into secondary recipients, BM from two single-HSC primary recipients was harvested and stained with antibodies against CD45.2-FITC (clone 104; Pharmingen, San Diego, California, USA) and CD45.1-phycoerythrin (CD45.1-PE) (clone A20). Approximately 18 × 10^6 CD45.2^ cells from each mouse were then sorted, divided in two, and injected into lethally irradiated recipients. Levels of hematopoietic chimerism were tested by staining of peripheral blood leukocytes of recipients at various time points with CD45.2-FITC and CD45.1-PE. For determination of hematopoietic lineage contribution, peripheral blood leukocytes were stained with anti--CD45.2-FITC and antibodies against CD3 (145-2C11), Thy-1 (30-H12), B220 (RA3-6B2), Gr-1 (RB6-2C5), and Mac-1 (M1/70). All lineage antibodies were directly conjugated to FITC or PE-Cy5 and purchased from eBioscience (San Diego, California, USA). Analysis of β-gal activity in hematopoietic cells of LysM-Cre/R26R mice was performed as previously reported (32).

Tissue injury models. Liver injury in mice transplanted with single Rosa26 cells was induced by addition of DDC (Sigma-Aldrich, St.
Louis, Missouri, USA) to the animals' feed at a concentration of 0.1% for 20 days at 6 months after transplantation.

**Liver immunohistology.** For whole-mount X-gal staining, liver lobes were fixed for 2 hours in 4% paraformaldehyde, and tissues were washed with PBS and moved to staining medium that contained 1 mg/ml of X-gal in buffer of 5 mmol/l K ferricyanide, 5 mmol/l K ferrocyanide, 2 mmol/l MgCl₂, 0.02% Nonidet P-40, and 40 mmol/l HEPES. X-gal staining of frozen liver samples was done as described previously (33) on 10-μm sections. Sections were then counterstained with light H&E or nuclear fast red. For FAH immunohistochemistry, liver was fixed in 10% neutral-buffered formalin, paraffin-embedded, and sectioned at 4 μm. FAH staining using a polyclonal rabbit antibody against rat FAH was performed as described (34). In order to quantify donor-derived hepatocytes in primary recipients, we analyzed 20 random sections from different liver lobes and reported the number of positive cells (large β-gal cells with abundant cytoplasm) per the total number of hepatocytes on the slides (this estimation was based on an average of 1,600 hepatocytes per square millimeter of mouse hepatic tissue).

**Biochemical measurements.** Amino acid levels were determined on a Beckman model 6300 amino acid analyzer.

**Acknowledgments**

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