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Report

Evidence against a Stem Cell Origin of New Hepatocytes in a Common Mouse Model of Chronic Liver Injury

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SUMMARY

Hepatocytes provide most liver functions, but they can also proliferate and regenerate the liver after injury. However, under some liver injury conditions, particularly chronic liver injury where hepatocyte proliferation is impaired, liver stem cells (LSCs) are thought to replenish lost hepatocytes. Conflicting results have been reported about the identity of LSCs and their contribution to liver regeneration. To address this uncertainty, we followed candidate LSC populations by genetic fate tracing in adult mice with chronic liver injury due to a choline-deficient, ethionine-supplemented diet. In contrast to previous studies, we failed to detect hepatocytes derived from biliary epithelial cells or mesenchymal liver cells beyond a negligible frequency. In fact, we failed to detect hepatocytes that were not derived from pre-existing hepatocytes. In conclusion, our findings argue against LSCs, or other nonhepatocyte cell types, providing a backup system for hepatocyte regeneration in this common mouse model of chronic liver injury.

INTRODUCTION

The adult liver is unique in its ability to efficiently regenerate after injury. Under most circumstances, liver function is restored through replacement of damaged hepatocytes by self-duplication of remaining hepatocytes. However, when hepatocyte proliferation is impaired—as under chronic injury conditions—other cells may contribute to liver regeneration by giving rise to hepatocytes (Itoh and Miyajima, 2014).

Liver stem cells (LSCs) have long been favored as the most likely alternative source of hepatocytes in the adult liver. In the classical view, LSCs are nonhepatocyte precursors of highly proliferative progenitor cells that can differentiate into both hepatocytes and biliary epithelial cells (BECs), thereby providing a backup system for liver regeneration (Duncan et al., 2009). In support of this view, cells that are bipotential in vitro can be isolated from the adult mouse liver (Dorrell et al., 2011; Huch et al., 2013; Shin et al., 2011). These cells exhibit markers of BECs, which accords with numerous studies locating LSCs in biliary structures, particularly at the interphase of bile ducts and hepatocyte plates (Itoh and Miyajima, 2014). However, specific markers of LSCs have not been identified, and therefore no direct evidence currently exists for a contribution from LSCs to hepatocytes in vivo.

In the absence of specific LSC markers, researchers have resorted to using broader lineage markers to delineate alternative cell sources of hepatocytes in vivo. Genetic fate-tracing studies in mice based on SRY (sex determining region Y) box 9 (Sox9), osteopontin (Opn), or hepatocyte nuclear factor 1 beta (Hnf1β) expression support that cells within the BEC population can differentiate into hepatocytes (Español-Suñer et al., 2012; Furuyama et al., 2011; Rodrigo-Torres et al., 2014). Other studies of fate tracing using a human glial fibrillary acidic protein (GFAP) promoter reported that stellate cells—a mesenchymal liver cell type at the center of liver fibrosis—can give rise to new hepatocytes (Michelotti et al., 2013; Swiderska-Syn et al., 2014; Yang et al., 2008). In addition, hematopoietic cells have been implicated as hepatocyte precursors, but these findings were later clarified to be due to cell fusion (Wang et al., 2003). Recent studies not only have challenged previous reports of stellate cells giving rise to hepatocytes (Mederacke et al., 2013), but also have raised doubt about the established concept of a subset of BECs being—or being able to act as—LSCs by giving rise to hepatocytes (Tarlow et al., 2014).

Because of these contradictory findings, the contribution of LSCs, or any nonhepatocyte cell type, to the formation of new hepatocytes in the chronically injured liver is uncertain. Here, we sought to resolve this uncertainty using our previously reported hepatocyte fate-tracing mouse model (Malato et al., 2011) and mouse models that afford highly specific labeling and therefore reliable fate tracing of BECs and mesenchymal liver cells.

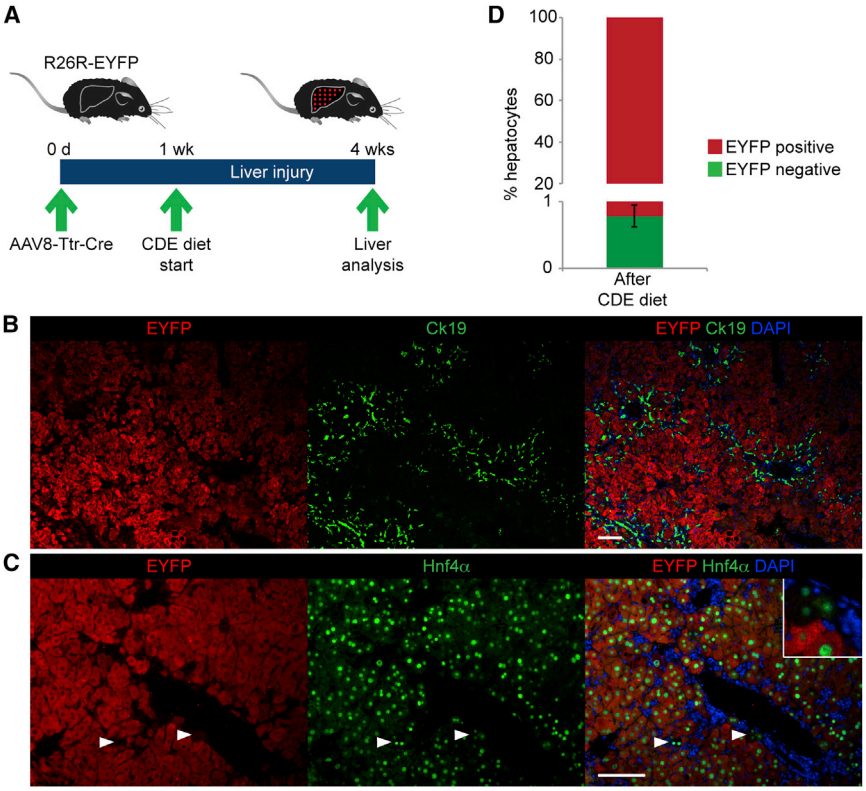

Figure 1. Presence of Unlabeled Hepatocytes in a Hepatocyte Fate-Tracing Mouse Model after Chronic Liver Injury

(A) The hepatocyte fate-tracing model was generated by injecting R26R-EYFP mice with AAV8-Ttr-Cre. Liver injury was induced by CDE diet feeding 1 week later. Livers were analyzed after 3 weeks of CDE diet feeding.

(B) Coimmunostaining for EYFP and Ck19 shows oval cell expansion characteristic for livers of mice after CDE diet feeding.

(C) Coimmunostaining for EYFP and Hnf4α shows EYFP-negative, Hnf4α-positive cells; i.e., non-fate-traced hepatocytes (arrowheads and inset).

(D) Quantification of non-fate-traced hepatocytes. Data are shown as mean ± SEM.

Scale bars, 100 μm. Representative images and results from three mice are shown. See also Figures S1 and S4B.

RESULTS

Hepatocyte Fate Tracing in Choline-Deficient, Ethionine-Supplemented Diet-Induced Chronic Liver Injury

To study the contribution of LSCs or other nonhepatocytes to new hepatocytes, we chose a mouse model of chronic liver injury caused by a choline-deficient, ethionine-supplemented (CDE) diet. The CDE diet was originally observed in rats, and subsequently in mice, to cause emergence of liver progenitor cells—called oval cells in rodents—from portal tracts, thereby mimicking ductular reactions observed in chronic liver diseases in humans (Akhurst et al., 2001; Shinozuka et al., 1978). Although other chronic liver injury models exist, we focused on CDE diet feeding because, for mice, it is the only model with which multiple research groups obtained direct evidence for the conversion of nonhepatocytes into hepatocytes (Español-Suñer et al., 2012; Rodrigo-Torres et al., 2014).

To determine the frequency at which new hepatocytes are formed from nonhepatocytes in CDE-diet-fed mice, we performed hepatocyte fate tracing. For this, we injected Cre recombinase reporter (R26R-EYFP) mice with an adenoassociated viral vector expressing Cre from the transthyretin promoter (AAV8-Ttr-Cre; Figure 1A). We showed previously that this nonintegrating vector affords specific and efficient reporter gene activation in hepatocytes but does not label BECs, stellate cells, macrophages, or endothelial cells in livers of R26R-EYFP mice (Malato et al., 2011). One week after genetically labeling hepatocytes with AAV8-Ttr-Cre, we started feeding mice the CDE diet. As previously reported (Español-Suñer et al., 2012; Rodrigo-Torres et al., 2014), the CDE diet was used for 3 weeks, after which we confirmed that a characteristic oval cell response was present—identified by the expansion of cytokeratin 19 (Ck19)-positive cells or Opn-positive cells in periportal regions (Figures 1B and S1A). Next, we analyzed the injured livers by coimmunostaining for EYFP and the hepatocyte markers hepatocyte nuclear factor 4 alpha (Hnf4α) and fumarylacetoacetate hydrolase (Fah) for the presence of EYFP-negative hepatocytes (Figures 1C and S1B). We found these non-fate-traced hepatocytes at a frequency of 0.76% ± 0.16% (Figure 1D). This result was in accord with results from previous studies showing that new hepatocytes originate—in small numbers—from a nonhepatocyte source, presumably LSCs, in mice with CDE-diet-induced chronic liver injury (Español-Suñer et al., 2012; Rodrigo-Torres et al., 2014).

Biliary Cell Fate Tracing in CDE-Diet-Induced Chronic Liver Injury

To determine the source of the observed non-fate-traced hepatocytes, we performed fate tracing of candidate cell populations. First, we tested the predominant view that LSCs capable of giving rise to hepatocytes reside in biliary structures (Español-Suñer et al., 2012; Furuyama et al., 2011; Rodrigo-Torres et al., 2014). Intriguingly, the frequency of non-fate-traced hepatocytes observed by us was similar to the frequency with which Opn-expressing cells were previously reported to give rise to hepatocytes in the same liver injury model (Español-Suñer et al., 2012). However, these results have been called into question by a recent Sox9-based fate-tracing study in which conversion of BECs into hepatocytes was not observed (Tarlow et al., 2014). To resolve this contradiction, we used a different BEC fate-tracing model, Ck19-CreER;R26R-RFP mice (Figures 2A). Direct RFP fluorescence combined with immunostaining for Opn after four tamoxifen (TAM) injections showed an overall BEC labeling efficiency of 10.5% ± 2.0%, with some bile ducts