Extrahepatic Cells Contribute to the Progenitor/Stem Cell Response Following Reduced-Size Liver Transplantation in Mice

LARS O. CONZELMANN,* IAN N. HINES,*,[1] MICHAEL KREMER,* ASHLEY W. PERRY,* JOHN J. LEMASTERS,*,† AND MICHAEL D. WHEELER*,‡,§

Center for Alcohol Studies, †Department of Cell Biology and Anatomy, ‡Department of Nutrition, and §Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

The extent to which extrahepatic cells participate in liver regeneration following transplantation is not known. Either full-size or reduced-size livers from wild-type mice were implanted into green fluorescent protein–positive (GFP^+) transgenic recipient mice to determine whether regenerated liver contained host-derived GFP^+ hepatic cells. After reduced-size liver transplantation, GFP^+ cells were localized to the portal zone of the liver lobule. Interestingly, GFP^+ cells stained for CD117, a marker for progenitor cells, beginning 2 days after transplantation. A significant number of GFP^+ $CD117^+$ cells were identified in donor livers after 28 days. GFP^+ cells comprised nearly 9% of the donor liver 28 days after reduced-size liver transplant. Moreover, GFP^+ cells also expressed the hepatic progenitor cell marker A6 and novel marker hepatic-specific antigen (HSA), as well as stem cell antigen-1 (Sca-1). Interestingly, some GFP^- cells also were stained for CD117 and A6, suggesting that both extrahepatic and intrahepatic stem cells were present and may have contributed to the regenerative response under these conditions. Reduced-size liver transplantation using GFP^+ transgenic mice supports the hypothesis that recipient-derived progenitor cells are present and may contribute to liver regeneration following transplantation. Exp Biol Med 232:571–580, 2007

Key words: GFP^+ transgenic mice; regeneration; CD117; biliary epithelia, α-fetoprotein; Sca-1

Introduction

Liver transplantation remains the primary therapy for a number of end-stage liver diseases, including those associated with hepatitis C virus infection, chronic alcohol consumption, and certain autoimmune liver diseases (1). Unfortunately, the number of viable donor organs is exceeded by the recipient population (2). This unbalanced donor-to-recipient ratio has led to the development of alternative transplant strategies, including living donor related liver transplantation and split liver transplant procedures (2, 3). These approaches have substantially expanded the donor pool from which recipients may draw. However, following partial or split liver procedures, grafts must not only survive but also proliferate to restore functional liver capacity.

A large body of literature exists describing the intricate mechanisms by which the liver restores functional capacity following major tissue loss. Early and vigorous proliferation of periportal hepatocytes replenishes lost hepatocytes, while parallel expansion of nonparenchymal cell populations rebuilds the lost hepatocellular architecture (1). This highly orchestrated response involves a number of soluble signals, including certain proinflammatory yet promitogenic cytokines like tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), as well as specific growth factors such as hepatocyte growth factor (4, 5). Together, soluble signals and defined cellular responses serve to rapidly replenish functional liver tissue both experimentally and clinically.

A growing body of data would suggest that damage to mature hepatic parenchyma may be detrimental to the regenerative response. For example, warm ischemia-reperfusion injury prior to partial hepatectomy significantly reduces the ability of the liver to function and regenerate (6–8). Indeed, increased production of oxidants, altered cytokine responses, and impaired expression of hepatoprotective, endothelial-derived, nitric oxide appear to contribute to this impairment of hepatocellular function and tissue

This study was supported in part by grants from the National Institute on Alcohol Abuse and Alcoholism, the National Institute of Diabetes & Digestive & Kidney Diseases, and Deutsche Forschungsgemeinschaft DFG, CO272/1–1.

[1] To whom correspondence should be addressed at Laboratory of Hepatobiology and Toxicology, Center for Alcohol Studies, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599. E-mail: ihines@med.unc.edu

Received August 10, 2006.
Accepted December 4, 2006.

1535-3702/07/2324-0571$15.00
Copyright © 2007 by the Society for Experimental Biology and Medicine

regeneration. Likewise, hepatosteatosis, or the accumulation of lipid within hepatocytes, also is associated with decreased regenerative capacity of hepatocytes (9, 10). Significant lipid accumulation impairs the function of important signaling molecules such as Ras, alters important cytokine signals, and disrupts normal cell cycle protein expression, leading to reduced hepatocellular proliferation (11, 12). Furthermore, steatosis may alter the function of certain inflammatory cells, including T lymphocytes, thereby exacerbating the inflammatory component in addition to inhibiting the proliferative response (13). In the setting of liver transplantation, and in particular reduced-size liver transplantation (RSLT), ischemic damage or use of compromised grafts may impair organ function, delay organ regeneration, and result in patient morbidity.

When mature hepatocyte proliferation is inhibited, a small subset of cells can be observed periportally in the canals of Herring that can proliferate and differentiate into hepatocytes and bile duct epithelial cells (14). These cells, often referred to as oval cells, represent an important alternative cell population for the restoration of tissue mass. Recent studies have demonstrated the presence of these cells in several models of liver injury and regeneration and have identified important cell surface markers, including CD117 or c-kit, stem cell antigen 1, and A6 (15, 16). This cell population shares characteristics with naive cells or stem-like cells, as well as certain hepatocyte markers, including α-fetoprotein (AFP) and albumin (17). The absolute source of this cell population is unclear. One hypothesis is that hepatic oval cells arise from an intrahepatic source directly in the canals of Herring. Alternatively, oval cells may migrate from extrahepatic sources, such as from the bone marrow or systemic circulation, to the liver, where they differentiate into both hepatocytes and bile duct epithelial cells (18, 19).

The origin and mechanisms of recruitment and differentiation of different types of hepatic progenitor cells in liver injury remains poorly understood, and good models to investigate such issues are limited. Thus, critical questions remain concerning the role of extrahepatic stem cells in liver regeneration. In particular, little is known regarding whether extrahepatic stem cells participate in regeneration of liver mass after liver transplantation. Here a liver transplantation model was developed to address the role of stem cells in liver regeneration following transplantation in which wild-type mouse livers were implanted into transgenic green fluorescent protein–positive (GFP^+) recipient mice. After both RSLT and full-size liver transplantation (FSLT), GFP^+ recipient-derived stem cells repopulated wild-type livers, differentiating into hepatocyte-like cells in the injured parenchyma.

Materials and Methods

Experimental Animals. Inbred 8- to 10-week-old male mice expressing GFP under control of the cytomegalovirus promoter on a C57Bl/6 background and C57Bl/6 wild-type mice were purchased from Jackson Laboratory (Bar Harbor, ME) and were allowed free access to laboratory chow and water. For surgery, donors and recipients were anesthetized with methoxyflurane (Metofane; Schering-Plough, Kenilworth, NJ). All mice were given humane care in compliance with institutional guidelines using protocols approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Orthotopic Liver Transplantation. Full-size orthotopic liver transplantation by modification of the procedure described by Qian *et al*. (20) and Conzelman *et al*. (21) was performed. After harvest of the liver, the infrahepatic vena cava and the portal vein were prepared with cuffs, and the explanted liver was stored in University of Wisconsin solution for 12 hrs at 4°C. Then the liver was rinsed with 4 ml lactated Ringer's solution and transplanted by connecting the suprahepatic vena cava with a running 10–0 nylon suture (Ethilon; Ethicon Inc., Somerville, NJ) and inserting the portal cuff into the recipient vessel. After securing the reconstructed portal vein with a 7–0 silk ligature, the liver was reperfused. The inferior vena cava was then reconnected. Estimated blood loss was less than 0.2 ml and was replenished with lactated Ringer's solution. Finally, the bile duct was anastomosed with an intraluminal stent (polyethylene tube PE10), and the abdomen was closed. The average time without a liver was 18 mins. For control of surgical stress, some animals were subjected to laparotomy without transplantation and are referred to as sham-operated controls.

RSLT. Orthotopic RSLTs were performed as described above with some modifications. Briefly, after explantation of the wild-type liver, the infrahepatic vena cava and the portal vein were prepared with cuffs. The left part of the median lobe, the left lateral lobe, and the papillary process then were removed to obtain a 50% reduced explanted liver. The half-sized liver was stored in University of Wisconsin solution for 1 hr at 4°C prior to transplantation. Again, the average time without a liver was 17 mins during the RSLT procedure.

Histologic Procedures. Livers were fixed by immersion in 10% buffered formalin, embedded in paraffin, and processed for histology. Tissue damage was assessed in hematoxylin and eosin–stained sections by estimating the proportion of necrotic areas.

5-Bromo-2′-Deoxyuridine (BrdU) Immunohistochemistry. BrdU was used to label DNA in the S phase of mitosis. One hour prior to sacrifice, BrdU (100 mg/kg in 0.3 ml of normal saline, ip; Sigma, St. Louis, MO) was administered. After sacrifice, livers were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections (7 μm) were deparaffinized, rehydrated, and hydrolyzed with 4 *N* HCl followed by mild proteolysis in proteinase K (20 μg/ml) for 30 mins at 37°C. Sections then were incubated with mouse anti-BrdU antibody (clone BMC

9318; Boehringer Mannheim, Mannheim, Germany) for 1 hr at room temperature. Antibody labeling was visualized using an Envision Peroxidase Staining Kit (DAKO, Carpinteria, CA).

Fluorescent Detection of GFP$^+$ Cells in Wild-Type Livers and Immunohistochemistry. Fluorescent microscopy at an excitation wavelength of 495 nm and a green emission barrier filter was used to identify GFP-positive cells. Prior to immunohistochemical staining, paraffin-embedded sections were deparaffinized and rehydrated through xylene and graded ethanol solutions. For GFP immunohistochemical analysis, sections were pretreated with trypsin for 20 mins at room temperature prior to staining. Sections then were incubated with an antibody against GFP (A.v. Living Colors; Clontech, Mountain View, CA) at a dilution of 1:200 for 2 hrs at room temperature. For CD117 staining, a 1:200 dilution of a polyclonal antibody specific for CD117 (c-kit; NeoMarkers, Freemont, CA), in Tris-buffered saline containing 0.5% Tween-20 (TTBS) and 1% bovine serum albumin (BSA) was used. For A6 staining, anti-A6 antibody was diluted 1:50 in 1% BSA-TTBS. For CK7 staining, a marker for biliary epithelial cells, anti-CK7 antibody (NeoMarkers) at a dilution of 1:200 in 1% BSA-TTBS was used. To stain cells along the hepatocellular lineage, anti–hepatic-specific antigen (anti-HSA; Cell Marque Corp., Austin, TX) and anti-AFP antibody was used at a dilution of 1:200 in 1% BSA-TTBS. For stem cell antigen-1 (Sca-1) expression, sections were first subjected to heat-induced epitope retrieval under steam in 10 m*M* sodium citrate buffer for 20 mins. Antibodies against mouse Sca-1 (clone D7; eBioscience, Camerilo, CA) at a dilution of 1:100 in 1% BSA-TTBS were then incubated with sections for 2 hrs at 37°C. For secondary staining of CD117, A6, CK7, and HSA, anti-mouse IgG or anti-rabbit IgG conjugated to Alexa 594 Red (Molecular Probes, Eugene, OR) was used, whereas AFP and GFP were visualized with the Envision Peroxidase Kit. Control staining was performed with secondary antibodies alone to account for nonspecific and background staining. For image analysis, 10 randomly selected low-power (×40) fields were captured for each liver section. Three liver sections per group were used for image analysis. Immunohistochemical image analysis was then performed using National Institutes of Health (Bethesda, MD) Image J software to determine the extent of staining.

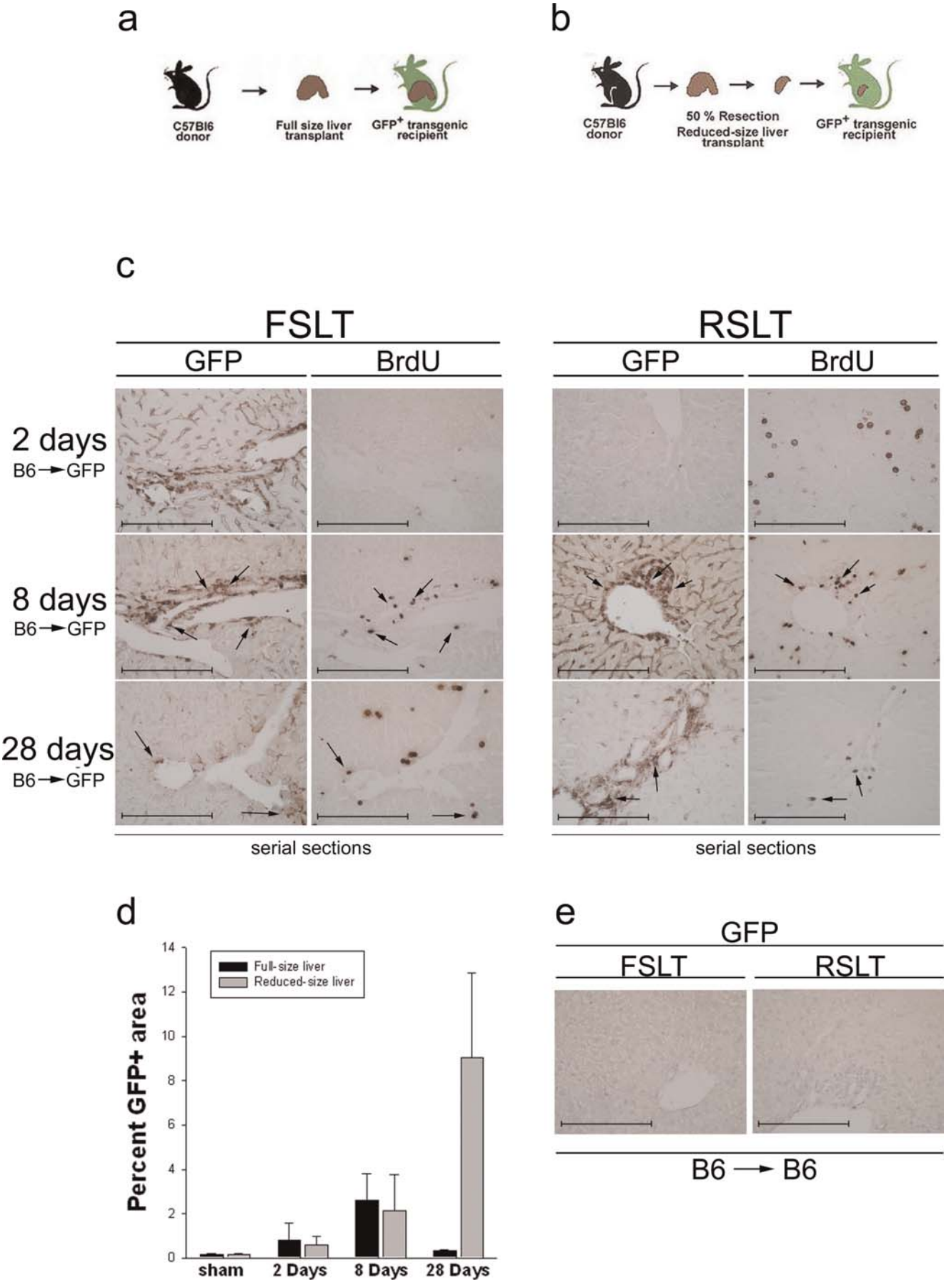
Statistical Analysis. All values are expressed as mean ± SEM. Statistical significance between two groups of data was evaluated by using an unpaired Student's *t* test with comparisons between multiple groups analyzed by ANOVA. Statistical significance was set at $P < 0.05$.

Results

Recipient-Derived Cells Are Present in the Transplanted Liver and Proliferate. The purpose of the current series of studies is to investigate the contribution of extrahepatic cells to the regenerative response within the transplanted liver. Following transplantation of both full-size (Fig. 1a, FSLT) and reduced-size (Fig. 1b, RSLT) liver allografts into GFP-transgenic mice, GFP$^+$ cells could be observed. As shown in Figure 1c, GFP-expressing cells were present as early as 2 days after transplantation in FSLT but not in RSLT. However, 8 days following either FSLT or RSLT, substantial numbers of GFP$^+$ cells could be observed in the donor liver. Interestingly, while GFP$^+$ cell numbers peaked at 8 days after transplantation with FSLT and returned to near control levels by 28 days, reduced-size liver allografts showed continued increases in GFP$^+$ cell populations out to 28 days after transplantation, data that are presented in Figure 1d. Importantly, GFP$^+$ cells were small and ovoid shaped and were arranged in close proximity to the portal triad, findings consistent with the previously described oval cell population (19). Control experiments in which C57Bl/6 livers, both full and reduced size, were transplanted into C57Bl/6 mice were conducted, and no GFP expression was observed (Fig. 1e).

To determine whether these cells might contribute to the regenerative response within the liver, colocalization studies were undertaken using BrdU as a marker of cellular proliferation. As shown in Figure 1c, BrdU-positive hepatocytes were numerous 2 days following RSLT but not FSLT. These findings are consistent with the necessity of a reduced-size liver allograft to proliferate to restore functional hepatocellular mass. At 8 and 28 days following transplant, BrdU$^+$ cells could be observed primarily periportally in both FSLT and RSLT. Moreover, using serial sections it was demonstrated that a number of BrdU$^+$ cells were also GFP$^+$ 8 days following either RSLT or FSLT. Interestingly, however, 28 days following FSLT, few GFP$^+$ cells could be observed proliferating, while numerous GFP$^+$ cells were BrdU$^+$ in the RSLT group. Together, these data demonstrate the presence of recipient cell populations following both FSLT and RSLT.

Recipient-Derived Cells Express CD117 and Sca-1, Putative Markers of Liver Progenitor Cells. Proliferation of recipient-derived cells within the transplanted liver would suggest that these cells are potentially a progenitor cell population. Their location and morphology are highly suggestive of an oval cell population. Several surface markers associated with naive cell populations, including CD117 and Sca-1, have been identified recently on oval cell populations (15, 16). We therefore used these markers to further characterize the recipient-derived cell populations in our model. As shown in Figure 2a, GFP expression could not be colocalized to CD117-expressing cells within the full-size liver allograft at any time point examined following transplant. In contrast, GFP$^+$ CD117$^+$ cells were present as early as 2 days after transplantation in reduced-size liver allografts, and they persisted and, in fact, peaked at 28 days after transplantation. Importantly, not all GFP$^+$ cells were positive for CD117, and not all CD117$^+$ cells were positive for GFP, suggesting that both intrahepatic cells and extrahepatic cells contribute to this


a
C57Bl6 donor
Full size liver transplant
GFP+ transgenic recipient
b
C57Bl6 donor
50 % Resection
Reduced-size liver transplant
GFP+ transgenic recipient
c
FSLT
RSLT
GFP
BrdU
2 days B6→GFP
8 days B6→GFP
28 days B6→GFP
serial sections
serial sections
d
Full-size liver
Reduced-size liver
Percent GFP+ area
0
2
4
6
8
10
12
14
sham
2 Days
8 Days
28 Days
e
GFP
FSLT
RSLT
B6 → B6

apparent progenitor cell response. Moreover, the $CD117^+$ cell response was more pronounced in the reduced-size liver allograft when compared to the full-size graft, although both were highest at 28 days after ligation. Also of importance is that in the wild-type to wild-type RSLTs, after 28 days CD117 expression was nearly identical to that observed at the similar time point in wild-type to GFP^+ recipient transplantation, with the exception that GFP was not observed (Fig. 2b).

To further evaluate the GFP^+ cell population within the recipient liver, serial sections were stained for GFP and Sca-1, an additional marker of liver progenitor cells (16, 22). As shown in Figure 3, Sca-1^+ cells appear in both full-size and reduced-size liver allografts as early as 2 days after ligation. These cells, located periportally, colocalize in both models, with GFP expression again suggesting that recipient-derived progenitor-like cells are present in the liver allograft. Interestingly, and in constrast to the pattern of CD117 expression, GFP^+ Sca-1^+ cells are present at 8 days after trasplantation in both full- and reduced-sized allografts at near equal levels and persist in both models for at least 28 days. Moreover, the majority, if not all, Sca-1^+ cells are GFP^+ in this model. Together, these data demonstrate the presence of extrahepatic GFP^+ cells expressing known progenitor cell markers, specifically CD117 and Sca-1, in the transplanted liver allograft. Additionally, these data highlight the potential differences in these cell populations, as Sca-1^+ cells appear regardless of the size of allograft, whereas extrahepatic $CD117^+$ cells appear only when liver mass is reduced.

Recipient-Derived Cells Differentiate Following RSLT. Progenitor cells can differentiate into biliary epithelial cells and hepatocytes (23). To determine whether extrahepatic GFP^+ progenitor cells differentiate into mature hepatocytes or biliary epithelial cells after RSLT, liver sections were stained against standard differentiation markers for hepatic progenitor cells (A6) and biliary epithelial cells (cytokeratin-7 [CK7]), as well as a novel marker of immature hepatocytes (HSA). HSA has been used as a marker for liver-derived tissues like hepatoblastoma, hepatocellular carcinoma, and hepatic adenoma (24). A6 has been described to be specific for biliary cells and proliferating progenitor cells and was recently used to characterize surface marker expression of mouse hepatic progenitor/oval cells (25, 26). At 2 and 8 days after RSLT, A6 expression was not observed in donor livers (data not shown). However, at 28 days, A6 expression was present in GFP^+ cells surrounding the portal region of the regenerating liver (Fig. 4a).

HSA was present in liver sections at 2 and 8 days after RSLT, although colocalization with GFP was not observed (data not shown). However, after 28 days, significant HSA^+ staining was present in the portal regions of the regenerated liver and colocalized in GFP-expressing cells (Fig. 4b). Interestingly, HSA was observed in both GFP^+ and GFP^- cells, suggesting that both intrahepatic and extrahepatic stem cells participate in the stem/progenitor cell response within the donor liver.

During development of the fetal liver, epithelial liver stem cells can differentiate along the hepatocyte or bile duct epithelial cell linage (23). CK7, a marker of biliary epithelium, was used to determine whether infiltrating cells were differentiating into biliary epithelium or share markers for both epithelium and hepatic lineage. Staining for CK7 was observed at 8 and 28 days after RSLT (Fig. 4c). However we observed no overlap between GFP^+ cells and CK7 expression, suggesting that extrahepatic stem cells were not differentiating into bile duct epithelium.

In addition to CK7, AFP also is expressed within the fetal liver. A number of studies have demonstrated its utility in identifying primitive cell populations within the adult liver. As shown in Figure 4d, limited numbers of small, round periportally located cells express AFP. Examination of serial sections reveals the co-expression of GFP within this cell population, a further indicator of a progenitor cell population. These findings suggest that extrahepatic cells are colonizing the liver and that these cells express markers consistent with hepatocyte-like cell populations.

Discussion

The important point of the findings here is first that a model of RSLT in the mouse was established. Second, this model was used to address the issue of the involvement of progenitor cells during liver regeneration. An unanswered question is whether hepatic progenitor cells arise from a quiescent cell within the liver or come from an extrahepatic source. It appears that these responses depend upon the type of liver injury (27).

It is clear that liver regeneration following resection alone involves little participation of a stem cell/progenitor cell response (28), except for conditions in which hepatocyte proliferation is inhibited, in which case hepatic progenitor/oval cell proliferation is induced (27, 29). Several reports suggest that progenitor cells are capable of

←

Figure 1. Contribution of extrahepatic cells during liver regeneration following transplantation. (a and b) Model of FSLT or RSLT in the mouse. A full-size liver (a) or 50% reduced-size liver (b) was transplanted from a C57Bl/6 mouse to a GFP transgenic recipient. (c) Immunohistochemical detection of GFP or BrdU 2, 8, or 28 days following FSLT or RSLT. Serial sections are provided to show colocalization of GFP and BrdU in these liver sections. Representative photomicrographs (magnification: ×400) are provided. Arrows indicate areas of colocalization. Scale bars represent 100 μm unless otherwise noted. (d) Quantitation of GFP^+ area within the full-size or reduced-size liver allograft 2, 8, or 28 days after transplantation. (e) Immunohistochemistry for GFP in C57Bl/6 mouse liver (full size and reduced size) transplanted to a C57Bl/6 wild-type (non-GFP) recipient. B6 indicates C57Bl/6 wild-type mouse. Color figure available in on-line version.

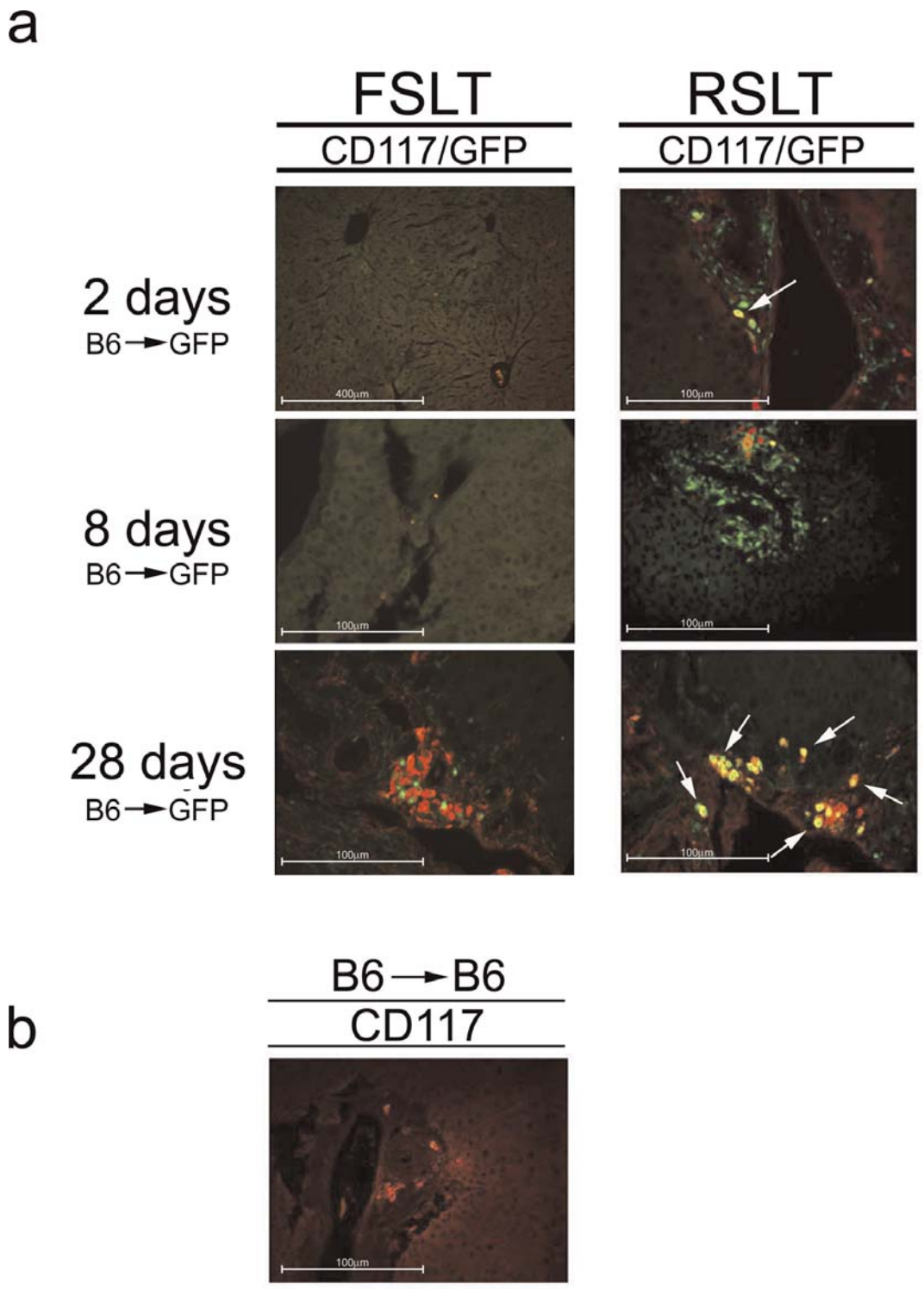


Figure 2. GFP^{+} cells infiltrating the reduced-size but not the full-size liver co-express CD117, a marker of hepatic progenitor cells. (a) Liver sections from FSLT or RSLT were stained immunofluorescently with anti-CD117 antibodies (red) and overlayed over GFP autofluorescence. Colocalization is shown in yellow. Arrows indicate double-positive cells exclusively in RSLT grafts. (b) Immunohistochemistry for CD117 in C57Bl/6 mouse liver (reduced size) 28 days following transplantation into a C57Bl/6 wild-type (non-GFP) recipient. Note similar numbers of CD117-positive cells in C57Bl/6 to GFP transplants. B6 indicates C57Bl/6 wild-type mouse. Representative photomicrographs (magnification: ×400, or ×100 for 2-day FSLT) are presented. Scale bars represent 100 μm unless otherwise noted. Color figure is available in on-line version.

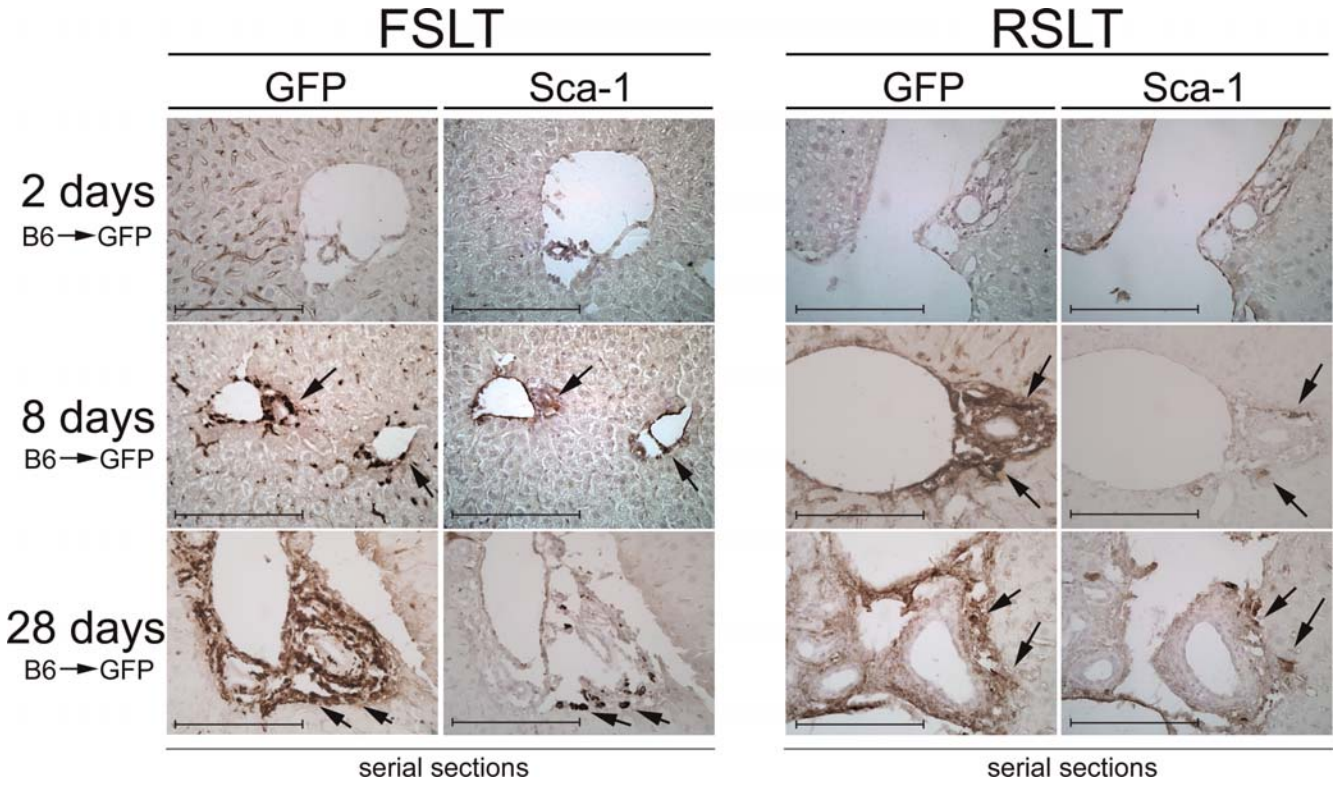


Figure 3. GFP$^+$ cells within the full-size and reduced-size transplanted liver co-express Sca-1, a marker of hepatic progenitor cells. Serial liver sections from FSLT and RSLT 2, 8, or 28 days after transplantation were stained with anti-GFP antibodies or anti–Sca-1 antibodies. Representative photomicrographs (magnification: ×400) are presented. Scale bars represent 100 μm unless otherwise noted. Arrows indicate areas of antigen colocalization. Color figure available in on-line version.

repopulating liver under specific conditions though these conditions have yet to be defined (30, 31). It seems clear, however, that hepatocyte injury and a regenerative response are both required for stem cell/hepatic progenitor cell proliferation.

In liver transplantation, necrosis of the implanted graft is expected, and subsequent regeneration follows. It is shown here that a subpopulation of periductal cells undergo a significant increase in proliferation following liver transplantation (Fig. 1). Further, it was demonstrated that a portion of these cells co-express GFP, indicating their extrahepatic origin. Liver transplantation also induced a population of cells expressing CD117, an established marker for hepatic progenitor cells (Fig. 2). Whether these hepatic progenitor cells arise from quiescent cells within liver or from an extrahepatic source such as bone marrow or hematopoietic stem cells is easily addressed using the model of mouse liver transplantation. However, because minimal cell proliferation occurs after whole-liver transplantation, a model of RSLT was developed in order to induce a robust regenerative response. The rationale is that any GFP$^+$ cells identified in donor livers would be from an extrahepatic source, such as a hematopoietic or bone marrow stem cell pool. Within 8 days after RSLT, the regenerating liver contained a remarkable number of GFP$^+$ CD117$^+$ cells, supporting the conclusion that extrahepatic cells, possibly bone marrow or hematopoietic stem cells, are involved in the regenerative response after RSLT. However, some CD117$^+$ progenitor cells were not GFP$^+$, indicating that another population of progenitor cells is already present within the liver. These intrahepatic progenitor cells are likely quiescent in normal liver and are stimulated to proliferate and express antigens associated with differentiation after transplantation. Some GFP$^+$ cells could represent inflammatory cell populations. While the current studies have not addressed the possibility, it is likely that some of the GFP$^+$ cells are lymphocytes or granulocytes that have been recruited to the ischemically damaged tissue. Further investigation is required to fully characterize the GFP$^+$ cell populations. It can be concluded from our data, which characterize these double-positive cells, that both intrahepatic progenitor cells and extrahepatic progenitor cells participate in the regeneration of the reduced-size liver grafts.

Many markers used for the identification of mouse stem/progenitor cells have not been characterized and seem to vary among studies. CD117 (c-kit) has been known to play a role in the proliferation, migration, and perhaps the differentiation of the early stem cell pool. Fujio *et al.* were the first to report that hepatic oval cells expressed stem cell factor and its receptor CD117 (32), and several groups have since used CD117/c-kit as a marker for hepatic progenitor

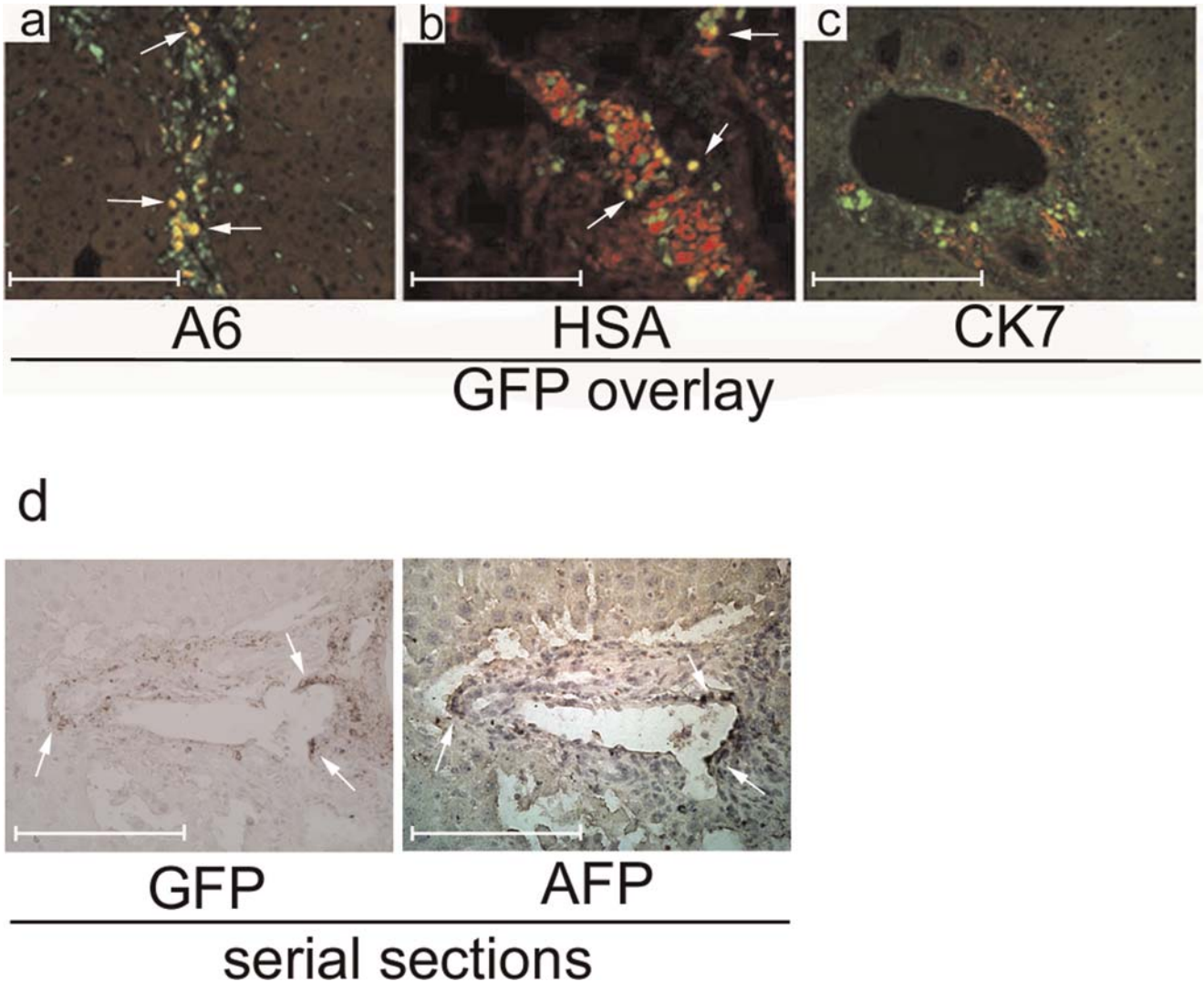


Figure 4. GFP$^+$ cells within the reduced-size liver transplant express markers of hepatic progenitor cells. Liver sections from mice 28 days following RSLT show colocalization of GFP with A6 (a), a marker of murine oval cells, and HSA (b), a marker of primitive hepatocytes, but not CK7 (c), a marker of biliary epithelial cells. (d) Serial liver sections 28 days following RSLT demonstrating colocalization of GFP with AFP periportally. Representative photomicrographs (magnification: ×400) are presented. Arrows indicate areas of antigen colocalization. Scale bars represent 100 μm unless otherwise noted. Color figure available in on-line version.

cell characterization and/or isolation. It was recently reported that c-kit$^+$ fetal hepatic progenitors also expressed AFP and albumin (33). Like CD117, Sca-1 has been used to characterize hematopoietic stem cells (22) and has recently been shown to be expressed in adult liver cell populations (34). The important point to this discussion is the fact that Sca-1 expression is observed in donor livers following transplantation and colocalizes to GFP (Fig. 3), further confirming that liver transplantation induces a progenitor cell response, and these cells may represent an influx of an extrahepatic (i.e., bone marrow or hematopoietic) cells. Perhaps more importantly, Sca-1$^+$ cell populations are, for the majority, GFP positive, indicating their extrahepatic source. Studies by Petersen *et al.* and other suggest that Sca-1$^+$ progenitor cells, which also express CD34, may represent an important early progenitor cell pool within the liver (25). Further, recent studies have identified Sca-1 on the surface of bone marrow–derived hepatic progenitor cells (35). Together, these data are supportive of Sca-1$^+$ cells as an extrahepatic cell population.

Although the issue of differentiation was not directly addressed in the experiments reported here, it was observed that GFP colocalized with several hepatocyte differentiation markers, HSA and A6, shortly following RSLT (Fig. 4). HSA is a marker for liver-derived tissues like hepatoblastoma, hepatocellular carcinoma, and hepatic adenoma (24). Notably, this is the first report that uses the HSA clone OCH1E5 antibody to evaluate hepatic stem cells. At 28 days after RSLT, GFP$^+$ cells stained strongly for HSA and A6, markers of hepatic progenitor cell differentiation. While some GFP$^+$ cells were HSA$^+$, most HSA$^+$ cells did not express GFP, and a similar observation was made with

respect to A6 staining. Expression of CK7, which is expressed on progenitor cells and bile duct epithelial cells, occurred only in a very few GFP^+ cells at any time after RSLT. There is an apparent increase in CK7 expression after transplantation, suggesting that biliary epithelial cells are indeed capable of proliferating and potentially differentiating into hepatocytes after transplantation. The increase in CK7 expression, however, does indicate that biliary epithelial cells are indeed proliferating under these conditions. The possibility that some liver-derived cells arose from biliary epithelia and began to differentiate into hepatocytes cannot be excluded by these experiments. Here, the data support the conclusion that GFP^+ cells take on phenotypes of the hepatocyte lineage, as indicated by HSA and A6 colabeling, and correlate with the appearance of AFP-expressing cells. Furthermore, *in vitro* experiments indicate that the differentiation of bone marrow–derived stem cells into AFP^+ or $albumin^+$ hepatocytes involves the temporal expression of CD117 and A6 during differentiation (25, 36).

It also was shown here that distinct periportal liver cells expressed AFP 28 days after RSLT (Fig. 4). It is reasonable that regeneration of the liver following RSLT involves hepatic progenitor cells that eventually express AFP, an early marker of hepatocellular lineage. The role of AFP in hepatocellular differentiation is not clear but is consistent with the progression of progenitor cell maturation into hepatocytes. Colocalization of AFP with GFP^+ cells in the reduced-size regenerating liver allograft further supports the notion that extrahepatic cells may contribute to the hepatic progenitor cell response in this model.

Several questions remain from findings in this study. First, what is the overall significance of the extrahepatic recruitment of potential progenitor cells? We believe that in this clinically relevant model of liver injury, these data further support the hypothesis that extrahepatic cells can be recruited to the damaged and regenerating liver. Using this model and others, we may gain a better understanding of the mechanisms involved in the recruitment, knowledge that will no doubt aid in the development of cell-based therapies for the treatment of liver disease. Second, what is the extent of the contribution of extrahepatic cells to the regenerative response in the context of liver transplantation? Based on our current results, it is apparent that some extrahepatic cells infiltrate the liver and express key markers of hepatic progenitor cells. However, few GFP^+ mature hepatocytes were observed 28 days after transplantation in the RSLT model. These data would suggest that while the cells are present, the overall proliferative response of mature hepatocytes within the liver is capable of restoring liver mass independent of extrahepatic progenitor cell populations. There are several potential reasons for this response. It is likely in the current model that extrahepatic progenitor cells infiltrate the mildly damaged liver and are poised to proliferate if needed (i.e., if the hepatic parenchyma is unable to meet the functional needs of the organism). Indeed, the presence of small GFP^+ hepatic progenitor–like cells 28 days after ligation may represent the original infiltrating cell population or a continually cycling cell population that is present in the event that hepatocyte proliferation becomes impaired. Future experiments using inhibitors of cell proliferation in combination with liver transplantation may aid in understanding the ultimate significance of extrahepatic as well as intrahepatic progenitor cell populations.

In conclusion, extrahepatic cells are observed in donor livers after RSLT. These data suggest not only that $CD117^+$ and $Sca\text{-}1^+$ cells exist within the regenerating liver, but that these cells can arise from an extrahepatic source and can contribute to hepatocyte and possibly biliary regeneration. It is important to reiterate the significance of the model and method of liver damage in stimulating the proliferative response and the subsequent differentiation of stem cells. Using the RSLT model, extrahepatic stem cell recruitment was observed. This approach allows the utilization of transgenic and knockout mice to address and validate the central hypothesis that extrahepatic stem cells are involved in liver regeneration following transplantation.

1. Fausto N, Laird AD, Webber EM. Liver regeneration. 2. Role of growth factors and cytokines in hepatic regeneration. FASEB J 9:1527–1536, 1995.
2. Florman S, Miller CM. Live donor liver transplantation. Liver Transpl 12:499–510, 2006.
3. Busuttil RW, Goss JA. Split liver transplantation. Ann Surg 229:313–321, 1999.
4. Tian Y, Jochum W, Georgiev P, Moritz W, Graf R, Clavien PA. Kupffer cell-dependent TNF-alpha signaling mediates injury in the arterialized small-for-size liver transplantation in the mouse. Proc Natl Acad Sci U S A 103:4598–4603, 2006.
5. Fausto N. Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. Hepatology 39:1477–1487, 2004.
6. Harada H, Hines IN, Flores S, Gao B, McCord J, Scheerens H, Grisham MB. Role of NADPH oxidase-derived superoxide in reduced size liver ischemia and reperfusion injury. Arch Biochem Biophys 423:103–108, 2004.
7. Harada H, Pavlick KP, Hines IN, Lefer DJ, Hoffman JM, Bharwani S, Wolf RE, Grisham MB. Sexual dimorphism in reduced-size liver ischemia and reperfusion injury in mice: role of endothelial cell nitric oxide synthase. Proc Natl Acad Sci U S A 100:739–744, 2003.
8. Harada H, Pavlick KP, Hines IN, Hoffman JM, Bharwani S, Gray L, Wolf RE, Grisham MB. Selected contribution: effects of gender on reduced-size liver ischemia and reperfusion injury. J Appl Physiol 91: 2816–2822, 2001.
9. Koneru B, Dikdan G. Hepatic steatosis and liver transplantation current clinical and experimental perspectives. Transplantation 73:325–330, 2002.
10. Selzner M, Clavien PA. Fatty liver in liver transplantation and surgery. Semin Liver Dis 21:105–113, 2001.
11. Leclercq IA, Field J, Farrell GC. Leptin-specific mechanisms for impaired liver regeneration in ob/ob mice after toxic injury. Gastroenterology 124:1451–1464, 2003.
12. Wheeler MD, Smutney OM, Check JF, Rusyn I, Schulte-Hermann R, Thurman RG. Impaired Ras membrane association and activation in PPARalpha knockout mice after partial hepatectomy. Am J Physiol Gastrointest Liver Physiol 284:G302–G312, 2003.

13. Kremer M, Hines IN, Milton RJ, Wheeler MD. Favored T helper 1 response in a mouse model of hepatosteatosis is associated with enhanced T cell-mediated hepatitis. Hepatology 44:216–227, 2006.
14. Sell S. The role of progenitor cells in repair of liver injury and in liver transplantation. Wound Repari Regen 9:467–482, 2001.
15. Crosby HA, Kelly DA, Strain AJ. Human hepatic stem-like cells isolated using c-kit or CD34 can differentiate into biliary epithelium. Gastroenterology 120:534–544, 2001.
16. Petersen BE, Grossbard B, Hatch H, Pi L, Deng J, Scott EW. Mouse A6-positive hepatic oval cells also express several hematopoietic stem cell markers. Hepatology 37:632–640, 2003.
17. Petersen BE. Hepatic "stem" cells: coming full circle. Blood Cells Mol Dis 27:590–600, 2001.
18. Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP. Bone marrow as a potential source of hepatic oval cells. Science 284:1168–1170, 1999.
19. Sigal SH, Brill S, Fiorino AS, Reid LM. The liver as a stem cell and lineage system. Am J Physiol 263:G139–G148, 1992.
20. Qian SG, Fung JJ, Demetris AV, Ildstad ST, Starzl TE. Orthotopic liver transplantation in the mouse. Transplantation 52:562–564, 1991.
21. Conzelmann LO, Zhong Z, Bunzendahl H, Wheeler MD, Lemasters JJ. Reduced-size liver transplantation in the mouse. Transplantation 76: 496–501, 2003.
22. van de RM, Heimfeld S, Spangrude GJ, Weissman IL. Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. Proc Natl Acad Sci U S A 86:4634–4638, 1989.
23. Sell S. Heterogeneity and plasticity of hepatocyte lineage cells. Hepatology 33:738–750, 2001.
24. Minervini MI, Demetris AJ, Lee RG, Carr BI, Madariaga J, Nalesnik MA. Utilization of hepatocyte-specific antibody in the immunocytochemical evaluation of liver tumors. Mod Pathol 10:686–692, 1997.
25. Petersen BE, Grossbard B, Hatch H, Pi L, Deng J and Scott EW. Mouse A6-positive hepatic oval cells also express several hematopoietic stem cell markers. Hepatology 37: 632–640, 2003.
26. Engelhardt NV, Factor VM, Medvinsky AL, Baranov VN, Lazareva MN, Poltoranina VS. Common antigen of oval and biliary epithelial cells (A6) is a differentiation marker of epithelial and erythroid cell lineages in early development of the mouse. Differentiation 55:19–26, 1993.
27. Golding M, Sarraf CE, Lalani EN, Anilkumar TV, Edwards RJ, Nagy P, Thorgeirsson SS, Alison MR. Oval cell differentiation into hepatocytes in the acetylaminofluorene-treated regenerating rat liver. Hepatology 22:1243–1253, 1995.
28. Fausto N, Campbell J. The role of haptocytes and oval cells in liver regeneration and repopulation. Mech Dev 120:117–130, 2003.
29. Alison MR, Golding M, Sarraf CE, Edwards RJ, Lalani EN. Liver damage in the rat induces hepatocyte stem cells from biliary epithelial cells. Gastroenterology 110:1182–1190, 1996.
30. Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L, Wang X, Finegold M, Weissman IL, Grompe M. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med 6:1229–1234, 2000.
31. Yavorkovsky L, Lai E, Ilic Z, Sell S. Participation of small intraportal stem cells in the restitutive response of the liver to periportal necrosis induced by allyl alcohol. Hepatology 21:1702–1712, 1995.
32. Fujio K, Evarts RP, Hu Z, Marsden ER, Thorgeirsson SS. Expression of stem cell factor and its receptor, c-kit, during liver regeneration from putative stem cells in adult rat. Lab Invest 70:511–516, 1994.
33. Minguet S, Cortegano I, Gonzalo P, Martinez-Marin JA, de AB, Salas C, Melero D, Gaspar ML, Marcos MA. A population of c-Kit(low)(CD45/TER119)- hepatic cell progenitors of 11-day postcoitus mouse embryo liver reconstitutes cell-depleted liver organoids. J Clin Invest 112:1152–1163, 2003.
34. Taniguchi H, Toyoshima T, Fukao K, Nakauchi H. Presence of hematopoietic stem cells in the adult liver. Nat Med 2:198–203, 1996.
35. Yamazaki S, Miki K, Takayama T, Hasegawa K, Sata M, Midorikawa Y, Aburatani H, Makuuchi M. Hepatic gene induction in murine bone marrow after hepatectomy. J Hepatol 44:325–333, 2006.
36. Wang J, Clark JB, Rhee GS, Fair JH, Reid LM, Gerber DA. Proliferation and hepatic differentiation of adult-derived progenitor cells. Cells Tissues Organs 173:193–203, 2003.