

# Cellular Origin of Regenerating Parenchyma in a Mouse Model of Severe Hepatic Injury

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**Several treatments in rodents, including administration of the alkylating agent dipin, followed by two-thirds partial hepatectomy in mice combine destruction of liver parenchyma with hepatocyte mitoinhibition. These treatments induce proliferation of bile epithelial-like cells (termed oval cells), development of foci composed of small hepatocytes, and eventual replacement of damaged parenchyma by healthy hepatocytes. It has been proposed that these oval cells represent transitional cells in a nonhepatocytic liver facultative stem cell lineage that can give rise to the small hepatocyte foci, and that these foci eventually become confluent and replace liver parenchyma. In this study, we used *in vivo* cell lineage marking in genetically chimeric livers to test the hypothesis that hepatocytes can serve as the precursor cell type to the small hepatocyte foci that develop in mouse liver after treatment with dipin plus partial hepatectomy. Although we do not exclude the possibility that some small hepatocyte foci may be stem cell-derived, we demonstrate that hepatocyte-derived foci are present after dipin-induced liver damage in mice. (*Am J Pathol* 2000, 157:561–569)**

Unlike rapidly renewing epithelia (eg, intestinal mucosa and epidermis), in which an active stem cell population continually initiates replacement of differentiated cells, the adult liver is a quiescent organ. However, despite this lack of a mitotically active cell population, liver mass can be restored rapidly after toxin- or surgical-mediated loss of liver parenchyma. For example, after two-thirds hepatectomy, the liver fully regains its original weight within 7–10 days. Liver mass is restored by a process of compensatory hyperplasia, in which differentiated hepatocytes in the remnant lobes respond to the functional deficit by exiting  $G_0$  and undergoing one or several rounds of DNA synthesis, followed by replication of non-parenchymal cells. Recent studies have demonstrated that hepatocytes have a remarkable replicative capacity,<sup>1–3</sup> suggesting that they may be considered a “committed stem cell” population, capable of giving rise only to additional hepatocytes.<sup>4,5</sup> In contrast, in a different

type of liver regeneration in which hepatocyte mitoinhibition is combined with severe loss or destruction of liver parenchyma, undifferentiated stem cells are proposed to give rise to new hepatocytes. Several protocols that induce this type of liver regeneration have been developed in the rat and mouse (reviewed in ref. 6). Subsequent to each of these treatments, a population of small cells with scant cytoplasm and ovoid nuclei (oval cells) is found next to biliary ductules.<sup>5–9</sup> Oval cells proliferate, forming cords and channels that connect to bile ducts, and migrate into the hepatic parenchyma. After liver regeneration is complete the oval cells are no longer observed. In the rat, models include administration of 2-acetylaminofluorene before and after two-thirds hepatectomy,<sup>10–21</sup> a single intraperitoneal injection of D-galactosamine,<sup>22,23</sup> and chronic feeding of a choline-deficient diet.<sup>24–30</sup> Mice treated with 1,4-bis[*N,N'*-di(ethylene)-phosphamide]piperazine (dipin) followed by two-thirds hepatectomy (as a mitotic stimulus) also exhibit an oval cell response.<sup>31–34</sup> Dipin is an alkylating agent that causes chromosomal breakage in cells undergoing DNA synthesis; therefore, hepatocytes that attempt to respond to the loss of liver parenchyma by re-entering the cell cycle may be irreversibly damaged. Oval cells arise 1–3 weeks after dipin treatment, with a maximum response beginning at 8–10 weeks after treatment. At that time basophilic hepatocytes can be observed adjacent to oval cells. Foci of these small hepatocytes increase in size, often gradually displacing pre-existing damaged hepatocytes. By 5–6 months after treatment, damaged parenchyma has been replaced by normal-appearing parenchyma, and it has been proposed that the progeny of these small hepatocytes repopulate the liver.

Several lines of evidence suggest that oval cells may be transitional cells in a facultative stem cell lineage that ultimately gives rise to mature hepatocytes in cases of severe hepatic injury. Oval cells are often found in tight association with intermediate cells that display morphological characteristics of both hepatocytes and biliary epithelial cells<sup>11,13,16,17,22,23,33,34</sup>; oval cells also can share tight junctions, desmosomes, and bile canalicular-like structures with neighboring hepatocytes.<sup>26,28,34</sup> Although oval cells are a heterogeneous population, a

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subset expresses hepatocyte-specific genes such as  $\alpha$ -fetoprotein and albumin<sup>11,12,15,17,18,21-23,25,30</sup>. Immunohistochemical staining has demonstrated that oval and biliary epithelial cell antigens are present on some hepatocytes after severe liver damage in rodents.<sup>13,32</sup> Cultured rat liver epithelial cells (oval-like cells) can serve as progenitor cells for normal hepatocytes or hepatocellular carcinomas after intrahepatic or subcutaneous transplantation.<sup>35-38</sup> Finally, and most importantly, when [<sup>3</sup>H]thymidine was used to prelabel oval cells in two different rat models of hepatocyte mitoinhibition, the label was found later in basophilic hepatocytes, suggesting that the hepatocytes may be progeny of the oval cells.<sup>11,12,20,22</sup> Taken together, these data suggest that a precursor-product relationship may exist between oval cells and hepatocytes.

Although there are persuasive data that support a role for oval cells as a transitional cell type in a lineage that can produce hepatocytes in adult liver, the evidence is not conclusive.<sup>10,14,24,27</sup> Limitations of previous approaches include 1) reliance on examination of fixed liver sections collected at multiple time points from different animals for morphological studies, 2) the possibility of altered cell lineage potential in cultured cells, and 3) the difficulty of interpreting *in vivo* labeling due to potential label transfer between cells or label dilution in replicating cells. For example, some investigators were unable to find intermediate cells associated with oval cells,<sup>27</sup> and others were unable to find evidence of label transfer from the oval cell fraction to hepatocytes.<sup>14</sup> Thus a principal difficulty in determining whether oval cells are part of a facultative stem cell lineage is that much of the evidence has been circumstantial; transitions of individual cells from one cell type to another cannot be observed in a live animal. In this study, we used *in vivo* marking of cell lineage based on a genetic difference (transgene status) between hepatocytes and nonhepatocytes to identify the cellular source of small hepatocyte foci in dipin-treated mouse liver. In particular, we wished to determine whether these new hepatocytes could be derived from hepatocytes that escape dipin-mediated lethality, rather than exclusively from a nonhepatocytic facultative stem cell population.

## Materials and Methods

### Transgenic Mice

Mice carrying a major urinary protein (MUP)-urokinase-type plasminogen activator (uPA) fusion transgene were used as hepatocyte recipients. Construction of the MUP-uPA transgene construct has been described (Weglarz et al, manuscript submitted for publication). Briefly, the transgene was generated by joining the MUP gene promoter to the uPA coding sequence and substituting 3' noncoding DNA, including the poly(A) addition signal from the human growth hormone gene. The fusion construct was microinjected into fertilized C57BL/6 strain mouse eggs using standard methods.<sup>39</sup> MUP-uPA mice were identified by polymerase chain reaction, using a forward probe specific for uPA, 5'-GCGATTCTGGAG-GACCGCTTATC-3', and a reverse probe specific for hu-

man growth hormone, 5'-TTAGGACAAGGCTGGT-GGGCACTG-3'. Genomic DNA extracted from tail tissue was amplified in a 25- $\mu$ l reaction mixture under the following conditions: 1) 92°C for 2 minutes; 2) 35 cycles of 45 seconds at 92°C, 1 minute at 60°C, and 1 minute at 72°C; and 3) 72°C for 5 minutes. Transgene DNA displayed an amplified product band of 300 bp on an agarose gel.

Transgenic mice expressing uPA in hepatocytes display hepatocellular lesions.<sup>1,40</sup> In albumin promoter-uPA mice (AL-uPA), a small fraction of hepatocytes physically delete transgene DNA. These transgene-deficient hepatocytes, liberated from the toxic effects of uPA expression, proliferate at the expense of remaining uPA-expressing cells, eventually leading to complete clonal repopulation of the liver by endogenous transgene-deficient hepatocytes. This model also has been used to achieve replacement of diseased liver by transplanted healthy hepatocytes.<sup>2</sup> Typically transplant recipients display between 20% and 80% repopulation by donor hepatocytes, with the remaining parenchyma composed of endogenous transgene-deficient hepatocytes. MUP-uPA transgenic mice develop liver disease resembling that described for AL-uPA transgenic mice and similarly permit extensive parenchymal repopulation by transplanted healthy hepatocytes (Weglarz et al, manuscript submitted for publication).

Hepatocyte donor mice carried the metallothionein (MT)-nLacZ transgene.<sup>2</sup> Expression from the MT enhancer/promoter is inducible in liver by heavy metals (zinc or cadmium). The MT-nLacZ transgene encodes  $\beta$ -galactosidase ( $\beta$ -gal) protein that localizes to the nucleus; cells that contain this protein can be detected both histochemically and immunohistochemically. MT-nLacZ mice were identified by polymerase chain reaction, using the forward probe 5'-CAGAGCGGGTAAACTGGCTCGGAT-TAG-3' and the reverse probe 5'-GACACCAGACCAAC TGGTAATGGTAGC-3'. Genomic DNA extracted from tail tissue was amplified in a 25- $\mu$ l reaction mixture under the following conditions: 1) 94°C for 3 minutes; 2) 35 cycles of 30 seconds at 94°C, 1 minute at 55°C, and 1 minute at 72°C; and 3) 72°C for 7 minutes. Transgene DNA displayed an amplified product band of 400 bp on an agarose gel.

Mice were housed in AAALAC-accredited facilities, and all husbandry and experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the School of Veterinary Medicine Animal Care and Use Committee. Certain transgenic mice used in these studies have been assigned the following genetic designations: MUP-uPA line 350-2, TgN(MupPlau)1Eps; MT-nLacZ line 379-4, TgN(Mt1nLacZ)4Eps.

### Hepatocyte Isolation and Cell Transplantation

To control for genetic background effects, all donor and recipient mice in these studies were (C57BL/6xFVB)F1 hybrids. Hepatocytes were isolated from adult mice carrying the MT-nLacZ transgene, using standard two-step

EDTA-collagenase liver perfusion.<sup>41</sup> The isolated cells were collected in L15 medium (Gibco) and centrifuged at 4°C for 1 minute at 440 × *g*, and the resulting cell pellet was resuspended in L15. A sample from each of three donor cell populations collected with this procedure was placed on a slide and examined microscopically. In those samples, hepatocytes comprised between 74% and 88% of all cells. Examination of Trypan blue-stained cells with a hemacytometer indicated that this procedure routinely yielded approximately 10<sup>7</sup> hepatocytes per mouse, with viability ranging from 60% to 90%. Cells were stored on ice. For transplantation, 2- to 5-week-old recipient mice were anesthetized with Avertin, and cells were surgically injected into recipient mouse spleen with a threaded-plunger syringe (product 81041; Hamilton Company, Reno, NV) with a 26-gauge needle. Each recipient animal received between 2.3 and 8.6 × 10<sup>5</sup> viable donor hepatocytes.

### *Dipin Treatment*

Dipin (1,4-bis[*N,N'*-di(ethylene)-phosphamide]piperazine) was synthesized at the All Union Pharmaceutical Research Institute, Russia (a gift of Dr. Valentina Factor). Dipin is an alkylating agent that induces chromosomal breakage in dividing cells. Mice over 19 weeks old (at least 15 weeks after transplant) were given 60–120 mg dipin/kg body weight in phosphate-buffered saline (PBS) by intraperitoneal injection; control mice were injected with PBS. Approximately 2 hours later, mice were anesthetized, and a two-thirds partial hepatectomy was performed by surgical removal of the left and median liver lobes.<sup>42</sup> Tissue removed at partial hepatectomy was fixed and saved for analysis. Mice were allowed to recover on a warm plate and were sacrificed between 2 and 23 weeks after the operation.

### *Tissue Procedures*

To induce MT-nLacZ expression in donor cells, recipient mice were given 25 mmol/L zinc sulfate in drinking water before partial hepatectomy or received a single intraperitoneal injection of cadmium sulfate (1 mg Cd<sup>2+</sup>/kg body weight) approximately 20 hours before sacrifice. Liver removed during two-thirds hepatectomy and after mice were killed by CO<sub>2</sub> euthanasia was subjected to histochemical and immunohistochemical analysis.

Separate pieces from each liver lobe were fixed at 4°C in 4% paraformaldehyde for 1–2 hours or submerged in OCT compound and frozen in liquid nitrogen for subsequent cryostat sectioning. One-half of each paraformaldehyde-fixed lobe was stained histochemically to identify LacZ-expressing donor cells on the liver surface, using the substrate 5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactoside (X-gal) (United States Biological).<sup>2</sup> Remaining paraformaldehyde-fixed tissues were transferred to 70% ethanol, embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E), and examined microscopically. Some paraformaldehyde-fixed, paraffin sections were rehydrated and stained immunohistochemically (see below) to identify  $\beta$ -gal-containing cells. Finally, tissue frozen in OCT compound was cryostat sectioned, fixed for 10 min-

utes at 4°C in 1.25% glutaraldehyde, and stained histochemically with X-gal to identify  $\beta$ -gal-containing cells.<sup>2</sup>

For immunohistochemistry to identify  $\beta$ -galactosidase, paraffin sections were stained with a polyclonal rabbit antiserum (7-063100; Eppendorf-5 Prime, Boulder, CO). Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol. The antiserum was diluted to between 1:50 and 1:150 in PBS plus 0.1% nonfat dried milk and applied overnight at 4°C. The secondary antibody was biotinylated anti-rabbit (HK336–9R; BioGenex, San Ramon, CA), and the label was peroxidase-conjugated streptavidin (HK330–5K; BioGenex). Color development was performed with diaminobenzidine peroxidase substrate (Sigma; D-4293).

The A6 rat monoclonal antibody (provided by Dr. Valentina Factor) was used on frozen sections to immunohistochemically detect cell membrane antigens on mouse oval and biliary epithelial cells.<sup>32,34</sup> Cryostat sections were thawed to room temperature and fixed in 1.25% glutaraldehyde for 10 minutes at 4°C or in 100% ethanol for 5 minutes at 4°C. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol. The A6 antibody was diluted by 1:30 to 1:100 in PBS plus 0.1% nonfat dried milk and applied overnight at room temperature. The secondary antibody was biotinylated anti-rat (HK393–9T, 1:10 dilution; BioGenex), and the label was peroxidase-conjugated streptavidin (HK330–5K, 1:10 dilution; BioGenex). Color development was performed with diaminobenzidine peroxidase substrate.

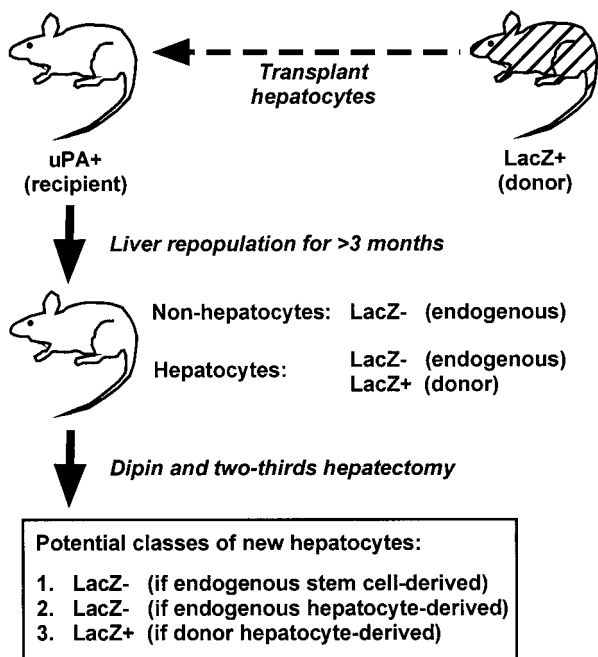
### *Quantitation of Parenchymal Repopulation by Donor Cells*

The percentage of the hepatic surface area occupied by blue-staining donor cells in pieces of whole liver was determined by computer-assisted image analysis with Image Pro software (Media Cybernetics Image Pro Plus Capture; Image Pro, Silver Spring, MD).<sup>43</sup>

The extent of donor cell repopulation in individual tissue cross sections was determined both for paraffin-embedded and cryostat sections. Slides were stained histochemically or immunohistochemically to identify LacZ-expressing donor hepatocytes and visualized under the microscope with an eyepiece reticle. For each reticle grid intersection point falling on a hepatocyte, the origin of that hepatocyte (donor or endogenous) was recorded. Microscope counts from at least 18 randomly placed grids (>2000 cells) were added together to determine the percentage of donor-derived hepatocytes in each liver examined.

### *Statistics*

Data were analyzed using GraphPad Prism version 2.0 (GraphPad Software, Inc., San Diego, CA). A two-tailed paired *t*-test was performed, with significance recognized when *P* < 0.05.



**Figure 1.** Experimental design (see text for details). Note that transplanted donor cell preparations are not composed exclusively of hepatocytes, and thus some nonparenchymal cells also will be transplanted. However, these cells are not stimulated to divide in recipient liver, so they represent at most a small fraction (<0.1%; see Discussion) of the endogenous nonparenchymal cell number, and their potential as a source of newly developing hepatocytes will be small.

## Results

### Experimental Design

The studies described below take advantage of the chimeric genetic status of parenchyma in repopulated MUP-uPA mouse liver (Figure 1).<sup>44</sup> Recipient livers display stable repopulation by two kinds of hepatocytes, endogenous cells and LacZ-marked donor cells, 3 months after transplant. In contrast, nonparenchymal cells in the recipient should be almost exclusively of recipient origin.<sup>45</sup> After repopulation was complete, mice were given dipin, followed by two-thirds partial hepatectomy. Livers were collected during the next 6 months to identify the source of the newly developing hepatocytes.

### Dipin Treatment Produces Hepatocellular Abnormalities in uPA Transgenic Mice

Livers were collected at various times after treatment from 44 MUP-uPA transgenic mice and examined for the

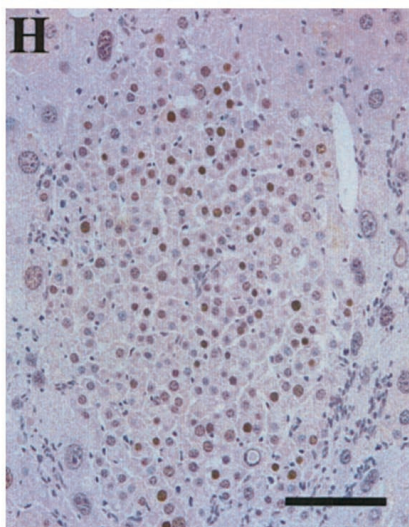
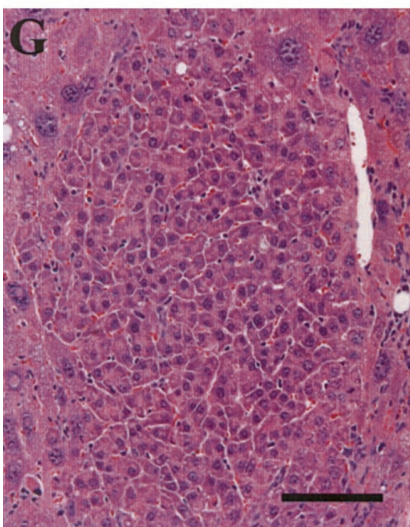
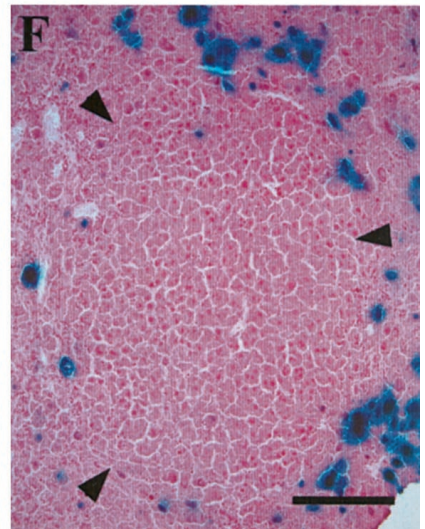
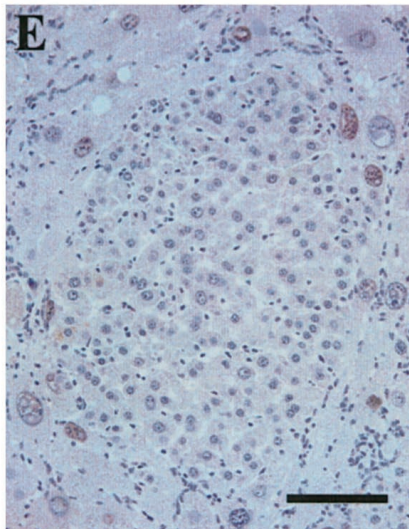
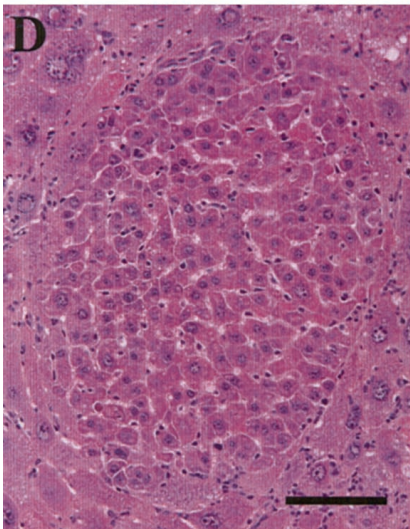
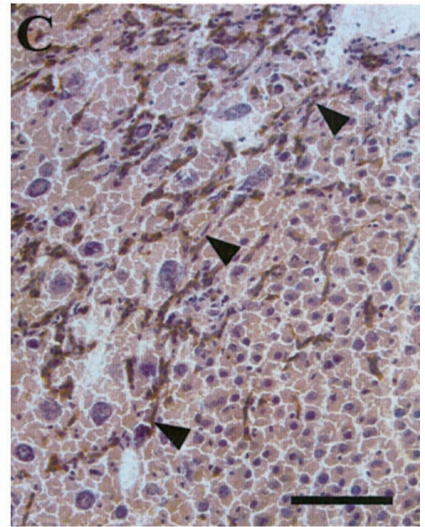
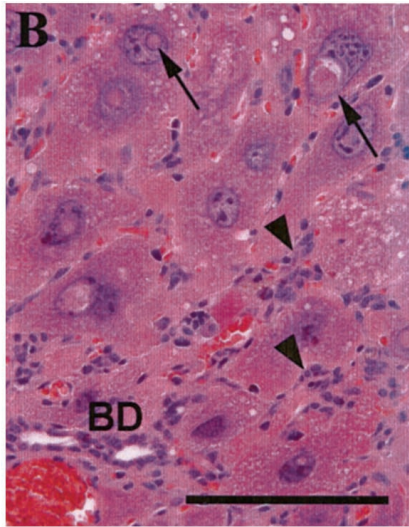
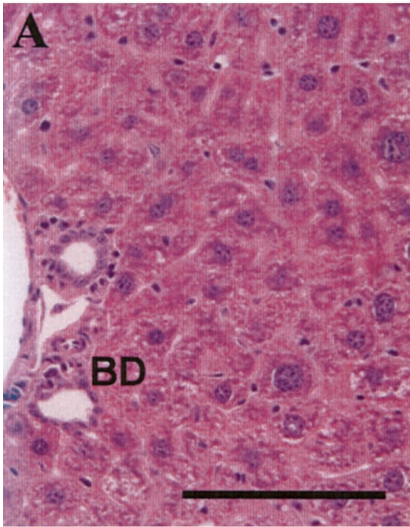
**Table 1.** Classification of Hepatic Phenotype in Dipin-Treated Mice

Classification of liver morphology	Time of sacrifice (weeks after dipin)				
	0-5	6-10	11-15	16-20	21-23
MUP-uPA					
All mice	4	9	11	10	10
Oval cells	1	6	8	7	4
Hepatocellular abnormalities	0	3	9	9	8
Normal liver	3	3	1	1	2
Nontransgenic					
All mice	0	1	9	8	3
Oval cells	0	3	4	0	
Hepatocellular abnormalities	0	1	4	1	
Normal liver		1	6	4	2

presence of morphological alterations (Table 1). After dipin treatment and hepatectomy, 59% of all dipin-treated MUP-uPA mice displayed oval cells extending into the parenchyma. Oval cells first were visible at 5 weeks after treatment, and the response could be observed in some mice throughout the 23-week duration of the experiment (Figure 2, A-C). Hepatocellular abnormalities were observed in 66% of MUP-uPA transgenic mice, beginning at 8 weeks after dipin treatment (Figure 2B), and included 1) hepatocyte pleiomorphism, including frequent hepatocytomegaly; 2) hepatocytes with large, deeply basophilic nuclei; 3) frequent hepatocyte cytoplasmic nuclear inclusions; 4) mitotic figures in some samples; and 5) development of variably sized foci composed of small, uniform-appearing hepatocytes (Figure 2, D-I). This response to dipin plus partial hepatectomy resembles that observed previously in mice, although the extent of the response was less than that reported by others.<sup>31-34</sup> However, one investigator (V. Factor, personal communication) routinely observed a similarly blunted response when mice over 2 months of age were treated with dipin plus partial hepatectomy.

In addition, four MUP-uPA control mice treated with PBS and subjected to partial hepatectomy displayed no evidence of either an oval cell response or hepatocellular abnormalities at 13-17 weeks after dipin treatment (Figure 2A). Furthermore, livers of 11 MUP-uPA transplant recipients not subjected to dipin treatment or partial hepatectomy were examined at 18 months after transplant. Only one displayed moderate hepatocyte pleiomorphism and increased hepatocyte mitosis, but none of the other hepatocellular abnormalities were observed in dipin-treated mice, indicating that the hepatic changes are not

**Figure 2.** Hepatic lesions in dipin-treated mice. **A:** Liver morphology in a PBS-treated mouse 13 weeks after hepatectomy. BD, bile duct. **B:** Liver morphology in a dipin-treated MUP-uPA mouse 15 weeks after hepatectomy. Dipin-induced abnormalities include hepatocytomegaly, frequent nuclear inclusions (arrows), and oval cells (arrowheads). BD, bile duct. **C:** Immunohistochemical staining with A6 monoclonal antibody of MUP-uPA liver collected 15 weeks after dipin treatment. In contrast to hepatocytes, oval cells and bile epithelial cells contain A6 antigen and stain dark brown. Arrowheads mark the edge of a focus of small hepatocytes. **D-F:** Endogenous focus of small hepatocytes. **D** and **E:** Adjacent sections of a small hepatocyte focus stained with H&E (**D**) and immunohistochemically with antiserum to  $\beta$ -gal (**E**). **F:** A separate focus (arrowheads) incubated with X-gal. Nuclear fast red counterstain. Hepatocytes in endogenous foci do not contain  $\beta$ -gal. **G-I:** Donor-derived focus of small hepatocytes. **G** and **H:** Adjacent sections of a small hepatocyte focus stained with H&E (**G**) and immunohistochemically with antiserum to  $\beta$ -gal (**H**). **I:** A separate focus (arrowheads) incubated with X-gal. Nuclear fast red counterstain. Hepatocytes in donor-derived foci contain  $\beta$ -gal that is detectable both immunohistochemically (brown stain, **H**) and histochemically (blue stain, **I**). Although blue staining in the focus in **I** appears to be less intense than staining in adjacent donor-derived parenchyma, this likely reflects the smaller size of hepatocyte nuclei in this focus. Fixation: **C, F,** and **I,** frozen tissue; all others, paraformaldehyde-fixed, paraffin-embedded tissue. Size bars: **F** and **I,** 200  $\mu$ m; all others, 100  $\mu$ m.



**Table 2.** Origin of Small Hepatocyte Foci in Dipin-Treated Mice with Genetically Chimeric Livers

Mouse	Weeks after dipin	Small hepatocyte foci: donor-derived/total	Donor-derived parenchyma*
5-15	15	10/47 (21%)	14%
5-17	15	8/52 (15%)	27%
2-88	17	5/33 (15%)	36%
7-20	17	8/34 (24%)	38%
13-6	20	5/28 (18%)	46%
4-9	22	2/11 (18%)	48%
Combined data		38/205 (19%)	35% <sup>†</sup>

\*See Materials and Methods for calculation.

<sup>†</sup>Mean.

simply age-related changes in MUP-uPA hepatocyte transplant recipients.

We were concerned that activation of a putative non-parenchymal stem cell lineage by dipin plus hepatectomy in transgenic recipients might be suppressed, because if endogenous nonhepatocytic stem cells could differentiate into hepatocytes they might begin to express the uPA transgene. To address this possibility, we examined liver from 21 nontransgenic transplant-recipient mice that had been treated with dipin plus hepatectomy at the same time as their transgenic littermates (Table 1). The presence of the transgene did not suppress lesion development, and in fact the response appeared somewhat reduced in nontransgenic relative to transgenic mice (33% displayed an oval cell response and 29% displayed hepatocellular abnormalities).

#### *Parenchymal Small Hepatocyte Foci Are Derived from Both Donor and Endogenous Cells*

As described above, dipin plus partial hepatectomy induced hepatocellular abnormalities in transgenic mice with chimeric liver parenchyma. These abnormalities included foci of small hepatocytes (Figure 2, D-I) that were uniform in appearance, which have been proposed to be the progeny of activated nonhepatocytic liver stem cells in this model.<sup>32-34</sup> To determine the origin of these new hepatocytes, livers from six mice that displayed distinct small hepatocyte foci and had a >10% donor cell repopulation were selected for detailed analysis of focus genotype. First, liver lobes from these animals were examined to compare the percentage of donor cell parenchymal repopulation at the time of partial hepatectomy and at the time of sacrifice. In each of three animals with reliable staining of whole tissue at both collection time points (Table 2, mice 5-17, 2-88, and 7-20), the proportion of donor-derived parenchyma stayed the same or was increased at the time of sacrifice relative to partial hepatectomy, indicating that donor-derived cells persist in parenchyma after dipin treatment plus partial hepatectomy (data not shown). For each mouse, tissue step sections separated by at least 0.5 mm were examined to identify foci of small hepatocytes. Foci of endogenous origin did not carry the *lacZ* transgene and failed to stain for  $\beta$ -gal (Figure 2, D-F). In contrast, small hepatocyte foci of donor cell origin contained the *lacZ* transgene and

stained either brown (via  $\beta$ -gal immunohistochemistry; Figure 2H) or blue (via X-gal histochemistry; Figure 2I). In each of the six mice examined, both donor cell- and endogenous cell-derived small hepatocyte foci were present (Table 2). A total of 38/205 (19%) of the small hepatocyte foci were of donor origin. This remains significantly less ( $P = 0.03$ ) than the 35% average parenchymal repopulation by donor cells observed in these animals (Table 2). For three mice, both frozen and paraformaldehyde-fixed tissues were informative. In this subset of mice, immunohistochemical analysis of paraformaldehyde-fixed tissue indicated that the average parenchymal repopulation by donor hepatocytes was 24% and that 14/72 (19%) of the small hepatocyte foci were donor-derived. Histochemical analysis of frozen tissue yielded similar results, with an average donor cell repopulation of 31% and 12/57 (21%) small hepatocyte foci of donor origin, indicating that these two methods of analysis gave comparable results.

Finally, Factor and colleagues<sup>32,34</sup> previously reported that occasional hepatocytes in a subset of small hepatocyte foci were reactive with the A6 antibody. In this study, 0/42 foci analyzed displayed hepatocyte staining with the A6 antibody; however, A6-positive oval cells were present both within parenchyma and in 18/42 of the small hepatocyte foci (Figure 2C).

#### *Discussion*

This study was designed to determine whether hepatocytes could serve as the precursors to foci of small hepatocytes that develop in mouse liver after treatment with the alkylating agent dipin followed by partial hepatectomy. These small hepatocyte foci had been proposed to arise from nonhepatocytic liver facultative stem cells.<sup>32-34</sup> Candidate sources of small hepatocyte foci in this model include 1) hepatocytes that have not been irreversibly damaged by dipin, in which case some small hepatocyte foci should consist of  $\beta$ -gal-containing (donor) hepatocytes and other foci should consist of  $\beta$ -gal-deficient (endogenous) hepatocytes; 2) proliferation and differentiation of a stem cell compartment, in which case the small hepatocyte foci should consist only of  $\beta$ -gal-deficient hepatocytes; or 3) a combination of both sources, in which case some foci are hepatocyte-derived

and others are stem cell-derived. We observed both  $\beta$ -gal-containing and  $\beta$ -gal-deficient small hepatocyte foci in all mice examined, indicating that some hepatocyte-derived small cell foci are present after dipin-induced damage in mice, a possibility noted by Factor and colleagues.<sup>34</sup> Our data do not allow us to distinguish between possibilities 1) and 3), because both donor cell- and endogenous cell-derived foci would be observed in either case. Thus we cannot exclude the possibility that some small hepatocyte foci may be stem cell-derived. In fact, the observation that the percentage of donor-derived parenchyma was typically higher than the percentage of donor-derived small hepatocyte foci (Table 2) is consistent with the generation of some foci via a stem cell-mediated pathway.

Interpretation of our results must take into account two important aspects of the experimental system. First, we must address the possibility that  $\beta$ -gal-containing foci of small hepatocytes are derived from transplanted stem cells. Numerical quantitation of the number of putative stem cells in rodent liver has not been published, although based on a morphological analysis of liver cell preparations, one investigator in this field has estimated a frequency of one stem cell per  $10^4$  to  $10^5$  hepatocytes (R. Faris, personal communication). A reasonable high estimate for the frequency of stem cells in the liver is one stem cell per  $10^3$  hepatocytes. In these experiments, approximately  $5 \times 10^5$  hepatocytes (together with contaminating nonparenchymal cells) are transplanted into the spleen. Assuming that at most 20% of the transplanted cells engraft in the liver<sup>2,46</sup> (Weglarz et al, manuscript submitted for publication), only 100 donor stem cells would seed a recipient liver. Because the liver has approximately  $10^8$  hepatocytes, the host liver would have 100,000 endogenous stem cells (assuming the same 1/1000 stem cell frequency). Therefore, at most 0.1% of the stem cells in a recipient liver should be donor-derived. If dipin-induced small hepatocyte foci were all stem cell-derived, only 0.1% should contain  $\beta$ -gal in our recipient mice. The actual value in this study was 200 times higher. It might be argued further that expansion of endogenous stem cell-derived foci would be selectively suppressed by reactivation of uPA transgene expression. However, if this were true, the frequency of lesions, including small hepatocyte foci, in MUP-uPA transgenic mice would be much lower than the frequency of lesions in dipin-treated nontransgenic littermates, which was not observed. A second concern is whether the response to Dipin plus partial hepatectomy is fundamentally different in MUP-uPA transgenic mice *versus* nontransgenic mice. In our study, the incidence of morphological abnormalities was higher in uPA-expressing transgenic mice relative to nontransgenic littermates. Although the reason for the increased response in MUP-uPA mice is not clear, the abnormalities that do arise in MUP-uPA and nontransgenic mice appear to be the same. In the two groups, morphological abnormalities appear at similar times after dipin treatment and have equivalent histological characteristics. The ratio between mice with hepatocellular abnormalities and mice with an oval cell response is comparable in the two groups. Overall, the progression of

liver changes observed in transgenic mice in our study recapitulates that reported for other mouse strains.<sup>32-34</sup> We also need to address the possibility that parenchymal foci in MUP-uPA transgenic mice are preneoplastic rather than regenerative, despite their resemblance to previously described dipin-induced foci. We examined 18-month-old MUP-uPA hepatocyte transplant recipients, in which donor hepatocytes had undergone approximately 12 cell doublings after transplantation. None of the 11 mice displayed foci of small hepatocytes, indicating that neither donor-derived nor endogenous parenchyma is inherently predisposed to lesion development in these mice. Furthermore, even serially transplanted hepatocytes with a history of at least 70 cell doublings do not appear to be at greater risk for the development of neoplastic changes, as demonstrated by Overturf and colleagues in fumarylacetoacetate hydrolyase null recipients.<sup>3</sup> Taken together, these data suggest that the parenchymal changes observed in MUP-uPA mice treated with dipin plus partial hepatectomy are not qualitatively different from those observed in nontransgenic mice.

Our finding that hepatocytes in dipin-treated liver can give rise to foci of small hepatocytes is consistent with reports that hepatocytes can escape growth-suppressive regimens in certain rat models of severe liver disease.<sup>10,11,14,22,23,47</sup> For example, in a study of D-galactosamine-induced injury in rat liver, [<sup>3</sup>H]thymidine labeling and *in situ* hybridization for a marker of cell proliferation were used to demonstrate that both oval cells and hepatocytes could proliferate after the administration of the toxic insult.<sup>23</sup> In a second study, flow cytometry was used to demonstrate that 5-bromodeoxyuridine was not transferred from the oval cell-containing nonhepatocyte fraction to the diploid hepatocyte population after treatment with 2-acetylaminofluorene plus two-thirds hepatectomy.<sup>14</sup> The authors suggested that proliferation of diploid hepatocytes may have been responsible for the increase in the size of the diploid hepatocyte fraction. However, other workers suggested subsequently that the 5-bromodeoxyuridine label may have been diluted to undetectable levels by cell division.<sup>20</sup> Finally, Gordon and colleagues examined parenchymal repopulation in rat livers treated with the pyrrolizidine alkaloid retrorsine followed by two-thirds partial hepatectomy.<sup>47</sup> They concluded that foci of small hepatocytes observed soon after hepatectomy were derived from hepatocyte-like progenitors rather than from oval cells.

The present study demonstrates the importance of differential cell marking when trying to establish cell lineage relationships *in vivo*. Without this, cellular transitions cannot be directly and unequivocally traced, a problem that has affected previous oval cell studies. A next important step is to determine the general applicability of our findings. Chimeric livers have been created in the rat by partially hepatectomizing pyrrolizidine alkaloid-treated animals and then transplanting hepatocytes.<sup>48,49</sup> Future studies of rat oval cell models can employ differential somatic cell marking to determine whether hepatocytes in this species also can give rise to regenerative foci of small hepatocytes, or whether all repopulation occurs via the putative nonhepatocytic stem cell pathway.

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