

Derivation of Hepatocytes From Bone Marrow Cells in Mice After Radiation-Induced Myeloablation

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Following a report of skeletal muscle regeneration from bone marrow cells, we investigated whether hepatocytes could also derive *in vivo* from bone marrow cells. A cohort of lethally irradiated B6D2F1 female mice received whole bone marrow transplants from age-matched male donors and were sacrificed at days 1, 3, 5, and 7 and months 2, 4, and 6 posttransplantation ($n = 3$ for each time point). Additionally, 2 archival female mice of the same strain who had previously been recipients of 200 male fluorescence-activated cell sorter (FACS)-sorted CD34⁺lin⁻ cells were sacrificed 8 months posttransplantation under the same protocol. Fluorescence *in situ* hybridization (FISH) for the Y-chromosome was performed on liver tissue. Y-positive hepatocytes, up to 2.2% of total hepatocytes, were identified in 1 animal at 7 days posttransplantation and in all animals sacrificed 2 months or longer posttransplantation. Simultaneous FISH for the Y-chromosome and albumin messenger RNA (mRNA) confirmed male-derived cells were mature hepatocytes. These animals had received lethal doses of irradiation at the time of bone marrow transplantation, but this induced no overt, histologically demonstrable, acute hepatic injury, including inflammation, necrosis, oval cell proliferation, or scarring. We conclude that hepatocytes can derive from bone marrow cells after irradiation in the absence of severe acute injury. Also, the small subpopulation of CD34⁺lin⁻ bone marrow cells is capable of such hepatic engraftment. (HEPATOLOGY 2000;31:235-240.)

The existence of hepatic progenitor cells, or stem cells, capable of regenerating both hepatocytes and cholangiocytes, has been avidly debated for many years. Early observations of cell proliferation in response to chemical hepatocarcinogenesis led to the suggestion that such a population existed,¹⁻³ and since then a large body of experimental evidence has sup-

ported this concept.⁴⁻⁸ An alternative view is that the small oval cells proliferating in these models represent proliferating ductular cells.⁹ However, later evidence indicated that some immature liver cell lines could differentiate into both duct cells and hepatocytes¹⁰ and, most recently, an extensive body of experimental work now documents the participation of a "bipotent" liver stem cell *in vivo*.⁴⁻⁸

The anatomic location of these cells has also been a subject of some controversy. Experimental work suggests that they lie within¹¹ or immediately adjacent to^{12,13} the canals of Hering, the anatomic juncture of the hepatocyte canalicular system, and the terminal branches of the biliary tree. This location has also been recently suggested by analysis of normal and diseased human livers.¹⁴ The morphology and phenotypes of the putative stem cells generally reflect such an origin: they are morphologically and phenotypically like small cholangiocytes, albeit with varying degrees of hepatocytic features depending on the models examined.¹⁻¹⁴

However, in experimental work by Yavorkovsky et al.,¹⁵ periportal necrosis of allyl alcohol toxicity resulted in a "null cell" proliferation that was negative not only for hepatocyte markers, but also for cholangiocyte markers. This could be explained in two ways: first, either the null cells derive from the canals of Hering and/or biliary tree, and lose cholangiocyte marker expression in the earliest stages of proliferation only to regain them after a short while or, second, that the cholangiocytes lining the most peripheral branches of the biliary tree are not the only source for these cells. That this second hypothesis might be correct was suggested by the work of Ferrari et al.,¹⁶ demonstrating skeletal muscle differentiation of injected bone marrow cells in response to myocyte injury, and suggesting that bone marrow-derived cells might function as progenitor cells for unexpected tissues. Thus, the present studies were initiated to determine if transplanted bone marrow cells could differentiate into epithelial cells of the liver. After initiation of these studies, Petersen et al.¹⁷ reported hepatocyte differentiation of transplanted bone marrow cells after acute, severe liver injury. In our studies showing bone marrow-derived hepatocytes, there is minimal, if any, liver injury, indicating that stem cells of bone marrow origin may take part in normal tissue renewal in the liver.

MATERIALS AND METHODS

Bone Marrow Transplantation. Three- to 5-week-old, syngeneic B6D2F1 male mice served as donors for age-matched female bone marrow recipients. Bone marrow transplantation was performed as previously described.¹⁸ After whole body irradiation with 1,200 cGy, the female recipients were injected via the tail vein with 20,000 male whole bone marrow cells. Three recipient mice were sacrificed on

Abbreviations: FISH, fluorescence *in situ* hybridization; PCR, polymerase chain reaction; DAPI, 4,6-diaminidino-2-phenylindole; mRNA, messenger RNA; FITC, fluorescein isothiocyanate; RNase, ribonuclease.

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days 1, 3, 5, and 7 posttransplantation, and at 2-month intervals, until 6 months posttransplantation. Cross-sections of each of the 2 largest lobes of the liver, taken at the greatest diameter from hilum to periphery, were formalin-fixed and paraffin embedded. Fluorescence *in situ* hybridization (FISH) for the Y-chromosome was used to detect donor-derived cells in 2 serial sections of these tissues as described later.

Additionally, 2 archival mice from a prior experiment were available for study.¹⁹ These were female mice transplanted with male donor cells according to the same transplantation protocol, but a restricted population of 200 flow-sorted, CD34⁺lin⁻, freshly isolated, male-derived bone marrow cells were used rather than whole bone marrow. These mice were also transplanted with 20,000 female donor-derived rotor off cells to provide short-term (30 days) reconstitution after lethal irradiation. The lineage markers used for depletion were B220, CD3, CD5, Gr1, Mac1, and Ter 119.²⁰ These mice were sacrificed at 8 months posttransplantation.

All research involving mice was approved by the Yale University Animal Care and Use Committee.

FISH for Y-chromosome. FISH for the Y-chromosome was performed as previously described.¹⁹ The Y-chromosome probe was produced using degenerate oligonucleotide primed polymerase chain reaction (PCR) from a murine Y-chromosome template. The oligonucleotide used was CCGACTCGAGNNNNNNATGTGG, and the program was as follows: 93°C for 5 minutes, 5 cycles of 94°C for 1 minute, 30°C for 1.5 minutes, and 72°C for 30 minutes, 35 cycles of 94°C for 1 minute, 62°C for 1 minute, and 72°C for 3 minutes, followed by 10 minutes at 72°C. The probe was labeled with digoxigenin by Nick Translation (DIG-Nick Translation Mix; Boehringer-Mannheim, Indianapolis, IN).

Male bone marrow engraftment was assessed by FISH on bone marrow smears as previously described.¹⁹ Engraftment of liver was assessed by FISH on 2 sequential 3-micron thick, formalin-fixed, paraffin-embedded tissue sections. After heat fixation at 60°C overnight, these slides were deparaffinized in xylene for 10 minutes, dried, placed in methanol/acetic acid (3:1) for 30 seconds, dried, then treated with proteinase K, 10 µg/mL, for 45 minutes at 37°C.

Probe was applied under sealed coverslips at 37°C, overnight. Detection was performed using anti-digoxigenin-rhodamine (Boehringer-Mannheim), and 4,6-diamidino-2-phenylindole (DAPI) was used as a nuclear counterstain.

The positive signal for Y-chromosomes was a large, often irregular signal, within a DAPI counter-stained nucleus (Fig. 1). With extensive washings, coarse debris that might have been mistaken for a positive signal were minimized. Such coarse debris, though rarely present, could be seen with multiple fluorescent filters, whereas true positives were visible only with the rhodamine filter. Liver from normal male and female mice served as positive and negative control tissues, respectively (Fig. 1A and B).

Double FISH for the Y-Chromosome and Albumin Messenger RNA. Slides containing 3-micron tissue sections were deparaffinized in xylene and pretreated for 14 minutes in 100 µg/mL proteinase K with 0.05% sodium dodecyl sulfate at 45°C. Genomic DNA probes were prepared based on messenger RNA (mRNA) sequence for mouse albumin (accession: AJ011413). Primer pairs were synthesized at positions 346-368/437-415, 236-258/359-336, and 517-539/648-624 in the albumin sequence and designed to yield very short products, 100 to 200 bp long. Each of these primer pairs yielded PCR products perfectly matching the theoretical length of the complementary DNA segment between the 2 primers. Obtaining the correct PCR product proved that the respective 2 primers belonged to the same exon and did not span an intron. Genomic PCR products were labeled by incorporation of digoxigenin-dUTP. The mouse Y-chromosome probe was also labeled by PCR amplification using biotin-dUTP. Each PCR product was then partially digested with deoxyribonuclease I (1.5-2.2 µg/mL final concentration) for 15 minutes at 21°C, followed by heating to 95°C for 2 to 4 minutes. For each slide, 20 ng dig-labeled albumin probe and 10 ng biotin-labeled Y-chromosome probe were precipitated together with mouse Cot1 DNA (GibcoBRL, Life Technologies, Frederick, MD), resuspended in 10 µL hybridization buffer (50% formamide) and denatured 5 minutes at 75°C. Slides were denatured for 8 minutes at 86°C and hybridized overnight at 37°C. Posthybridization washes were done at 37°C, followed by antibody detection, using 10-µg protein

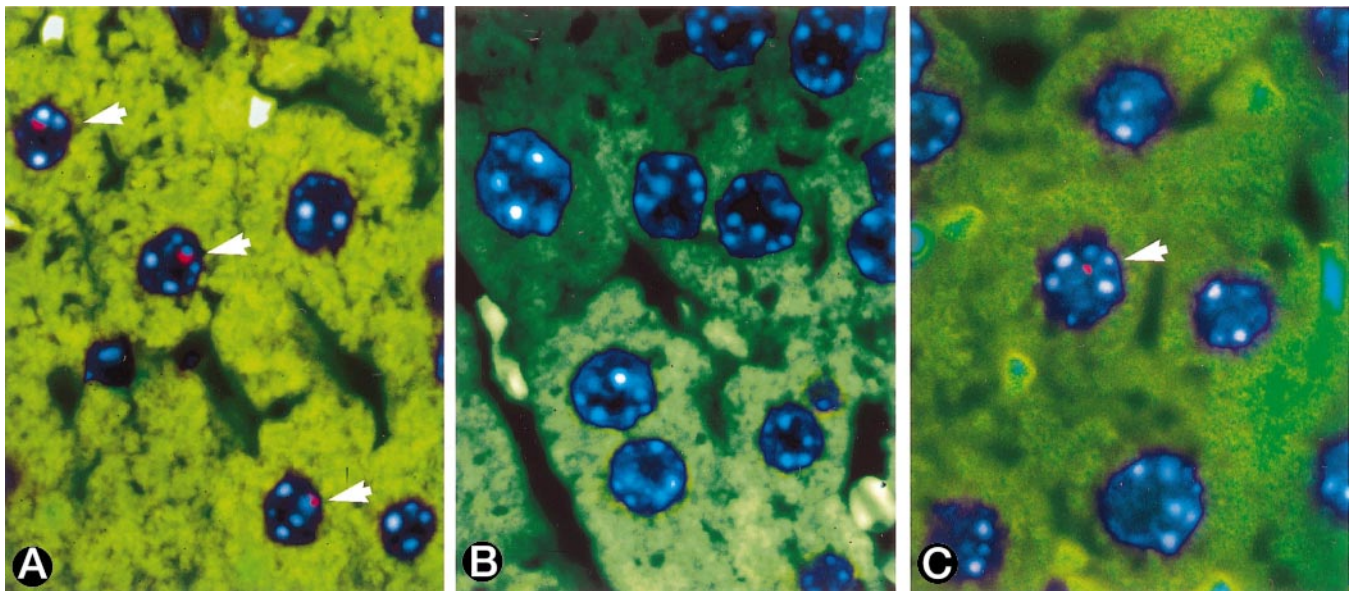


FIG. 1. (A) Normal male mouse liver stained with DAPI (blue) for nuclear chromatin and rhodamine (red) for Y-chromosome FISH. Hepatocyte cytoplasm autofluorescent green (FITC filter). Polygonal hepatocytes arranged in cell cords, with large nuclei are evident. The red-labeled Y-chromosomes, seen as bright comma-shaped bodies in nuclei (arrowheads), are identifiable in approximately half of the nuclei as the nuclei are only partially sampled in a 3-micron tissue section. The yellow-white structures represent brightly autofluorescent erythrocytes. (Original magnification $\times 60$.) (B) Normal female mouse liver stained as in panel A. No Y-chromosomes are identified. (Original magnification $\times 60$.) (C) Liver from a female mouse sacrificed 6 months after lethal irradiation and transplantation with male whole bone marrow, stained as in panel A. One hepatocyte nucleus contains a Y-chromosome (arrowhead) indicative of bone marrow cell origin. (Original magnification $\times 60$.) Note: Due to artifacts of merging 3 separately filtered images for each of the illustrations that were captured at slightly different exposures in the organs examined, the cytoplasmic autofluorescence varies from one tissue sample to the next.

solutions in 4× sodium chloride sodium citrate. The first detection step included mouse antidigoxigenin and equal amounts of avidin-fluorescein isothiocyanate (FITC) mixed with avidin Cy5; the second step included sheep antimouse Cy3. After washing, slides were mounted in DAPI antifade. The FITC signal enabled visualization of the albumin mRNA signals whereas the Cy5 signal (infrared) was used to provide better signal to noise ratios during image capturing (tissue autofluorescence is higher through the green filter than the Cy5 filter).

Protease digestion for double FISH staining was more extensive than that required for single FISH analysis, resulting in poorer tissue morphology and a different, more dot-like Y-chromosome signal compared with the single FISH assay. Negative controls included liver tissue treated with ribonuclease (RNase) A (Sigma, St. Louis MO; 10 µg/mL, at 37°C, for 50 minutes) before hybridization with the mRNA probe and staining of tissues expected to contain no *de novo* synthesis of albumin, including skeletal muscle, spleen, esophagus, stomach, small and large intestines, pancreas, kidney, heart, and skin.

Image Analysis. Images were taken using an Olympus Provis (Tokyo, Japan) microscope equipped with a cooled CCD camera (Quantix Corp., Cambridge, MA) and specialized software (PSI Inc., League City, TX). Confocal microscopy was performed using a Biorad MRC1024 Scanning Confocal Microscope (Biorad, Hercules, CA) with a krypton/argon laser. Autofluorescence was excited at 488 nm, and emission was collected above 515 nm. The rhodamine signal was excited at 568 nm and emission collected above 585 nm. Images were pseudocolored using image processing software (Adobe Photoshop, San Jose, CA).

Quantitation of Bone Marrow-Derived Cells. Cell counts for bone marrow were obtained using slides stained by the single FISH staining technique described previously, combining 60× images of DAPI-stained nuclei, FITC auto-fluorescence, and Cy3.5 for Y-chromosome signals. For double FISH staining (for Y-chromosome and albumin mRNA), the Y-chromosome was visualized with the Cy3.5 filter then captured, while albumin mRNA was visualized with the FITC filter, then captured with the Cy5 filter. Fields were selected randomly and focused under a DAPI filter, then captured using that filter. Images were then sequentially captured using the Cy3.5 filter (Y-chromosome) and FITC (cytoplasmic autofluorescence) or Cy5 (albumin mRNA) filters without changing focus, thus ensuring the same plane of analysis. All three filtered images were then digitally combined (DAPI, Cy3.5, FITC for single Y-chromosome staining; DAPI, Cy3.5, Cy5 for double staining) and cell counts derived from these combined images.

Hepatocytes were identified in 2 ways. With single FISH for the Y-chromosome, hepatocytes were large polygonal cells, arranged in plates, with large, round nuclei, and characteristic bright green FITC-type cytoplasmic autofluorescence caused by bilirubin metabolic products (Fig. 1). With double FISH staining for the Y-chromosome and albumin mRNA, 1 or 2 bright green dots, representing albumin gene transcription centers²⁰ identified hepatocytic nuclei (Fig. 2A). Although a finely stippled, cloud-like signal for albumin mRNA could be seen in the hepatocyte cytoplasm in these slides, it was difficult to associate a given nucleus with this diffuse, poorly localized signal; therefore, only the appearance of intranuclear albumin mRNA transcription centers was used to define a nucleus as hepatocytic.

To compensate for undercounting of Y-positive nuclei due to partial nuclear sampling in tissue sections, cell counts were normalized to the percentage of Y-positive cells seen in normal male tissue. This adjustment was performed on the basis that 3-micron thin sections were analyzed, whereas hepatocyte nuclei in mice are approximately 6 to 8 microns in diameter. Thus, nuclei are only partially sampled in tissue sections and some male nuclei will not have demonstrable Y-chromosomes in these sections. To compensate for this artifact of sectioning, we counted Y-chromosomes in a similarly sectioned normal male mouse and found that 51% of hepatocytes had Y-chromosomes. This provided a correction factor (dividing by .51) for undercounting of Y-positive hepatocytes

because of the partial sampling of hepatocyte nuclei in tissue sections. It should be noted that 96% to 100% of nuclei of male bone marrow cells stained positively with this Y-chromosome probe when whole cell smears were examined, indicating that Y-chromosomes did consistently stain when present on the slide.

RESULTS

Data are summarized in Table 1. Bone marrow smears of mice receiving whole bone marrow transplants showed a predominantly host (female) bone marrow at posttransplantation day 1, followed by the development of an essentially acellular bone marrow at day 3. At day 5, engraftment by the male donor cells is seen in the bone marrow with a normal-appearing cellularity by smear on day 7. Bone marrow engraftment was demonstrable by FISH for Y-chromosomes at day 5 posttransplantation, substantially accomplished by day 7, and remained intact for the duration of the study (up to 6 months), between 98% and 100%, in all whole bone marrow transplant recipients. In the 2 animals transplanted with CD34⁺lin⁻ cells, engraftment was 100% in 1 and 18% in the other at 8 months posttransplantation. Fifty-one percent of hepatocyte nuclei in male control tissues were positive for the Y-chromosome, indicative of partial nuclear sampling in the 3-micron thick tissue sections examined (Fig. 1A). Female control liver was negative for Y-chromosome staining (Fig. 1B). No staining for albumin mRNA was seen in any nonhepatic tissues (data not shown), or in liver after predigestion with RNase (Fig. 2B).

No Y-positive hepatocytes were identified in animals sacrificed at days 1, 3, or 5 posttransplantation. One animal at day 7 showed rare Y-positive hepatocytes. Y-positive hepatocyte

TABLE 1. Number of Y-Positive Nuclei in Female Mice Transplanted With Male Bone Marrow Cells

Mouse	Time Post-BMT	Trans-planted Cells	Bone Marrow Cell Count (%)	Hepatocytes	
				Cell Count (%)	Adjusted %
Normal Male	—	none	200/200 (100%)	267/523 (51%)	100
1	1 d	wbm	ND	0	0
2	1 d	wbm	5/223 (2.2%)	0	0
3	1 d	wbm	3/219 (1.4%)	0	0
4	3 d	wbm	ND	0	0
5	3 d	wbm	scant cells	0	0
6	3 d	wbm	scant cells	0	0
7	5 d	wbm	ND	0	0
8	5 d	wbm	203/204 (100%)	0	0
9	5 d	wbm	211/213 (99%)	0	0
10	7 d	wbm	ND	0	0
11	7 d	wbm	207/207 (100%)	1/1,000 (.1%)	.2
12	7 d	wbm	209/215 (97%)	0	0
13	2 mo	wbm	223/223 (100%)	4/1,016 (.39%)	.76
14	2 mo	wbm	215/218 (99%)	6/1,002 (.60%)	1.2
15	2 mo	wbm	209/210 (100%)	9/1,024 (.88%)	1.7
16	4 mo	wbm	202/203 (100%)	7/1,017 (.69%)	1.4
17	4 mo	wbm	202/202 (100%)	8/1,005 (.80%)	1.6
18	4 mo	wbm	214/214 (100%)	4/1,012 (.40%)	1.2
19	6 mo	wbm	218/220 (99%)	6/1,023 (.59%)	.77
20	6 mo	wbm	215/216 (99%)	7/1,006 (.70%)	1.4
21	6 mo	wbm	207/211 (98%)	11/1,019 (1.1%)	2.2
archival 1	8 mo	CD34 ⁺ lin ⁻	552/552 (100%)	5/1,036 (.48%)	.94
archival 2	8 mo	CD34 ⁺ lin ⁻	112/623 (18%)	2/1,007 (.19%)	.37

Abbreviations: BMT, bone marrow transplantation; wbm, whole bone marrow; ND, not done.

nuclei were more readily identified in all female recipients sacrificed at 2, 4, 6, and 8 months posttransplantation (Figs. 1C, 2, and 3). Y-positive white blood cells were identified in hepatic sinusoids, which served as an internal positive control beginning at day 5. Confocal microscopy confirmed that the Y-chromosome signal was actually within the nucleus, rather than nonspecific debris overlying the tissue section (Fig. 3). The Y-positive signal with confocal microscopy takes on a dot-like appearance rather than the linear or comma shape often seen in thicker sections, because it represents serial, thin cross-sections of the linear chromosome. Quantitative analysis indicates that up to 2.2% of hepatocytes in the 2 largest lobes were donor derived (Table 1).

Single Y-positive hepatocytes were located both near portal tracts and farther out in the parenchyma and were always clearly part of liver cell plates. Donor-derived cholangiocytes were not identified. Histological examination of livers of all mice failed to show parenchymal necroses, increased or abnormal mitoses, oval cell proliferations, fibrosis, inflammation, or other features of acute or chronic liver injury and repair.

DISCUSSION

The present experiments show that bone marrow cells transplanted from male donors to syngeneic recipients are able to localize in the two largest lobes of the liver, differentiating into mature hepatocytes carrying the Y-chromosome. The presence of a facultative liver stem cell compartment has been documented in both animals⁴⁻⁸ and humans.^{14,16,21,22} However, the exact anatomic location of these cells has been difficult to ascertain. The presence of blast-like cells in the bile ducts of rats treated with carcinogens has been clearly documented.²³ However, there is equally compelling evidence for the early participation of periductular cells after carcinogen exposure.¹³ The present results support the concept of an extrabiliary bone marrow origin of primitive liver stem cells. Thus, during restorative liver proliferation there may be participation of hepatocytes, liver-derived ductular stem cells, and a more primitive bone marrow-derived stem cell.

Petersen et al.¹⁷ have also recently reported the derivation of hepatocytes from bone marrow cells in rats but with broader parenchymal replacement by marrow-derived cells. Regeneration in those experiments was elicited through carbon tetrachloride and 2-acetylaminofluorene toxicity. Such a combination results in a primary activation of a stem cell compartment through extensive parenchymal injury and concomitant inhibition of hepatocyte regeneration; thus, it is not surprising that more widespread parenchymal replacement by donor-derived cells took place.

In our model, differentiation of bone marrow cells into hepatocytes occurred after irradiation. Livers exposed to low-level radiation have been reported to show only increased mitoses as late as 1 year after exposure without overt necrosis or oval cell proliferation at any time,²⁴ in keeping with histological findings in the liver tissue examined here. Thus, at most, the irradiation in this study has caused only slight perturbations in normal liver cell turnover, perhaps not inducing damage or requiring repair processes. Regeneration of hepatocytes in response to a mild injury is generally thought to occur by division of mature hepatocytes without invoking a progenitor cell maturation process.²⁵ The lack of clustering and regional replacement of parenchyma by mar-

row-derived hepatocytes in our experimental animals is most likely caused by repopulation by mature hepatocytes, which predominates after radiation exposure. Our data therefore raise the possibility that movement and maturation of marrow-derived, hepatic progenitor cells is not limited to a response to severe liver injury, but may occur after mild liver injury or perhaps even during normal, baseline tissue renewal.

Neither the source of the engrafting cells nor the mechanism of engraftment as hepatocytes is known. The marrow-derived hepatocytes may derive from progeny of transplanted cells that have first engrafted within the recipient's bone marrow or may enter directly into the liver after intravenous injection in the days after transplantation. Our inability to detect such cells in the liver during the first few days posttransplantation could be because of a lack of hepatocytic differentiation within this first week. The relatively static percentage engraftment over the months of the study might reflect this peripheral route of entry of these cells into the liver without new cells localizing in the liver from subsequently engrafted bone marrow at later times.

The mechanism of entry and differentiation of the transplanted cells within the liver cell plates might also occur in different ways. Circulating, marrow-derived cells might enter the liver cell plates directly from the sinusoidal circulation. Incorporation of mature hepatocytes into the liver cell plates after vascular injection has been already reported.²⁶ Alternatively, given previous demonstrations that oval cells derive from the canals of Hering^{11,14} and that Petersen et al.¹⁷ showed not only hepatocyte, but oval-cell derivation from marrow cells, we can speculate that marrow cells might enter the liver through the circulation, translocate across basement membranes into the canals of Hering and/or the terminal branches of the biliary tree, and differentiate into cytokeratin 19-positive small cholangiocyte-like cells. Proliferation and differentiation into hepatocytes would follow, in proportion to the type and extent of hepatocyte injury. This second hypothesis is consistent with several independent findings: the identification of c-kit on the canals of Hering in normal livers¹⁴ and in regenerating cells in massive hepatic necrosis,^{14,22} perhaps representing persistence of the original bone marrow phenotype; and the ultrastructural identification of cells with morphologies consistent with a hepatic stem cell adjacent to, but not within the terminal branches of the biliary tree, possibly capturing the moment just before the entry of bone marrow-derived cells into the biliary tree proper.^{13,27}

Our data from 2 archival cases indicate that at least some of the marrow-derived cells with hepatic differentiation potential are CD34⁺lin⁻, leaving open the question of whether they are hematopoietic or stromal cells, or both, because both populations contain CD34-positive cells.^{28,29} Either way, the differentiation of the donor cells into hepatocytes represents repopulation of epithelial cells, usually thought of as endodermally derived, from mesodermal tissue, further showing the wide differentiation capacity of adult cells already shown in other experiments.^{16,17,30,31} Further experiments must be performed to characterize which subpopulations of marrow cells can give rise to hepatic stem cells.

In summary, after irradiation of recipient animals and intravascular injection of whole bone marrow cells, we show movement of these cells into the liver where they differentiate into hepatocytes. Moreover, CD34⁺lin⁻ subpopulations of marrow cells can also accomplish this regenerative response.

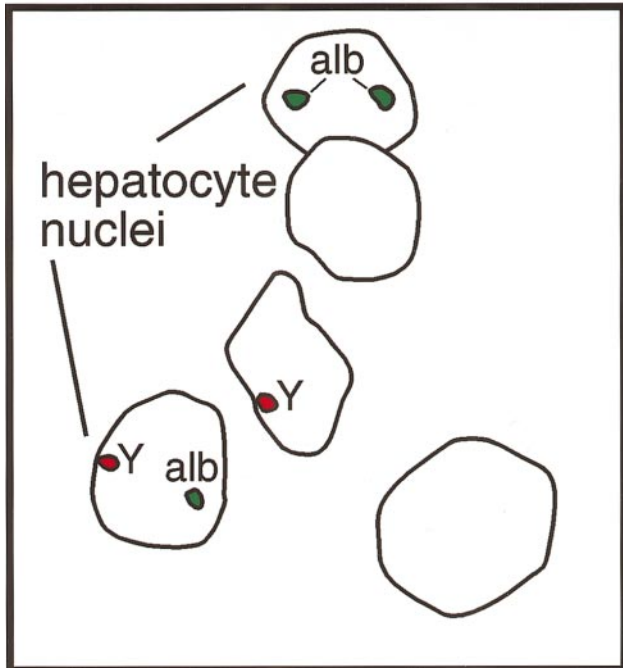
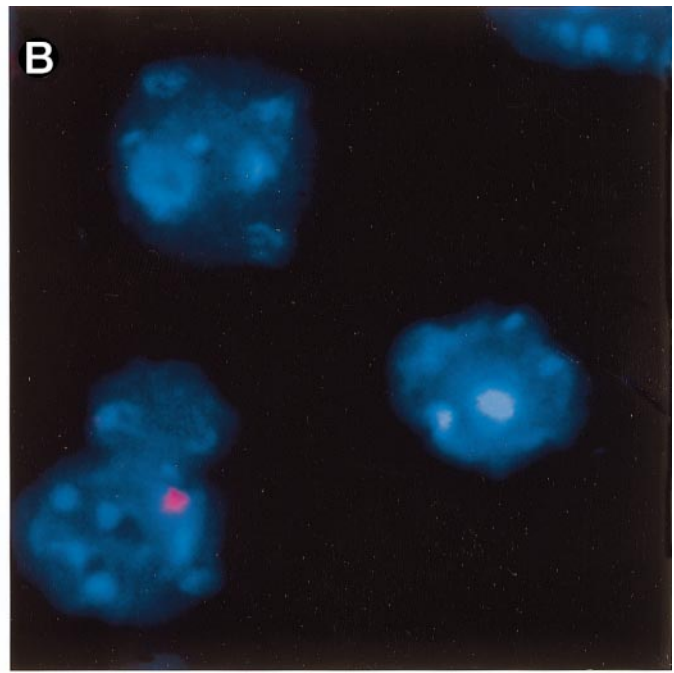
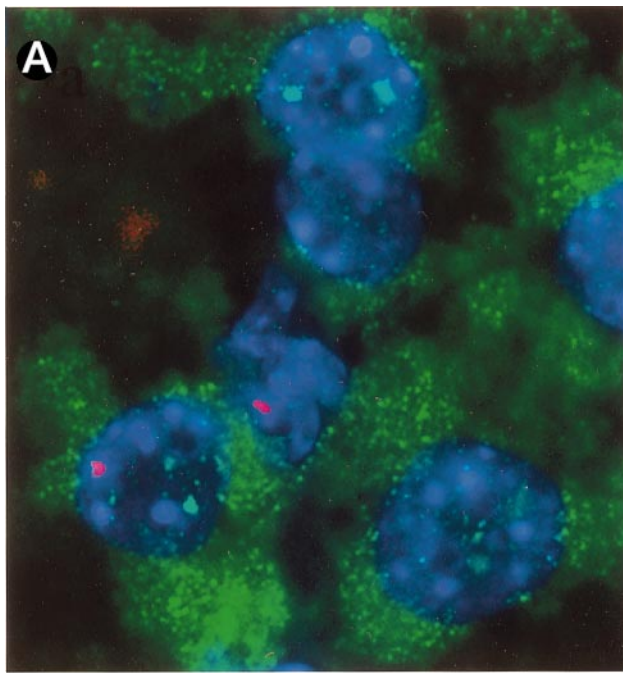


FIG. 2. Double FISH staining for the Y-chromosome and albumin mRNA in the liver of a female mouse 8 months after bone marrow transplantation with CD34⁺lin⁻ male bone marrow cells, with and without RNase predigestion. (A) Bright green dots in blue-stained hepatocyte nuclei indicate transcription centers for albumin mRNA. The finely stippled, bright green staining surrounding the nuclei is cytoplasmic albumin mRNA. One hepatocyte (lower left) also contains a red-labeled Y-chromosome indicating that it is a male bone marrow-derived hepatocyte in this female mouse. The other Y-positive nucleus, without an associated albumin mRNA signal, may be a partially sampled hepatocyte nucleus in which the albumin transcription site is out of the plane of section, a white blood cell, or a sinusoidal lining cell. (B) After RNase predigestion, all signal for albumin mRNA was eliminated in another section from this same mouse. With the combination of this treatment with the elimination of cytoplasmic autofluorescence by extensive protease digestion necessary for double staining, there are no features remaining to identify which nuclei these cells represent, although location in the tissue section, round shape, and relatively large size suggest that some may be hepatocyte nuclei. However, in the absence of cytoplasmic features or mRNA, it is uncertain what type of cell contains a Y-chromosome. (Original magnifications $\times 100$.)

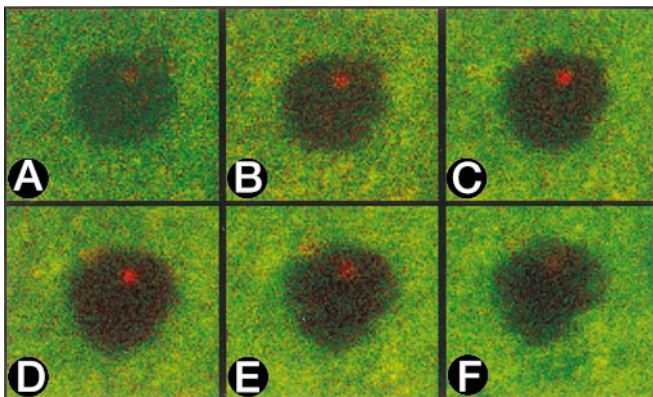


FIG. 3. Female mouse liver, 8 months after bone marrow transplantation with CD34⁺lin⁻ male bone marrow cells. The staining scheme was performed as in Fig. 1. Confocal imaging every 0.15 microns shows that the red signal for the Y-chromosome represents chromosomal material within the nucleus, rather than nonspecific fluorescent debris overlying the tissue. The Y-chromosome-positive nucleus in this mid-zonal hepatocyte indicates bone marrow cell origin. Note: the DAPI filter is not used in these confocal micrographs. (Original magnification $\times 63$.)

The relatively small although significant number of hepatocytes that are marrow-derived in this study probably reflects the predominance of mature hepatocyte replication in the model used. Comparison of our data with other published data regarding the derivation of hepatocytes from bone marrow cells suggests that the extent of hepatic engraftment by bone marrow cells depends on the nature and extent of hepatic injury.

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REFERENCES

- Wilson JW, Leduc RH. Role of cholangioles in restoration of the liver of the mouse after dietary injury. *J Pathol Bacteriol* 1958;76:441-449.
- Sell S. Distribution of alpha-fetoprotein and albumin containing cells in the livers of Fischer rats fed four cycles of N-2-fluorenylacetamide. *Cancer Res* 1978;38:3107-3113.
- Sell S, Sala-Trepat JM, Sargent TH, Thomas K, Nahon JL, Goodman TA, Bonner J. Molecular mechanisms of control of albumin and alpha-fetoprotein production: a system to study the early effects of chemical hepatocarcinogens. *Cell Biol Int Rep* 1980;4:134-254.
- Sell S, Leffert JL. An evaluation of cellular lineages in the pathogenesis of experimental hepatocellular carcinomas. *HEPATOLOGY* 1982;2:77-86.
- Thorgeirsson SS. Hepatic stem cells in liver regeneration. *FASEB J* 1996;10:1249-1256.
- Marceau N. Epithelial cell lineages in developing, restoring, and transforming liver: evidence for the existence of a "differentiation window." *Gut* 1994;35:294-196.
- Grisham JW. Hepatic epithelial stem-like cells. *Verh Dtsch Ges Pathol* 1995;79:47-54.
- Fausto N, Lemire JM, Shiojiri N. Cell lineages in hepatic development and the identification of progenitor cells in normal and injured liver. *Proc Soc Exp Biol Med* 1993;204:237-241.
- Grisham JW, Porta EA. Origin and fate of proliferated hepatic ductal cells in the rat: electron microscopic and autoradiographic studies. *Exp Mol Pathol* 1964;3:242-261.
- Grisham JW. Cell types in long term propagable cultures of rat liver. *Ann N Y Acad Sci* 1980;349:128-137.
- Faktor VM, Radaeva SA. The formation of oval-cell ducts during hepatic carcinogenesis in mice. Its relationship to the pre-existing canals of Hering. *Ontogenez* 1992;23:407-418.
- Sell S, Osborn K, Leffert H. Autoradiography of "oval cells" appearing rapidly in the livers of rats fed N-2-fluorenylacetamide in a choline devoid diet. *Carcinogenesis* 1981;2:7-14.
- Sell S, Salman J. Light- and electron microscopic autoradiographic analysis of proliferating cells during the early stages of chemical hepatocarcinogenesis in the rat induced by feeding N02 fluorenylacetamide in a choline-deficient diet. *Am J Pathol* 1984;114:287-300.
- Theise ND, Saxena R, Portmann BC, Thung SN, Yee H, Chiriboga L, Kumar A, et al. The canals of Hering and hepatic stem cells in humans. *HEPATOLOGY* 1999;30:1425-1433.
- 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* 1995;21:1702-1712.
- Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossug, Mavilio F. Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* 1998; 279: 1528-1530.
- Petersen BE, Bowen WC, Patrene KD, Mars MW, Sullivan AK, Murase N, Boggs SS, et al. Bone marrow as a potential source of hepatic oval cells. *Science* 1999;284:1168-1170.
- Sharkis SJ, Wiktor-Jedrzejczak W, Ahmed A, Santos GW, McKee A, Sell KW. Antitheta-sensitive regulatory cell (TSRC) and hematopoiesis: regulation of differentiation of transplanted stem cells in W/Wv anemic and normal mice. *Blood* 1978;52:802-817.
- Donnelly DS, Zelteman D, Sharkis S, Krause DS. Functional activity of murine CD34+ and CD34- hematopoietic stem cell populations. *Exp Hematol* 1999;27:788-796.
- Dirks RW. RNA molecules lighting up under the microscope. *Histochem Cell Biol* 1996;106:151-166.
- Roskams T, Desmet V. Ductular reaction and its diagnostic significance. *Semin Diagn Pathol* 1998;15:259-269.
- Baumann U, Crosby HA, Ramani P, Kelly DA, Strain AJ. Expression of the stem cell factor receptor c-kit in normal and diseased pediatric liver: identification of a human hepatic progenitor cell? *HEPATOLOGY* 1999;30: 112-117.
- Novikoff PM, Yam A, Oikawa I. Blast-like cell compartment in carcinogen-induced proliferating bile ductules. *Am J Pathol* 1996;148:1473-1492.
- Travis EL, Peters LJ, McNeill J, Thames HD Jr, Karolis C. Effect of dose-rate on total body irradiation: lethality and pathologic findings. *Radiation Oncol* 1985;4:341-351.
- Sell S, Ilic Z. *Liver Stem Cells*. Austin: Landis Publications, 1997.
- Gupta S, Bhargava KK, Novikoff PM. Mechanisms of cell engraftment during liver repopulation with hepatocyte transplantation. *Semin Liver Dis* 1999;19:15-26.
- DeVos R, Desmet V. Ultrastructural characteristics of novel epithelial cell types identified in human pathologic liver specimens with chronic ductular reaction. *Am J Pathol* 1992;140:1441-1450.
- Krause DS, Ito T, Fackler MJ, Smith OM, Collector MI, Sharkis SJ, May WS. Characterization of murine CD34, a marker for hematopoietic progenitor and stem cells. *Blood* 1994;84:691-701.
- Baumhueter S, Dybdal N, Kyle C, Lasky LA. Global vascular expression of murine CD34, a sialomucin-like endothelial ligand for L-selectin. *Blood* 1994;84:2554-2565.
- Pereira RE, O'Hara MD, Laptev AV, Halford KW, Pollard MD, Class R, Simon D, et al. Marrow stromal cells as a source of progenitor cells for nonhematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta. *Proc Natl Acad Sci U S A* 1998;95:1142-1147.
- Bjornson CR, Rietze RL, Reynolds BA, Magli MC, Vescovi AL. Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. *Science* 1999;283:534-537.