Cells of the hepatic side population contribute to liver regeneration and can be replenished by bone marrow stem cells

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Background and Objectives. The aim of this study was to determine whether Hoechst effluxing *side population* cells isolated from murine liver represent hepatic stem cells, and to examine whether hepatic side population cells arise from bone marrow side population cells.

Design and Methods. Side population cells were isolated from murine liver by flow cytometry after Hoechst staining and injected directly into murine livers of animals pre-treated with the hepatotoxin 3,5 diethoxy carbonyl-1, 4-dihydrocollidine (DDC). Y-chromosome *in situ* hybridization was used to track donor cells in the livers. In addition, bone marrow side population cells were stably engrafted into the hematopoietic system of sublethally irradiated recipients and CD45 alleleic staining and Y-chromosome *in situ* hybridization were used to track side population cell progeny in the liver.

Results. In vitro, $CD45^{pos}$ and $CD45^{neg}$ hepatic SP cells gave rise to hematopoietic colonies and mixed colonies of hematopoietic and hepatic differentiation. After orthotopic liver cell transplantation, donor hepatic side population cells contributed to the regeneration of mature liver parenchyma and bile duct epithelium. After transplantation of bone marrow side population cells, both $CD45^{pos}$ and $CD45^{neg}$ hepatic side population cells were partially derived from donor stem cells and could be recruited to repair liver damage after treatment with DDC.

Interpretation and Conclusions. These findings introduce hepatic side population cells as a facultative liver-regenerating population, reveal interchangeability of tissue stem cells at the level of the side population, and suggest that bone marrow-derived side population cells might be exploited for the repair of diseased or damaged liver.

Key words: stem cell biology.

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The liver is an organ with extensive regenerative capacity based on two distinct mechanisms. After limited hepatocellular damage, mature hepatocytes expand extensively.[1-3] After more serious damage, or when hepatocyte-based tissue regeneration is severely impeded, tissue is replaced through the proliferation and maturation of a phenotypcially defined pool of primitive precursor cells. These stem cells have been characterized as oval cells because of their distinct morphology, and they possess the capacity for bi-directional differentiation to hepatocytes and bile duct epithelium.[4-6] Thus, in contrast to regenerative tissues whose repair processes exclusively involve stem cells, the liver features a pool of facultative stem cells that are recruitable on demand.[7,8] The concept that the oval cell is the most primitive stem cell in liver was challenged by recent experimental models showing that bone marrow-derived stem cells had sufficient plasticity to contribute to hepatic regeneration.[9-13] These observations left unexplored the phenotypic and functional inter-relationships between marrow- and liver-derived hepatic progenitor cells. This knowledge is critical if marrow and hepatic stem cells are to be effectively exploited in therapy for liver disorders. Among the methods used to isolate hematopoietic stem cells (HSCs) in the mouse, staining with Hoechst 33342 dye and selection of the side population (SP) of cells with the highest efflux capacity produces a high purity primitive cell population.[14] Cell populations with the SP phenotype were subsequently found in other tissues such as skeletal muscle,[15,16] and showed a potential for differentiation beyond their organ of origin.[16] These observations prompted us to analyze hepatic tissue for the prevalence of SP cells, to clarify the functional role of these cells in the liver stem cell pool, and to evaluate their phenotypic and functional relationships with bone marrow SP cells.[4]

Design and Methods

Animals

Animal studies used wild type or C57Bl/6-Rosa26 transgenic mice (Jackson Labs), and followed *Good Animal Practice* guidelines after approval by the local animal ethics committee. In some transplantations, donors and recipients differed in their expression of CD45.1/CD45.2 alleles so that donor- and host-derived cells could be distinguished by FACS. 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC; Aldrich) was added to the animals' feed at a concentration of 0.1% and fed to mice for 10 days before and after transplantation.

Preparation of the hepatic side population

Hepatic cell suspensions were prepared as follows. For all hepatic SP cell phenotyping and transplantation studies, livers of anesthetized 3- to 6-month-old mice were perfused through the portal vein with 100 μg/mL collagenase solution (Liberase, Roche) at 2 mL/min at room temperature. In those experiments in which the donor contribution to the hepatic SP cells was analyzed after bone marrow transplantation, the liver was mechanically minced, and the fragments digested with pronase (1 mg/mL; Sigma) for 1 h at 37°C with constant agitation. The perfusion or digestion product was washed several times and strained through a 70-μm mesh then stained with 5 μg/mL Hoechst 33342 (Sigma) for 90 min at 37°C in DMEM, 2% FCS, followed by propidium iodide (2 μg/mL), and kept at 4°C until sorting. Sorted cell populations were recovered in 100% FCS and kept at 4°C until processing. A 350 nm argon-laser was used to excite Hoechst 33342, and emission was collected on a MoFlow (Cytomation) at 405/30 and 670/30 nm, as described elsewhere;[14,17] also, *http://www.bcm.tmc.edu/genetherapy/goodell*).

The SP was selected by gating consecutively on the small cell population of the hepatic cells, and then on the SP (Figure 1). Immunofluorescence staining was performed at 4°C immediately after Hoechst staining with the use of labeled antibodies. The monoclonal antibodies associated with hematopoietic stem and progenitor cells for flow cytometry were: CD34 biotin (clone RAM34), CD45 FITC or biotin (clone 30-F11), Thy-1 (CD90.2 FITC, clone 30-H12), and c-Kit (CD117 FITC, clone 2B8; all from Pharmingen). $CD45^{neg}$ and $CD45^{pos}$ SP cells were sorted in one step by staining the whole hepatic population with Hoechst and CD45-FITC. An average of 31,500±11,280 (SD) purified SP cells (n=11) were obtained from the liver of a 3- to 6- month old C57/Bl6J mouse of either gender (approximately 5×10^7 cells per liver). Immunohistochemical analysis of 10-μm frozen liver sections with an antibody against albumin (polyclonal rabbit antialbumin, 1:50; Dako) or immunoglobulin control (rabbit immunoglobulin fraction, 1:50, Dako) was performed after biotin blocking (Avidin/Biotin blocking kit; Vector) with an indirect immunoperoxidase system according to the manufacturer's instructions. Tissue staining with a monoclonal antibody against desmosomal proteins (ZK31, 1:100; Sigma;)[18] and the negative control (mouse IgG1, 1:100; Immunotech) was performed with a kit (MOM; Vector) for the detection of mouse primary antibodies on mouse tissues after biotin blocking. The rat monoclonal antibody A6 against bile duct epithelia was a generous gift from N. Engelhardt (1:10)[19] and was visualized with a peroxidase-labeled mouse-adsorbed polyclonal anti-rat antibody (Southern Biotechnology Associates).

In vitro assays

To assess the *in vitro* proliferative capacity of SP cells, we seeded the relevant cell populations into methylcellulose medium (M3236, M3435, Stem Cell Technologies) at 2×10^4 cells/3 mL of medium in 3.5 cm dishes, followed by incubation at 37°C in 5% CO_2. The cytokines recombinant murine (rm) interleukin-3 (IL-3) (10 ng/mL), rm interleukin-6 (IL-6) (10 ng/mL), rm stem cell factor (SCF) (50 ng/mL), rm erythropoietin EPO (3 units/mL), rm transforming growth factor α (TGF-α) (10 ng/mL), rm epidermal growth factor (EGF) (10 6 ng/mL) (Stem Cell Technologies, Genzyme) and nicotinamide (10 mM) (Sigma) were added as described in the text. Colony growth was scored and photodocumented on days 14 and 21. For immunophenotyping of cultured cells, the colonies were picked, spun onto slides and stained by an indirect immunoperoxidase procedure (*see above*). Primary and nested reverse transcriptase polymerase chain reactions (RT-PCR) for albumin were performed according to standard protocols (annealing temperature 55°C, 35 cycles) with primers spanning at least one intron to avoid amplification of contaminating DNA sequences (mAlb us 1: GATCCACAAGCTCCTGACAG; mAlb us 2: CTCTGATGGTCAAAGTCCTG; mAlb ds1: TGCTTCACGGCATCTTCCTG; mAlb ds 2: GCCAAGTGTCTTCCAGTACG). FAH staining was performed as previously described.[11]

Transplantation assays

Donor male Rosa26 C57Bl/6 cells were suspended in PBS (final volume, 50 μL), and injected directly into the livers of anesthetized female syngeneic recipients during laparotomy. The animals were fed a DDC-containing diet for 10 days, before and after transplantation, and were sacrificed for analysis 6 weeks later. Engrafted cells were identified in hepatic tissue by their cytoplasmic β-galactosidase activity with X-gal or fluorescein di-β-Dgalactopyranoside (FDG, Molecular Probes, Inc.) as substrates,[20] as well as by *in situ* hybridization with a probe that recognizes repetitive sequences on the *Mus musculus* Y-chromosome (pY353/B),[21] kindly provided by R. Behringer (MD Anderson Cancer Center). The 1.6-kb DNA probe was random labeled with digoxigenin (Roche), purified and used in a final concentration of 10 nmol/μL. *In situ* hybridization on paraffin sections was performed as described elsewhere.[22] For fresh frozen liver tissues, 10-μm sections were fixed in Histochoice (Amresco) for 20 min, washed in PBS twice, digested with proteinase K (5 μg/mL, Roche) for 10 min, washed in PBS, dehydrated in alcohol, denatured in hybridization buffer for 15 min at 75°C, dehydrated in an ice-cold alcohol series, and kept on ice until addition of the labeled probe. The probe was denatured separately at 100°C for 15 min, chilled to 4°C, added to the slides, and incubated overnight

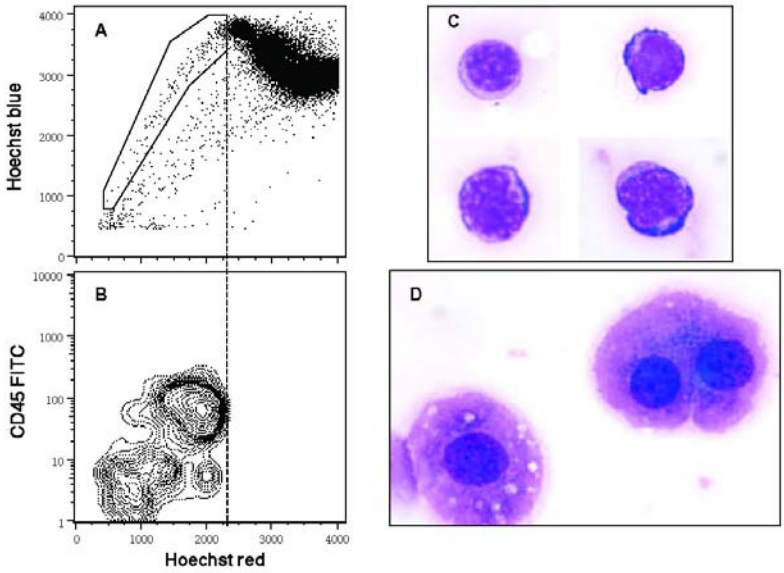

Figure 1. Hoechst 33342 staining profile and phenotype of SP cells. The side population (SP) accounts for an average of 1% of all nucleated cells in the linear window of Hoechst 33342 staining (A). This population expresses CD45 in the region of highest Hoechst 33342 fluorescence, delineated by emission above 670 nm on the X-axis (*Hoechst red*) only, and lacks CD45 expression in the region of lowest Hoechst 33342 fluorescence (B). Sorted hepatic SP cells, spun on slides by cytocentrifugation and stained with Wright-Giemsa, are small cells with scant azurophilic, agranular cytoplasm and a blastoid nucleus: they contrast with the cytoplasm-rich, partially binucleated hepatocytes (C, D, 100×).

at 37°C. The slides were then washed and the bound probe detected with an alkaline phosphatase coupled antidigoxigenin antibody, with NBT/BCIP used as the chromogen (Roche). The slides were subsequently stained with Nuclear Fast Red (Vector) and antibodies as indicated. To quantify the potential of a particular sample, we counted the number of Y-chromosome-positive cells in three different fields for each 100 cells studied. When there were few positive events per slide, the total number of Y-chromosome-positive cells per total number of cells on the slide were reported (estimation based on an average of 1,568 cells per mm^2 of mouse hepatic tissue). To assess the potency of hepatic SP cells for hematopoietic reconstitution, we directly injected sorted liver-derived SP cells from male CD45.2 animals intravenously into female CD45.1 recipients after total body-irradiation (10 Gy) from a ^{137}Cs source, given in two fractions at least 2 h apart. In tests for radioprotection, some groups of animals were transplanted with hepatic SP alone, whereas long-term repopulation capacity was analyzed after 2×10^5 female CD45.1 whole bone marrow cells had been co-injected with the test population to secure short-term hematopoietic recovery. Engraftment was assessed by FACS at 7 and 10 months posttransplantation, using an antibody against CD45.2, and by PCR on 300 ng peripheral blood DNA yielding a product of 249 bp to detect the mouse Y-chromosome-specific SRY gene (35 cycles at 55° C annealing temperature; modified from Lavrovsky *et al.*).[23]

Results

Prevalence and phenotype of hepatic SP cells

Hoechst 33342 staining separated the mononuclear cell preparations of whole murine livers into a population of Hoechst 33342 bright-staining non-SP cells and a minority SP characterized by low Hoechst 33342 fluorescence (Figure 1A). The SP cells accounted for approximately 1% of all mononuclear cells. Sorted SP cells were small, blast-like mononuclear cells with scant azurophilic, agranular cytoplasms (Figure 1C), in marked contrast to the large, partially binuclear hepatocytes found in the non-SP population (Figure 1D). By immunophenotyping, a mean 75.5±16.0% (SD) of the hepatic SP cells expressed the hematopoiesis-associated antigen CD45, while cells with the highest efflux potential, found at the lower tip of the SP tail, were enriched for $CD45^{neg}$ cells (Figure 1B). Distinct subpopulations of hepatic SP cells, whether positive or negative for CD45, expressed the stem cell markers CD34 (3.7±4.1%), c-kit (12.3±6.8%), Sca-1 (48.9±15%) and Thy-1 (48±16.2%), on both $CD45^{pos}$ and $CD45^{neg}$ cells. By immunocytology, hepatic SP cells were negative for the mature hepatocyte marker fumaryl acetoacetate hydrolase (FAH), and the biliary epithelium markers cytokeratin 19 and A6. Thus, hepatic SP cells resemble their bone marrow counterparts in both prevalence and general morphologic features, but they are phenotypically more heterogeneous.[14,17]

In vitro growth of hepatic SP cells

To test the clonogenicity and differentiation potential of hepatic SP cells, we analyzed their *in vitro* growth characteristics in a methylcellulose culture assay. The mean number of colonies generated from 2×10^4 fresh hepatic SP cells was 5.7±2.9 (SD) in methylcellulose supplemented with rmIL-3, rmIL-6, rmSCF, and rhEPO (Figure 2A). A similar result was obtained with IL-3 alone, but the colonies were smaller and less dense in appearance. In cultures supplemented with classical stimulants

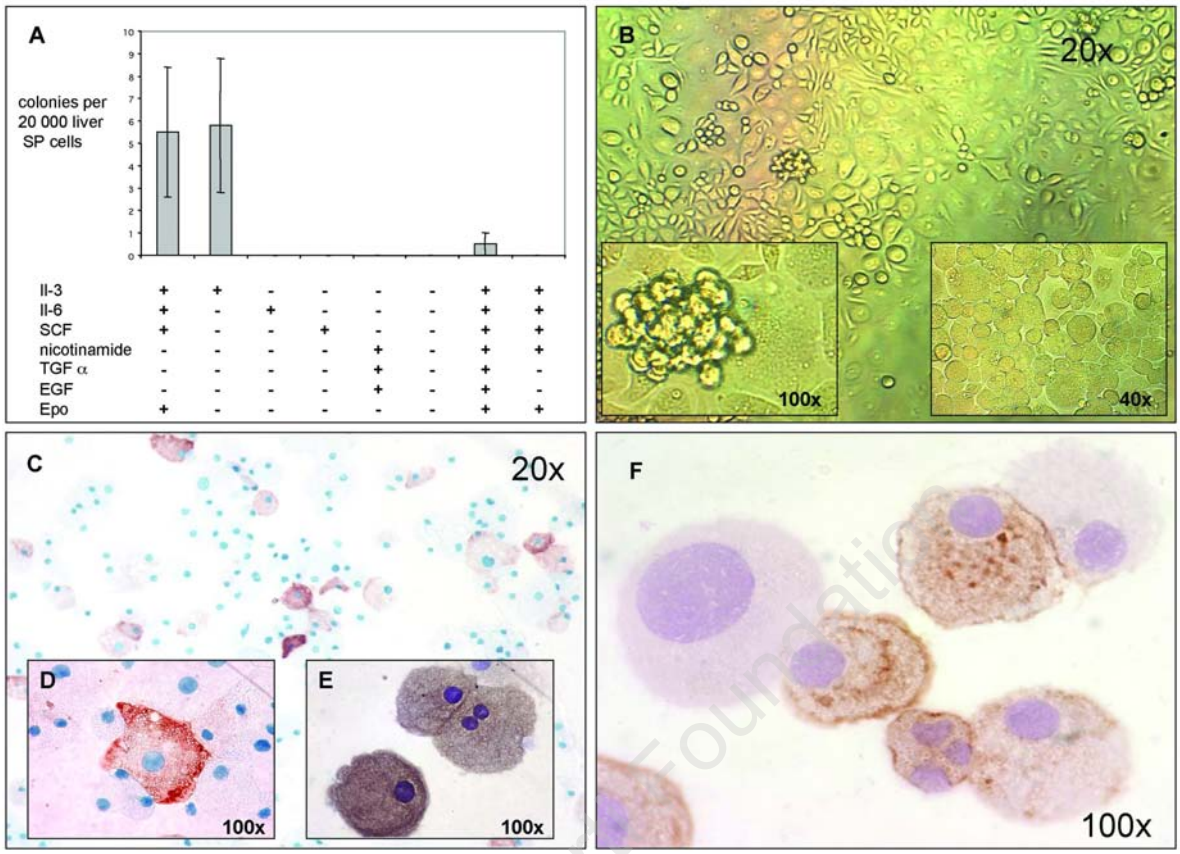

Figure 2. ***In vitro*** **growth characteristics of hepatic SP cells. Hepatic SP cells showed clonogenic growth in a methylcellulose-based semisolid assay when stimulated with rmIL-3, rmIL-6 and rmSCF, but not after stimulation with rm-TGF-α, rmEGF 22 and nicotinamide (A). One third of the colonies derived from hepatic SP cells were a heterogeneous mixture of spindle-shaped polygonal adherent cells and clusters of semiadherent small cells as well as very large adherent cells on day 21 of cell culture (B). Within single colonies some large cells stained positive for FAH (C, D) and albumin (E), whereas the small cells stained in part positive for CD45 (F).**

for growth of hepatocytes,[24] rmEGF, rmTGF-α and nicotinamide, there was no obvious growth. Moreover, when TGF-α, EGF or nicotinamide was added after rmIL-3, rmIL-6, rmSCF and EPO, only slight proliferation was seen, suggesting an inhibitory effect of the hepatocyte growth factors on early progenitor growth. Comparison of colony-forming potential between $CD45^{pos}$ and $CD45^{neg}$ hepatic SP cells failed to reveal any appreciable difference (data not shown). Among the cells stained with Hoechst 33342, only those with an SP phenotype showed growth in methylcellulose. Unsorted hepatic parenchymal cells did survive in the assay, but only rarely formed colonies (3 colonies per 200,000 Hoechst 33342- unstained liver cells plated). In general, the colonies of densely growing round cells appeared similar to those derived from bone marrow SP cells; however, about 30% of the hepatic SP-derived colonies consisted of a mixture of polygonal adherent cells and clusters of semiadherent small cells as well as very large cells (Figure 2B). Within the same colonies, immunophenotyping identified cells positive for both markers of hepatocytic (FAH and albumin; Figures 2D and 2E) and hematopoietic (CD45, Figure 2F) differentiation; by RT-PCR, cells with these mixed colonies expressed albumin (*data not shown*). However, experiments in which male and female cells were mixed and plated indicated that at least some of the colonies containing both hematopoietic cells and hepatocytes were derived from the deposition of two cells (*data not shown*). Thus, hepatic SP cells have moderate colony-forming potential *in vitro*, required growth stimulation with early acting hematopoietic growth factors, and showed a differentiation pattern that was partly hematopoietic.

Liver regeneration from hepatic SP cells

To evaluate the potency of hepatic SP cells for liver regeneration, we performed orthotopic hepatic SP cell transplantation. In preliminary experiments, no contribution from hepatic SP cells was

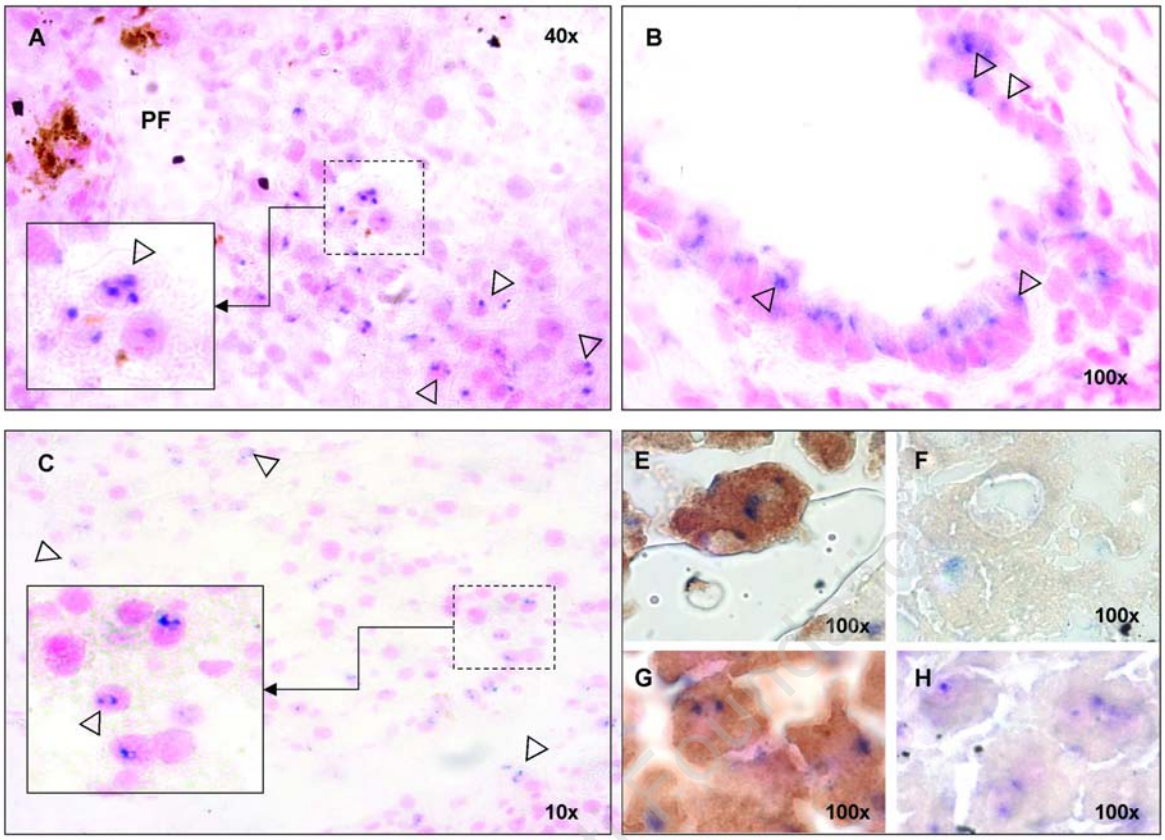

Figure 3. Engraftment of hepatic SP cells into liver parenchyma. Six weeks after local injection of hepatic SP cells, the progeny of male donor cells were detected by *in situ* hybridization for the Y-chromosome. Donor-derived cells were detected in clusters associated with the periportal field (PF, A), in the bile duct epithelium (B), and diffusely distributed in the liver plate (C). Representative male donor cells positive for the Y-chromosome are pointed out by arrowheads. Hepatic and epithelial differentiation of the engrafted cells was shown by combining the *in situ* hybridization with immunohistochemistry using a polyclonal antibody against albumin (E) and a monoclonal antibody (ZK31) against epithelial desmosomes (G) in comparison to the respective isotype controls (F, H).

seen when these were transplanted directly into non-pretreated livers. Thus, to favor recruitment of primitive hepatic progenitors over hepatocytes, we transplanted the hepatic SP cells into the livers of mice treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) for 10 days before and after cell transplantation. This treatment severely impedes parenchymal cell-driven regeneration, allowing more primitive cells to contribute to tissue repair.[25-27] By transplanting donor cells from male Rosa26 transgenic mice into female wild-type recipients, we were able to use β-galactosidase enzyme activity and *in situ* hybridization with a Y-chromosome probe as markers to track the progeny of the engrafted cells. Six weeks after orthotopic cell transplantation, donor-derived cells had engrafted as mature hepatocytes and as bile duct epithelium (Figure 3, Table 1). As markers for the site of cell injection were not available, we screened the whole liver for donor cell contribution. Positive cells were thus found in distinct areas, which consisted both of regions with diffusely distributed as well as clustered positive cells (Figure 3). The extent of donor cell contribution in these areas of highest engraftment ranged from 3.6% to 22.0% in different experiments; a low level of parenchymal engraftment was also observed adjacent to these areas of highest donor cell contribution (Table 1). The concentrations and morphologic features of the engrafted cells did not differ whether $CD45^{pos}$ or $CD45^{neg}$ SP cells were transplanted (Table 1). Finally, engraftment was negligible in control animals transplanted with equivalent numbers of non-SP hepatic cells (Table 1). These experiments show that locally transplanted hepatic SP cells survived and expanded in the livers of DDC-treated animals, competing efficiently with endogenous liver stem cells, and contributing to the regeneration of the major components of hepatic parenchyma. The phenotypic similarity of hepatic SP cells to their bone marrow counterparts, as well as their ability to give rise to hematopoietic colonies *in vitro*,

Table 1. Engraftment of hepatic SP cells after local injection into the liver of DDC treated mice.

Animal	*Transplanted population*	*Cell number ×10⁻²*	*Hepatic injury (DDC treatment)*		*% Engraftment (mean±SD)*	
			Donor	*Recipient*	*Highest*	*Adjacent area*
1	SP	60	–	+	18.8±7.7	0.7±0.8
2	SP	200	–	+	5.3±1.5	1±1.7
3	non-SP	200	–	+	0	0
4	SP	500	+	+	5.3±0.6	1.3±0.6
5	SP	1000	+	+	22±2.5	3.3±2.5
6	non-SP	550	+	+	2 / 150,000	ND
7	non-SP	1000	+	+	5 / 400,000	ND
8	SP CD45^{pos}	94	–	+	3.6±1.5	0
9	SP CD45^{pos}	50	–	+	31.6±2.5	0
10	SP CD45^{neg}	33	–	+	10±5	0.3±0.6
11	SP CD45^{neg}	2	–	+	13±1	0
Normal male					77.4±4.5	
Normal female					0	

SP cells or non-SP cells were injected directly into liver lobes. After 6 weeks, livers were examined for extent of engraftment by Y chromosome in situ hybridization or β-galactosidase expression (animal 3). Normal male and female controls were employed during each Y-chromosome in situ staining. Engraftment was quantified only in the parenchyma, although substantial engraftment could also be observed in the periportal areas. Engraftment is reported as the mean percentage of Y-chromosome-positive cells determined from three 100-cell fields in the field of highest positivity and in an adjacent area, as indicated. In experiments 6 and 7 the amount of engraftment from non-SP cells was very low, so that the number of positive cells is given per estimated total number of cells on the slide (see Methods).
DDC, 3.5-diethoxycarbonyl-1,4-dihydrocollidine.

prompted us to assess the potency of hepatic SP cells for hematopoietic reconstitution *in vivo*. When 200,000 female CD45.1 bone marrow cells were co-transplanted with 500 to 1000 male hepatic SP cells to secure short-term hematopoietic recovery, hepatic SP-derived cells could be consistently detected over a period of 10 months in 3 out of 5 animals by PCR for mouse Y-chromosome-specific sequences (*data not shown*) but not by flow cytometry (limit of detection around 1%). All animals injected with hepatic SP cells alone (500 to 60,000 donor cells) died within 4 weeks after transplantation indicating the absence of substantial hematopoietic activity even when high cell numbers were given. Thus, hepatic SP cells have, at best, a low capacity to contribute to long-term hematopoiesis.

Recruitment of bone marrow SP cells for liver regeneration

The phenotypic and functional similarities between liver and bone marrow SP cells piqued our interest in the interchangeability of the stem cell pools in these tissues. In order to track the potential flow of stem cells from the bone marrow to the liver, we first marked the bone marrow stem cell pool in experimental animals. Bone marrow SP cells, distinguishable by CD45 polymorphism, sex mismatch or β-galactosidase expression, were purified and transplanted into sublethally irradiated recipients. Four to 12 months after stable hematopoietic chimerism had been confirmed by analysis of peripheral blood, the animals were treated for 10 days with DDC and the liver tissues were analyzed at specific time points (Figure 4, Table 2). Whole bone marrow was highly engrafted in all but one animal (mean 90.6%), with lower engraftment levels seen in the bone marrow SP and hepatic SP compartments (means 40% and 24%, respectively). The level of engraftment in the hepatic SP that were CD45^{pos} approximated that of the bone marrow SP, except in the one animal that received no liver damage treatment (animal 1, Table 2). Analysis of the chimerism in two animals transplanted with bone marrow SP cells from β-galactosidase transgenic donors revealed that the bone marrow SP also continued to the CD45^{neg} hepatic SP compartment (Table 2). Thus, SP cells originally purified from the bone marrow contributed to regeneration of both CD45^{pos} and CD45^{neg} liver SP after DDC treatment. Within the architecture of the liver, the contribution of bone marrow-SP-derived cells was found at low levels in the hepatocytes of the parenchyma (black arrowheads on hepatocytes in Figure 5A and

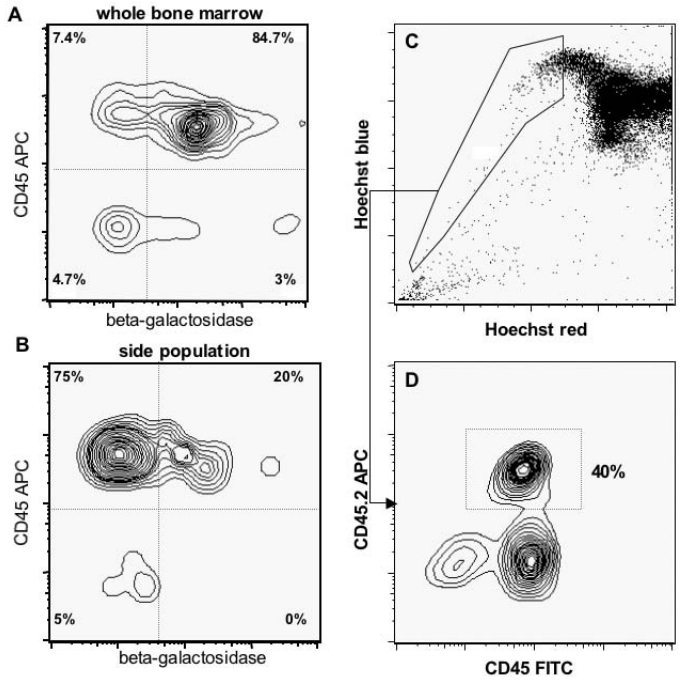

Figure 4. Contribution of transplanted bone marrow SP cells to hepatic and bone marrow stem cell chimerism. Female C57Bl/6 mice with the CD45.1 allele were transplanted with 3×10^3 bone marrow SP cells from male syngeneic animals with the CD45.2 allele or β-galactosidase transgene expression detected by fluorescein di-ß-D-galactopyranoside (FDG) staining (Panel A). The mice were stable hematopoietic chimeras 4 to 12 months after transplantation. Following treatment with DDC, the proportion of donor-derived CD45.2 positive cells within hepatic SP was 40% in this animal on day 1 after DDC (Panels C and D). In the bone marrow, the majority (here 84.7% at day 10 after DDC) of the $CD45^{pos}$ mononucleated cells were donor-derived, as detected by transgenic β-galactosidase expression in panel A. In contrast, both the $CD45^{pos}$ and the $CD45^{neg}$ cells in the bone marrow SP were mainly host-derived (here 75% and 5% of all mononuclear cells, respectively; panel B).

Table 2. Bone marrow SP engraftment and contribution to hepatic tissue.

					Chimerism		
Animal	*Time post-transplant (months)*	*Time post DDC (days)*	*WBM (%)*	*BM-SP (%) (%)*	*$CD45^{pos}$ hepatic SP (%)*	*$CD45^{neg}$ hepatic SP (%)*	*Liver parenchyma*
1	4	0	97	64	5.7	14.5	121/~39,200
2	4	10	92	21	13	9.5	428/~65,000
3	12	1	92*	ND	40	ND	747/~58,800
4	12	14	74	43	31	ND	690/~117,600
5	12	36	98	32	28	ND	110/~58,800

*Engraftment from 3×10^3 bone marrow SP cells to whole bone marrow, bone marrow SP, and $CD45^{pos}$ hepatic SP was determined by flow cytometry as the percentage of $CD45.2^{pos}$ cells in the CD45.1 host. In transplants #1 and 2, the contributions of donor-derived cells to the $CD45^{neg}$ hepatic SP were estimated by β-galactosidase positivity. The extent of male donor-derived contribution to hepatic parenchyma was estimated by the number of large, ZK31-positive cells with nuclear Y-chromosome signals per the estimated total number of nucleated cells on the slide (based on 1568 cell nuclei per mm^2 liver tissue section). Substantially higher chimerism was observed in peri-portal regions (compare Figure 5). Normal untransplanted male and female mice were co-stained every experiment to ensure fidelity of the Y-chromosome in situ technique. ND: not done; *PB chimerism; WBM: whole bone marrow; BM-SP: bone marrow-SP.*

B, < 0.37% on average, Table 2), and at higher levels in the periportal fields (Figure 6). Dissection of periportal engraftment in serial sections revealed Y-chromosome-positive cells in clusters of small periductular cells (Figure 6A and B). Within these clusters, only the epithelial cell layer of the bile duct stained positive with the antibody A6, commonly used to identify oval cells,[19] (also black arrowhead in Figure 6C); whereas the periductular cells represented an admixture of $CD45^{pos}$ and $CD45^{neg}$ cells (open arrowheads in Figure 6D). The $CD45^{pos}$ periductular cells were neither T- nor B-cells, as they failed to stain with antibodies against CD5 or B220 (*data not shown*). Thus, after stable bone marrow engraftment, the progeny of bone marrow SP cells partially replenished the liver stem cell pool, from where they were recruited for regeneration of the damaged liver.

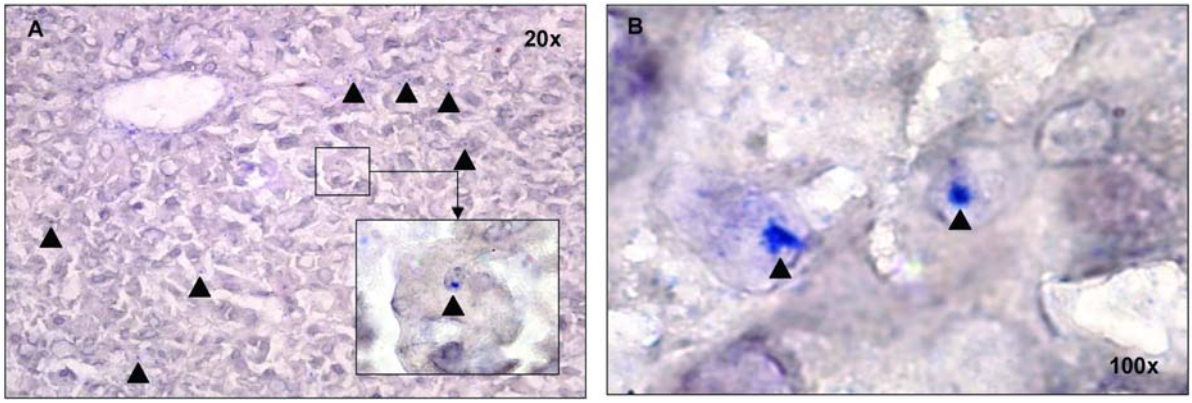

Figure 5. Contribution of transplanted bone marrow SP cells to hepatic tissue regeneration after hepatic injury. In female C57Bl/6 mice with stable hematopoietic chimerism 12 months after transplantation with bone marrow SP cells from male syngeneic animals, the hepatic tissue was analyzed for donor-derived cells by Y-chromosome *in situ* hybridization. Engrafted cells were detected predominantly in the periportal field (Figure 6), but also in the hepatic plate (panel A, B; black arrowheads). All slides were immunostained with a monoclonal antibody against the desmosomes of epithelial cells (ZK31) and immunoperoxidase reaction with DAB (nickel enhanced). Representative donor-derived cells with the blue/purple nuclear Y-chromosome hybridization signal are indicated by arrowheads.

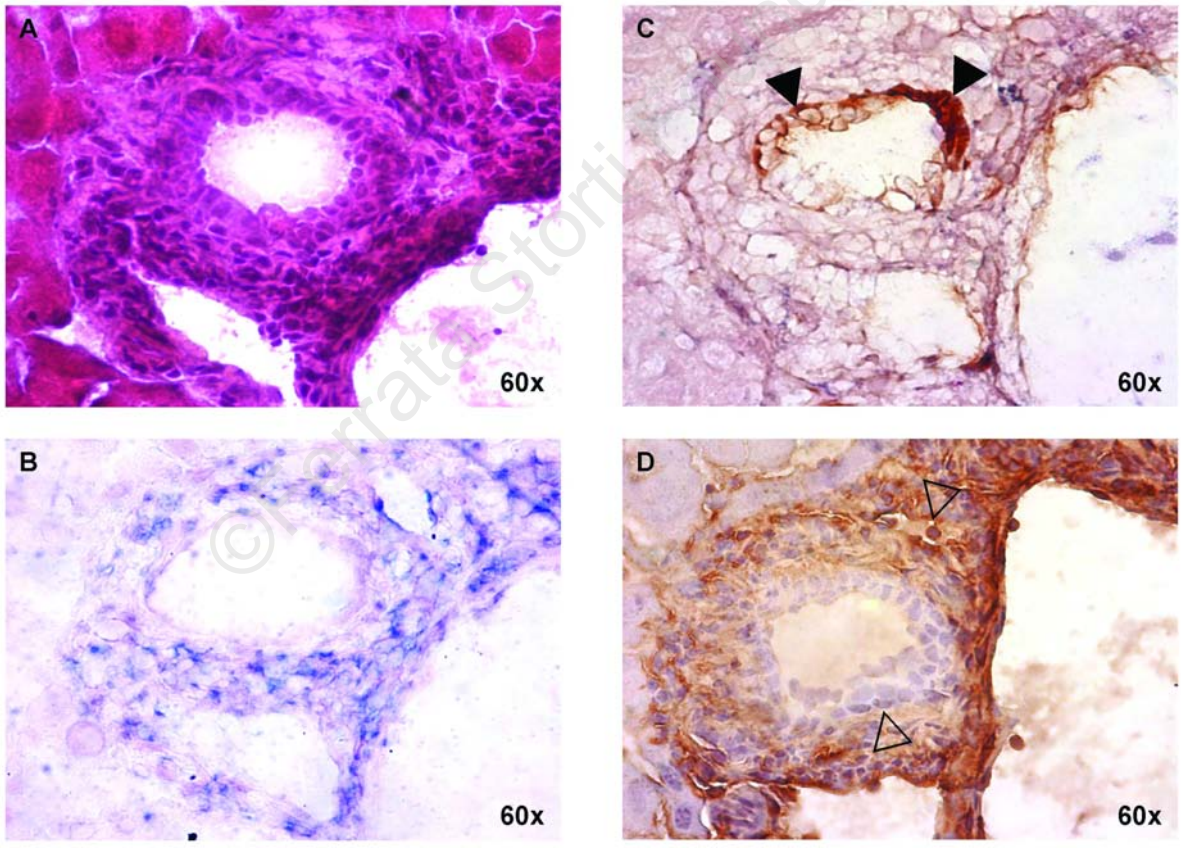

Figure 6. Origin of periductular oval cells from transplanted bone marrow SP cells. Female C57Bl/6 mice with stable hematopoietic chimerism after transplantation with bone marrow SP cells from male congeneic animals were treated with DDC for 10 days, and frozen sections stained with hematoxylin/eosin (A) and Y-chromosome *in situ* hybridization (B) after a recovery of 10 days. The bile duct epithelium was stained with the rat monoclonal antibody A6, commonly used to identify oval cells, and visualized by DAB reaction through an anti-rat horse radish peroxidase conjugate (C, black arrowheads). In an immunostain with an anti-CD45 24 antibody (D), the oval cells were found to be an admixture of $CD45^{pos}$ and $CD45^{neg}$ cells (open arrowheads, D).

Discussion

The integrity of most highly regenerative organs is maintained by the tightly regulated formation of parenchyma from a pool of local primitive stem cells. Recent studies have called attention to the similarities among stem cell pools in different tissues, including the expression and function of c-kit, CD34, and Thy-19,[28-30] Among those features common to stem cells from different sources, bone marrow and skeletal muscle share a small population of cells, termed the side population (SP), which are distinguished by their high efflux capacity for Hoechst 33342.

In murine bone marrow, SP cells represent a minute population of $CD34^{low/neg}$ stem cells that contain virtually all of the long-term marrow engraftment capacity.[14,17] In mouse liver, we consistently found a minority population of cells with both morphologic and phenotypic similarities to bone marrow SP cells. *In vitro* testing of these putative stem cells revealed a low colony-forming efficiency in response to early-acting hematopoietic growth factors, especially IL-3, and an inhibitory effect of growth factors known to be effective for the stimulation of mature hepatocytes, such as EGF, TGF-α, and nicotinamide.[24] The appreciably weaker colony-forming capacity of hepatic versus bone marrow SP cells was presumably due to an intrinsic bias of the assay, favoring detection of cells that retain the ability to respond to hematopoietic growth factors. We also found evidence for mixed hematopoietic and hepatocellular differentiation in one-third of the colonies derived from hepatic SP cells; however, we cannot exclude the possibility that these colonies were derived from two cells.

When transplanted into recipient mice that had been treated with DDC, hepatic SP cells made a substantial contribution to the regeneration of liver parenchyma and bile duct epithelial cells, suggesting that they can function as facultative stem cells. Expression of CD45 by these SP cells did not appear to have functional significance, since $CD45^{pos}$ and $CD45^{neg}$ subpopulations engrafted with similar efficiencies into hepatic parenchyma and were equally clonogenic *in vitro.* Since the progeny of hepatic SP cells were integrated into both the hepatic plate and bile duct epithelium, we suggest that such stem cells are functionally related to the morphologically and phenotypically distinctive oval cell population, which also supplies precursors for these two components of liver tissue.[4,5,7,8]

Hepatic SP cells, in contrast to bone marrow SP cells, contributed only marginally to hematopoietic reconstitution when transplanted after radiation-induced myeloablation (*data not shown*). Whether this result reflects a true limitation in differentiation capacity, a propensity for quiescence, or a limitation of the *in vivo* assay system chosen to detect hematopoietic differentiation of liver-derived stem cells is unclear, but will need to be resolved in future studies.

The ability of bone marrow cells to contribute to the regeneration of liver has been recently reported[9,10,12,13,] and Lagasse *et al.* convincingly showed that prospectively isolated hematopoietic stem cells, identified on the basis of cell surface markers, could differentiate into hepatocytes in a genetic liver regeneration model.[11] Using a different method of stem cell isolation, we provide evidence that a liver stem cell population can be replenished by bone marrow-derived stem cells. We show that transplanted bone marrow hematopoietic stem cells, purified on the basis of Hoechst dye efflux, generate hepatic stem cells (SP cells) and ultimately hepatocytes and biliary epithelium. In addition to the differentiation into bile duct epithelium and hepatic cells, differentiation of bone marrow- or liver-derived SP cells into Küppfer cells might account for the few positive cells seen in the liver plate. The contribution of SP cells to the cells of the liver stroma, such as Küppfer and Itoh cells does, however, remain to be elucidated in detail in further studies.

SP-like cells have now been identified in bone marrow,[14,17] skeletal muscle,[15,16] and liver. The finding presented here, that SP cells of the liver can be derived from their counterparts in the bone marrow, suggests that SP cells of additional tissues may also be ultimately derived from the bone marrow. Transplanted SP cells may colonize many tissues after transplantation, contributing to both a $CD45^{pos}$ and $CD45^{neg}$ SP cell compartment. Where, within the hepatic tissue architecture, the hepatic SP cells are located remains to be clarified. This may point to the hematopoietic stem cell as the cell which can replenish multiple dispersed stem cell populations throughout the animal. This finding is consistent with the observation that purified bone marrow stem cells can contribute to the formation of skeletal muscle,[16] epithelial cells of the gastrointestinal and respiratory tract,[31] cardiac muscle, and vascular endothelial cells.[32,33]

Also notable is the observation that although engraftment of bone marrow SP cells to the whole bone marrow was very high (average 90%), the contribution to the SP cell population of bone marrow and liver was significantly lower (40% and 24%, respectively), indicating that the majority of host SP cells persisted long-term after transplantation, but did not feed into the pool of expanding and maturating progenitors. The role of these functionally quiescent subsets of the side population in tissue regeneration remains to be elucidated. The full potential of hepatic SP cells for liver regeneration was apparent in animals receiving DDC treatment, which partially abrogates mature hepato-

cyte-based regeneration and induces compensatory regeneration via hepatic oval cells. In this model, we found a predominant engraftment of donor bone marrow SP-derived cells in the periductular fraction of the small cell in the periportal field (Figures 5 and 6). In the rat, Petersen *et al.* described the expansion of Thy-1 positive cells in the portal region during oval cell-driven liver regeneration after 2-acetylaminofluorene (2-AAF) induced hepatic injury.[38] In the same model Omori *et al.* described the transient expression of CD34 in the hepatic oval cells during stem cell driven regeneration of hepatocytes.[39] Furthermore, periportal necrosis induced by allyl alcohol intoxication led to parenchymal regeneration originating from small intraportal stem cells, which were negative for markers of hepatocyte and bile duct precursors (*null cells*).[34-36] Based on this observation, Sell proposed that primitive periductular cells constitute the cell fraction into which bone marrow-derived stem cells could integrate in the situation of toxically induced liver regeneration.[37] Our findings on the phenotype and the engraftment pattern of SP cells are consistent with this hypothesis and suggest that the tissue niche in which the bone marrow-derived hepatic SP cells and/or their progeny harbor is the periductular space in the periportal area. However, as SP cells are currently solely defined by their Hoechst 33342 staining profile on the flow cytometer, the question of their exact tissue location in the liver remains open.

In a clinical perspective, it is intriguing that hepatic SP cells already *in situ* as well as the injected SP cells were able to grow, expand, and functionally integrate under the conditions of a severely damaged liver microenvironment. In conclusion, the SP phenotype defines a minority population of primitive stem cells in adult murine liver that possesses considerable potential for liver regeneration in the setting of impaired regrowth and can be replenished from the bone marrow SP compartment. Thus, either hepatic or marrow SP cells might provide suitable candidates for liver cell transplantation, offering both a graft population capable of homing to diseased hepatic tissue and a target for transplanting hepatic stem cells after *ex vivo* gene therapy.

References

1. Overturf K, al-Dhalimy M, Ou CN, Finegold M, Grompe M. Serial transplantation reveals the stem-cell-like regenerative potential of adult mouse hepatocytes. Am J Pathol 1997; 151:1273-80.
2. Rhim JA, Sandgren EP, Degen JL, Palmiter RD, Brinster RL. Replacement of diseased mouse liver by hepatic cell transplantation. Science. 1994;263:1149-52.
3. Rajvanshi P, Kerr A, Bhargava KK, Burk RD, Gupta S. Efficacy and safety of repeated hepatocyte transplantation for significant liver repopulation in rodents. Gastroenterology 1996;111:1092-102.
4. Farber E. Similarities in the sequence of early histologic changes induced in the liver of rats by ethionine, 1-acetylaminofluorene and 3'-methyl-4-dimethylaminoazobenzene. Cancer Res 1956;16:142-8.
5. Evarts RP, Nagy P, Marsden E, Thorgeirsson SS. A precursor-product relationship exists between oval cells and hepatocytes in rat liver. Carcinogenesis 1987;8:1737-40.
6. Koukoulis G, Rayner A, Tan KC, Williams R, Portmann B. Immunolocalization of regenerating cells after submassive liver necrosis using PCNA staining. J Pathol 1992;166:359-68.
7. Sell S. Is there a liver stem cell? Cancer Res 1990;50:3811-5.
8. Alison MR, Golding M, Sarraf CE. Liver stem cells: when the going gets tough they get going. Int J Exp Pathol 1997;78: 365-81.
9. Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, et al. Bone marrow as a potential source of hepatic oval cells. Science 1999;284:1168-70.
10. Alison MR, Poulsom R, Jeffery R, Dhillon AP, Quaglia A, Jacob J, et al. Hepatocytes from non-hepatic adult stem cells. Nature 2000;406:257.
11. Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L, et al. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med 2000;6:1229-34.
12. Theise ND, Badve S, Saxena R, Henegariu O, Sell S, Crawford JM, et al. Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology 2000;31:235-40.
13. Theise ND, Nimmakayalu M, Gardner R, Illei PB, Morgan G, Teperman L, et al. Liver from bone marrow in humans. Hepatology 2000;32:11-6.
14. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 1996; 183:1797-806.
15. Jackson KA, Mi T, Goodell MA. Hematopoietic potential of stem cells isolated from murine skeletal muscle. Proc Natl Acad Sci USA 1999;96:14482-6.
16. Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, et al. Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature 1999;401: 390-4.
17. Goodell MA, Rosenzweig M, Kim H, Marks DF, DeMaria M, Paradis G, et al. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat Med 1997;3:1337-45.
18. Lang AB, Odermatt BF, Ruettner JR. Monoclonal antibodies to human cytokeratins: application to various epithelial and mesothelial cells. Exp Cell Biol 1986;54:61-72.
19. 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 1993;55:19-26.
20. Bonnerot C, Nicolas JF. Application of LacZ gene fusions to postimplantation development. Methods Enzymol 1993; 225:451-69.
21. Bishop CE, Hatat D. Molecular cloning and sequence analysis of a mouse Y chromosome RNA transcript expressed in the testis. Nucleic Acids Res 1987;15:2959-69.
22. Eckert JW, Buerkle CJ, Major AM, Finegold MJ, Brandt ML. In situ hybridization utilizing a Y chromosome DNA probe. Use as a cell marker for hepatocellular transplantation. Transplantation 1995;59:109-11.
23. Lavrovsky Y, Song CS, Chatterjee B, Roy AK. A rapid and reliable PCR-based assay for gene transmission and sex determination in newborn transgenic mice. Transgenic Res 1998; 7:319-20.
24. Mitaka T. The current status of primary hepatocyte culture. Int J Exp Pathol 1998;79:393-409.
25. Solomon HK, Frank HJ. Disturbance in porphyrin metabolism caused by feeding diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3, 5-dicarboxylate. Cells 1958:583-5.
26. Yokoo H, Harwood TR, Racker D, Arak S. Experimental pro-

duction of Mallory bodies in mice by diet containing 3,5-diethoxycarbonyl-1,4-dihydrocollidine. Gastroenterology 1982;83:109-13.
27. Preisegger KH, Factor VM, Fuchsbichler A, Stumptner C, Denk H, Thorgeirsson SS. Atypical ductular proliferation and its inhibition by transforming growth factor β1 in the 3,5-diethoxycarbonyl-1,4-dihydrocollidine mouse model for chronic alcoholic liver disease. Lab Invest 1999;79:103-9.
28. 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 1994;70:511-6.
29. 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;30:112-7.
30. Crosby HA, Kelly DA, Strain AJ. Human hepatic stem-like cells isolated using c-kit or CD34 can differentiate into biliary epithelium. Gastroenterology 2001;120:534-44.
31. Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 2001;105:369-77.
32. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001;410:701-5.
33. Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, Majesky MW, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 2001; 107:1395-402.
34. 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;21:1702-12.
35. Yin L, Lynch D, Sell S. Participation of different cell types in the restitutive response of the rat liver to periportal injury induced by allyl alcohol. J Hepatol 1999;31:497-507.
36. Sell S. Electron microscopic identification of putative liver stem cells and intermediate hepatocytes following periportal necrosis induced in rats by allyl alcohol. Stem Cells 1997; 15:378-85.
37. Sell S. Heterogeneity and plasticity of hepatocyte lineage cells. Hepatology 2001;33:738-50.
38. Petersen BE, Goff JP, Greenberg JS, Michalopoulos GK. Hepatic oval cells express the hematopoietic stem cell marker Thy-1 in the rat. Hepatology 1998;27:433-45.
39. Omori N, Omori M, Evarts RP, Teramoto T, Miller MJ, Hoang TN, et al. Partial cloning of rat CD34 cDNA and expression during stem cell-dependent liver regeneration in the adult rat. Hepatology 1997;26:720-7.

Pre-Publication Report & Outcomes of Peer Review

Contributions

All authors: conception and design, analysis and interpretation of data, drafting the article, final approval of the version to be published.

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Conflict of interest: none.

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Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Vicente Vicente, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Professor Vicente and the Editors. Manuscript received August 16, 2002; accepted February 11, 2003.

In the following paragraphs, the Deputy Editor summarizes the peer-review process and its outcomes.

What is already known on this topic

It has been demonstrated that bone marrow-derived stem cells have sufficient plasticity to contribute to hepatic regeneration.

What this study adds

This study adds evidence that bone marrow derived side population (SP) cells might be used for the repair of damaged liver. Thus, the SP cells might provide suitable candidates for liver cell transplantation.