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Clonal identification and characterization of self-renewing pluripotent stem cells in the developing liver

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Using flow cytometry and single cell–based assays, we prospectively identified hepatic stem cells with multilineage differentiation potential and self-renewing capability. These cells could be clonally propagated in culture where they continuously produced hepatocytes and cholangiocytes as descendants while maintaining primitive stem cells. When cells that expanded in vitro were transplanted into recipient animals, they morphologically and functionally differentiated into hepatocytes and cholangiocytes with reconstitution of hepatocyte and bile duct structures. Furthermore, these cells differentiated into pancreatic ductal and acinar cells or intestinal epithelial cells when transplanted into pancreas or duodenal wall. These data indicate that self-renewing pluripotent stem cells persist in the developing mouse liver and that such cells can be induced to become cells of other organs of endodermal origin under appropriate microenvironment. Manipulation of hepatic stem cells may provide new insight into therapies for diseases of the digestive system.

Introduction

The enormous regenerative capacity of the liver after partial hepatectomy or chemical injury is well known. In rodents, liver weight returns to normal within a few weeks even after loss of up to two-thirds of total liver mass (Fausto and Webber, 1994). Remarkable regenerative potential is also retained in hematopoiesis. Hematopoietic stem cells (HSCs)* certainly exist in bone marrow where they self-renew and differentiate along all hematopoietic lineages. Sophisticated isolation methods have recently identified a highly probable HSC candidate; a single such cell can reconstitute bone marrow (Osawa et al., 1996). By analogy with hematopoiesis, liver regeneration can be regarded as mediated by proliferation and differentiation of hepatic stem cells. However, it remains unclear how the liver is regenerated and what cells are involved in such regeneration. Overturf et al. (1997) inferred from serial transplantation studies the presence in adult mouse liver of cells capable of dividing more than 60 times; they ascribed this great reconstitutive ability to hepatic stem/progenitor cells. However, examination in greater detail strongly indicated that fully differentiated hepatocytes but not progenitors divided intensively after cell transplantation. In addition, the reconstitutive capacity of serially transplanted hepatocytes was as high as that of freshly isolated hepatocytes (Overturf et al., 1999). Of major importance in understanding proliferative processes in the liver is to recognize that fully differentiated hepatocytes themselves possess great growth potential and that stem cells may not be required for liver regeneration (Michalopoulos and DeFrances, 1997).

By contrast, it is believed that in the developing liver both hepatocytes and cholangiocytes differentiate from a common cell component, the hepatoblast (Shiojiri, 1984; Shio-

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*Abbreviations used in this paper: APC, allophycocyanin; EGFP, enhanced green fluorescent protein; ED, embryonic day; H-CFU-C, hepatic colony-forming unit in culture; HGF, hepatocyte growth factor; HNF, hepatocyte nuclear factor; HSC, hematopoietic stem cell; mAb, monoclonal antibody; MC, medium colony; PI, propidium iodide; RT, reverse transcriptase; SC, small colony.

Key words: liver; hepatocyte; stem cell; self-renew; endoderm

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jiri et al., 1991; Fausto, 1994). It was reported that fetal rat liver cells transplanted into retrorsine-treated liver reconstituted bile duct and hepatocyte structures (Dabeva et al., 2000). This result suggested that the donor cell population included at least bipotent hepatic stem/progenitor cells. However, it was not possible to determine if the regenerated structures had differentiated from stem cells or from lineage-committed cells. Recently, by combining FACS® and in vitro clonal analysis bipotent hepatic progenitor cells have been isolated from rats or mice (Kubota and Reid, 2000; Suzuki et al., 2000). Although these data demonstrated that isolated cell is a possible candidate for the hepatic stem cell in the developing liver, its self-renewal potential and multiple differentiation capability remain largely unanswered. For this reason, isolated progenitor cells have never been identified as hepatic stem cells.

Stem cells are generally defined as clonogenic cells capable of both self-renewal and multilineage differentiation (Till and McCulloch, 1961; Metcalf and Moore, 1971). During development and regeneration of a given tissue, such cells give rise to nonself-renewing progenitors with restricted differentiation potential and finally to functionally mature cells. In the study reported here, using in vitro single cell–based assays, we further enriched candidate hepatic stem cells and clonogenically identified cells with self-renewing capability and multilineage differentiation potential. These cells could be clonally propagated in culture for >6 mo where they continuously produced hepatocytes and cholangiocytes as descendants. The value of stem cells expanded in vitro is expected to be great not only in conventional studies of their differentiation or self-renewing potential but also in therapy, for example, with virus-mediated gene transfer or as theoretically unlimited sources of cells. Furthermore, upon cell transplantation these cells differentiated not only into hepato-biliary lineage cells but cells in other organs of endoderm origin such as pancreas and intestine. Thus, the stem cells that we isolated may actually be primitive endodermal stem cells persisting in fetal mouse liver. Alternatively, these findings may reflect lineage plasticity or transdifferentiation of hepatic stem cells.

Results

Flow cytometric fractionation of fetal mouse liver cells

We have reported previously that cells in embryonic day (ED) 13.5 fetal mouse liver cells, which coexpress CD49f and CD29 (α6 and β1 integrin subunits) but do not express c-Kit (stem cell factor receptor), CD45 (leukocyte common antigen), or TER119 (a molecule resembling glycophorin and exclusively expressed on immature erythroid cells), are the best candidate hepatic stem/progenitor cells (Suzuki et al., 2000). We provisionally designated as "hepatic colony-forming unit in culture" (H-CFU-C) the class of individual cells that over 5 d in culture gave rise to a relatively large colony containing >100 cells. Sorting for c-Kit$^-$ CD49f$^+$ CD29$^+$ CD45$^-$ TER119$^-$ cells has achieved 34.9-fold enrichment of H-CFU-C compared with total fetal liver cells. In order to further enrich the yield of H-CFU-C and thereby to permit clonal analysis of this cell class and examine such cells' capacity for self-renewal and differentiation, we attempted in the present study to sort for cells expressing c-Met, the hepatocyte growth factor (HGF) receptor. During mammalian organogenesis, HGF and c-Met interaction mediates a signal exchange between mesenchymal and epithelial cells in the developing liver (Hu et al., 1993; Johnson et al., 1993; Schmidt et al., 1995). In embryonic mice lacking HGF, placental defects and liver abnormalities were observed (Uehara et al., 1995). In addition, HGF was a critical requirement for the proliferation of H-CFU-C; its absence was not compensated for by EGF or other cytokines (Suzuki et al., 2000; unpublished data). These findings led us to speculate that cells expressing c-Met have an essential role in the developing mouse liver.

We fractionated c-Kit$^-$ CD45$^-$ TER119$^-$ cells (5.05 ± 1.72%) in ED 13.5 fetal mouse livers into six subpopulations by using antibodies against c-Met and CD49f in combination: (a) c-Met$^+$ CD49f$^{+/high}$ cells (0.05 ± 0.01%); (b) c-Met$^+$ CD49f$^{+/low}$ cells (0.30 ± 0.05%); (c) c-Met$^+$ CD49f$^-$ cells (0.02 ± 0.02%); (d) c-Met$^-$ CD49f$^{+/high}$ cells (0.14 ± 0.03%); (e) c-Met$^-$ CD49f$^{+/low}$ cells (1.90 ± 0.33%); and (f) c-Met$^-$ CD49f$^-$ cells (1.19 ± 0.45%) (Fig. 1 A). Under clonal density culture conditions of 30 cells/cm^2, H-CFU-Cs were found mostly in the c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cell subpopulation (Fig. 1 B). Sorting for c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells achieved 560-fold enrichment in H-CFU-C compared with total fetal liver cells. These results were not limited to ED 13.5 fetal mice. In both ED 11.5 and ED 15.5 mouse liver, H-CFU-C were also found in the same cell subpopulation (ED 11.5, 1.54 ± 0.02%; ED 15.5, 0.21 ± 0.02%) (Fig. 1, A and B).

Characterization of c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells

High enrichment in H-CFU-C permitted efficient culture of clone-sorted c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells for analyses of self-renewal and differentiation potential. Cells identified on clone sorting by flow cytometry were cultured in individual wells of laminin-coated 96-well plates. To ascertain that single cells have been deposited, we always examine each well to confirm the presence of a single cell under the microscope after clone sorting. Once a cell sorter is adjusted for optimal setting before the experiment, we seldom find wells with more than two cells after clone sorting. In one series of experiments, we found one well that had two cells out of 4,000 wells. We have never found three cells in a well. When we found these wells, we excluded them from samples for analysis.

As in clonal density culture, relatively large colonies (>100 cells) derived from H-CFU-C in a truly clonal manner were observed (Fig. 2, A–C). Not only H-CFU-C colonies were present; colonies containing 50–100 cells (medium colonies [MCs]) and colonies containing <50 cells (small colonies [SCs]) also were seen. These different sizes of colonies, H-CFU-C colonies, MCs, and SCs, were formed by 5.88 ± 3.57%, 6.56 ± 3.19%, and 26.7 ± 6.41% of sorted cells, respectively (average of 75 plates [7,200 wells]; 15 independent experiments).

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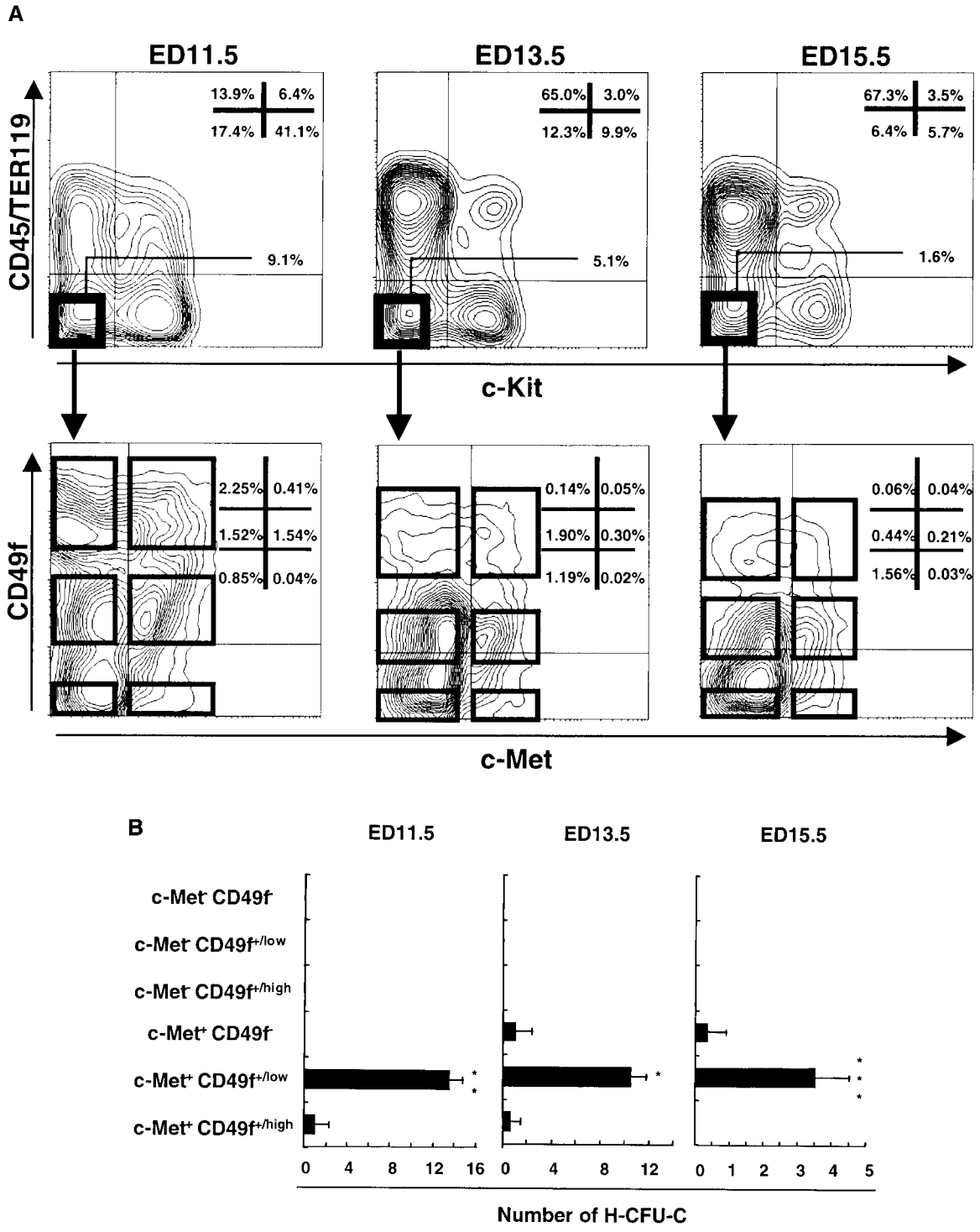

Figure 1. **Flow cytometric analysis of fetal mouse liver cells.** (A) c-Kit$^-$ CD45$^-$ TER119$^-$ cells among ED 11.5, ED 13.5, and ED 15.5 fetal mouse liver cells were fractionated by c-Met and CD49f expression. Sorting gates were then set for c-Met$^-$ CD49f$^-$, c-Met$^-$ CD49f$^{+/low}$, c-Met$^-$ CD49f$^{+/high}$, c-Met$^+$ CD49f$^-$, c-Met$^+$ CD49f$^{+/low}$, and c-Met$^+$ CD49f$^{+/high}$ subpopulations. The percentage of fractionated cells is shown in the upper right. Representative data from six independent experiments are shown. (B) Numbers of H-CFU-C per 3×10^2 cells in each cell subpopulation derived from ED 11.5, ED 13.5, and ED 15.5 fetal mouse livers. This graph shows the average of 18 dishes for each cell subpopulation in six independent experiments ($n = 6$). *$P < 0.001$; **$P < 0.005$; ***$P < 0.01$.

To characterize the colonies, we stained colony-constituent cells at days 5 and 21 with antibodies against albumin or cytokeratin 19 as described (Suzuki et al., 2000). At day 5 of culture, immunocytochemistry revealed that most SCs were composed of cells expressing either albumin or cytokeratin 19 (Fig. 2, D and E). In contrast, cells expressing neither formed most H-CFU-C colonies (Fig. 2 F) except for a few multicolor-stained colonies (Fig. 2 G). Cells in H-CFU-C

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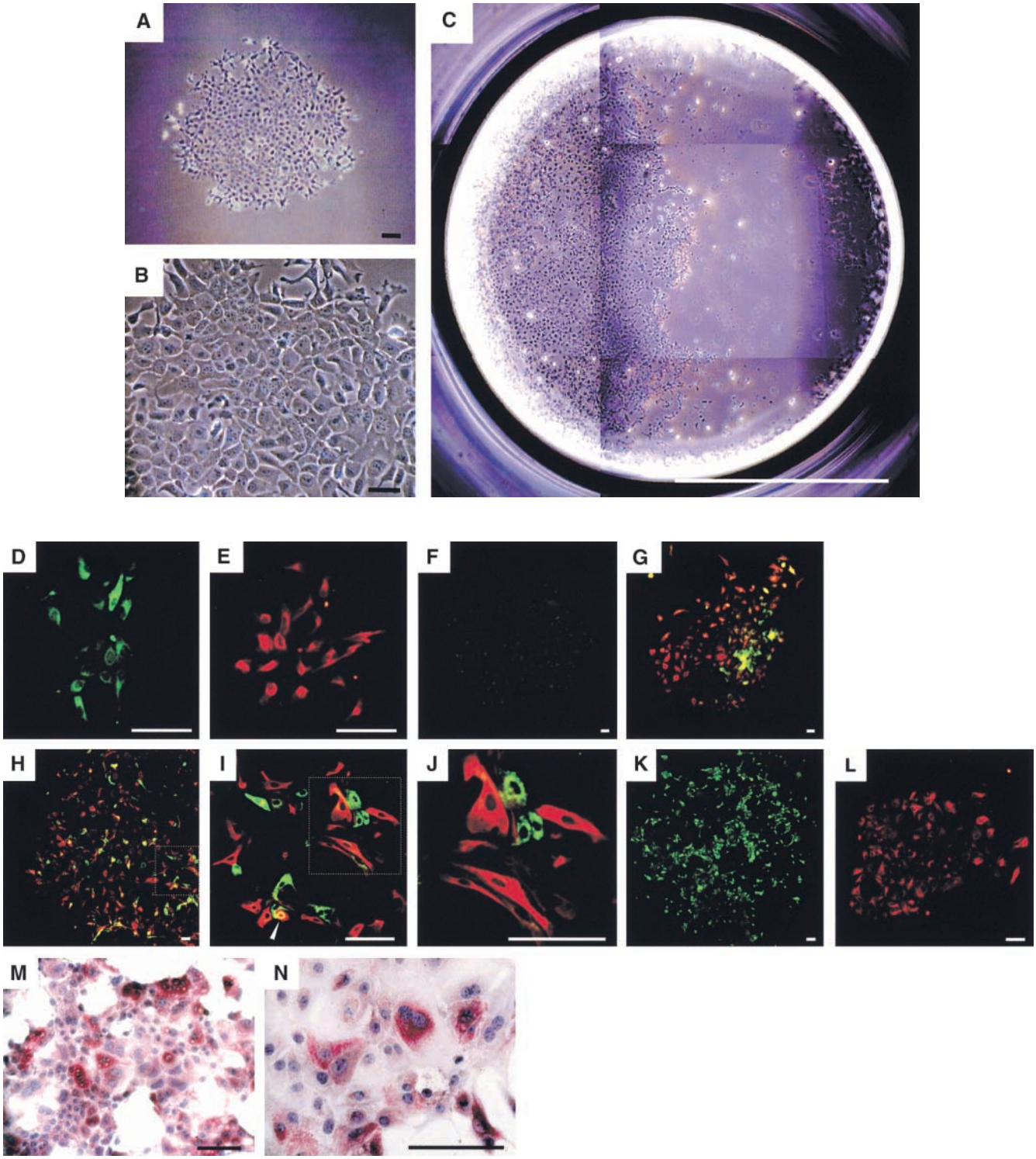

Figure 2. **In vitro multilineage colony formation from a sorted c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cell.** (A and B) Single cell culture of c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells was performed on laminin-coated 96-well plates for 5 d, and then H-CFU-C colonies were determined. (C) Some H-CFU-C formed even larger colonies when cultured for 21 d. Immunocytochemical staining was conducted after 5 (D–G) or 21 d (H–L) of culture. Cells stained green for single-positive cells marking for albumin or red for cytokeratin 19. After 5 d of culture, most small colonies were composed of cells positive for only albumin (D) or cytokeratin 19 (E). Although most H-CFU-C colonies were formed by cells expressing neither marker (F), some of them included both cells expressing one marker and cells expressing the other (G). After 21 d of culture, most H-CFU-C colonies included both cells expressing albumin and cells expressing cytokeratin 19 (H–J), whereas a few of them were composed of cells that marked only for albumin (K) or for cytokeratin 19 (L). We also observed cells expressing both markers at once (I, arrowhead, yellow). I and J are magnified pictures of the regions surrounded by broken lines in H and I. (M and N) Periodic acid–Schiff staining revealed that most H-CFU-C gave rise to functionally mature hepatocytes, containing abundant glycogen stores, after 21 d of culture. Bars: (A and D–N) 100 μm; (B) 50 μm; (C) 2.5 mm.

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Table I. **Immunocytochemical analysis of varying differentiation potential among various sized colonies**

	Cells in a colony				
Colony type	**Bipotent Alb- and Ck19-positive cells**	**Unipotent Alb-positive cells**	**Unipotent Ck19-positive cells**	**No expression of Alb and Ck19**	**Number of colonies examined**
	(%)	*(%)*	*(%)*		
5-d culture					
H-CFU-C	7 (22.6)	1 (3.2)	5 (16.1)	18 (58.1)	31
medium	8 (18.2)	0 (0)	18 (40.9)	18 (40.9)	44
small	6 (9.2)	15 (23.1)	30 (46.2)	14 (21.5)	65
21-d culture					
H-CFU-C	20 (76.9)	1 (3.85)	1 (3.85)	4 (15.4)	26
medium	9 (45.0)	3 (15.0)	2 (10.0)	6 (30.0)	20
small	7 (12.3)	7 (12.3)	9 (15.8)	34 (59.6)	57

Clone-sorted c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells were cultured for 5 or 21 d, after which double staining for albumin and cytokeratin 19 markers was conducted. At day 5, most H-CFU-C progeny had differentiated into neither hepatocytes nor cholangiocytes. However, after 21 d of culture most H-CFU-C had given rise to cells of both lineages. In contrast, cells in SC differentiated rapidly into either hepatocytes or cholangiocytes at day 5 and rarely into both. Cells in half of the SC survived up to 21 d in culture, but they had already stopped growing and expressing albumin or cytokeratin 19. MCs showed patterns of differentiation intermediate between those of H-CFU-C colonies and those of SCs.

colonies continued to proliferate intensively for ~2 wk and then reached near plateau. By day 21, most H-CFU-C eventually gave rise to cells: (a) marking only for albumin; (b) marking only for cytokeratin 19; (c) marking for both albumin and cytokeratin 19; and (d) marking for neither albumin nor cytokeratin 19 (Fig. 2, H and J). However, a few H-CFU-C appeared already to have been committed at planting, since they generated only hepatocytes or cholangiocytes (Fig. 2, K and L). Periodic acid–Schiff staining (Lillie and Harold, 1976) showed that several cells derived from H-CFU-C were functionally mature hepatocytes with abundant glycogen stores at day 21 (Fig. 1, M and N). The differentiation potential of cells forming MCs appeared intermediate between that of H-CFU-C and of cells forming SCs. The cell profiles of colonies at day 5 and 21 are summarized in Table I. These results of FACS® clone-sorting experiments suggest that most SCs were formed by lineage-committed cells with limited potential for growth and differentiation, whereas H-CFU-C were capable of intensive growth and multilineage differentiation.

Clonal expansion and self-renewal capability of H-CFU-C in culture

We next used subcloning experiments to test the self-renewal potential of H-CFU-C. Single c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells were clone sorted and individually cultured. This yielded several large H-CFU-C colonies (Fig. 2 C), which we then replated onto new culture dishes. Over 2–3 wk, about half of subcultured clones gradually expanded, finally to become confluent. These clonally expanding subcultured cells then again underwent clone sorting and single cell culture. Surprisingly, many resorted cells (~15%) formed large colonies and at day 21 had characteristics of multipotent cells on immunocytochemical and reverse transcriptase (RT)–PCR analysis (Fig. 3, A–C, and Table II). Sequential immunocytochemical analysis of colonies formed by resorted cells showed that most colonies observed at day 3 (83.3%; $n = 12$ colonies assessed), at day 5 (93.8%; $n = 16$), at day 8 (77.8%; $n = 9$), and at day 14 (72.7%; $n = 11$) contained cells expressing neither albumin nor cytokeratin 19. However, on observation of colonies at day 21 most colonies (68.0%; $n = 25$) were formed of both cells expressing albumin and cells expressing cytokeratin 19. Furthermore, albumin-positive cells with two nuclei and cytokeratin 19–positive cells forming duct-like structures appeared on day 21 of culture after resorting (Fig. 3, A–C). Expression of several genes found in functionally mature hepatocytes was detected in resorted cell colonies (Table II). Of interest is that expression of *c-kit*, *CD34*, and *thy-1* became detectable in some of these colonies (Table II). These results may suggest that oval cells, mark for c-Kit, CD34, and Thy-1 (Fujio et al., 1994; Omori et al., 1997; Petersen et al., 1998; Matsusaka et al., 1999) and are considered candidate hepatic stem/progenitor cells in adult liver (Sell and Dunsford, 1989; Fausto, 1994; Thorgeirsson, 1996; Crosby et al., 2001), are close descendants of H-CFU-C.

Transmission EM of the progeny of a reclone-sorted cell conducted as described (Suzuki et al., 2000) showed cells to be present that were largely occupied by well-developed ovoid mitochondria and that were attached closely to adjacent cells by intracellular tight junctional complexes (Fig. 3 D). These cells' borders defined luminal spaces densely decorated with microvilli, structures strongly resembling bile canaliculi between mature hepatocytes. The cells' cytoplasm also contained abundant glycogen. These observations demonstrated that cells among the progeny of resorted cells retained several morphologic and functional characteristics of hepatocytes. In addition to hepatocyte-lineage cells, we found many well-defined duct-like structures constituted of 4–15 neatly aligned cells. These cells were characterized by a relatively large nucleus to cytoplasm ratio, numerous short microvilli, junctional complexes between adjacent cells, and a nucleus (frequently notched) that lay at the pole opposite to the apparent luminal space (Fig. 3, E and F). These observations indicated that resorted cells gave rise to cells capable of forming bile duct-like structures. The various morphologic and functional characteristics described above clearly demonstrate that the progeny of single resorted cells could reconstitute hepatocyte or cholangiocyte microstructures in vitro. The results

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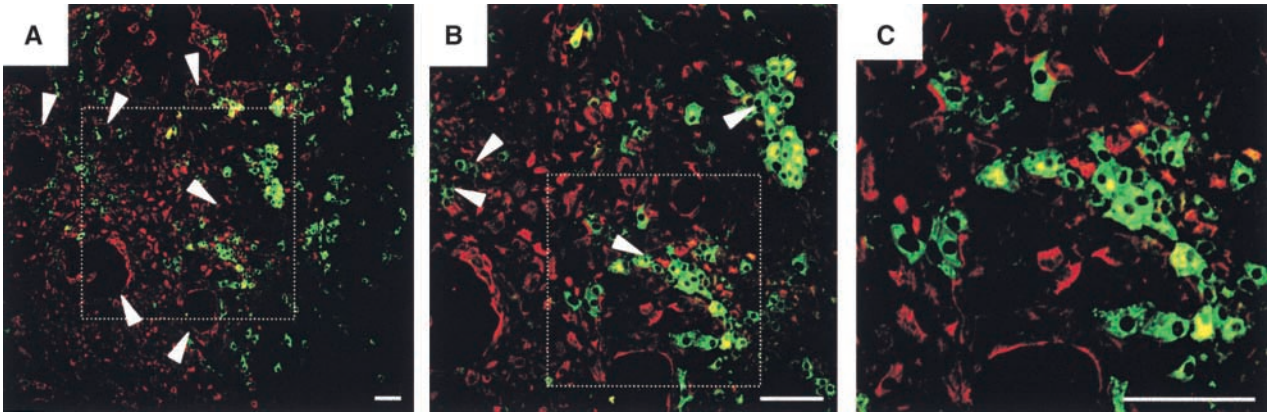

Figure 3. **Multipotency of reclone-sorted progeny derived from a single H-CFU-C.** Subcultured cells derived from an H-CFU-C were clone-sorted again, and subjected to single cell culture. (A–C) Resorted cells gave rise to albumin-positive hepatocytes (green) or cytokeratin 19–positive cholangiocytes (red) after 21 d of culture. As upon primary culture, they gave rise to albumin and cytokeratin 19 double positive cells (yellow) and to double negative cells. Duct-like structures formed by cells expressing cytokeratin 19 (A, arrowheads) and binucleate cells (B, arrowheads) interpreted as mature hepatocytes were regularly observed. (D) Ultrastructural study of the progeny of reclone-sorted cells found many ovoid mitochondria and glycogen rosettes in their cytoplasm. Adjacent cells also formed well-defined narrow lumina with numerous microvilli and intracellular tight junctional complexes (arrows). These structures resembled hepatic bile canaliculi. (E and F) Bile duct-like structures formed by neatly aligned cells were morphologically characterized by frequently notched nuclei at the basal aspects, a relatively large nucleus to cytoplasm ratio, numerous short microvilli projecting into luminal regions (F, arrowheads), and close attachment with juxtaluminal junctional complexes between adjacent cells (F, arrows). Bars: (A–C) 100 μm; (D) 2 μm; (E and F) 10 μm.

of immunocytochemical, RT-PCR, and ultrastructural analysis thus established that H-CFU-C in the phenotypically defined c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cell subpopulation have self-renewal potential and the capability of multilineage differentiation in vitro.

In vivo self-renewal of H-CFU-C in the developing mouse liver

Because the self-renewing cells described here were isolated after expansion in culture for a relatively long period, it was possible that their self-renewal characteristics did not reflect their behavior in vivo. To clarify whether such cells are generated in vivo by division of cells with similar properties, we examined the in vivo self-renewing capability of H-CFU-C without in vitro explantation. To assay whether H-CFU-C were self-renewing in vivo, pregnant mice were administered the thymidine analogue BrdU 17 h before harvest of fetal mouse liver cells (ED 12.75 to ED 13.5). Unfractionated total fetal liver cells and c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells sorted by FACS® were directly fixed, stained, and analyzed for BrdU incorporation by FACS®. Nearly 100% of both total fetal liver cells (93.0 ± 2.75%; $n = 3$) and c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells (96.0 ± 1.07%) incorporated BrdU over the 17-h in vivo pulse (Fig. 4 A). In addition, sorted cells cultured for 10–12 h and frozen sections from fetal liver after BrdU exposure were immunostained using an anti-BrdU antibody. As with results of FACS® analysis, most cells that had successfully attached (91.3 ± 1.61%) marked on immunostaining as did cells in histologic sections of liver (Fig. 4, B–E). To confirm that H-CFU-C isolated from fetal liver after BrdU exposure retained multilineage differentiation potential, we clone-sorted c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells and cultured them for 21 d. BrdU administration did not alter the number of H-CFU-C colonies, MCs, and SCs or their growth potential in vitro. Nor was there any significant change in FACS® profiles between BrdU-treated and normal mice. We then double stained H-CFU-C colonies to evaluate albumin and cytokeratin 19 expression and counted colonies with multilineage differentiation potential. In three independent experiments, we found 50, 36, and 45 colonies arising from 96 clonally cultured cells. Immunocytochemical examination of 23, 20, and 29 colonies, respectively, showed that 8, 7, and 10 colonies, respectively, continued to be multipotent in culture. The proportion of cells with multilineage differentiation potential was thus 8.68 ± 1.59% (the average of 8.33%, 7.29%, and 10.4%) in sorted c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells; it was 19.2 ± 3.12% (the average of 16.0%, 19.4%, and 22.2%) in sorted successfully attached cells. Since 96% of sorted c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells and 91% of cultured cells incorporated BrdU in vivo, at least half of the c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells retained multilineage differentiation potential after BrdU incorporation. These data clearly demonstrated that at least half of the c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells underwent self-renewing divisions in ED 12.75 to ED 13.5 developing mouse liver.

In vivo differentiation and tissue reconstitution potential of clonally expanding H-CFU-C

To determine whether clonally expanding H-CFU-C in culture could generate both hepatocytes and cholangiocytes in vivo after transplantation, we injected them into the spleen of mice subjected to severe hepatic disruption by carbon tetrachloride treatment. To distinguish donor cells from recipient cells, the implanted H-CFU-C had been marked genetically with enhanced green fluorescent protein (EGFP) by retrovirus infection. The concentrated vesicular stomatitis virus pseudo-typed retrovirus allowed high transduction fre-

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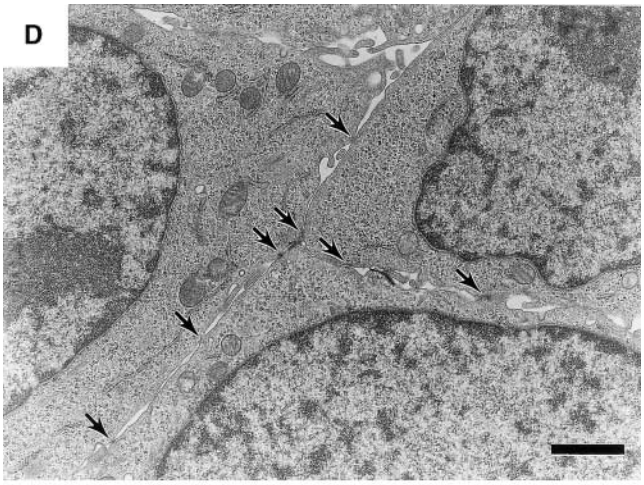

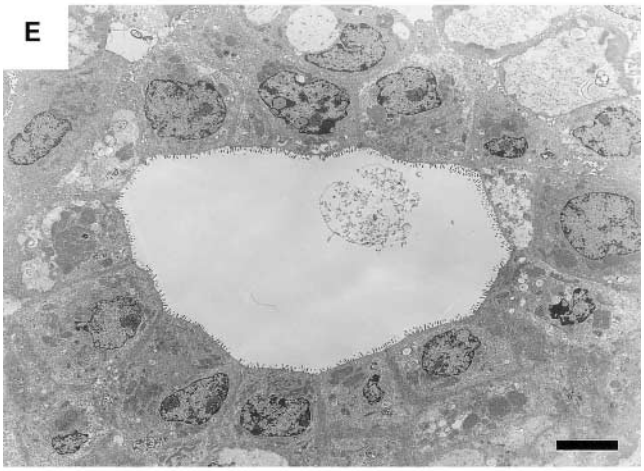

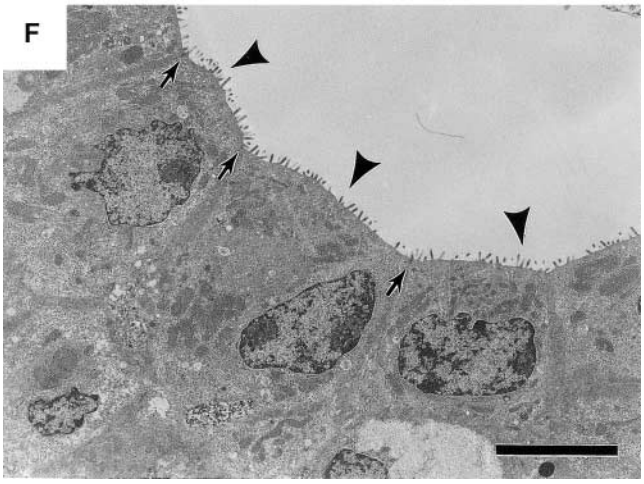

Figure 3 (continued)

quencies, and >95% of cells highly expressed EGFP after a single round of infection.

At 38 d posttransplant, in all five recipient mice donor cells engrafted more efficiently in periportal regions and diffused along hepatic cords (Fig. 5 A). Serial sections stained for albumin showed that many but not all donor cells had given rise to hepatocytes (Fig. 5 B). In spleens, many bile duct-like structures composed of cholangiocytes expressing bile duct-specific cytokeratins (Pinkus et al., 1985) and containing abundant mucin granules adjoining the luminal space were found (Fig. 5, C and D). Engraftment was confirmed even 6 mo after transplantation.

Since a common cell of origin has been proposed for primitive epithelial cells isolated from liver and pancreas (Rao et al., 1989; Bisgaard and Thorgeirsson, 1991; Chen et al., 1995; Deutsch et al., 2001; Zulewski et al., 2001), the developmental potential of H-CFU-C was further investigated by RT-PCR analysis and by transplanting them into pancreatic and intestinal environments. We isolated and propagated H-CFU-C clones from fetal livers (ED 13.5) of EGFP transgenic mice (Okabe et al., 1997). The expression of pancreatic endocrine and exocrine lineage markers became detectable in the progenies of H-CFU-C (Fig. 5 E). Furthermore, the expression of intestinal and gastric markers was also detected (Fig. 5 E).

Two clones were randomly selected, and the cells were transplanted into liver, pancreas, and intestine. As expected, cells of both clones differentiated into hepatocytes or cholangiocytes in recipient livers after regenerative induction by either carbon tetrachloride for hepatocytes or DAPM for bile duct epithelial cells (Fig. 5, F and G). Surprisingly, when those cells were injected into pancreas they integrated into and formed pancreatic ducts (Fig. 5, H and I) and acinar cells (Fig. 5, J and L) at 4 mo after transplant. Furthermore, upon injection into duodenal wall those cells integrated into intestinal epithelium and reconstituted intestinal villi and crypts (Fig. 5 M). Some of them differentiated into goblet cells with the capacity to produce mucin as indicated by alcian blue staining (Paulus et al., 1993) (Fig. 5 M, insets).

Discussion

Self-renewal of H-CFU-C in vitro and in vivo

In vitro clonal subculture analysis showed that a single sorted H-CFU-C generated many individual daughter cells, giving rise both to binucleate albumin-positive hepatocytes and cytokeratin 19–positive cholangiocytes that formed duct-like structures. Study on the secondary colonies by transmission EM also showed that they were capable of differentiating hepatocytes, forming bile canaliculi-like structures with luminal spaces occupied by microvilli, and into cholangiocytes, forming well-organized bile duct-like structures with luminal membranes covered with short microvilli. Moreover, when we conducted recloning experiments using cells derived in turn from a single daughter cell we obtained similar results. These data clearly show that H-CFU-C underwent self-renewing divisions while retaining multilineage differentiation potential in vitro. Most colonies derived from progeny of an H-CFU-C expressed neither albumin nor cytokeratin 19 for up to 14 d in culture. However, by day 21 of culture they gave rise to cells in two lineages, each expressing one of these markers. This pattern of differentiation parallels that of primarily cultured H-CFU-C; even when propagated for longer periods, colony-repopulating cells normally began to proliferate while expressing neither of these markers and slowly generated lineage-committed progeny in vitro.

Table II. **Expression of lineage marker genes in colonies formed after reclone sorting**

	Laminin-coated dish													Type IV collagen–coated dish										
Colony number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**%**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**%**
Hepatocyte markers																									
albumin	+	+	+	+	+	+	+	+	+	+	+	+	100.0	+	+	+	+	+	+	+	+	+	+	+	100.0
α-fetoprotein	+	+	+	+	+	+	+	+	−	+	+	+	91.7	+	+	+	+	+	+	+	+	+	+	+	100.0
α-1–antitrypsin	−	+	−	−	−	−	−	−	−	+	−	−	16.7	−	−	−	+	−	+	−	−	+	−	+	36.4
glucose-6-phosphatase	+	+	+	+	+	+	+	+	+	+	+	+	91.7	+	+	+	+	+	+	−	+	−	+	+	81.8
dipeptidylpeptidase IV	+	+	+	+	+	+	+	+	+	+	+	+	100.0	+	+	+	+	+	+	+	+	+	+	+	100.0
Cholangiocyte marker																									
cytokeratin 19	+	+	+	+	+	+	+	+	+	+	+	+	100.0	+	+	+	+	+	+	+	+	+	+	+	100.0
thymosin β4	+	+	+	+	+	+	+	+	+	+	+	+	100.0	+	+	+	+	+	+	+	+	+	+	+	100.0
billary glycoprotein	+	+	+	+	+	−	+	+	+	+	+	+	91.7	+	+	+	+	+	+	+	+	+	+	+	100.0
γ-glutamyltranspeptidase	+	+	+	+	−	+	+	−	+	+	+	+	83.3	+	+	+	+	+	−	+	+	−	+	+	81.8
vinculin	+	+	+	+	+	+	+	+	+	+	+	+	100.0	+	+	+	+	+	+	+	+	+	+	+	100.0
Miscellaneous																									
cytokeratin 18	+	+	+	+	+	+	+	+	+	+	+	+	100.0	+	+	+	+	+	+	+	+	+	+	+	100.0
cytokeratin 8	+	+	+	+	+	+	+	+	+	+	+	+	100.0	+	+	+	+	+	+	+	+	+	+	+	100.0
HNF-1	+	+	+	+	+	+	+	+	+	+	+	+	100.0	+	+	+	+	+	+	+	+	+	+	+	100.0
HNF-3α	+	+	+	+	+	+	+	+	+	+	+	+	100.0	+	+	+	+	+	+	+	+	+	+	+	100.0
HNF-3β	+	+	+	+	+	+	+	+	+	+	+	+	100.0	+	+	+	+	+	+	+	+	+	+	+	100.0
HNF-3γ	+	+	+	+	+	+	+	+	+	+	+	+	100.0	+	+	+	+	+	+	+	+	+	+	+	100.0
HNF-4	+	+	+	+	+	+	+	+	+	+	+	+	100.0	+	+	+	+	+	+	+	+	+	+	+	100.0
TTR	+	+	+	+	+	+	+	+	+	+	+	+	100.0	+	+	+	+	+	+	+	+	+	+	+	100.0
c-met	+	+	+	+	+	+	+	+	+	+	+	+	100.0	+	+	+	+	+	+	+	+	+	+	+	100.0
HPRT	+	+	+	+	+	+	+	+	+	+	+	+	100.0	+	+	+	+	+	+	+	+	+	+	+	100.0
Hepatocyte functional genes																									
tryptophan-2, 3-dioxygenase	+	−	−	−	+	−	+	−	−	−	−	−	25.0	+	+	−	−	+	+	−	+	−	+	−	54.5
glutathione *S*-transferase	−	−	+	+	−	−	+	−	+	+	+	+	58.3	−	−	−	+	+	−	+	−	+	+	−	54.5
glutamine synthetase	−	+	+	−	−	+	−	+	+	−	+	−	50.0	+	+	−	+	−	+	+	+	−	+	+	72.7
Oval cell–related genes																									
c-kit	−	+	−	−	+	−	−	+	−	+	−	+	41.7	−	+	−	−	+	+	−	+	−	+	+	54.5
CD34	−	+	+	−	−	−	−	−	+	−	−	−	25.0	+	−	−	+	+	−	−	−	−	+	−	36.4
thy-1	+	+	−	−	+	−	−	−	−	+	−	−	33.3	−	−	−	+	+	+	−	−	−	+	+	45.5

23 individual colonies (12 colonies cultured on laminin-coated and 11 on type IV collagen–coated 96-well plates) were examined by RT-PCR at day 21. Frequencies of positive colonies are indicated.

HSCs can reconstitute all types of blood cells in sublethally irradiated mice without being cultured in vitro. Transplantation of a single stem cell demonstrated clearly that such cells could generate a subpopulation of their own cell type by self-renewing divisions in vivo (Osawa et al., 1996). Although the H-CFU-C described here have the potential to self-renew in vitro, it has not been confirmed directly that this property reflects the potential for self-renewing cell division in vivo. It has not yet been technically possible to transplant a single H-CFU-C and thereby directly to analyze the self-renewing potential of such a cell in vivo. However, by combining in vivo BrdU incorporation with in vitro functional assays of c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells we found that at least half the population of such cells is capable of self-renewing cell division in the ED 12.75 to ED 13.5 developing liver. This result also demonstrates that self-renewing H-CFU-C is very rare even in early liver development. The numbers of H-CFU-C present in the fetal liver increase at most sevenfold through ED 11.5 to ED 13.5 and then remain steady until ED 15.5. We concluded that H-CFU-C are mostly self-renewing early in liver organogenesis and that they then divide more slowly, shifting to the production of committed progenitors that, in their turn, proliferate and differentiate relatively rapidly in subsequent liver development.

H-CFU-C differ from previously described hepatic stem-like cell lines

Like other tissue-specific stem cells, H-CFU-C can maintain themselves in culture and continuously give rise to hepatocytes and cholangiocytes. Although several bipotent hepatic stem-like cell lines have been isolated from normal and malignant hepatic tissues (Grisham et al., 1993; Pack et al., 1993), evidence has been lacking as to whether they normally resided in liver or accidentally emerged after the process of immortalization in culture. In the present work, we repeatedly isolated and clonally propagated large numbers of H-CFU-C from fetal mouse liver. In the c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cell subpopulation, nearly 60% of H-CFU-C were subcultured and propagated in vitro with few morphological and functional differences among them. We also found the same cell subpopulation to be enriched in H-CFU-C in various mouse strains and propagated H-CFU-C routinely. After cell transplantation, even into mice with immunodeficiency, we have never found abnormal development and tumor formation by donor-derived cells. These results strongly suggest that H-CFU-C do not appear transiently, are not strain specific, and are not the product of transformation; instead, they normally exist in the cell subpopulation within developing mouse livers that is phenotypically distinguished as c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$.

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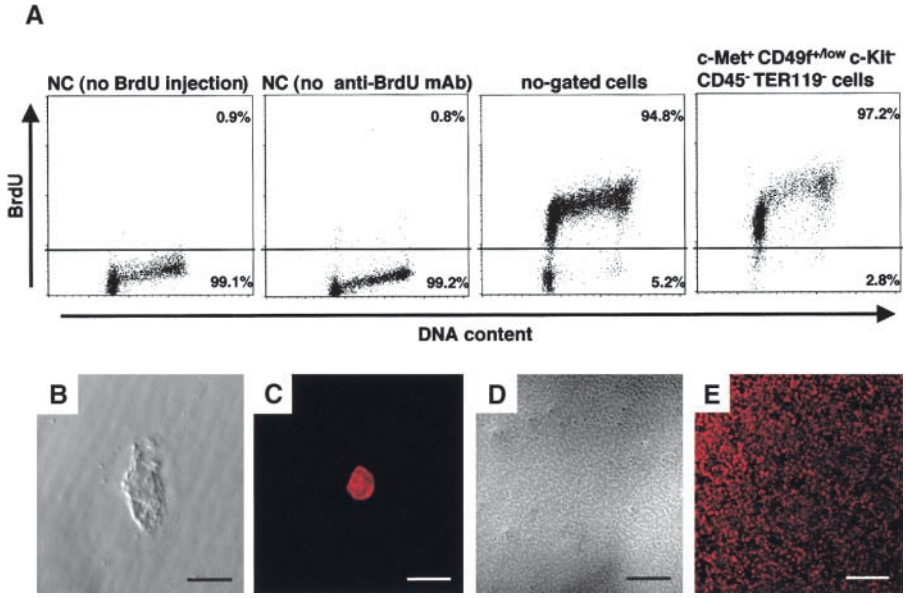

Figure 4. **H-CFU-C incorporated BrdU in vivo while retaining the capacity for multilineage differentiation.** (A) FACS® analysis of cells that had incorporated BrdU after pulse labeling from ED 12.75 to ED 13.5. Most sorted unfractionated and c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells isolated from BrdU-treated mice were BrdU positive. Representative data are shown. (B and C) 10–12 h after c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells were plated; most attaching (but not dividing) cells were BrdU positive. (D and E) In sections of BrdU-treated ED 13.5 fetal mouse liver, almost all liver cells were BrdU positive. Bars: (B and C) 25 μm; (D and E) 100 μm.

H-CFU-C may represent a primitive pluripotent stem cell persisting in the developing mouse liver

Sequential histologic analyses suggested that the hepatoblasts in ED 13.5 developing mouse liver have already expressed albumin and α-fetoprotein and that these cells still were capable of differentiation into cholangiocytes (Shiojiri et al., 1991). Our data show that hepatic stem cells defined as H-CFU-C exist in the developing liver without expression of both hepatocyte and cholangiocyte lineage markers. It also demonstrated that such cells were much fewer than hypothesized previously and their frequency appear to decrease as gestation advances. These findings may suggest that H-CFU-C differ from hepatoblasts that express several lineage markers and largely occupy the developing liver.

We transplanted cells derived from clone-sorted H-CFU-C. Although we did not conduct single H-CFU-C transplantation, our results show that cells derived from a single H-CFU-C reconstituted hepatocytic, bile-ductal, pancreatic, and intestinal structures after in vivo differentiation into cells of each lineage. Especially in pancreas and intestine, they differentiated into cells in pancreatic ducts and cells residing near the base of crypt in intestinal epithelium, generally thought as pancreatic or intestinal epithelial stem cells. In this cell transplantation study, few EGFP-positive islet-forming cells were seen in vivo. However, expression of *insulin*, *glucagon*, and *somatostatin*, marker for pancreatic β, α, and δ cells, was detectable by RT-PCR in culture of those clones. The adult pancreatic environment may not support differentiation of islet-forming cells from H-CFU-C or it may be that an adequate tissue injury is required for islet reconstitution. Alternatively, islet-specific transgene silencing may have occurred during the differentiation process. We also found the expression of *fabp-2 secretin*, *GIP*, *CCK*, *gastrin*, and of *pepsinogen F* expressed in intestine and/or stomach. These results suggest that a few self-renewing pluripotent stem cells remained in the later gestational stage even after the specification of liver, pancreas, stomach, and intestine from endoderm layer. Although there is no evidence that there is a hierarchy of lineage progression between hepatoblasts and more primitive cells, H-CFU-C could be considered as the equivalent to a pluripotent endodermal stem cells maintained by their own self-renewal capability in the developing liver.

Pluripotent stem cells: their importance to stem cell biology and therapeutic strategies

The cell-based study of stem cells in mammalian solid organs is generally considered difficult because the constituent cells of these organs adhere tightly to one another and because cells of many lineages are present. Progress in organ-specific stem cell biology has been correspondingly slow. Cell populations enriched in prospectively identified liver stem cells can provide fundamental understanding of the characteristics of such cells such as what signals determine their lineage commitment, what genes are driven when differentiation or self-renewal occurs, and whether they divide symmetrically or asymmetrically in vivo or in vitro. They also provide a powerful tool and information for developing therapeutic strategies such as gene therapy, cell therapy, and the treatment of organ failure by using manipulated somatic stem cells or embryonic stem cells. In this study, to trace donor-derived cells after cell transplantation we infected hepatic stem cells with a retrovirus and marked them for EGFP expression in vitro; single virus infection reproducibly achieved nearly complete tagging. This highly efficient gene transfer method may supply a tool for examination of critical genes in differentiation or self-renewal. In addition, gene-modified stem cells are theoretically useful for clinical gene therapy. Identification of self-renewing pluripotent stem cells that can be propagated in vitro could allow us to describe endodermal cell lineages precisely and reveal the molecular mechanisms involved in self-renewal and differentiation.

Materials and methods

Flow cytometry

Single cell suspensions of liver cells were prepared from Balb/cA ED 13.5 fetal mice (CLEA) as described (Suzuki et al., 2000). Dissociated liver cells were incubated on ice for 30 min with biotinylated anti-CD45, TER119 monoclonal antibodies (mAbs) (PharMingen), and anti–c-Met mAb (Upstate Biotechnology). After three washes with staining

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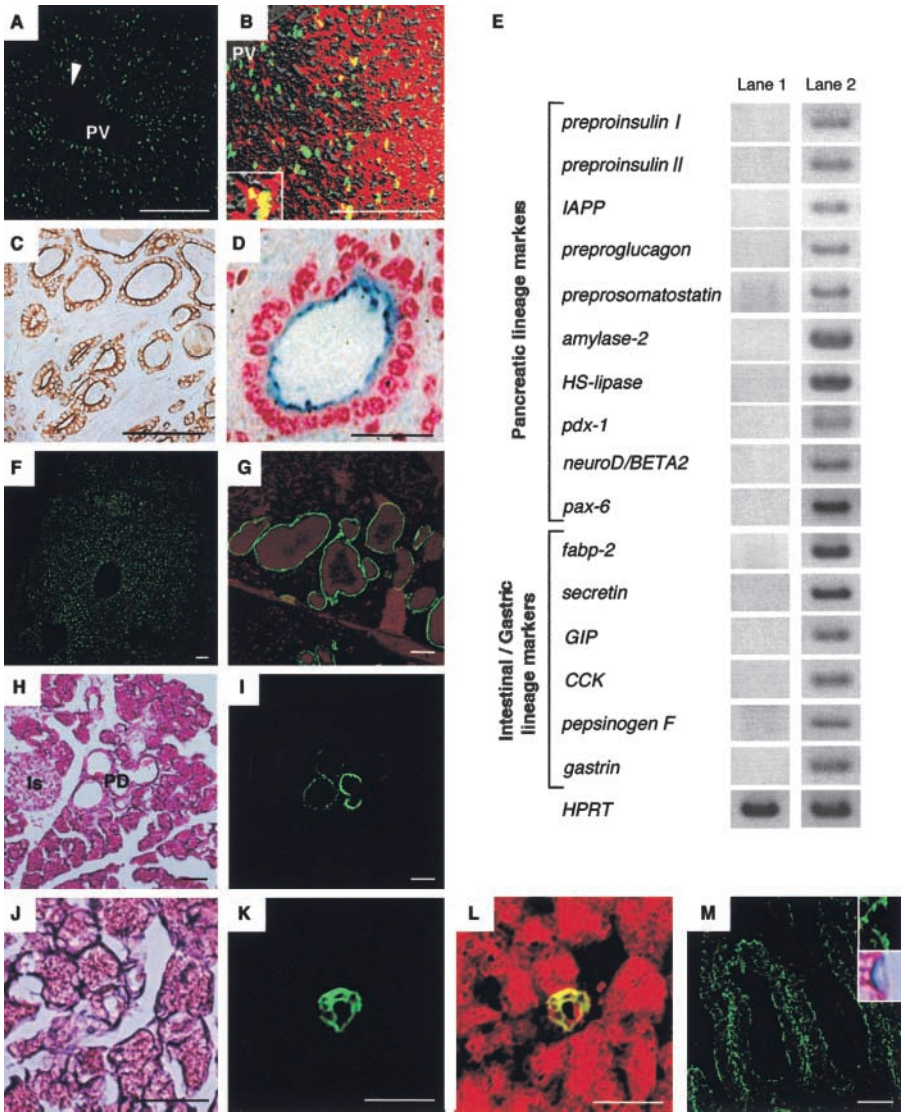

Figure 5. **Clonally expanding H-CFU-C in culture reconstituted liver, pancreas, and intestine in vivo.** (A–D) 38 d after transplantation of EGFP-tagged H-CFU-C, both livers and spleens were fixed and sectioned. (A) Many EGFP-positive donor cells are seen engrafted in hepatic cords of periportal zones but not in bile ducts (arrowhead). (B) Overlay image of EGFP, phase picture, and serial section stained by albumin (red) clearly shows that many but not all EGFP-positive donor cells had differentiated into albumin-positive hepatocytes (yellow). A detail is shown at higher magnification in the inset. (C) In the spleens of recipient mice, many bile duct-like structures were formed by keratin-positive donor-derived cholangiocytes. (D) Alcian blue staining (pH 2.5) (Mowry, 1963) also showed that the component cells contained abundant mucin near luminal membranes (counterstain, nuclear fast red). (E) Pancreatic, intestinal, and gastric marker expression were not detected in the sorted c-Met$^+$ CD49f$^{+/low}$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells (lane 1). However, they became detectable in the progeny of a single H-CFU-C after several months of culture (lane 2). (F and G) A H-CFU-C clone isolated from EGFP transgenic fetal mice differentiated into (F) hepatocytes (6 mo posttransplant) or (G) cholangiocytes (2 mo posttransplant) in recipient livers after either carbon tetrachloride or DAPM treatment. (H–M) Differentiation was also seen in cells constituting pancreas (4 mo) and duodenum (2 mo). (H and I) Pancreatic ducts; (J–L) amylase-positive acinar cells (L, yellow); (M) intestinal vili and crypts. Insets in M show EGFP-positive goblet cells stained in alcian blue. (H and J) Hematoxylin eosin stain. PV, portal venule; PD, pancreatic duct; Is, islet. Bars: (A, F, G, and M) 100 μm; (H–L) 50 μm; (D) 2.5 μm.

medium (PBS containing 3% FBS), cells were incubated with phycoerythrin-conjugated anti-CD49f mAb (PharMingen), FITC-conjugated anti–mouse IgG_{2a} mAb (PharMingen), allophycocyanin (APC)-conjugated anti–c-Kit mAb (PharMingen), and streptavidin-labeled Texas red (GIBCO BRL) on ice for 30 min. For cells from EGFP transgenic mice, we used mAbs for CD45 (Cy-chrome), c-Kit (APC), TER119 (APC), CD49f (phycoerythrin), c-Met, and mouse IgG_{2a} (biotinylated) (PharMingen), and streptavidin-labeled Texas red. Finally, cells were washed three times and resuspended in staining medium containing propidium iodide (PI) (5 μg/ml). The labeled cells were analyzed and separated with FACS®Vantage (Becton Dickinson). Gating was implemented based on negative control staining profiles.

In vitro colony assay

For low density culture analysis, sorted cells were plated on laminin-coated 6-well plates (Becton Dickinson) at a density of 30 cells/cm^2 and cultured in our fresh standard medium (Suzuki et al., 2000). For single cell culture analysis, we used standard medium 50% supplemented with medium conditioned by 7-d culture of nonsorted (total) fetal liver cells. Both culture media included human recombinant HGF (50 ng/ml) (Sigma-Aldrich) and EGF (20 ng/ml) (Sigma-Aldrich). Viability of sorted cells exceeded 90% as assessed by trypan blue exclusion. Residual erythrocytes, debris, doublets, and dead cells were excluded by forward scatter, side scatter, and PI gating. The number of colonies was determined after 5 d of culture; H-CFU-C is defined as a colony containing >100 cells.

RT-PCR

Detection of hepatocyte or cholangiocyte marker gene expression by RT-PCR was conducted as described (Suzuki et al., 2000). Hepatocyte differentiation markers included *albumin, α-fetoprotein, α-1-antitrypsin, glucose-6-phosphatase,* and *dipeptidylpeptidase IV*. Cholangiocyte differentiation markers included *cytokeratin 19, thymosin β4* (5′-TGTCCAGCGCAGGCACTTG-3′ and 5′-CAAAGATGTCCTGCAGGATG-3′), *biliary glycoprotein, γ-glutamyltranspeptidase,* and *vinculin*. Miscellaneous markers included *cytokeratin 18, cytokeratin 8, hepatocyte nuclear factor (HNF)-1* (5′-AAGCTGGTCTCAGCCACGG-3′ and 5′-CTGAGGTGAAGACCTGCTT-3′), *HNF-3α* (5′-GTCGCAAGGACCCCTCAGG-3′ and 5′-CTTGAAGTCCAGCTTGTGCTG-3′), *HNF-3β* (5′-CTTCTCCGTGTCAGGAGCAC-3′ and 5′-CTGGGTAGTGCATGACCTG-3′), *HNF-3γ* (5′-TCTGCCACCACTACAGCTGC-3′ and 5′-CGCTGCTAGGATGCATTAAGC-3′), *HNF-4* (5′-CTTCCAAGAGCTGCAGATTG-3′ and 5′-CTTGTAGGATTCAGATCCCG-3′), *transthyretin* (5′-TGGTATTTGTGTCTGAAGCTG-3′ and 5′-TTAATAAGAATGCTTCACGGC-3′), *c-met,* and *hypoxanthine phosphoribosyltransferase* as a positive control. Mature hepatocyte functional gene expression was assessed using *tryptophan-2, 3-dioxygenase* (5′-TGCGCAAGAACTTCAGAGTGA-3′ and 5′-AGCAACAGCTCATTGTAGTCT-3′), *glutathione S-transferase* (5′-AAGTGATGGGAGTCTGATGTT-3′ and 5′-TTCTTTGCTGACTCAACACAT-3′), and *glutamine synthetase* (5′-AGTTACCTGAGTGGAACTTTG-3′ and 5′-TTCGCACACCCGATGCAAGAT-3′). Oval cell–related gene expression was assessed using *c-kit* (5′-CCCAAGACGTAACAGCTTCTG-3′ and 5′-CAGTCTCGTACATGACCACAG-3′),

CD34 (5′-TCCTGATGAACCGTCGCAGTTG-3′ and 5′-TGTCAGCCACCACATGTTGTC-3′), and *thy-1* (5′-AGAAGGTGACCAGCCTGACA-3′ and 5′-AATGAAGTCCAGGGCTTGGA-3′). For analysis of pancreatic, intestinal, or gastric marker gene expression, PCR was conducted using primers for β cell markers: *preproinsulin I* (5′-CTGTTGGTGCACTTCTACC-3′ and 5′-GCAGTAGTTCTCCAGCTG GT-3′), *preproinsulin II* (5′-TCAAGCAGCACCTTTGTGGTT-3′ and 5′-GTTGCAGTAGTTCTCCAGCTG-3′), and *islet amyloid polypeptide* (5′-TGTCCTCCTCATCCTCTCTGT-3′ and 5′-TATGTATTCGATCCCACGTTG-3′); α cell marker, *preproglucagon* (5′-ATTTACTTTGTGGCTGGATTG-3′ and 5′-TGTCAGTGATCTTGGTTTGAA-3′); δ cell marker, *preprosomatostatin* (5′-CTCTGCATCGTCCTGGCTTT-3′ and 5′-CAGGATGTGAATGTCTTCCAG-3′); exocrine lineage cell markers, *amylase-2* (5′-AGTACCTGTGGAAGTTACCT-3′ and 5′-ACACAAGGGCTCTGTCAGAA-3′) and *hormone-sensitive lipase* (5′-TCTTCTTCGAGGGTGATGAA-3′ and 5′-TACCTTGCTGTCCTGTCCTT-3′); pancreatic transcriptional factor genes, *pancreas duodenal homeobox 1* (5′-TTACAAGCTCGCTGGGATCAC-3′ and 5′-AGGTCACCGCACAATCTTGCT-3′), *neuroD/BETA2*, and *paired box transcription factor 6* (Jensen et al., 2000); intestinal or gastric lineage cell markers, *intestinal fatty acid binding protein* (5′-ATTCGACGGCACGTGGAAAGT-3′ and 5′-AAGAATCGCTTGGCCTCAACT-3′), *secretin* (5′-AAGACACTCAGACGGAATGT-3′ and 5′-TGGTTGTTTCAGTCCACTCT-3′), *gastric inhibitory peptide*, *cholecystokinin*, *gastrin* (Jensen et al., 2000), and *pepsinogen F* (5′-ACCTAGACCTGGTCTACATTG-3′ and 5′-AGTGAAGCTCTCCATGGTAGT-3′).

BrdU experiment

BrdU (50 μg/g body weight) (Sigma-Aldrich) dissolved in 200 μl PBS with 0.007 M NaOH (Morrison et al., 1999) was injected intraperitoneally at harvest –17, –15, –13, –3.25, –2.5, –1.75, and –1 h. Additionally, from the first injection onward drinking water given the mice contained 2 mg/ml of BrdU. After the flow cytometric cell separation, sorted cells were fixed in 70% ethanol. After washing with PBS including 0.05% polyoxyethylene (20) sorbitan monolaurate (Tween 20; Wako), the cells were treated with hydrochloric acid (4N) and neutralized in 0.1 M sodium tetraborate (pH 8.5) (Sasaki et al., 1987, 1988). The cells were then washed and stained with FITC-conjugated anti-BrdU antibody (Becton Dickinson) at room temperature for 30 min. Finally, after the cells were resuspended and incubated at 37°C for 30 min in PBS containing RNase A (15 μg/ml; Wako) and PI (5 μg/ml), analysis of the labeled cells was conducted by FACS®Calibur (Becton Dickinson). Short-term cultured nondivided cells and fetal liver frozen sections were stained with anti-BrdU antibody (Becton Dickinson) as described (Raff et al., 1988) using Cy3-conjugated goat anti–mouse IgG as a second antibody for visualization.

Retrovirus infection

A retroviral vector pGCsapEGFP (MSCV) and the virus producing cell line PG13/GCsapEGFP (MSCV) are described elsewhere (Kaneko et al., 2001). To increase the viral titer and widen the host range of infection, the supernatant of PG13/GCsapEGFP (MSCV) clones was used to infect the packaging cell line 293gpg, which expresses the vesicular stomatitis virus G protein under the tetracycline inducible system (tet off system) (Ory et al., 1996). EGFP-expressing 293gpg cells were sorted by FACS®Vantage and expanded for subsequent experiments. For the collection of vesicular stomatitis virus G pseudotype virus, 293gpg/GCsapEGFP (MSCV) cells were maintained in tetracycline-free medium for 48–60 h before harvest. The harvested supernatant was centrifuged at 6,000 *g* for 16 h at 4°C to concentrate the virus. The virus pellet was finally resuspended in STEM PRO-34 SFM (GIBCO BRL) and stored at −80°C until used. The estimated titer of the concentrated retrovirus was 10^7 EGFP-expressing cells/ml on HeLa cells. For marking of H-CFU-C with EGFP, 25 μl of the concentrated virus supernatant was added to cultures in which cells had grown up to 40–50% confluence in 2.5 ml of standard medium with 5 μg/ml protamine sulfate (Sigma-Aldrich) followed by "spinoculation" (Kotani et al., 1994). Residual virus was excluded by washing the cells with PBS and changing the medium after 24 h. Frequency of EGFP-positive cells was assayed by FACS®Vantage.

Cell transplantation

After the initiation of culture, we maintained H-CFU-C in culture by replating them every 7 d. Donor cells were usually prepared from cells obtained at these passage points; both H-CFU-C and their progeny were administered. We trypsinized, washed, and resuspended 2×10^6 GFP-tagged H-CFU-C in standard medium (100 μl). They were then injected intrasplenically into recipient mice (Balb/cA, 4 wk old; $n = 5$) (CLEA) under anesthesia. We also injected standard medium without cells as a negative control ($n = 5$). For pretransplant conditioning of recipient mice, we induced acute liver damage by subcutaneous injection of 2 ml/kg carbon tetrachloride dissolved in olive oil. 2 d afterward, cell transplantation was conducted. The same number of H-CFU-C–derived EGFP transgenic mice was injected intrasplenically into recipient mice (C57BL/6, 4 wk old; $n = 5$) (CLEA) after carbon tetrachloride or 4,4′-methylene dianiline (4,4′-diaminodiphenylmethane) (80–120 mg/kg; Wako) treatment for hepatocyte or bile duct disruption (Kanz et al., 1992). They were also injected into pancreas ($n = 4$) through common bile duct and into wall of duodenum ($n = 5$).

Immunohistochemistry

Recipient liver, pancreas, and duodenum were fixed in 4% phosphate-buffered paraformaldehyde overnight at 4°C and embedded in OCT compound. Cryostat sections of the liver were stained with rabbit antialbumin (Biogenesis) as the primary antibody and Cy3-conjugated goat anti–rabbit IgG (Jackson ImmunoResearch Laboratories) as the secondary antibody. Sections of pancreas were stained with rabbit antiamylase (Sigma-Aldrich) and Cy3-conjugated goat anti–rabbit IgG. Stained tissues were viewed by using a ZEISS LSM510 laser scanning microscope. For analysis of cholangiocyte differentiation, spleens from recipient animals were fixed in 10% phosphate-buffered formalin, dehydrated in ethanol and xylene, and embedded in paraffin wax at 58–60°C. After deparaffinization and rehydration of sections, they were stained with rabbit antikeratin antibody (Dako) and colorized (LSAB-2 kit; Dako) according to the manufacturer's instructions.

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