Proliferation and Differentiation of Fetal Liver Epithelial Progenitor Cells after Transplantation into Adult Rat Liver

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To identify cells that have the ability to proliferate and differentiate into all epithelial components of the liver lobule, we isolated fetal liver epithelial cells (FLEC) from ED 14 Fischer (F) 344 rats and transplanted these cells in conjunction with two-thirds partial hepatectomy into the liver of normal and retorsine (Rs) treated syngeneic dipeptidyl peptidase IV mutant (DPPIV⁻) F344 rats. Using dual label immunohistochemistry/*in situ* hybridization, three subpopulations of FLEC were identified: cells expressing both α -fetoprotein (AFP) and albumin, but not CK-19; cells expressing CK-19, but not AFP or albumin, and cells expressing AFP, albumin, and cytokeratins-19 (CK-19). Proliferation, differentiation, and expansion of transplanted FLEC differed significantly in the two models. In normal liver, 1 to 2 weeks after transplantation, mainly cells with a single phenotype, hepatocytic (expressing AFP and albumin) or bile ductular (expressing only CK-19), had proliferated. In Rs-treated rats, in which the proliferative capacity of endogenous hepatocytes is impaired, transplanted cells showed mainly a dual phenotype (expressing both AFP/albumin and CK-19). One month after transplantation, DPPIV⁺ FLEC engrafted into the parenchyma exhibited an hepatocytic phenotype and generated new hepatic cord structures. FLEC, localized in the vicinity of bile ducts, exhibited a biliary epithelial phenotype and formed new bile duct structures or were incorporated into pre-existing bile ducts. In the absence of a proliferative stimulus, ED 14 FLEC did not proliferate or differentiate. Our results demonstrate that 14-day fetal liver contains lineage committed (unipotential) and uncommitted (bipotential) progenitor cells exerting different repopulating capacities,

which are affected by the proliferative status of the recipient liver and the host site within the liver where the transplanted cells become engrafted. These findings have important implications in future studies directed toward liver repopulation and *ex vivo* gene therapy. (Am J Pathol 2000, 156:2017–2031)

The liver originates from the gut endoderm. On embryonic day (ED) 8.5 in the mouse and 1 day later in the rat, primitive epithelial cells of the foregut contact the cardiac mesoderm and form the liver diverticulum.^{1–6} These cells proliferate extensively, invade the septum transversum, begin to differentiate, and, on ED 9.5 in mice and ED 10.5 in rats, acquire the morphological appearance of immature liver epithelial cells (hepatoblasts), expressing first α -fetoprotein (AFP) and then albumin.^{6,7} Following the expression of these and other hepatic markers, including also cytokeratins (CKs), most authors conclude that hepatoblasts are bipotential cells, capable of differentiating along the hepatocytic or bile duct epithelial cell lineage.^{7–11} At ED 15–16, the rat liver already contains committed immature hepatocytes and bile duct epithelial cells.^{7,8,12,13} In both rats and humans, embryonic hepatoblasts in large vascular spaces also form primitive ductal structures, which ultimately give rise to the intrahepatic bile ducts.^{14–16}

A number of transcription, signaling, and growth factors have been identified that play an essential role in gut endoderm differentiation and fetal liver development. These include factors that bind to the GATA DNA sequence motif (GATA), signal transducers and activators of transcription (STATs), hepatocyte nuclear factors (HNF)-3- α and - β , HNF-4, HNF-6, and certain fibroblast growth factors (FGFs).^{17–25} However, the mechanisms by which primitive pluripotential endodermal cells undergo hepatic specification and how bipotential hepatoblasts differentiate further into hepatocytes and bile duct epithelium remain largely unknown.

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Studies in the adult liver have also provided strong evidence for the existence of putative liver stem cells, ie, undifferentiated liver epithelial cells that can be activated to proliferate and differentiate into hepatocytes or bile duct epithelial cells.^{26–28} These cells are thought to reside within or adjacent to the canals of Hering. Unlike stem cells in other tissues, such as bone marrow, skin, and intestine, which undergo continuous renewal, liver stem-like cells are facultative; they comprise a quiescent compartment of dormant cells that is activated only if the regenerative capacity of hepatocytes is impaired. Attempts have been made to identify their counterpart in fetal liver,^{7,28–31} and it has been suggested that the dormant stem-like cells originate most probably from bipotential fetal liver epithelial progenitor cells.^{28,32,33}

To explore the ability of fetal liver epithelial progenitor cells (FLEC) to proliferate and differentiate into hepatocytes (Hc) and bile duct epithelial cells (BDEC) and become incorporated into structural components of the liver lobule, we have used a cell transplantation approach to monitor the fate of these cells under different experimental conditions. Cells were transplanted into the liver of a syngeneic mutant Fischer 344 (F344) rat strain, deficient in the exopeptidase dipeptidyl-peptidase IV (DPPIV).³⁴ Because this enzyme is expressed in both Hc and BDEC, the genetically DPPIV-deficient F344 rat is an excellent model to follow the proliferation, lobular distribution, and morphological appearance of transplanted wild-type (DPPIV⁺) hepatic cells.^{34–38}

When normal liver is subjected to partial hepatectomy (PH), liver regeneration occurs through proliferation of pre-existing mature hepatocytes.^{39–40} However, when rats are treated with retrorsine (Rs), this pyrrolizidine alkaloid is taken up by hepatocytes and metabolized to a bioactive form, which alkylates cellular DNA. This interferes with cell cycle progression and leads to inability of hepatocytes to proliferate.^{41–43} In the present study, we used both normal and Rs-treated DPPIV[−] rats to follow the proliferation, lineage progression, and differentiation of transplanted ED 14 FLEC cells. This was evaluated by their morphological appearance, histochemical expression of DPPIV, and expression of markers specific for Hc or BDEC, using dual label immunohistochemistry and *in situ* hybridization (ISH). Our results demonstrate that FLEC are a heterogeneous population of cells with a single or dual phenotype (unipotential or bipotential) and that their lineage commitment and proliferative activity varies depending on the engraftment site and functional status of the host liver.

Materials and Methods

Materials

Rs and diaminobenzidine (DAB) were purchased from Sigma Chemical (St. Louis, MO). The Vectastain Elite ABC kit was from Vector Laboratories (Burlingame, CA). Rabbit anti-rat red blood cells IgG was from Rockland (Gilbertsville, PA). Radioactive ³⁵S-UTP (SJ603) and CK-19 antibody (RPN 1165) were obtained from Amer-

sham Life Science Products (Arlington Heights, IL). CK-14 antibody (NCL-LL002) was from Novocastra Laboratories (United States distributor, Vector Laboratories). OV-6 monoclonal antibody was a generous gift from Dr. S. Sell (Albany Medical College, Albany, NY). Digoxigenin RNA labeling mix and anti-digoxigenin-POD, Fab fragments were from Boehringer Mannheim (Indianapolis, IN). Autoradiographic emulsion, type NBT2, was purchased from Eastman Kodak Company (New Haven, CT). Dr. N. Fausto (University of Washington, Seattle, WA) kindly provided plasmid BAF700, used for synthesis of the fetal form of AFP mRNA riboprobe.

Animals and Animal Treatment

Normal Fischer rats (F344) were purchased from Charles River Laboratories (Wilmington, MA). Mutant DPPIV-deficient (DPPIV[−]) F344 rats were obtained from the Special Animal Core Facility of the Liver Research Center, Albert Einstein College of Medicine. All studies with animals were conducted under protocols approved by the Animal Care Use Committee of the Albert Einstein College of Medicine and were in accordance with National Institutes of Health guidelines. Rs treatment of the animals was as described previously.³⁸ In all experiments, cell transplantation recipients were female DPPIV[−] F344 rats. For experiments in which cell transplantation recipients were treated with retrorsine, rats weighing 90 to 100 g were given two intraperitoneal injections of Rs, 2 weeks apart, each of 30 mg/kg body weight. One month after the second injection, animals were subjected either to two-thirds PH and transplantation or to transplantation without PH. For stimulation of cell proliferation with triiodothyronine (T3), 1 day before cell transplantation and every week thereafter, animals received subcutaneous injections of T3 (Sigma) at a dose of 400 µg/100 g body wt, for a total of four T3 injections.

Isolation of FLEC

Fourteen-day FLEC from DPPIV⁺ animals were isolated by a modification of the procedure of Sigal et al.¹² In brief, fetal livers were placed in ice-cold modified Hanks' balanced salt solution (HBSS; Gibco BRL, Grand Island, NY) without Ca²⁺, containing 0.8 mmol/L MgCl₂ and 20 mmol/L HEPES, pH 7.4, and then triturated gently several times in modified HBSS containing 1 mmol/L EGTA. After centrifugation for 5 minutes at 450 × *g* at 4°C, the pellet was suspended in modified HBSS containing 0.2% collagenase, 0.07% DNase, and 1 mmol/L CaCl₂. Digestion was carried out for 15 minutes at 37°C, with gentle trituration every 5 minutes. The reaction was stopped by adding an equal volume of modified HBSS containing 1 mmol/L EGTA and fetal bovine serum at a final concentration of 10%. The cell suspension was filtered through a 45-µm nylon mesh, and cells were collected by centrifugation as above. The cell pellet was washed twice with modified HBSS/0.1% bovine serum albumin, centrifuged, and suspended at a concentration of 10⁷ cells/ml. The cell suspension was subjected to two rounds of panning

with rabbit IgG against rat red blood cells (Rockland), as described.¹²

Transplantation of FLEC

A total of 1.5 to 3.0×10^6 cells in a volume of 0.5 ml (of which approximately 15% or 2.25 to 4.50×10^5 cells were judged to be FLEC by detection of AFP mRNA using ISH of small aliquots fixed to cytospin slides) were infused into the liver through the portal vein immediately after two-thirds PH. Control animals received only cell transplantation. Four to five animals, including control animals, were used for each time point. The proliferation and differentiation of FLEC in the liver of the recipients was analyzed 1, 2, and 4 weeks after cell transplantation. The livers of T3-treated animals were analyzed 4 weeks after cell transplantation.

Histochemical Detection of DPPIV, γ -Glutamyl Transpeptidase (γ -GT), and Glucose-6 Phosphatase (G6-P)

To detect DPPIV⁺ transplanted cells in the liver of DP-PIV⁻ F344 rats, histochemical staining was carried out as described previously.³⁷ γ -GT was detected by the method of Rutenburg et al⁴⁴ as described previously⁴⁵ and G-6P by the method of Teusch,⁴⁶ with modifications described previously.⁴⁷

Immunohistochemical Detection of CK-19, CK-14, and Oval Cell (OV)-6 Antigen

Immunohistochemical detection was performed after 10 minutes' fixation in cold 4% paraformaldehyde (PFA) prepared in phosphate buffered saline (PBS). The slides were washed in PBS and then in PBS/0.1% Triton X-100. Endogenous peroxidase was blocked for 5 minutes with 5 mmol/L periodic acid, and the sections were washed for 30 minutes with 3 mmol/L sodium borohydride in PBS. Further blocking was performed according to instructions in the Vectastain ABC Elite kit (including biotin/avidin blocking). CK-19 antibody (RPN 1165) at a dilution of 1:10, CK-14 antibody (NCL-LL002) at a dilution of 1:20, and OV-6 antibody at a dilution of 1:100 were applied for 2 hours at room temperature. Biotinylated anti-mouse IgG (BA-2001) was used as a secondary antibody in combination with the Vectastain ABC kit. Peroxidase activity was developed by diaminobenzidine (DAB) staining.

ISH and Dual Immunohistochemistry/ISH Labeling

ISH was conducted on frozen sections as described previously.⁴⁵ ISH of cells collected on plus charged slides (Fisher Scientific, Springfield, NJ) by the cytospin method, including several additional steps before acetylation. In brief, after fixation, washing, and dehydration, the slides were rehydrated for 10 minutes in PBS/5

mmol/L MgCl₂ and permeabilized for 20 minutes in 0.1% Triton X-100, prepared in the same buffer. The slides were then washed for 5 minutes with the same buffer, treated for 5 minutes at room temperature with 5 μ g/ml of Proteinase K (in 0.1 mol/L Tris/HCl, pH 8, and 5 mmol/L EDTA), washed for 3 minutes with 0.2% glycine, fixed again for 5 minutes in PFA, washed with buffer, and acetylated.

Dual ISH labeling was performed with ³⁵S-labeled rat albumin antisense riboprobe and digoxigenin-labeled AFP antisense riboprobe. For detection of digoxigenin-labeled RNA hybrids, anti-digoxigenin POD (Fab fragment) was applied and peroxidase activity revealed by DAB.⁴⁵ The slides were dehydrated, dipped in autoradiographic emulsion (NBT2), and exposed for 1 week to detect autoradiographic grains representing albumin mRNA.

For combined (dual) immunohistochemistry and ISH labeling, frozen sections were first processed with CK-19 antibody, peroxidase activity developed with DAB, and ISH was then performed with ³⁵S-labeled AFP or albumin antisense riboprobe as described previously.⁴⁷ After washings and dehydration, the slides were exposed with autoradiographic emulsion for 1 to 3 days and stained with hematoxylin.

Screening for Y Chromosome Marker Sry in Female Rats Transplanted with FLEC

This procedure followed the protocol described by An et al.⁴⁸ First, a Sry fragment was amplified from rat genomic DNA by polymerase chain reaction, using primers homologous to the mouse sry gene. The amplified product of 459 bp was cloned into pGEM T-Easy vector (Promega, Madison, WI). Screening for Y chromosome DNA in female livers transplanted with FLEC was carried out using rat Sry primers as follows: Primer 1, Rat Sry (5'-CATC-GAAGGGTTAAAGTGCCA-3') and primer 2, Rat Sry-R (5'-ATAGTGTGTAGGTTGTTGTCC-3'). These primers amplify a stretch of 104 bp nested within the 459-bp fragment. Rat liver DNA from the recipient livers and control male and female rats was purified using the DNEasy kit (Qiagen Inc., Valencia, CA). Serial dilution of the DNA samples beginning with 50 ng of DNA per reaction were prepared in a 50- μ l reaction mixture containing 2.5 mmol/L MgCl₂, 0.4 μ mol/L of each primer, 0.2 mmol/L of each dNTP, and 1 unit of Platinum Taq polymerase (Gibco BRL, Grand Island, NY). Amplification conditions included a 3-minute incubation at 94°C, followed by 32 cycles of 1 minute at 94°C, 1 minute at 58°C, and 1 minute at 72°C, and a final termination step of 7 minutes at 72°C. The product was resolved on a 2% agarose gel.

Results

Characteristics of Isolated FLEC

Rat liver at ED 14 contains immature epithelial cells and a large number of hematopoietic cells at different stages of

Figure 1. Phenotypic characteristics of ED 14–15 rat FLEC. Fetal liver cells were isolated as described in Materials and Methods, washed, and collected on slides. **A:** AFP mRNA expression (the fetal form), evaluated by ISH to ^{35}S antisense riboprobe (cells exhibiting autoradiographic grains) in ED 14 FLEC. **B:** Albumin mRNA expression, evaluated by ISH to digoxigenin-labeled antisense riboprobe (cells exhibiting dark color) in ED 14 FLEC. **C:** γ -GT, histochemical staining (cells exhibiting dark color) in ED 15 FLEC. **D:** CK-19, immunohistochemical staining (cells exhibiting dark color) in ED 15 FLEC. Original magnification, $\times 200$.

differentiation. We have analyzed cells isolated from ED 14–15 fetal liver for expression of AFP, albumin, G-6P, γ -GT, CK-19, CK-14, OV-6, and DPPIV. The percentage of FLEC in the total cellular suspension was approximately 15%, determined as the number of cells expressing AFP mRNA (Figure 1A) or albumin mRNA (Figure 1B). None of the isolated ED 14 liver cells expressed G-6P, CK-14, OV-6, or DPPIV (data not shown). However, expression of γ -GT and CK-19 was clearly observed and increased on ED 15 (Figure 1, C and D).

To further characterize the phenotype of immature liver epithelial cells, we tested for coexpression of AFP, albumin, and CK-19 in isolated ED 12 and ED 14 FLEC. The analyses were carried out on cytopspins of isolated cells to allow better evaluation of co-expressed markers in individual cells. Coexpression of AFP and albumin in isolated fetal liver cells was determined by dual label ISH (digoxigenin-labeling of antisense riboprobe specific for the fetal form of AFP mRNA and ^{35}S -labeling of antisense riboprobe for albumin mRNA). The results showed that in ED 12 and ED 14 liver, all cells expressing the fetal form of AFP mRNA coexpressed albumin mRNA (Figure 2, A and B). Coexpression studies using immunohistochemistry for detection of CK-19 and ISH for AFP mRNA showed that most FLEC expressed only AFP mRNA (Figure 2C), some cells expressed both AFP mRNA and

CK-19 (Figure 2C), and a third population of cells expressed only CK-19 (Figure 2D). Dual label immunohistochemistry for CK-19 and ISH for albumin mRNA confirmed this observation, as most FLEC expressed only albumin mRNA, some cells coexpressed albumin mRNA and CK-19, and a third, smaller group of cells coexpressed albumin mRNA and CK-19 (Figure 2, E and F). Dual labeling of isolated 12-day fetal liver cells revealed the same heterogeneity (Figure 2, G and H).

These results demonstrated the existence of at least three phenotypically distinct subpopulations of epithelial cells in 12- to 14-day fetal rat liver. The first group of cells expressed AFP and albumin mRNA but not CK-19. This was the largest subpopulation, comprising roughly 75% of FLEC. The second group ($\sim 15\%$ of total) expressed CK-19, but not AFP or albumin mRNA. The third and smallest group of cells ($\sim 10\%$ of total) expressed both AFP and albumin mRNAs and CK-19.

Proliferation and Differentiation of FLEC in the Liver of Adult Syngeneic Animals

To follow the proliferation and differentiation of FLEC, we transplanted isolated ED 14 cells into the liver of normal and Rs-treated rats subjected to two-thirds PH. Differen-

Figure 2. Dual phenotypic characteristics of ED 12 and ED 14 FLEC. Fetal liver cells were isolated as described in Materials and Methods, washed, and collected on slides. Dual ISH for AFP mRNA (brown color) and albumin mRNA (autoradiographic grains) of ED 12 (**A**) and ED 14 (**B**) FLEC. All cells expressing AFP mRNA are also positive for albumin mRNA. **C** and **D**: Immunohistochemistry (brown color) for CK-19 combined with ISH for AFP mRNA (autoradiographic grains). The majority of cells express only AFP mRNA (arrows in **C** and **D**). Some cells expressing AFP mRNA also express CK-19 (arrowhead in **C**), others express only CK-19 (small arrow in **D**). **E** and **F**: Immunohistochemistry (brown color) for CK-19 combined with ISH for albumin mRNA (autoradiographic grains). Most cells express only albumin mRNA (arrow in **E**). Some cells express only CK-19 (arrow in **F**) and others express both albumin mRNA and CK-19 (small arrow in **F**). **G** and **H**: Immunohistochemistry of ED 12 FLEC for CK-19 (brown color) combined with ISH for albumin mRNA (autoradiographic grains). Most cells express only albumin mRNA (arrows in **G** and **H**). Some cells express only CK-19 (small arrow in **G**) and others express both albumin mRNA and CK-19 (arrowheads in **G** and **H**). Original magnifications, $\times 400$ (**A-C**, **E**, **F**, and **H**) and $\times 200$ (**D** and **G**).

Figure 3. Differentiation of rat FLEC in normal adult regenerating liver. FLEC were isolated from the liver of ED 14 rat DPPIV⁺ fetuses and transplanted into the liver of mutant DPPIV⁻ female rats, as described in Materials and Methods. One, 2 and 4 weeks later, the livers were removed and frozen sections were stained for histochemical detection of DPPIV enzyme activity (red color). **A:** One week after transplantation, scattered cells, diffusely stained for DPPIV, were identified. **B:** Two weeks after transplantation, FLEC in the parenchyma (zones 2 and 3) both expanded in number and differentiated into mature hepatocytes. **C:** Two weeks after transplantation, some cells in the regions of bile ducts (zone 1) had differentiated into BDEC (diffuse staining for DPPIV). **D:** One month after transplantation, larger clusters of morphologically fully differentiated Hc were observed. Fully mature bile duct structures comprised of either transplanted cells or a mixture of transplanted and host cells were also present (data not shown). Original magnification, $\times 400$.

tiation of the cells was monitored morphologically and phenotypically, using DPPIV expression to detect transplanted cells. DPPIV cannot be detected in the liver before ED 16–17 by enzyme histochemistry (our findings), immunoblot,⁴⁹ or indirect immunofluorescence⁵⁰ methods. Therefore, detection of this enzyme served as a marker for both proliferation and lineage progression of transplanted FLEC. In ED 16 liver and thereafter, fetal hepatocytes show diffuse membranous staining for DPPIV. However, in adult liver, hepatocytes show a distinctive and unique expression pattern for DPPIV; it is localized to the apical (bile canicular) domain of the plasma membrane. On the other hand, mature BDEC still show diffuse membranous staining for DPPIV, so that these two phenotypically distinct liver epithelial cell types can be readily distinguished.

One week after transplantation of FLEC into the liver of normal adult rats, cells scattered throughout the parenchyma were diffusely stained for DPPIV (Figure 3A). This suggested that the cells were not fully differentiated. Two weeks after transplantation, cells in the parenchyma (zones 2 and 3) acquired an hepatocytic morphology with canalicular expression of DPPIV (Figure 3B),

whereas others in the regions of bile ducts (zone 1) differentiated into biliary epithelial cells (Figure 3C). One month after transplantation, larger clusters of DPPIV⁺ cells, on average 2–7 per cm² in random tissue sections, formed morphologically fully differentiated Hc (Figure 3D). In addition, 1 to 2 clusters per cm² exhibited a fully differentiated bile duct epithelial cell morphology (data not shown).

In Rs-treated rats, the kinetics and proportions of various cells repopulating the liver was different from that observed in normal rats. One week after transplantation into Rs-treated animals, many foci or clusters of small epithelial cells with diffuse membranous staining for DPPIV were observed (Figure 4A). Two weeks after transplantation, large foci of DPPIV⁺ hepatocytes were already present in the liver parenchyma (Figure 4B), and once again, DPPIV⁺ bile duct structures were identified in portal regions (Figure 4C). One month after transplantation of FLEC into Rs-treated rats, numerous foci of DPPIV⁺ mature hepatocyte were observed (50–120/cm² in random tissue sections). These foci were beginning to fuse into very large DPPIV⁺ structures, taking over a substantial portion of the liver parenchyma (Figure 4D).

Figure 4. Differentiation of rat FLEC in Rs-treated adult regenerating liver. FLEC were isolated from the liver of ED 14 rat DPPIV⁺ fetuses and transplanted into the liver of mutant DPPIV⁻ female rats treated with Rs, as described in Materials and Methods. One, 2 and 4 weeks later, the livers were removed and frozen sections were stained for histochemical detection of DPPIV enzyme activity (red color). **A:** One week after transplantation, small groups of cells diffusely stained for DPPIV, were found. **B and C:** Two weeks after transplantation, DPPIV⁺ cells formed larger clusters of hepatocytes that were beginning to show canalicular staining for DPPIV (**B**), or BDEC in the bile duct region, as evidenced by diffusely stained DPPIV⁺ small epithelial cells in bile duct-like structures (**C**). **D:** One month after transplantation, numerous clusters of morphologically fully differentiated Hc were observed. Original magnifications, $\times 200$ (**A-C**) and $\times 100$ (**D**).

Although the number of hepatocytic clusters increased considerably in Rs-treated compared to untreated liver after PH and FLEC transplantation, the number of bile duct structures remained unchanged (1–3/cm² in random tissue sections).

To determine whether transplanted cells exhibiting a fully differentiated hepatocyte morphology had lost markers specific for hepatoblasts and acquired markers specific for mature hepatocytes, serial sections from livers taken 1 month after transplantation were processed for DPPIV, CK-19, γ -GT, or AFP mRNA. As shown in Figure 5, DPPIV⁺ hepatocytes did not express CK-19 (Figure 5, A and B), γ -GT (Figure 5, C and D), or AFP mRNA (Figure 5, E and F), although proliferating small epithelial cells in Rs-treated animals express all three of these markers.⁴⁷ The FLEC that differentiated into Hc were functional and expressed very high levels of albumin mRNA (Figure 6, A and B), and G-6P (Figure 6, C and D); the latter is not expressed in ED 14 FLEC. Bile duct structures formed by transplanted cells retained expression of CK-19 (not shown) and also became positive for OV-6 (Figure 6, E and F), another marker that is not expressed in ED 14 FLEC, but is expressed after ED 16 in biliary epithelial lineage-committed cells, as well as in mature BDEC.

These results demonstrate that immature FLEC can proliferate and differentiate into mature Hc and BDEC in regenerating liver of adult syngeneic animals. As noted above, repopulation of Rs-treated liver by transplanted cells occurred much more rapidly than that observed in normal liver. On the other hand, the frequency of appearance of bile duct structures originating from transplanted FLEC was proportionally higher in normal livers.

Proliferation and Differentiation of Immature FLEC into Hc and BDEC Occurs Only in Liver Subjected to a Proliferative Stimulus

In experiments described above, we transplanted 14 day FLEC into normal or Rs-treated adult liver subjected to two-thirds PH. In control experiments, in which PH was not performed, we did not detect DPPIV⁺ cells (Hc or BDEC). In other experiments, animals were subjected to two-thirds PH 1 week after cell transplantation and were then kept for an additional 1, 2, or 4 weeks. All livers (those removed at the time of PH and the regenerated liver 1, 2, or 4 weeks after PH) were analyzed to detect DPPIV⁺ cells. Again, no DPPIV⁺ cells were found either in

Figure 5. Transplanted cells lose markers of undifferentiated FLEC. Fetal liver cells were isolated from ED 14 rat DPPIV⁺ fetuses and transplanted in conjunction with PH into the liver of mutant DPPIV[−] female rats treated with Rs, as described in Materials and Methods. Serial sections were processed for DPPIV histochemical staining, shown by transplanted cells exhibiting dark staining in a membranous (bile canalicular) distribution in **A**, **C**, and **E** and for CK-19 by immunohistochemical staining, shown as darkly colored cells in **B**; γ -GT by histochemical staining, shown as darkly colored cells in **D** and AFP mRNA by ISH, shown as autoradiographic grains, which are negative, in **F**. The clusters of transplanted cells are surrounded by arrows. As shown in these serial sections taken 1 month after cell transplantation, DPPIV⁺ hepatocytes are negative for γ -GT, CK-19, and AFP mRNA. Original magnifications, ×40 (**A–D**) and ×200 (**E**, **F**).

the liver removed at the time of PH (1 week after cell transplantation) or in the regenerated liver 1, 2, or 4 weeks after PH. Serial sections from the liver removed 1 week after cell transplantation were also analyzed for clusters of AFP mRNA expressing cells and results were again negative. From these data, we conclude that undifferentiated ED 14 FLEC do not proliferate and differentiate in quiescent liver.

That the proliferative status of the liver is crucial for the proliferation and differentiation of transplanted cells was confirmed by an experiment in which Rs-treated recipient animals received 4 doses of triiodothyronine (T3) instead of PH (see Methods). Recently, we reported that T3 is an alternate mitogen for transplanted adult hepatocytes in Rs-treated liver.⁵¹ One month after transplantation of FLEC, small clusters of DPPIV⁺ mature hepatocytes (Fig-

Figure 6. Transplanted cells acquire phenotypic markers of differentiated hepatocytes. FLEC were isolated from the liver of ED 14 rat DPPIV⁺ fetuses and transplanted in conjunction with PH into the liver of mutant DPPIV⁻ female rats treated with Rs, as described in Materials and Methods. One month after cell transplantation, livers were removed and serial sections were processed for DPPIV histochemical staining, (cells with dark staining in a membranous distribution and highlighted by **arrows**) in **A**, **C**, and **E**. **B**: ISH for albumin mRNA (autoradioactive grains) in the same region as the DPPIV⁺ Hc in **A**. This region shows a cluster of transplanted cells with high albumin mRNA expression (circumscribed by **arrows**). **D**: Histochemical staining for G-6P (dark color) expressed in Hc originating from transplanted cells in the same large cluster, which fills the microscopic field. **F**: Immunohistochemical staining for OV-6 (dark color) in epithelial cells within mature bile ducts, some of which are also positive for DPPIV (examples of dual positive cells are highlighted by **arrows** in **E** and **F**). Original magnifications, $\times 200$ (**A**, **B**, **E**, and **F**) and $\times 100$ (**C** and **D**).

ure 7A) or bile duct structures (Figure 7B) were detected in the liver of T3-treated recipients. Although the proliferation of transplanted cells was modest in T3-treated compared to PH treated rats (compare Figures 4D and 7A), this result showed clearly that the recipient liver needs to be activated (subjected to a regenerative stimulus) for

proliferation and differentiation of immature fetal cells to occur.

To determine whether undifferentiated (DPPIV⁻) FLEC remained in the liver, we transplanted FLEC into four normal female rats not subjected to PH and four normal female rats subjected to PH at the time of cell transplan-

Figure 7. Differentiation of rat FLEC in adult Rs-treated liver stimulated with T₃. FLEC were isolated from the liver of ED 14 rat DPPIV⁺ fetuses and transplanted into the liver of Rs-treated mutant DPPIV⁻ female rats and stimulated with T₃, as described in Materials and Methods. Four weeks later, the livers were removed and frozen sections were stained for histochemical detection of DPPIV. **A:** Cluster of transplanted cells differentiated morphologically into mature hepatocytes (area surrounded by arrowheads). **B:** Small bile duct (denoted by large arrow), originating from transplanted cells. Original magnification, $\times 200$.

tation. After 2 weeks, the livers were removed and DNA was isolated from each liver. It was expected that transplanted cells (originating from fetal liver) would be approximately 50% male and 50% female. The presence of male cells was detected by polymerase chain reaction amplification of the *Sry* gene located on the Y chromosome. As shown in Figure 8, some male fetal cells remained in the liver after transplantation in the absence of PH. However, their number was substantially lower than that present in the liver after PH (Figure 8, lanes 1–3 versus lanes 4–6). These cells did not express DPPIV, suggesting that they did not undergo hepatocyte lineage progression in the absence of a liver regenerative stimulus or, alternatively, that they were not of epithelial origin.

Preferential Differentiation of Lineage-Committed FLEC in Normal Adult Liver and of Uncommitted FLEC in Rs-Treated Liver

To determine the proliferative capacity and lineage commitment of the three subpopulations of FLEC described in

Figure 8. Detection and amplification of the rat *sry* gene located on the Y chromosome in transplanted cells. DNA was isolated from the liver of female rats 2 weeks after FLEC transplantation. A 104-bp fragment of the *sry* gene located on the Y chromosome was amplified, as described in Materials and Methods, and resolved on a 2% agarose gel. **Lanes 1–3:** Amplified fragments from 50, 5, and 0.5 ng, respectively, of recipient DNA, isolated 2 weeks after transplantation. **Lanes 4–6:** Amplified fragments from 50, 5 and 0.5 ng, respectively, of recipient DNA, isolated 2 weeks after transplantation from animals subjected to PH. **Lane 7:** Amplified fragment from 50 ng of control female DNA. **Lanes 8–12:** Amplified fragments from 50, 5, 0.5, 0.05, and 0.005 ng DNA from male F344 rats diluted into control female DNA. **Lane 13:** control tube with no DNA. **Lane 14:** Molecular weight markers.

Figure 2, we studied their differentiation in normal and Rs-treated regenerating liver. For this purpose, we analyzed the recipient liver at early time points, ie, 1 and 2 weeks after cell transplantation, following expression and coexpression of AFP mRNA and CK-19 in transplanted cells, which were identified in serial sections by DPPIV enzyme activity.

One week after transplantation of FLEC into normal adult rat liver, scattered DPPIV⁺ cells in the parenchyma were usually AFP mRNA⁺ and CK-19⁻ (Figure 9, A and B). Very infrequently, we found DPPIV⁺ cells that expressed a dual phenotype, ie, they expressed AFP mRNA and CK-19 (Figure 9, C and D). In the periportal region, transplanted DPPIV⁺ cells also expressed CK-19 but did not express AFP mRNA (Figure 9, E and F). The proportion of expanding cells with a single or dual phenotype reflected their relative abundance in the isolated FLEC preparations. Two weeks after transplantation, AFP mRNA expression was still positive, but reduced, in transplanted cells in the parenchyma, and the cells were negative for CK-19 (Figure 9, G and H). DPPIV⁺/CK-19⁺ BDEC did not express AFP mRNA (data not shown).

Different results were obtained in Rs-treated rats. One week after FLEC transplantation, DPPIV⁺ cells formed clusters in the parenchyma expressing AFP mRNA. The larger hepatocytic clusters also expressed CK-19 and thus were bipotential (Figure 10, A and B). However, CK-19 expression was lower in transplanted cells than in endogenous biliary epithelial cells (see arrowhead in Figure 10B). Other DPPIV⁺ clusters (Figure 10C) were comprised of a mixed cell population (CK-19⁺ and CK-19⁻) expressing AFP mRNA (Figure 10D, arrows) and a few clusters of transplanted cells expressed only AFP mRNA (arrowhead). Expression of AFP mRNA was higher in unipotential than in bipotential cells (see Figure 10D). Two weeks after cell transplantation, overall expression of AFP mRNA was reduced, although AFP mRNA was still expressed in both transplanted hepatocytes and biliary epithelium, with the signal being weaker in the latter (Figure 10E and F). AFP mRNA expression in CK-19⁺ hepatocytes in the parenchyma (Figure 10G and H, ar-

Figure 9. Differentiation of lineage committed FLEC in regenerating liver of normal adult F344 rats. FLEC were isolated from the liver of ED 14 rat DPPIV⁺ fetuses and transplanted in conjunction with PH into the liver of mutant DPPIV⁻ female rats not treated with Rs. One week (**A-D**) and 2 weeks (**E-H**) after transplantation, livers were removed and serial sections prepared. Histochemical detection of DPPIV (red color) is shown in **A, C, E, and G** and dual immunohistochemical detection of CK-19 (brown color) and ISH for AFP mRNA (autoradiographic grains) is presented in serial sections in **B, D, F, and H**. **B:** The cluster of AFP mRNA⁺ cells does not express CK-19. **D:** AFP mRNA expressing cells do express CK-19. **F:** None of the CK-19⁺ cells forming bile duct structures (arrow) express AFP mRNA. **G and H:** Decreased expression of AFP mRNA and absent expression of CK-19 in transplanted cells that differentiated into hepatocytes (arrow). Original magnification, $\times 400$.

Figure 10. Differentiation of lineage uncommitted FLEC in regenerating liver of Rs-treated adult F344 rats. FLEC were isolated from the liver of ED 14 rat DPPIV⁺ fetuses and transplanted in conjunction with PH into the liver of mutant DPPIV⁻ female rats treated with Rs. One week (**A-D**) and 2 weeks (**E-H**) after transplantation, livers were removed and serial sections processed. Histochemical detection of DPPIV (red color) is shown in **A, C, E, and G** and dual immunohistochemical detection of CK-19 (brown color) and ISH for AFP mRNA (autoradiographic grains) is shown in **B, D, F, and H**. **B:** Transplanted cells, shown in **A**, formed a large cluster of AFP mRNA⁺ and CK-19⁺ cells, shown in **B**. The expression of CK-19 is lower than in endogenous small epithelial cells (arrowhead in **B**). **D:** A mixed population of transplanted cells, expressing AFP mRNA. Those with lower expression of AFP mRNA also express CK-19 (arrows). The cells with higher expression of AFP mRNA do not express CK-19 (arrowhead). **F:** Transplanted cells, forming bile duct structures, express both CK-19 and AFP mRNA (arrows). Expression of AFP mRNA is generally lower in CK-19⁺ duct cells than that in Hc. Also note that CK-19⁺/AFP⁻ duct cells of host origin are also present (arrowheads). **H:** Expression of AFP mRNA in CK-19⁺ Hc (arrow) decreased faster than that in Hc not expressing CK-19 (arrowhead). Original magnifications, $\times 400$ (**A-F**) and $\times 200$ (**G** and **H**).

row) was lower than in hepatocytes expressing only AFP mRNA (Figure 10G and H, arrowheads).

From these studies, it appears that most FLEC that proliferate and differentiate after transplantation into normal liver are of a single phenotype; they express either AFP/albumin or CK-19 and behave like unipotential progenitor cells, differentiating into Hc or BDEC, respectively. In contrast, the bulk of the cells that proliferate and differentiate in Rs-treated liver behave like bipotential progenitor cells; they show a dual phenotype, expressing both AFP and CK-19, and differentiate into either hepatocytes or bile duct epithelial cells. This indicates that the bipotential cells have a higher proliferative capacity than unipotential cells. Because the same cell preparations were used for transplantation into both normal and Rs-treated liver, we conclude that the proliferative status of the recipient liver controls proliferation and lineage progression of the various subpopulations of FLEC.

Discussion

Results reported in this study represent the first demonstration that 14-day immature FLEC can proliferate extensively and differentiate in the liver of adult animals into morphologically and phenotypically mature Hc and BDEC. However, terminal differentiation and expansion of ED 14 FLEC occurs only in a liver subjected to PH or another proliferative stimulus (such as T3 administration). Fetal liver cells cannot complete terminal differentiation in the quiescent adult liver, normal or Rs-treated. Under these circumstances, most of the transplanted cells are eliminated, although some appear to remain as undifferentiated, dormant stem-like cells that cannot readily be activated. Thus, it can be speculated either that regenerating liver provides the necessary environment, factors, and signals for hepatoblasts to proliferate and differentiate, or that the quiescent liver inhibits these processes. We favor the former hypothesis, as both adult hepatocytes³⁸ and fetal hepatocytes after ED 16 (Sandhu J, Dabeva MD, Petkov PM, Hurston E, Shafritz DA, unpublished results) survive and undergo modest proliferation in Rs-treated liver in the absence of PH.

Preferential Proliferation and Differentiation of Committed FLEC in Normal Liver and Uncommitted FLEC in Rs-Treated Liver

Studying the antigenic profile of ED 12 fetal liver cells, Hixson et al⁵² found three major subpopulations: one expressing only HBD.1, another expressing only OC3, and a third expressing both markers. The authors suggested that all ED 12 cells are transitional and bipotential, and that they originate from a common pre-ED 12 precursor. That hepatic tissue of ED 12 is composed of bipotential epithelial cells that give rise to Hc and BDEC also has been suggested by others studying the differential expression of cytokeratins, AFP, albumin, cell surface markers, and kinetics of appearance of liver-specific markers in the developing rat embryo,^{7-9,29,30} kinetics of

increased expression of one marker and loss of another marker in human embryos,¹⁶ and differential expression of these markers in cell lines under the influence of different promoting agents.^{8,11,13}

From our study, we cannot conclude that there is a specific precursor/product relationship between the different subpopulations of FLEC, as we have observed all three subpopulations from ED 12 up to birth (data not shown). However, our data strongly suggest that commitment toward the hepatocytic or bile duct lineage occurs either very early during formation of the liver diverticulum (before ED 12), or that there is not a single FLEC precursor, as CK-19⁺ cells not expressing AFP may have a separate developmental origin. As demonstrated by cell transplantation, a substantial proportion of ED 14 FLEC are already committed to one or the other lineage, hepatocytic (AFP⁺/albumin⁺) and bile ductular (CK-19⁺). In normal regenerating liver, most of the AFP⁺ transplanted cells, which were scattered throughout the parenchyma, did not express CK-19. CK-19⁺ transplanted cells were found specifically in zone 1 of the liver lobule, as part of bile duct structures. Since on the same sections we observed AFP⁻/CK-19⁺ BDEC and AFP⁺/CK-19⁻ Hc, it is highly unlikely that the differences found in gene expression patterns are due to technical factors or that the cells have lost their dual phenotype 1 week after transplantation. (The expression of AFP in the neonatal liver decreases gradually and shuts off only after 4 weeks of age.)

The third subpopulation of FLEC has a dual phenotype (AFP⁺/albumin⁺ and CK-19⁺) and the cells behave like bipotential progenitors of Hc and BDEC. Our studies provide direct evidence for differentiation of epithelial progenitor cells with dual markers into Hc and BDEC after transplantation into the regenerating liver of Rs-treated animals. These cells exhibit a significantly higher proliferative capacity than endogenous liver cells, taking over approximately 20% of the liver mass within 1 month after cell transplantation and PH.

FLEC as a Source for Liver Cell Transplantation

The results in the present study demonstrate that immature FLEC in the environment of adult regenerating liver can proliferate, differentiate, and express genes characteristic of adult hepatocytes/bile duct epithelial cells. This strongly suggests the potential use of these cells for transplantation and *ex vivo* gene therapy. A few attempts have been made to transplant ED 18 and older fetal liver cells into the spleen or on solid supports implanted intraperitoneally.⁵³⁻⁵⁶ In all these cases, fetal hepatocytes engraft, proliferate to some extent, and perform liver-specific biochemical functions. Isolated fetal hepatocytes from late gestation, when transplanted intraportally into Nagase analbuminemic rats, engraft, expand, and give partial correction of serum albumin when an hepatic regenerative stimulus (portal branch ligation) is also applied.⁵⁷ Several studies also report successful engraftment and differentiation of early fetal liver tissue or cell suspensions after transplantation into ectopic sites.^{7,35,58}

However, engrafted liver tissue masses at ectopic sites do not expand very much, and it is unlikely that such limited liver transplantation will have broad therapeutic application.

The present study suggests that immature FLEC may represent a preferred source of hepatic cells for transplantation compared to adult hepatocytes for the following reasons: 1) FLEC are small (10–12 μm) and their intraportal injection is better tolerated than transplantation of mature hepatocytes (20–35 μm); 2) the number of injected cells we have used for the current experiments is ~5 times lower than the number of adult hepatocytes used for liver repopulation at the same efficiency in our previous study;³⁸ 3) due to their small volume, FLEC are not trapped in the periportal region, where the highest concentration of transplanted adult hepatocytes is observed,⁵⁹ and they move easily through the sinusoids, reaching zone 3 of the liver lobule. This increases the seeding and repopulating efficiency of the transplanted FLEC compared to hepatocytes; 4) immature FLEC possess sufficiently high proliferative capacity that they can repopulate the normal regenerating liver in the absence of Rs treatment; and finally, 5) FLEC differentiate morphologically and phenotypically into both mature hepatocytes and bile duct epithelial cells, which is not observed after hepatocyte transplantation. Since early fetal liver epithelial progenitor cells selectively proliferate in the normal liver in response to a regenerative stimulus (or hepatic parenchymal loss), they differentiate into mature hepatocytes and bile duct epithelial cells, and they become incorporated into the host liver lobule as part of normal hepatocytic cords and bile duct structures, this suggests that fetal liver cell transplantation represents an attractive method to restore functional liver tissue.

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