Isolation of human progenitor liver epithelial cells with extensive replication capacity and differentiation into mature hepatocytes

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Summary

The liver can regenerate itself through the progenitor cells it harbors. Here we demonstrate isolation of epithelial progenitor/stem cells from the fetal human liver, which contains a large number of hepatoblasts. Progenitor liver cells displayed clonogenic capacity, expressed genes observed in hepatocytes, bile duct cells and oval cells, and incorporated genes transferred by adenoviral or lentiviral vectors. Under culture conditions, progenitor cells proliferated for several months, with each cell undergoing more than forty divisions, but they retained normal karyotypes. Progenitor cells differentiated into mature hepatocytes in mice with severe combined immunodeficiency, both when in an ectopic location and when in the liver itself. Cells integrated in the liver parenchyma and proliferated following liver injury. An abundance of progenitor cells in the fetal human liver is consistent with models indicating depletion of progenitor/stem cells during aging and maturation of organs. The studies indicate that isolation of progenitor cells from fetal organs will be appropriate for establishing novel systems to investigate basic mechanisms and for cell and gene therapy.

Key words: Stem, Progenitor, Cells, Epithelial, Fetal, Human, Liver

Introduction

The self-renewal capacity of the liver is well established in models of hepatic resection, injury and carcinogenesis (Alison, 1998; Michalopoulos and DeFrances, 1997; Fausto, 2000). Although proliferation of mature hepatocytes alone restores the liver after partial hepatectomy and hepatocytes can repopulate the liver repeatedly (Michalopoulos and DeFrances, 1997; Overturf et al., 1997), under appropriate situations poorly differentiated 'oval cells' arise in the liver and can generate hepatic lineages (Farber, 1956; Evarts et al., 1987; Coleman et al., 1997; Yasui et al., 1997). Although stem/progenitor cells from adult organs, for example, the pancreas and bone marrow, may also generate mature hepatocytes (Dabeva et al., 1997; Petersen et al., 1999; Theise et al., 2000; Lagasse et al., 2000), such cells are rare in the adult liver.

The engraftment of transplanted cells in the liver drew significant interest from the field of cell therapy (Gupta et al., 1995; Gupta et al., 1999). Transplanted cells regulate gene expression physiologically and can proliferate in the liver (Gupta et al., 1999b; Grompe et al., 1999). However, despite the early promise of cell therapy (Fox et al., 1998), the scarcity of donor human livers, the absence of proliferation in cultured hepatocytes and the poor viability of hepatocytes after cryopreservation impose restrictions. We hypothesized that use of fetal cells will overcome these problems and help advance cell and gene therapy. Fetal liver cells are largely diploid, whereas maturing hepatocytes exhibit increasing polyploidy, which attenuates cell proliferation and eventually produces cell senescence (Sigal et al., 1995a; Sigal et al., 1999; Gorla et al., 2001). Progenitor cells from the fetal rat liver can generate both hepatocytes and bile duct cells in animals (Sigal et al., 1995b; Sandhu et al., 2001). These considerations suggested to us that the fetal human liver will be an appropriate source of stem/progenitor cells. The human liver arises from the foregut endoderm after four weeks of gestation and develops rapidly, such that bile is produced by 14 weeks. During this gestational period, hepatic cells express hepatocyte markers, for example, albumin, alphafetoprotein (AFP), α-1 microglobulin, glycogen, glucose-6-phosphatase (G-6-P) and Hep-Par-1, and biliary markers, for example, gamma glutamyl transpeptidase (GGT), dipeptidyl peptidase IV (DPPIV), cytokeratin (CK)-19 and Das-1-monoclonal antibody-reactive antigen, (Haruna et al., 1996; Badve et al., 2000). Human hepatoblasts express these markers throughout the second trimester (20-24 weeks), despite significant development of the fetal liver, which offered opportunities to isolate and study large numbers of progenitor cells. Here we demonstrate the survival of progenitor cells in long-term cultures, which indicates that similar opportunities to investigate cellular mechanisms could be developed by isolating progenitor cells from additional fetal organs.

Materials and Methods

Fetal human livers

Tissues were from programs at Albert Einstein College of Medicine under approval from the Committee on Clinical Investigations

(institutional review board). The fetal age was established by standard clinical parameters. Donors were negative for human immunodeficiency and hepatitis B viruses. Cells were isolated from livers of 17-24 week's gestation.

Cell isolation and culture

The liver was irrigated at 37°C with sterile buffer A (10 mM HEPES, 3 mM KCl, 130 mM NaCl, 1 mM NaH_2PO_4-H_2O and 10 mM glucose, pH 7.4; unless specified chemicals were from Sigma Chemical Co., St. Louis, MO), followed by buffer B (5 mM $CaCl_2$, 0.03% collagenase, from Worthington Biochemical Corp., Lakewood, NJ). Liver was next passed through wide bore syringe and incubated in buffer B for 30-40 minutes at 37°C. Dissociated cells were collected periodically at 4°C, passed through 80 μm dacron mesh, washed twice in buffer A and pelleted by centrifugation at 500 ***g*** for 5 minutes at 4°C. The cell pellet was resuspended in Dulbecco's minimal essential medium with 5 μg/ml insulin, 5 μM hydrocortisone, 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B (DMEM; Life Technologies Inc., Rockville, MD). Cell viability and number were determined with 0.2% trypan blue dye using Neubauer hemocytometer. 4×10^3 cells/cm^2 tissue culture plastic were incubated with DMEM containing 10% fetal bovine serum (FBS, Atlanta Biologicals Inc., Norcross, GA) in 5% CO_2. The medium was changed daily for 3 days after removing non-adherent cells with phosphate buffered saline, pH 7.4 (PBS); subsequently the media was changed every 3 days. Loosely adherent cells were removed with trypsin-EDTA diluted 1:10 in PBS for 8-10 min at 37°C on each occasion. For subpassaging, near-confluent cultures were split 1:3 using half-strength trypsin-EDTA for 6-10 minutes at 37°C.

Cryopreservation

1×10^6 cells were frozen per 200 μl freezing mixture (University of Wisconsin solution, FBS and dimethylsulfoxide in 7:2:1 ratios (v/v), respectively) in cryofreezing containers (Nalge Nunc International, Rochester, NY). Cells were stored at –80°C for one day and transferred to liquid nitrogen.

Clonogenic assays

Autologous fetal liver cells from various cell cultures were irradiated to 80 Gray and attached overnight to tissue culture plastic at 3×10^4 cells/cm^2 with 60 μl/cm^2 of rat-tail collagen overlayed for 4-6 hours. Collagen was extracted from the tail of several F344 rats. The tails were broken at joints and tendons were pulled out with a hemostat, disrupted and exposed to ultraviolet light for 24 hours. To solubilize, 1 g tendon was stirred for 48 hours in 300 ml acetic acid (diluted 1:1000) followed by filtration through sterile cheesecloth and storage at 4°C. Test cells in DMEM were placed on top of the feeder cell-collagen overlay under limiting dilutions, and medium was replaced regularly for 3 weeks. Colonies were stained with crystal violet and glycogen, G-6-P, GGT, and DPPIV activities were analyzed histochemically, as described previously (Ott et al., 1999b).

Cell proliferation

Doubling times were determined in 1×10^4 cells plated per cm^2 under triplicate conditions followed 1, 3, 5 and 7 days later by analysis of cell numbers during exponential cell growth, as recommended previously (Wieder, 1999). Cell ploidy was analyzed by flow cytometry, senescence-associated β-galactosidase (SABG) activity by histochemistry and p21 expression by immunostaining, as described previously (Sigal et al., 1999; Gorla et al., 2001). Growth factor responses were tested in 1×10^5 cells per 35 mm dish. F344 rat hepatocytes were isolated by standard collagenase perfusion. Growth factors were 10 ng/ml hHGF (Genentech Inc., South San Francisco, CA) and 20 ng/ml TGF-α and EGF (Sigma). 1 μCi [^{3}H]thymidine was added after 47 hours for 1 hour, and ^{3}H-thymidine incorporation into DNA was measured as described previously (Gorla et al., 2001).

Telomerase content

A commercial PCR TRAP assay was used according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). HepG2 cells served as positive controls. 2×10^5 cells were lysed in 200 μl, and 3 μl of the extract, corresponding to 3,000 cells, was used. All reactions were in triplicate and repeated twice. The extracts were heated for 15 minutes at 65°C for inactivating telomerase activity.

Cytogenetic analysis

Cells under 50-60% confluency were exposed to 4 μg/ml colchicine for 12 hours, lysed in 0.075 M KCl for 30 minutes at room temperature, pelleted by centrifugation and fixed in methanol and glacial acetic acid (3:1 v/v) for 20 minutes at 4°C. Metaphases were prepared on glass slides and stained with Giemsa according to standard procedures. Image analysis used the Cytovision Software (Applied Imaging, Santa Clara, CA).

Gene expression analysis

We used anti-human albumin (A6684, HSA-11 clone, Sigma), α-1 antitrypsin (BioGenex Corp., San Ramon, CA), CK-19 (RPN 1165, Amersham Pharmacia Biotech Inc., Piscataway, NJ), CK-8 (Vector Labs. Inc., Burlingame, CA) and AFP (Sigma), orosomucoid, and plasminogen activator inhibitor, type-1 (PAI-1) (Accurate Chemical and Scientific Corp., Westbury, NY). Flow cytometry used ethanol-fixed cells suspended in 1% bovine serum albumin (BSA) in PBS. Primary antibodies were diluted 1:10 in DIFTAGS (Shandon Lipshaw, Pittsburgh, PA) and then 1:100 in PBS with 2% goat serum and 1% BSA. Anti-albumin was diluted in PBS alone. Cells were stained for 2 hours at 4°C and analyzed by FITC-conjugated antibodies using FACScan (Becton Dickinson). For western blotting, cells were lysed in 50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS and 0.1% Triton-X at 4°C. Proteins in supernatant were measured by Bio-Rad assay (Cambridge, MA) and resolved by 8-12% SDS-PAGE. PVDF membrane (Amersham) blots were blocked with 5% non-fat milk in Tris-buffered saline containing Tween-20; except for CK-19, which used 1% BSA in PBS-Tween-20. After primary antibody incubation, a peroxidase-conjugated secondary antibody was used for enzymatic chemiluminiscence (Amersham). For demonstrating novel proteins, cells were pulsed with 1 μCi ^{35}S-cysteine and ^{35}S-methionine for 30 minutes and serum-free DMEM was chased for one hour. Cellular proteins were extracted with 10% trichloroacetic acid, solubilized in sodium hydroxide, resolved in 7.5% SDS-PAGE followed by autoradiography. Relevant bands excised from Coomassie blue stained gels were analyzed by peptide mass spectroscopy.

Gene transfer studies

The Adβgal vector was from the Genetic Engineering Core (Albert Einstein College of Medicine, Bronx, NY) and recombinant retrovirus and lentivirus were used as described previously (Gagandeep et al., 1999; Ott et al., 1998; Zahler et al., 2000). Transient transfections with luciferase plasmids expressing hepatitis B virus (HBV) enhancer I and preS1 promoter or simian virus (SV)40 enhancer and promoter were as described before (Ott et al., 1999a).

Cell transplantation

Cells passaged 3, 5 and 8 times were attached to microcarrier beads (Cytodex 3^{TM}, Amersham Pharmacia) by incubating 1×10^6 cells per

1 ml of swollen beads overnight at 37°C. 1×10^6 cells from three separate livers were injected intraperitoneally (i.p.), subcutaneously (s.c.) or intrasplenically into severe combined immunodeficiency mice in the Balb/c background (SCID) as described earlier (Gupta et al., 1999a). Ten days after intrasplenic cell transplantation, one group of mice (n=7) was treated with three doses at 10 day intervals of 1.45 ml/kg carbon tetrachloride in mineral oil (1:1 v/v).

Immunohistochemistry

Glycogen, DPPIV, GGT and G-6-P activities were stained as described previously (Ott et al., 1999b). To colocalize glycogen and CK-19, cells were fixed with 4% paraformaldehyde and endogenous peroxidase, then quenched with 3% H_2O_2 in methanol for 30 minutes. After glycogen staining (24), cells were blocked with 10% goat serum for 30 minutes at 37°C and incubated with 20 nanogram CK-19 antibody (A53-B/A2, Santa Cruz Biotechnologies, Santa Cruz, CA) for 90 minutes at 37°C. Antibody binding was localized by biotinylated goat anti-mouse IgG (Sigma) using the avidin-biotin complex (Vector) and diaminobenzidine (DAKO). Cryosections were probed with anti-human albumin (Sigma) and α-1 microglobulin after blocking peroxidase with Power Block™ (BioGenex), and antibody binding was detected with a peroxidase system.

In situ hybridization

5 μm cryosections were fixed in paraformaldehyde, and paraffin-embedded sections were dewaxed. Slides were rinsed in 2×SSC for 30 minutes at 37°C, denatured in 70% formamide for 2 minutes at 80°C and hybridized with a digoxigenin-labeled total human DNA probe (Oncor, P5080-DG.5, Vysis Inc., Downers Grove, IL) after denaturing for 5 minutes at 80°C. Hybridization was performed overnight at 37°C followed by washes in 50% formamide and 2×SSC. Sections were incubated with alkaline-phosphatase-conjugated anti-digoxigenin (Roche, 1093274) for 1 hour at room temperature, and the color was developed with either BCIP/NBT or Fast Red substrate (Sigma B5655 and F4523, respectively). Bile canalicular ATPase and G-6-P were colocalized histochemically as described previously (Gupta et al., 1995; Gupta et al., 1999). To determine the number of transplanted cells in the recipient liver, tissues subjected to in situ hybridization were analyzed by morphometric methods. The liver volume of several mice was determined. The number of transplanted cells was counted in defined areas of liver sections and converted into cells/mm^3, as the tissue thickness was already known (Rajvanshi et al., 1996). To estimate transplanted cell fractions surviving in the mouse liver after various intervals, we used the number of transplanted cells 2 hours after cell transplantation as the denominator.

Electron microscopy

Tissues were fixed in 2.5% glutaraldehyde in cacodylate buffer, post-fixed in osmium tetroxide and stained with 1% uranyl acetate before embedding in plastic. Ultrathin sections were examined under a Phillips transmission microscope.

Statistics

Data are expressed as means±s.d. The significance was analyzed by the Student's t-test or Mann-Whitney rank correlation tests with the SigmaStat software (Jandel Scientific, San Rafael, CA). P values <0.05 were considered significant.

Results

We isolated 0.8×10^7-2×10^8 mononuclear cells per fetal liver with a viability of 99-100% (Table 1). The initial cell population was an obvious mixture of hematopoietic and epithelial cells. For instance, red blood cells were copious, although these were rapidly depleted under culture conditions. It was noteworthy that the cells were quite robust and showed excellent capacity for initiating long-term cultures over several weeks to months, including after multiple subpassages. However, as these cultures were established by plating relatively large numbers of isolated cells ($4\text{-}5\times10^5$ nucleated cells per cm^2), without fractionating cell subpopulations, it was not known whether all or only some cultured cells were capable of generating long-term cultures.

Table 1. Establishment of primary fetal liver cell cultures

Fetal liver (gestational age)	Number of cells isolated	Primary cultures established	Number of cell subpassages before the culture was discontinued
#112097 (17 weeks)	80×10^6	Yes	4
#1210971 (19 weeks)	200×10^6	Yes	ND
#1210972 (23 weeks)	240×10^6	Yes	ND
#21198 (24 weeks)	150×10^6	Yes	15
#32598 (20 weeks)	120×10^6	Yes	12
#4298 (22 weeks)	125×10^6	Yes	13
#43098 (24 weeks)	225×10^6	Yes	16
#61898 (24 weeks)	175×10^6	Yes	ND
#112498 (23 weeks)	190×10^6	Yes	8
#210991 (20 weeks)	200×10^6	Yes	15
#210992 (22 weeks)	225×10^6	Yes	13
#7899 (24 weeks)	90×10^6	Yes	10
#72899 (19 weeks)	110×10^6	Yes	ND
#9899 (21 weeks)	200×10^6	Yes	13
#12700 (24 weeks)	150×10^6	Yes	13

Fetal progenitor liver cells exhibited unique morphological and gene expression profiles

Early characterization of the initial cell isolate showed that virtually half of the cells expressed the biliary marker, GGT, along with hepatocyte markers G-6-P, glycogen and DPPIV (Fig. 1A). These cells showed typical epithelial morphology. During prolonged primary culture, non-adherent or loosely adherent cells, including hematopoietic cells, became depleted. In this situation over 2 to 4 weeks, epithelial cells proliferated in dishes with copious cytoplasm, forming a prominent rounded or oval shaped nuclei and complex cytoplasmic organization on ultrastructural analysis (Fig. 1B). These cells exhibited a tendency to form heaps and retained expression of hepatocytic genes, such as G-6-P and glycogen, and biliary genes, such as DPPIV and GGT (Fig. 1C). Remarkably, during serial subpassaging in long-term culture over several months, cell morphology altered from an obviously hepatocyte-like shape in primary cultures to cells that were flatter and spindlier. These findings were observed in cells isolated from different livers and from cells cultured at different times following cryopreservation from the same liver. To determine whether liver-type function was still expressed in these subpassaged cells, we initially analyzed in situ expression of liver genes. These studies demonstrated that despite 2, 3, 5, 8 or more passages, fetal cells expressed G-6-P, GGT, glycogen and DPPIV in patterns similar to cells in primary cultures (Fig. 1D). Expression of hepatocyte (glycogen, G-6-P) and biliary markers (GGT, DPPIV) in large number of these cells suggested that many cells were coexpressing these markers, which is in agreement with bilineage gene expression. To

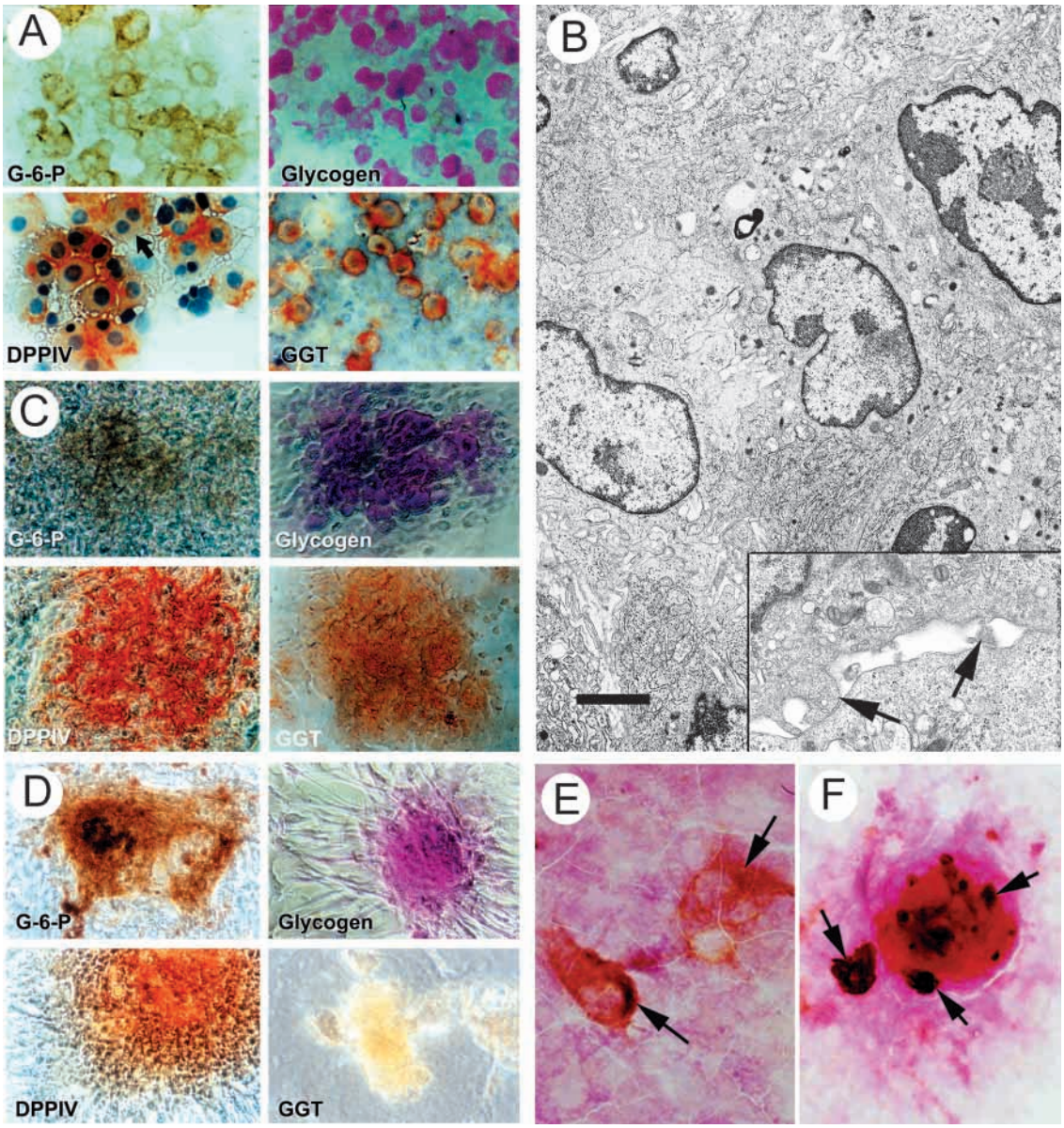

Fig. 1. Selected properties of progenitor liver cells in culture. (A) shows G-6-P, glycogen, DPPIV and GGT expression immediately after isolation of hepatoblasts. The arrow in the lower left panel indicates a binucleated hepatoblast. (B) Electron microscopy of cultured cells in the third passage (P3) showing epithelial morphology with characteristic nuclei and prominent nucleoli. The inset shows microvilli (arrows) and bile canalicular-type structure in two adjacent cells, which was in agreement with the epithelial morphology of the cells. Bar, 2 μm. (C) Primary culture of isolated cells with expression of various liver markers. These cells had been in culture for 3 weeks with depletion of non-adherent cells from the culture. (D) The fifth passage culture, showing heaped up cells expressing liver genes, which is identical to primary cultures. (E and F) show colocalization of glycogen (purple color) and CK-19 (brown color, arrows) in P1 progenitor cells arranged as monolayers (E) or cell heaps (F). The cells were isolated from livers #21198, #32598 and 210991.

verify this possibility, coexpression of glycogen and CK-19, which is expressed in mature bile duct cells, was examined in cultured cells (Fig. 1E,F). These experiments showed that 5-10% of cultured cells coexpressed glycogen and CK-19. The prevalence of cells in these cultures with expression of additional biliary markers, such as GGT and DPPIV, was far greater (see below), which presumably was in agreement with the usual expression of CK-19 only in mature bile duct cells.

To further analyze gene expression in cultured cells, western blots were used (Fig. 2). Primary cells expressed genes observed in hepatocytes, for example, albumin, AFP, ASGR, orosomucoid and α-1 microglobulin, as well as biliary genes, for example, CK-19. Moreover, cells displayed additional markers found in hepatoblasts and oval cells, such as CK-8 and PAI-1. The presence of PAI-1 in our cells was suggested initially by metabolic labeling with [^{35}S]. This showed abundant expression of a ~43 kDa protein in cell lysates and medium. Peptide mass spectroscopy and western blotting verified that the protein was PAI-1 (Fig. 2C). The overall pattern of how various liver genes were expressed in cultured cells is summarized in Table 2. It is noteworthy that most markers were expressed during long-term culture of cells,

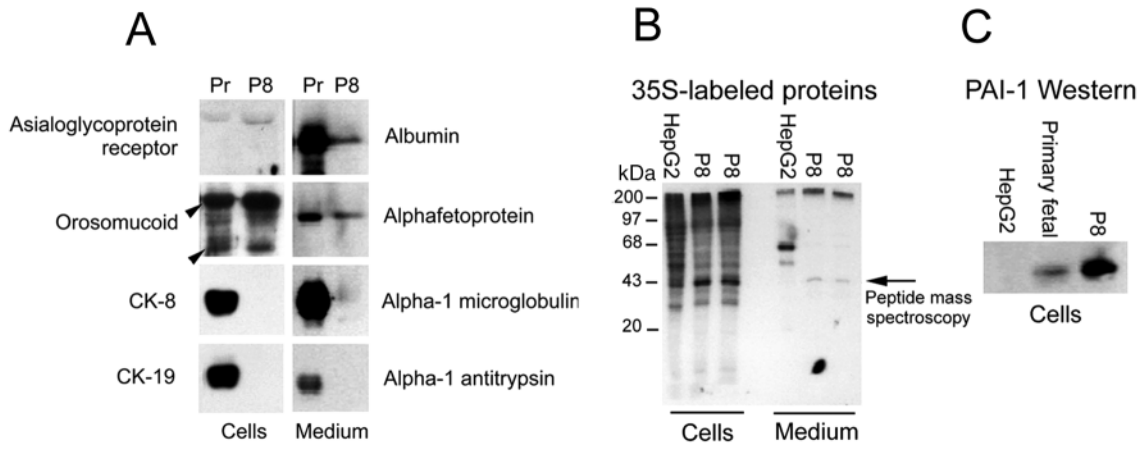

Fig. 2. Gene expression in cultured progenitor cells. (A) Western blots showing liver gene expression in primary cells (Pr) and eighth passage cells (P8), including cell lysates or culture medium. α-1 antitrypsin, CK-8 and CK-19 were not expressed in eighth passage cells. Arrowheads indicate monomeric and dimeric forms of orosomucoid. (B) PAI-1 expression with SDS-PAGE showing metabolic labeling with ^{35}S methionine and cysteine (left panel). Compared with HepG2 cancer cells, eight passage progenitor cells showed increased 43 kDa protein expression. Peptide mass spectroscopy suggested that this band was PAI-1, which was verified by western blotting (C), which showed PAI-1 expression in primary and subpassaged progenitor cells but not in HepG2 cells (right panel).

although the overall magnitude of gene expression altered somewhat in late cell passages.

Analysis of cell proliferation and selected cell-senescence-associated parameters

Analysis of subpassaging capacity showed that the cells could be subpassaged repeatedly, up to 15-16 times over 8-10 months. To determine the fraction of cells capable of clonogenic growth, we studied colony formation under limiting dilution conditions after depleting non-adherent cells over 3 weeks in P0 cultures and subpassaging cells once (P1). The studies utilized cells from two fetal livers (#7899 and #12700). When cells were plated on tissue culture plastic alone, no cell colonies formed. However, after fetal cells were plated on top of irradiated autologous cells (the same cells as those being tested), which served as feeders, single cell colonies appeared with efficiencies ranging from 5 to 30% (Fig. 3A). These cell colonies exhibited the properties of the original cells plated, as shown by liver gene expression, with the presence of G-6-P, glycogen, DPPIV and GGT in substantial proportions of cells (>80-90%). As our culture conditions were devoid of supplemental growth factors, this clonogenic ability presumably reflected either intrinsic properties of cells or release of paracrine growth factors in culture conditions. To examine whether cultured cells were responsive to supplemental growth factors, we studied HGF, EGF and TGFα, which are hepatic growth factors, in primary (P0) fetal cells and fetal cells subpassaged three (P3) or 10 times (P10) (donor livers were #21198, #32598 and #7899). Primary rat hepatocytes were used as controls to establish growth factor activity in DNA synthesis assays. After exposure to HGF,

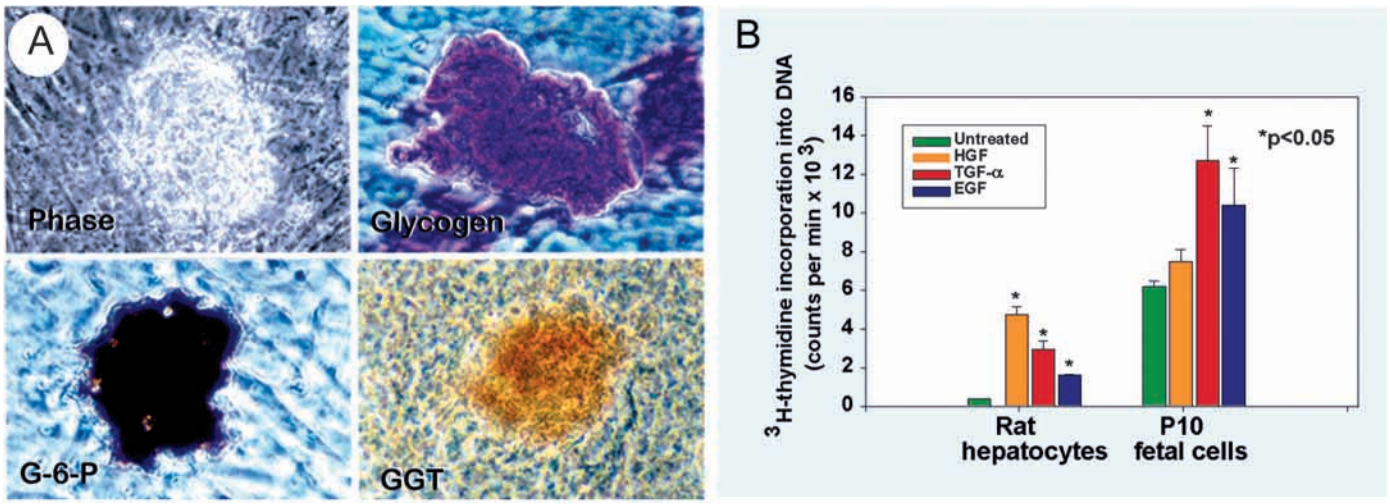

Fig. 3. Proliferation in cultured progenitor cells. (A) Cell colonies formed by third passage progenitor cells under limiting dilution conditions. The top left panel shows phase microscopy. Other panels show glycogen, G-6-P and GGT expression. (B) Growth factor responsiveness of progenitor cells. DNA synthesis rates following stimulation with HGF, TGFα and EGF increased in rat hepatocytes but progenitor cells responded to TGFα and EGF only.

Table 2. Gene expression profiles in fetal liver cells

Protein tested	Cell passages (% of cells with gene expression) P0	P1-4	P6-9	P10-15
Albumin†	+++	+	+	+
α-fetoprotein†	+	ND	+	ND
α-1 microglobulin†	+++	+	++	+
α-1 antitrypsin†	++	ND		ND
Asialoglycoprotein receptor†	+	ND	+	ND
CK-8†	++	ND	ND	ND
CK-19†	++	ND	–	–
DPPIV*	++	++	+	+
	(80-90%)	(50-70%)	(50-70%)	(30-40%)
G-6-P*	++	++	+	+
	(90-100%)	(70-80%)	(70-80%)	(70-80%)
GGT*	+++	+	+	+
	(80-90%)	(50-70%)	(50-70%)	(30-40%)
Glycogen*	++	++	++	++
	(90-100%)	(80-90%)	(80-90%)	(80-90%)
Orosomucoid†	+++	ND	++	ND
PAI-1†	++	ND	+	ND

The data are from cells isolated from two to six separate fetal livers. ND, not done. Western blot analysis was with cells from livers #112498 and #210991.

*Histochemical detection (graded from 3+, maximal, to 0, undetectable).

†Western blotting (graded from 3+, maximal, to 0, undetectable).

Table 3. Recovery of fetal liver cells following cryopreservation

Fetal liver number	State of cells before cryopreservation	Cells viable after recovery (>80% attached on plastic culture dishes)	Long-term cultures established after cells thawed (>three passages)
#1210972	Primary cells	Yes	Yes
#21198	Primary cells	Yes	Yes
#32598	Primary cells	Yes	Yes
#32598	2nd passage (P2)	Yes	Yes
#32598	4th passage (P4)	Yes	Yes
#4298	Primary cells	Yes	Yes
#43098	Primary cells	Yes	Yes
#43098	1st passage (P1)	Yes	Yes
#43098	3rd passage (P3)	Yes	Yes
#43098	8th passage (P8)	Yes	Yes
#210991	Primary cells	Yes	Yes
#210991	2nd passage (P2)	Yes	Yes
#210991	6th passage (P6)	Yes	Yes
#9899	Primary cells	Yes	Yes
#9899	3rd passage (P3)	Yes	Yes
#9899	4th passage (P4)	Yes	Yes
#9899	6th passage (P6)	Yes	Yes

TGFα and EGF, rat hepatocytes showed four- to 12-fold greater DNA synthesis, $P<0.001$ (Fig. 3B). In contrast, DNA synthesis was unchanged in P0 fetal cells exposed to these growth factors. However, subpassaged progenitor cells were responsive to TGFα and to EGF, but not to HGF, with up to 2.4-fold greater DNA synthesis, $P<0.01$. These findings were verified in an additional experiment and were in agreement with the alteration in either cell autonomous behavior or composition of cell cultures with respect to the release of specific growth factors by other cells.

Analysis of cells isolated from two separate livers showed that cell-doubling times ranged from ~59-70 hours during extensive subpassaging of cells for up to 12 passages, with longer doubling times observed in late passages (donor livers #9899 and 12700). The number of cells recovered from confluent dishes remained virtually constant during long-term culture, although after plating 5×10^5 cells per cm^2 in 100 mm cell culture dishes, early passages required 2-3 weeks, and later passages required 6-8 weeks for cells to become confluent in culture dishes. Nonetheless, we did not observe a greater prevalence of cells with polyploidy, immunostainable p21 expression or appearance of SABG in late cell passages. Moreover, we found telomerase activity to be two- to five-fold above controls, $P<0.01$, in fetal cells. Telomerase activity remained detectable, despite 16 subpassages (in cells isolated from liver #43098), which represent approximately 50 or more population doublings. Detailed cytogenetic analysis of primary (P0), intermediate (P3-5) and late passages (P10-13) showed cells with only normal chromosomal complement and structures. These findings suggested that our cell isolation and culture procedures provided genetically normal cell populations with extensive replication capacity.

Also, it was noteworthy that our cells were highly viable following release from culture dishes and serial subpassaging, including, after repeated cryopreservation, >80% of cells attaching to culture dishes following thawing and producing long-term cultures (Table 3).

Differentiation of fetal cells into mature hepatocytes

To establish the differentiation potential of progenitor cells in vivo, cells were first transplanted intraperitoneally in SCID mice. To localize transplanted cells in the peritoneal cavity, cells subpassaged 3, 5 and 8 times from two separate livers (#21198, #32598) were attached to microcarrier beads. Within 3 weeks, vascularized conglomerates developed and transplanted fetal cells that formed confluent masses (Fig. 4A). The presence of transplanted cells was verified by in situ hybridization using a human-specific DNA probe that visualized cell nuclei (Fig. 4B,C). Transplanted cells expressed G-6-P, glycogen, α-1 microglobulin and albumin (Fig. 4D-G). Electron microscopy of microcarriers recovered after 3 weeks showed characteristic hepatocytic morphology in transplanted cells, with microvilli on the apical surface, rounded nuclei, prominent nucleoli and bile canaliculi (Fig. 4H).

Also, no tumors were formed in scid mice 3 ($n=6$) and 5 months ($n=4$) after s.c. or i.p. injection of P8 subpassaged cells from these two livers, which was again in agreement with the absence of cell transformation under our culture conditions.

Progenitor cells expressed introduced genes and engrafted into the mouse liver

Incubation of cells with Adβgal-containing bacterial β galactosidase (LacZ) gene transduced ~100% cells. The use of amphotropic retrovirus or lentivirus vectors to express lacZ or green fluorescence protein, respectively, transduced 5-40% cells, with gene expression during five subsequent subpassages. The cells correctly regulated transfected HBV sequences (luciferase expression versus mock-treated cells was three- to four-fold greater, $P<0.05$), although SV40 sequences showed eight- to 16-fold greater luciferase activity, $P<0.05$.

After intrasplenic transplantation, primary P0 cells, as well

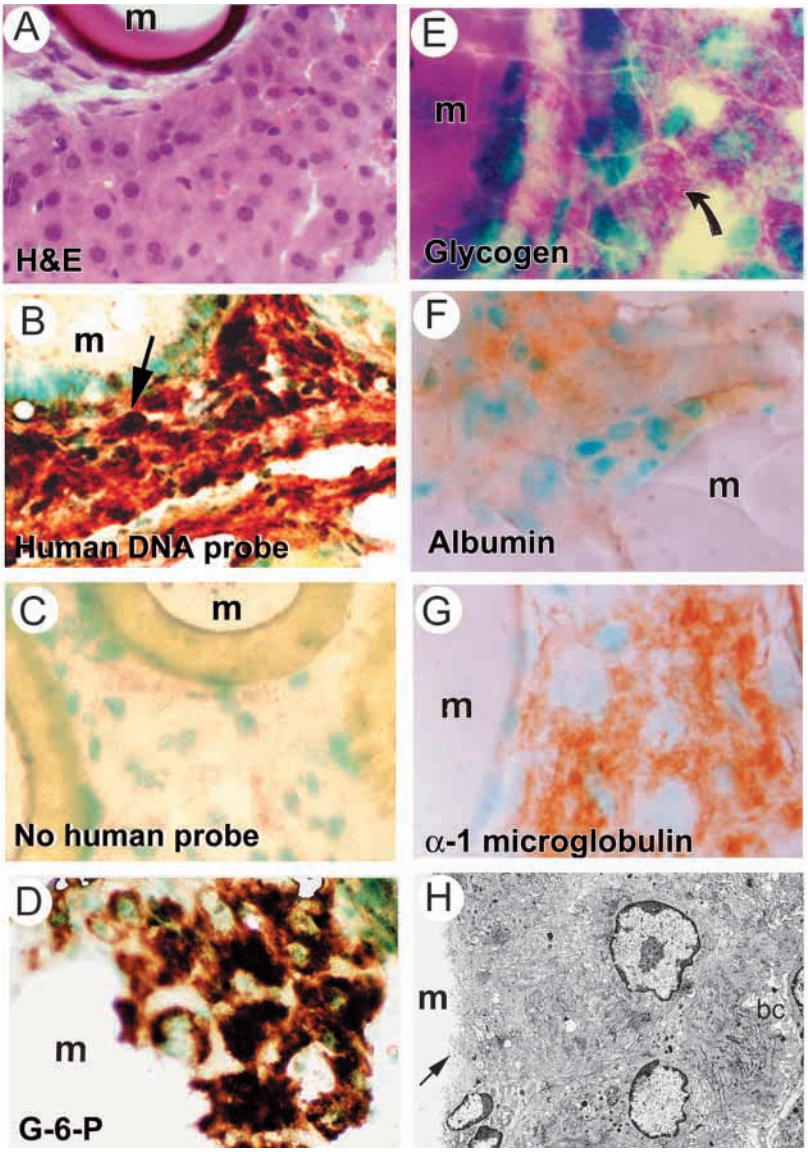

Fig. 4. In vivo fate of progenitor liver cells. Eighth passage cells are shown 3 weeks after transplantantion into the peritoneal cavity. (A) Confluent mass of transplanted cells adjacent to microcarrier bead (m), with typical hepatocyte morphology (H&E stain). (B) In situ hybridization of contiguous sections with human DNA probe visualized cell nuclei, whereas negative control tissues, where the probe was omitted, showed no signal (C). Transplanted cells contained G-6-P (D), glycogen (E), α-1 microglobulin (F) and albumin (G). The arrow in E points toward glycogen granules. (H) shows electron micrography of transplanted cells adhering to a microcarrier bead (m). The cells display epithelial morphology with microvilli on the apical surface (arrow) plus nuclei, nucleolus, abundant mitochondria and bile canaliculi (bc), which are ypical of hepatocytes (original magnification ×2,500).

as P5 and P8 subpassaged cells, survived in the SCID mouse liver. As expected, transplanted cells were in portal spaces 1 hour and 1 day after cell transplantation. Subsequently, transplanted cells entered liver plates and became integrated in the liver parenchyma (Fig. 5A-E). Moreover, transplanted cells showed proliferative capacity in animals treated with CCl4 (Fig. 5F,G). Detailed morphometric analysis of cell engraftment in multiple animals showed that transplanted cells constituted 1.5% of the mouse liver after 1 hour (Table 4). Subsequently, 14 to 55% of these transplanted cells engrafted in the liver, which reconstituted <1% of the host mouse liver. However, after CCl_4, transplanted cells proliferated and their numbers increased significantly (control animals, n=3, $6.3\pm4.2\times10^3$ cells versus $1.6\pm1.1\times10^4$ cells per liver in CCl_4-treated mice, n=7, p=0.02, t-test). This represented up to approximately an approximately five-fold increase in transplanted cell numbers, which was in agreement with more than one round of cell division in transplanted cells following CCl4 injury.

Discussion

The findings indicate that large numbers of highly viable progenitor epithelial cells can be isolated from fetal human liver. These cells exhibited unique properties, including the capacity to replicate extensively, to express genes observed in hepatoblasts and oval cells, including AFP, GGT, CK-8, CK-19, CD34 and PAI-1 (Allison, 1998; Bisgaard et al., 1998), and to produce hepatocytes in immunodeficient mice. The presence of cells expressing both glycogen and CK-19 in our cultures was in further agreement with bilineage gene expression in cells. Taken together, this evidence of extensive replication capacity, presence of oval cell markers and coexpression of biliary and hepatic lineage markers indicate that it is possible to designate our cells as possessing progenitor phenotype. Our major goal was to induce differentiation in these cells along the hepatocyte lineage for obvious implications in cell and gene therapy. Notwithstanding the lack, at present, of methods for inducing biliary differentiation, we were less interested at this stage in differentiating our cells into mature bile duct cells.

Replication of progenitor cells in culture, with retention of

Table 4. Progenitor cell engraftment in SCID mouse liver

Time after cell transplantation	Transplanted cells per liver (10-20 separate areas analyzed in two mice each; 1.2 cm^3 total liver volume)	% of liver replaced by transplanted cells (assuming 60% hepatocytes in 100 million cells in mouse liver)	Engraftment efficiency transplanted (% cells detected after 1 hour)
1 hour	82110	0.14	100
	91580	0.15	107
1 day	31581	0.05	36
	36318	0.06	43
3 days	12632	0.02	14
	4737	0.01	7
10 days	4737	0.01	7
	1580	0.003	2

differentiation capacity, would be helpful in a variety of biological studies. Our progenitor cells were obviously not derived from single cells. Nonetheless, these cells showed significant clonogenic capacity, which indicates that single-cell-derived colonies can possibly be expanded for further analysis. It is noteworthy that the normal adult liver, which contains replicatively quiescent cells, is devoid of telomerase activity (Tahara et al., 1995). By contrast, our cells expressed telomerase, which could be beneficial for continued cell division (Kobayashi et al., 2000). It was noteworthy that after ~50 population doublings, our cells showed decreasing proliferation. By contrast, mature hepatocytes are difficult to maintain and expand in cell culture (Reid and Jefferson, 1994; Block et al., 1996; Runge et al., 2000). We did not incorporate hepatic growth factors (except those present in fetal bovine serum), lipids and extracellular matrix components, which are required for culturing mature hepatocytes (Reid and Jefferson, 1994). Also, unlike murine embryonic liver cells, our progenitor cells survived and proliferated without requiring feeder cells (Rogler, 1997). Specific manipulations, including the release of cells with low trypsin/EDTA concentrations, were aimed at limiting cell membrane injury and selective removal of loosely adherent cells (Herring et al., 1983). It is noteworthy that hydrocortisone inhibits proliferation of fibroblasts and erythroid/granulocyte-macrophage hematopoietic progenitor cells, whereas insulin promotes hepatocyte attachment (Hoshi et al., 1987; Papoff et al., 1998); these observations were refelcted in our culture conditions. Although mature hepatocytes dedifferentiate in culture with rapid loss of tissue-specific genes, such as albumin, ASGR, etc., (Reid and Jefferson, 1994), our fetal epithelial cells expressed liver genes despite extensive culture. Moreover, our cells correctly regulated HBV enhancer/promoter, which requires the presence of multiple hepatic transcription factors (Ott et al., 1999a). Furthermore, the mitogenic responsiveness of our progenitor cells to TGFα and EGF was in agreement with oval cell responses, as shown previously with F344 rat-derived cells (Gupta et al., 1992).

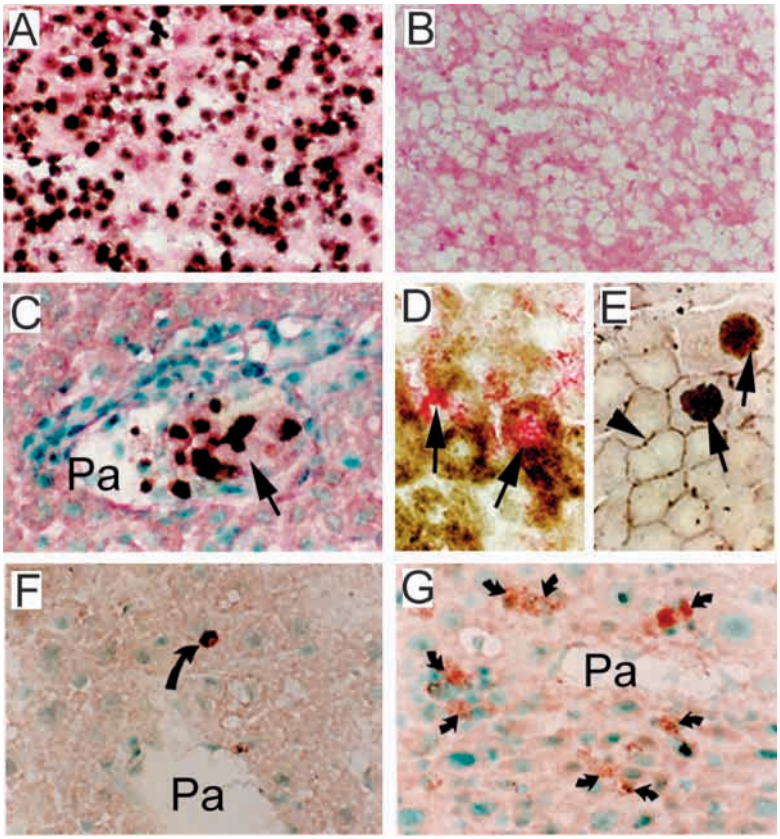

Fig. 5. Progenitor cells engrafted and proliferated in the mouse liver. (A) In situ hybridization showing nuclear localization of the signal in fetal human liver. (B) Negative control tissue showing absence of hybridization when the probe was omitted. (C) shows transplanted cells with dark nuclear staining (arrow) in a portal area (Pa) of recipient mouse liver after 24 hours. (D and F) show integration of transplanted cells in the liver six weeks after transplantation with transplanted cells localized by in situ hybridization (arrows) and histochemistry showing either G-6-P expression (D) or bile canalicular ATPase activity (arrowheads, E). (F) Only occasional transplanted cells were observed as shown six weeks after cell transplantation. (G) shows the increased number of transplanted cells six weeks after repeated CCl_4 treatment to induce cell proliferation. Panels C, F and G were counterstained with methyl green.

Although indefinite cell replication has been induced in somatic cells by expressing the SV40 T antigen or the catalytic subunit of telomerase, it is unresolved whether genetic transformation will induce greater susceptibility for cancer (Kobayashi et al., 2000; Farwell et al., 2000). By contrast, our progenitor cells were genetically unperturbed despite more than 40 to 50 population doublings over 16-18 subpassages. Such proliferation capacity in our cells indicates that cells isolated from a single fetal liver could potentially generate billions or even trillions of cells; whereas only 1-10 billion hepatocytes are required for treating an adult person and proportionately fewer cells will be necessary for treating a child. Therefore, expansion of progenitor cells in culture will facilitate novel clinical applications and help alleviate organ shortages. If highly efficient permanent gene transfer, such as those using lentiviral or retroviral vectors, were combined with effective strategies to repopulate the liver extensively, ex vivo liver gene therapy will once again become attractive. In this context, integration and differentiation of our fetal liver cells in the parenchyma of the mouse liver indicate that use of such cells will be appropriate for liver repopulation. Our data shown here indicate that significant proportions of transplanted cells were lost in mice shortly after transplantation. These findings were not surprising because a large fraction of transplanted cells sequestered in portal vein radicles and hepatic sinusoids undergoes phagocytotic clearance, even in syngeneic recipients (Gupta et al., 1999a). These cell losses constitute removal of approximately 70-80% of all transplanted cells, as also observed in our studies shown here. However, it is unclear at present whether human cells are at a survival disadvantage in the mouse liver compared with rodent cells. Interspecies differences in growth factors, extracellular matrix components, cell-cell interactions or other factors, could potentially regulate survival of human hepatocytes in the mouse liver. Of course, these findings do not exclude the possibility that our cells will

show far superior engraftment in the human liver. Nonetheless, it will be of great interest to establish how engraftment of human cells in the mouse liver may be improved, because this will be relevant for developing novel models of human disease, as well as establishing reproducible bioassays to test the properties of human hepatocytes before use in cell or gene therapy. In addition, under suitable situations, transplanted hepatocytes proliferate significantly in rodents, and the mouse liver can be repopulated virtually completely with transplanted cells (Grompe et al., 1999). In this respect, proliferation of our fetal cells in the mouse liver following CCl_4-induced hepatotoxicity was in agreement with the properties of rodent hepatocytes (Gupta et al., 1999a). Therefore, the clinical implications of our findings should be obvious for cell and gene therapy, especially when coupled with our data showing excellent recovery of cells following cryopreservation, which should greatly facilitate banking of cells for use at short notice.

The intraperitoneal bioassay used here was effective in demonstrating differentiation of progenitor cells into hepatocytes. This in vivo assay should be helpful for analyzing progenitor cell subpopulations, including analyzing progenitor cells for quality controls prior to clinical use. The availability of human progenitor cells capable of extensive proliferation, such as ours, will facilitate development of bioartificial liver (BAL) devices, which are being tested for liver failure, but are limited to porcine hepatocytes or less effective cell lines. Seeding of BAL devices with primary adult hepatocytes has been limited by their inability to proliferate. Additional applications of human progenitor liver cells concern development of novel models for pathophysiological studies, drug discovery systems and drug toxicity studies.

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