Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Mouse Fetal Liver Cells in Artificial Capillary Beds in Three-Dimensional Four-Compartment Bioreactors

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Bioreactors containing porcine or adult human hepatocytes have been used to sustain acute liver failure patients until liver transplantation. However, prolonged function of adult hepatocytes has not been achieved due to compromised proliferation and viability of adult cells *in vitro*. We investigated the use of fetal hepatocytes as an alternative cell source in bioreactors. Mouse fetal liver cells from gestational day 17 possessed intermediate differentiation and function based on their molecular profile. When cultured in a three-dimensional four-compartment hollow fiber-based bioreactor for 3 to 5 weeks these cells formed neo-tissues that were characterized comprehensively. Albumin liberation, testosterone metabolism, and P450 induction were demonstrated. Histology showed predominant ribbon-like three-dimensional structures composed of hepatocytes between hollow fibers. High positivity for proliferating cell nuclear antigen and Ki-67 and low positivity for terminal dUTP nick-end labeling indicated robust cell proliferation and survival. Most cells within these ribbon arrangements were albumin-positive. In addition, cells in peripheral zones were simultaneously positive for α -fetoprotein, cytokeratin-19, and c-kit, indicating their progenitor phenotype. Mesenchymal components including endothelial, stellate, and smooth muscle cells were also observed. Thus, fetal liver cells can survive, proliferate, differentiate, and function in a three-dimensional perfusion culture system while maintaining a progenitor pool, reflecting

an important advance in hepatic tissue engineering.
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Orthotopic liver transplantation is the treatment of choice for end-stage liver disease.^{1,2} However, in some instances, such as acute or acute-on-chronic liver failure, temporary extracorporeal liver support may address the shortage of donor organs.^{1,3–5} Various systems, including our own,⁶ have entered clinical investigation.⁷ However, certain technical and cell source issues are still in debate.⁸ We addressed technical issues by developing a hollow fiber-based four-compartment bioreactor technology, enabling decentralized oxygenation with low solute gradients and high-performance mass exchange.⁹ Many cell types have been investigated to address the cell source issue.¹⁰ Despite limited availability, primary human liver cells, isolated from explanted donor organs discarded from transplantation programs, would be a good cell source for addressing human liver metabolism. Other cell types currently being investigated include adult porcine hepatocytes and human hepatoma cells.^{11–14} With the current study we are investigating the potential use of fetal hepatocytes.

Early developing livers are composed of bipotential progenitors expressing c-kit, albumin, α -fetoprotein (α -FP), and cytokeratin-19 (CK-19) that possess enormous proliferation activity.^{15,16} The population of these bipotential progenitors decreases as liver development proceeds.¹⁷ Also, several other mesenchymal components exist in developing liver and may be imperative for its physiological growth and development including stellate and endothelial cells.^{18–21} We have previously used embryonic mouse livers from E10 stage and successfully regulated their differentiation using exogenous growth factors, repeatedly highlighting their stemness.^{15,16,22,23} In the present study using the mouse animal model with

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mouse fetal liver cells, we begin our analysis by identifying a stage of liver development that might hold cells that possess features of both adult differentiated cells as well as a population of undifferentiated hepatic progenitors. By using E17 mouse liver cells, we examine their behavior in terms of survival, growth, function, and tissue formation in a three-dimensional perfusion culture model based on four-compartment hollow fiber membrane bioreactors.

Materials and Methods

Bioreactor

The novel bioreactor contains three independent hollow fiber membrane systems (compartments 1 to 3), which are interwoven repetitive subunits. The extracapillary space forms a compartment in which up to 10^9 cultured cells are housed (compartment 4). Two of the membrane systems are made of polyether-sulfone capillary systems (Membrana, Wuppertal, Germany) with a molecular weight cutoff of 400,000 d. Hydrophobic multilaminate hollow fiber membrane systems (MHF; Mitsubishi, Tokyo, Japan) enable gas supply. The total cell compartment volume is 8 ml. The capillary network of this multicompartment bioreactor serves three functions: medium inflow, cell oxygenation/carbon dioxide removal, and medium outflow. As a result of interweaving and decentralized medium/plasma exchange gas supply can be provided for the cultured cells.^{24,25} Sterilization is performed with ethylene oxide.

Liver and Cell Isolation

Pregnant female ICR mice were used for all experiments performed and purchased from Charles River (Wilmington, MA). Animal use and experimentation was performed under the strict guidelines of the Institutional Animal Use and Care Committee at the University of Pittsburgh School of Medicine and the National Institutes of Health. Fetal livers were harvested at E11, E14, E17, and E18 stages of gestational development. Although the livers from these stages and the adult stage were used for RNA and Affymetrix analysis as described, the E17 livers were also used for cell isolation for bioreactor inoculation.

Four pregnant females yielding a total of 40 to 50 embryos were used to dissect livers for each independent bioreactor run. The isolated livers were agitated for 30 minutes at 37°C in 0.3% collagenase (Sigma, St. Louis, MO), prepared in Eagle's modified essential medium (EMEM) with HEPES (Cambrex, Walkersville, MD) with intermittent pipetting. Next, the cells were passed through a cell strainer (BD Bioscience, Bedford, MA) and washed twice by centrifugation ($85 \times g$, 5 minutes, 4°C). The pellet was resuspended in William's medium E-based culture medium (Heparmed Biochrom, Berlin, Germany) that was supplemented with amino acids, essential fatty acids, insulin, glucagon, transferrin, selenium, L-glutamine, 3% fetal calf serum, gentamicin, and amphotericin B. Cell yields were calculated by compar-

ing the wet weight of the untreated organ buds with that of the cells after isolation. The cell viability was determined by trypan blue exclusion and ranged from 70 to 80%. E17 livers were isolated and fixed in 10% formalin followed by paraffin embedding and sectioning for immunohistochemical characterization of these livers for comparison with the bioreactor-cultured cells.

Bioreactor Cultures

The experiments were performed using pooled E17 liver cells from multiple embryos for each bioreactor run. Cell suspensions were inoculated into bioreactors and cultured throughout periods of 3 weeks. Four independent bioreactor cultures were performed. An additional 5-week experiment was performed to investigate long-term albumin synthesis and P450 activity. The cultures were continuously perfused at a flow rate of 30 ml/minute. Fresh medium was continuously added to the perfusion circuit, initially at a flow rate of 2 ml/hour, and waste medium was removed from the circuit at the same rate. Because the cultures exhibited a continuous demand of glucose starting at day 2, the feed rate was increased up to 8 ml/hour for the remaining culture period. The flows of compressed air and carbon dioxide in the gas compartment were maintained at 30 ml/minute. Partial pressures of oxygen and carbon dioxide and acid/base status were regularly measured. The carbon dioxide content was adjusted to decreasing levels throughout culture time for keeping the medium pH between 7.3 and 7.5.

The metabolic activity of the cells inside the bioreactors was characterized on a daily basis by measuring lactate dehydrogenase, aspartate aminotransferase, and glutamate dehydrogenase release, and by measuring the concentrations of glucose, lactate, and albumin in the recirculating medium, using standard clinical analysis methods. Metabolic activities of the cultures, as measured by glucose consumption, lactate production, and albumin synthesis, increased during the first culture week and then remained relatively stable up to week 5 (glucose and lactate data not shown). The levels of lactate dehydrogenase and transaminases (aspartate aminotransferase and glutamate dehydrogenase) were markedly elevated during the first culture days, then continuously decreased throughout the remaining culture period (data not shown).

Measurement of CYP3A Activity and Inducibility

We performed one 5-week bioreactor culture to assess the long-term cytochrome P450 activity and inducibility. On culture days 11 and 27, we measured the CYP3A activity by challenging the cells with testosterone (Sigma). Infusion of fresh medium and removal of waste medium was stopped, and testosterone was added to the bioreactor perfusion circuit to a final concentration of 0.25 mmol/L. Medium samples were removed from the circuit every 30 minutes for 60 minutes and stored at -20°C. The concentration of 6 β -hydroxytestosterone (a testosterone metabolite) in the medium was measured by high

performance liquid chromatography as described previously.²⁶ To induce CYP3A activity, pregnenolone carboxitrile (Sigma) was added to the culture medium for 5 days (culture days 27 through 34) at a final concentration of 25 nmol/L. A testosterone challenge was performed as described above on day 34, and the postinduction 6 β -hydroxytestosterone formation rate was measured.

Bioreactor Tissue Preparation

At the end of the culture period of ~21 days, the bioreactors were opened and tissue specimens were immediately fixed in 10% formalin. The order of the sampled tissue representing the depth in the bioreactor was noted to address the cell distribution within the bioreactor. One or two wet preparations were examined under a microscope to examine the cell organization. After overnight fixation the samples were embedded in paraffin and used for 4- μ m sections. Representative materials from various depths of each bioreactor were used for further analysis.

Histological and Immunohistochemical Analysis

Hematoxylin and eosin (H&E) staining was performed on sections from bioreactor cultures to evaluate tissue architecture and organization. Subsequently, immunohistochemistry was performed using an indirect immunoperoxidase procedure as previously described.¹⁵ Bioreactor samples were characterized using primary antibodies against α -FP and desmin from Santa Cruz Biotechnology (Santa Cruz, CA); c-kit from Oncogene (Boston, MA); albumin, CK-19, and α -smooth muscle actin (α -SMA) from DAKO (Carpinteria, CA); vimentin from Chemicon (Temecula, CA); and isolectin B4 from Vector Laboratories (Burlingame, CA). The secondary antibodies were from Chemicon and the signal was detected using the ABC Elite kit (Vector Laboratories). For negative controls, the sections were incubated with secondary antibodies only. Control samples from livers at E17 stage were examined similarly.

For proliferation assays we used antibodies against proliferating cell nuclear antigen (Signet Laboratories) and the Ki-67 marker for cells in S phase of the cell cycle (Santa Cruz Biotechnology). The ApopTag peroxidase kit (Intergen Company, Purchase, NY) was used to detect apoptosis; terminal dUTP nick-end labeling (TUNEL)-positive apoptotic nuclei were detected by the presence of brown staining. Slides were viewed on a Zeiss upright research microscope (Axioskop 40) and digital images were obtained on a Nikon Coolpix 4500 camera. Collages were prepared using the Adobe Photoshop 5.0 software.

Affymetrix Gene Expression and Analysis

Fresh-pooled livers ($n > 4$) from E11, E14, E17, E18, E19 (*in utero*), and adult stage (3 months old) were used for isolating and purifying RNA by the Qiagen RNeasy kit (Qiagen, San Diego, CA) that was used for cRNA prep-

aration and for generating a biotinylated cRNA probe from each of the developmental stages as described previously.²³ Briefly, 5 μ g of total RNA were used in the first strand cDNA synthesis with T7-d(T)₂₄ primer (GGC-CAGTGAATTGTAATACGACTCACTATA GGGAGGCGG-(dT)₂₄) by Superscript II (Life Technologies, Inc., Rockville, MD) and the second strand cDNA synthesis was performed at 16°C by adding *Escherichia coli* DNA ligase, *E. coli* DNA polymerase I, and RnaseH in the reaction followed by T4 DNA polymerase addition and purification by phenol/chloroform and ethanol precipitation. The ME-*G*Ascript system (Ambion, Inc., Austin, TX) was used for *in vitro* transcription reaction to produce biotin-labeled cRNA for Affymetrix chip hybridization (U74A). Fragmented 15 to 20 μ g of cRNA were hybridized with a pre-equilibrated Affymetrix chip at 45°C for 14 to 16 hours followed by initial low stringency washes in 6 $\times$ sodium chloride/sodium phosphate/ethylenediamine tetraacetic acid, 0.01% Tween 20, 0.005% antifoam-containing buffer followed by washes in a stringent buffer (100 mmol/L morpholine-ethane-sulfonic acid (MES), 0.1 mol/L NaCl, and 0.01% Tween 20) and staining with streptavidin phycoerythrin, biotinylated mouse anti-avidin antibody and restaining with streptavidin phycoerythrin. Next, the chips were scanned for hybridization signals in a HP ChipScanner (Affymetrix Inc., Santa Clara, CA) and the final analysis was performed using Affymetrix microarray suite 5.0 software. The data were exported and organized in Excel spreadsheet (Microsoft Office application) to examine changes in various *cytochrome P450* genes. Some positive markers of differentiation aided in validating results by acting as internal controls. These changes were representative from multiple livers from the same stages of development and were also analyzed for statistical significance.

Statistical Analysis

We presented data from four independent bioreactor experiments. For each experiment, four mice and 40 to 50 fetal livers were pooled. Representative materials from six sections each from eight bioreactor locations were analyzed. For immunohistochemical stains, numbers of positive cells were counted from each section to calculate the percentage of positive cells. Due to some background staining care was taken to only include the cells showing nuclear positivity for proliferating cell nuclear antigen (PCNA) and Ki-67 for quantitative assessment. A comparable analysis was performed with E17 fetal tissue liver as well. Cells were carefully counted in three representative high-power fields and averaged and compared for statistical significance using the Student's *t*-test. The value of less than 0.05 was considered to be statistically significant. The comparisons were also made separately between the E17 livers and ribbon structures or E17 livers and all positive sheet/ribbon arrangements for further improving the interpretation of the immunohistochemical stains.

Table 1. Gene Expression of Metabolism-Related Genes during Mouse Liver Development

Gene	E11	E14	E17	E18	A
Albumin	20251.4	46789.7	85228.3	92631	95749.1
α FP	3067.1	2755.8	6584.8	5563.2	33.1
C-kit	549.8	382.9	320.2	390.9	98
Glycerolphosphate dehydrogenase 1	138.7	410.5	1073.7	1126.3	2033.6
NADP-dependent isocitrate dehydrogenase	972.5	928.4	1654.2	1688	3807.1
Acetyl coenzyme A dehydrogenase	1099.5	927.5	7649.4	10631.7	5308.2
Paroxysmal phytanoyl-CoA α -hydroxylase	847.1	831.7	5538.1	6659.4	20729.1
CYP450 oxidoreductase	796	517.4	1785.7	2210	4875.2
Steroid cytochrome p450 7- α hydroxylase	264.8	243	1479.1	977.9	13487.6
CYP 3a13	220.5	113.8	864.9	1388.2	1176.1
CYP 3a16	7	9.4	1675.6	1956.8	28592.8
CYP 4a10	73.4	46.3	420.8	730.8	2996.1
CYP 7a1	14.9	15.6	340.3	400.8	2927.9
CYP 2c37	74.9	56.8	908.7	1098.3	15109.4
CYP 2d9	401.3	178.5	2871.9	3667.3	9681.1
CYP 2d10	258.4	529.2	1721.6	2562.9	16071.7
CYP 2d11	481.4	665.4	2733.5	4292.2	34428.8
Haptoglobin	1362.9	606.7	13664.1	12467.2	42768.2
Apolipoprotein A-1	8539.8	24299.4	73048.9	82593.5	94589.7
Glycogen storage disease type 1b protein	396.6	634.4	3037.8	5894	5779.5

E, Embryonic day of mouse gestational development; A, adult liver (3 months old).

Results

E17 Fetal Liver Cell Population

In the search for a suitable cell source for bioreactor studies, we chose the mouse animal model. Our aim was to use a cell source with the potential of becoming functional hepatocytes while possessing progenitor properties to provide a continuous replenishing source of hepatocytes *in vitro*. To this end we examined the gene expression pattern from pooled whole livers from E11, E14, E17, E18, and adult livers using the Affymetrix U47A mouse gene chip. The analysis revealed multiple genes displaying temporal expression. Although the known expression patterns of various genes such as albumin and α -FP from previous studies were invaluable to validate the current analysis by acting as internal controls, several other gene expression patterns revealed stage-specific properties. When comparing the stage-specific expression, high expression of α -FP (hepatic progenitor and immature hepatocyte marker) and albumin (progenitor, immature, and mature hepatocyte marker) was observed at E17 stage (Table 1). At the same time the E17 hepatocytes displayed high c-kit (progenitor marker) expression compared to the adult. In addition, the analysis revealed multifold (ranging from 4- to 20-fold) increases in gene expression of a number of metabolic enzymes such as glycerol phosphate dehydrogenase, isocitrate dehydrogenase, acetyl coenzyme A dehydrogenase, peroxisomal phytanoyl-CoA α -hydroxylase, CYP450-oxidoreductase, steroid cytochrome p450 7- α hydroxylase from E14 to E17 indicating initiation of function by the resident hepatocytes (Figure 1A). Furthermore, apart from albumin, an increase in synthetic activity of resident hepatocytes from E11/E14 to E17 is also reflected by a many fold increase in the gene expression of haptoglobin (22-fold), apolipoprotein A-1 (fourfold), glycogen storage disease type 1b protein (fourfold), and others (Figure 1A). Similarly, many cytochrome P450s that also discern initi-

ation of function in resident hepatocytes undergo a many fold increase from E11 and E14 to E17 stage, while the most prominent changes are observed in CYP450 3a13, 3a16, 4a10, 7a1, 2c37, 2d9, 2d10, and 2d11 (Figure 1B). These gene expression studies situate the resident hepatocytes at E17 stage between the early progenitors and fully differentiated hepatocytes with intermediate differentiation and stemness, thus making them an attractive cell source for investigation in bioreactor applications.

Tissue Histology in Bioreactors

Five independent bioreactor experiments were performed and data presented is representative of these experiments. After ~21 days of culture with active cells, the bioreactors were opened for tissue analysis. Grossly, robust tissue-like presence was observed that surrounded the interwoven hollow fiber systems. A wet preparation from the cells fixed in formaldehyde showed the presence of ribbon-like arrangements that extended between the hollow fibers (Figure 2A). H&E staining from paraffin sections confirmed the presence of these tissue-like structures that traversed the hollow fibers (Figure 2B). The cells forming these structures possessed mainly hepatocytic morphology with a few ductal structures with flattened cells lining the edges of the ribbon (Figure 2, C and D). Also, there appeared to be sinusoidal-like spaces that were visible between one to two layers of hepatocytes, and ran perpendicular to the hollow fibers reflecting the medium flow in these spaces. These spaces appeared to be lined with endothelial cells in many but not all instances (Figure 2D).

Fetal Hepatocytes Show Robust Proliferation and Minimal Apoptosis in Bioreactors

Sections were examined for cell proliferation. PCNA staining exhibited a high number (>95%) of cells in cell

Figure 1. Microarray analysis identifies a multifold increase in gene expression of several genes that reflect synthetic and metabolic function of hepatocytes. **A:** A graph from a representative gene array analysis demonstrates a notable increase in the expression of many genes such as of *albumin*, *glycerol phosphate dehydrogenase*, *acetyl coenzyme A dehydrogenase*, *cytochrome p450 oxidoreductase*, *apolipoprotein A-1*, *glycogen storage disease protein 1B*, and *haptoglobin* from E14 to E17 stage (area highlighted), demonstrating acquisition of adult hepatocyte-like synthetic and metabolic functions. **B: Left:** a similar analysis for several cyp450s (3a13, 3a16, 4a10, 7a1, 2c37, 2d9, 2d10, 2d11) reveals their low expression in E11 and E14 livers with notable up-regulation in E17 liver (highlighted), although these are still low as compared to their respective adult levels. **Right:** Enlarged highlighted area from the **left** panel demonstrating a 5- to 100-fold increase in gene expression of these cyp450s in livers from E14 to E17 stage.

cycle as demonstrated by nuclear positivity (Figure 2E). Most of the cells that were negative for PCNA were the flattened cells lining the edges of the ribbon-like structures. A minority of the cells toward the middle of the ribbon-like structures were PCNA-negative as well (Figure 2F). Another observation was a more intense PCNA positivity of the cells in the peripheral zones in the ribbons as compared to less intensely stained cells in the middle region of these structures. Ki-67 staining was next used to identify cells specifically in S phase of

cycle or actively proliferating cells. Only the cells in the peripheral zones of the ribbon structures were Ki-67-positive in their nuclei whereas the cells occupying the middle areas were consistently Ki-67-negative (Figure 2G). High-magnification view also substantiates Ki-67 nuclear positivity in the peripheral area of ribbon structures (Figure 2H). Although background cytoplasmic staining was an issue, only cells with nuclear Ki-67 staining were counted for quantitative analysis that revealed ~20% of all cells to be in S phase. Although

Figure 2. Histology, proliferation, and apoptosis assay of the fetal hepatocytes cultured in bioreactors for 21 days. **A:** A wet mount of the fixed material under an inverted microscope reveals island of hepatocyte-like cells (**arrow**) that extend from one hollow fiber (**arrowhead**) to the other. **Inset** displays a ribbon-like structure formation. **B:** H&E staining (low power) shows a few cell-thick ribbon structures (**white arrow**) that attach to the hollow fibers (**arrowhead**). Primitive neosinusoidal channels (**arrow**) can be observed in these structures. **C:** H&E staining reveals hepatocytes (**black arrowhead**) within the newly formed tissue. The neosinusoidal spaces (**arrow**) and duct structure (**white arrowhead**) can also be observed. **D:** Another representative H&E staining also reveals ductal structure (**white arrowhead**) and some flattened cells (**arrow**) lining the edge of the tissue. **E:** PCNA immunohistochemistry identifies several proliferating cells (**arrow**) in ribbon arrangements. Note the negative cells (**arrowhead**) with flattened morphology at the edges of the tissue. **F:** High-power view also shows PCNA-positive cells (**arrow**) in ribbon structures with a few cells in the center and the flattened cell lining staining negative (**arrowhead**). **G:** In an adjacent section to **E**, several Ki-67-positive cells (**arrow**) are observed in mainly the peripheral area of ribbon-like structures. The cells in the middle part are negative (**arrowhead**). The flattened cells that line the edge of the tissue are negative (**white arrowhead**). **H:** High-power view also emphasizes the differential distribution of Ki-67-positive cells (**arrow**) toward the periphery of ribbon structures, with predominantly negative (**arrowhead**) cells toward the center. **I:** Some areas of ribbon structures show a more uniform PCNA positivity along the entire width with several cells intensely positive for PCNA (**arrow**). **J:** An adjacent section of the same area as **I** also showed Ki-67-positive cells (**arrow**) occupying the entire width of this part of the ribbon structure. **K:** Very few apoptotic nuclei (**arrow**) are observed among the predominantly negative cells (**arrowhead**) in ribbon-like tissue arrangements by TUNEL staining. **L:** In the sheet-like arrangement also, <5% of all cells were TUNEL-positive (**arrow**) with the remaining negatively staining cells (**arrowhead**). Original magnifications: $\times 4$ (**B**); $\times 20$ (**C–E**, **G**, **I–L**); $\times 40$ (**F**, **H**).

this pattern was the predominant type, a few ribbon-like structures displayed a more uniform and heavy PCNA positivity along the entire thickness of these

structures barring the flattened cells lining either edge (Figure 2I). In the corresponding adjacent section, staining for Ki-67 was similarly observed along the

entire width of the ribbon structures (Figure 2J) indicating heavy proliferation in these areas.

Lastly, we examined the survival of the fetal liver cells in the bioreactors using TUNEL staining to detect apoptotic nuclei. Less than 5% of cells within various arrangements were TUNEL-positive and no zonal preference was observed especially in the ribbon-like structures (Figure 2K). However, although a few sections showed ~10 to 15% TUNEL-positive cells, some sections also displayed less than 1% TUNEL positivity. Furthermore, a comparable number (<5%) of TUNEL-positive cells were seen in the ductal arrangements as well (Figure 2L).

Differentiation Analysis Reveals Specific Cellular Configuration and Maintenance of Progenitor Population within the Bioreactors

The next step in characterization was to confirm the cell type in the neo-tissue arrangements in the bioreactors by immunohistochemistry using known cell lineage markers in liver biology. The initial analysis was aimed at identifying the cellular arrangements within the ribbon-like and sheet-like structures. After that, conclusions were based on the examination of consecutive sections for various markers such as albumin, α -FP, c-kit, and CK-19. Immunohistochemistry for albumin that stains for mature hepatocytes, immature hepatocytes, and bipotential progenitors revealed that almost all of the cells in the ribbon-like structures were albumin-positive, except the cells at the edges (Figure 3A). Next, cell staining for α -FP, which is a marker of immature hepatocytes and bipotential progenitors, was examined. A distinctive pattern of staining was observed with α -FP-positive cells in peripheral zones of the ribbons (toward both edges) with the center part comprised of negatively staining cells (Figure 3D). C-kit is another marker for hepatic progenitor cells. We observed a pattern very similar to that of α -FP with cells in the peripheral zones being strongly positive for c-kit in ribbon structures (Figure 3G). CK-19 is a marker of differentiated biliary epithelial cells as well as bipotential progenitors. Although we were unable to detect typical bile duct-like structures with tall columnar cells, several cells at the periphery of ribbons were CK-19-positive (Figure 3J). To directly address the relationship of these cells, which were positive for various markers, to each other, the adjacent sections were examined. Several layers in the ribbon-like structures were albumin-positive (Figure 3B). High magnification displayed clear hepatocyte morphology of these albumin-positive cells, which occupy these structures with a few negative cells toward the middle of this representative section (Figure 3C). An adjacent section showed a subset of these albumin-positive cells to be α -FP-positive (Figure 3E). These cells occupied more peripheral zones in the ribbons, whereas the cells toward the middle zone that were albumin-positive were negative for α -FP (Figure 3, E and F). A comparable pattern was also observed for c-kit positivity, in which the majority of the positively staining cells were located in the peripheral region (Figure 3, H and I). CK-19 positivity also paralleled

α -FP and c-kit with positive cells situated toward either side in ribbon-like structures (Figure 3, K and L). This demonstrates a unique arrangement of fetal hepatocytes within the observed predominant ribbon-like arrangements in the bioreactor cultures. It appears that while the more differentiated albumin-only positive hepatocytes are seen in the middle zones of the ribbons, the more undifferentiated cells, simultaneously positive for albumin, α -FP, c-kit, and CK-19, occupied the peripheral zones.

A minority of these ribbon-like structures showed areas that were not only positive for albumin in their entire thickness (not shown), but were concurrently positive for α -FP (Figure 3M), c-kit (Figure 3N), and CK-19 (Figure 3O) in adjacent sections. These areas also showed a more uniform PCNA and Ki-67 positivity as already discussed previously (Figure 2, I and J). However, there were a few other isolated sheet-like structures or tissue islands that were comprised of either all progenitors or differentiated cells based on their staining characteristics for the above discussed markers (not shown). Such patterns depicted a minority of spontaneous cellular arrangements within the bioreactors.

Distinct Nonhepatocytic, Mesenchymal Cell Arrangements within Bioreactor Cultures

The cultures were also examined for other cell types native to the normal liver. The cultures were analyzed for endothelial cells and stellate cells, specifically, and for other mesenchymal components. The ribbon-like and tissue island structures were subjected to isolectin-B4 staining that detects endothelial cells. The flattened cells at the edges of these structures were strongly positive for isolectin, whereas the hepatocytes were negative (Figure 4A).

Next, the tissues were examined for stellate cells. Desmin staining revealed some areas in the ribbon arrangements that contained positive cells (Figure 4B). Again, the majority of the cells were negative for desmin indicating only a small yet persisting population of stellate cells in these cultures. The positively staining cells were larger than the nonstaining counterparts and exhibited a punctate pattern (Figure 4C). However, several areas in both forms of ribbon-like arrangements were completely devoid of any desmin-positive cells (Figure 4D). We also examined the cultures for smooth muscle cells or myofibroblasts that are mostly derived from the stellate cells. A minority of the areas in the bioreactors showed strong α -SMA positivity where dense fibrotic bands were clearly evident between hepatocytes (Figure 4E). Most of the arrangements showed minimal to no staining for α -SMA (Figure 4F). Vimentin staining was next performed to examine the neo-tissue formation for additional mesenchymal components. Most of the areas were negative with only an occasional positive cell (Figure 4, G and H). However a few isolated areas had additional positively staining cells (Figure 4I).

Figure 4. Bioreactor cultures also display mesenchymal differentiation. **A:** Isolectin-staining positive cells, with flattened morphology (arrowhead), are observed at the edges of this sheet-like arrangement of cells that is composed of many negatively staining epithelial cells (arrow). **B:** Desmin staining reveals an area predominantly composed of nonepithelial cells to have several desmin-positive stellate cells (arrowhead). Most other cells were negative (arrow). **C:** A high-power view of **D** reveals a large stellate cell with punctate desmin staining (arrowhead). **D:** Several other areas in ribbon formations were devoid of desmin-positive cells as seen in low magnification in left panel and high power in right panel that display mainly epithelial cells (arrow). **E:** α-SMA staining shows certain areas of the culture to contain myofibroblasts (arrowhead) interspersed between negatively staining epithelial cells (arrow). **F:** Most areas in ribbons had only minimal α-SMA positivity (inset and arrowhead) whereas most other epithelial cells were clearly negative (arrow). **G:** Vimentin staining was negative in most of the ribbon arrangements (arrow). **H:** High-power view also shows predominantly negatively staining cells for vimentin (arrow) with very few faintly positive cells that have hepatocyte morphology (arrowhead) and might represent EMT. **I:** Only a few areas showed more numbers of vimentin-positive cells (arrowhead and inset at left). Other areas showed larger stellate cells to be positive for vimentin as well (right). Original magnifications: × 20 (A, B, F, G); × 40 (C, E, inset in F, H).

Figure 3. Differentiation of fetal hepatocytes in three-dimensional capillary beds after 21 days in culture. **A:** Albumin-positive cells (arrowhead) are seen occupying most of the ribbon structures. Negative cells (arrow) are flattened cells that line the edge of the tissue. **B:** Another similar ribbon-like structure showing most cells to be albumin-positive. **C:** High power of **B** reveals albumin-positive hepatocytes (black arrowhead) several of them arranged being traversed by neosinusoidal structures (white arrowhead) and lined at either edge by flattened albumin-negative cells (white arrow). **D:** α-FP-positive cells (black arrowhead) are observed toward either peripheral zone in the ribbon structures. The cells in the middle zone (black arrow) and the flattened cell lining at the edge (white arrow) stains negative for α-FP. **E:** An adjacent section to **B** displays a subset of albumin-positive cells, mostly toward either periphery (arrowhead) to be α-FP-positive. Again the flattened cells toward the edges are negative. **F:** High power of **E** clearly shows α-FP-positive cells toward the periphery (arrowhead) and the negative cells toward the middle of the ribbon-like structures (black arrow). The flattened endothelial cells are α-FP-negative (white arrow). **G:** C-Kit-positive cells (arrowhead) are observed toward the periphery with negatively staining hepatocytes (black arrow) in the central region and flattened cells at the edges (white arrow). **H:** An adjacent section to **B** and **E** also demonstrates c-kit-positive cells toward the periphery (arrowhead) and negative cells in the middle zone (black arrow) and at the edges (white arrow). **I:** High power shows intensely staining c-kit-positive cells toward the side of ribbons (arrowhead) surrounding the negative hepatocytes in the middle (black arrow). Flattened cells at edges were c-kit-negative (white arrow). **J:** CK-19-positive cells (arrowhead) are observed at the peripheral zones in ribbon structures whereas the negative cells (black arrow) occupy the middle zone. Again the flattened cells at the edge were negative (arrowhead). **K:** An adjacent section to **B**, **E**, or **H**, also shows predominant CK-19-positive cells toward the side (arrowhead) whereas cells in the middle (black arrow) or edges (white arrow) are negative. **L:** A high power of this area reveals hepatocyte-like cells at the periphery to be CK-19-positive (arrowhead) and in the central region to be negative (arrow). **M:** Minority of ribbon-like structures displayed most cells (almost entire thickness) to be α-FP-positive (arrowhead) with a few negatively staining cells (black arrow). Flattened cells at edges were always negative (white arrow). **N:** An adjacent section to **M** shows the same cells to be also positive for c-kit (arrowhead) throughout the thickness of such ribbon structures. Again a few cells were negative (black arrow) and the cells at edges were negative as well (white arrow). **O:** Another adjacent section to **M** and **N** displaying similar distribution of CK-19-positive cells (arrowhead) with a few negatively staining cells interspersed in between (black arrow) or observed at edges (white arrow). Original magnifications: ×20 (A, B, D, E, G, J, K, M–O); ×40 (C, F, I); ×60 (L).

Figure 6. Comparison of cell populations after culture of E17 liver cells in bioreactors for 21 days. A significant increase in the number of PCNA- and Ki-67-positive cells after the culture of E17 cells indicates robust cell proliferation ($P < 0.01$). No adverse increases in TUNEL-positive cell numbers were observed after the culture of E17 liver cells indicating apoptosis within physiological limits and good cell viability of fetal hepatocytes in the bioreactors. Although the numbers of albumin-positive cells remained unaltered, a significant increase was observed in the numbers of α -FP-, c-kit-, and CK-19-positive cells ($P < 0.05$). Although α -FP positivity was comparable to the E17 livers in the ribbon-like structures, tissue islands were composed of either all positive or all negative cells (* $P < 0.05$; + cells are counted in tissue islands that were composed of all positive cells for α -FP, c-kit, and CK-19).

Comparison with E17 Livers for Mechanism of Differentiation

To understand the development of the observed phenotype within the bioreactors, we examined and compared the proliferation, apoptosis, and differentiation status of the resident cells within the bioreactors to the E17 livers that were the source of the inoculated population. Approximately 25 to 30% of resident hepatocytes in E17 livers were PCNA-positive (Figure 5A). On analysis for Ki-67 ~10 to 15% of cells were Ki-67-positive (Figure 5B). These observations were significantly different from the ones in bioreactors that displayed ~95% and 25% of PCNA and Ki-67-positive cells respectively ($P < 0.01$), indicating robust proliferation of the fetal hepatocytes in the bioreactors (Figure 6). TUNEL immunohistochemistry for E17 livers as

well as bioreactors displayed comparable numbers of apoptotic nuclei that ranged from 5 to 10% indicating physiological cell death during this stage of liver development (Figure 6).

Most of the E17 liver cells were albumin-positive (Figure 5C). This was comparable to the number of albumin-positive cells in the bioreactors thus showing an insignificant change between these two stages (Figure 6). Although high gene expression for the α -FP gene was observed in the E17 livers, the number of α -FP-positive cells was ~50% of all cells (Figure 5D). When compared to the number of cells in the bioreactors that were arranged in ribbons in the predominant peripheral zones, this number was higher but statistically insignificant (Figure 6). However, if the number of positive cells from E17 livers are compared to the sheet-like arrangements and ribbons composed of positively staining cells in their entire thickness (~90% positive cells), the increase is statistically significant in the bioreactor cultures ($P < 0.05$) (Figure 6) highlighting an overall expansion of these cells. Although most cells in E17 liver were negative for c-kit expression (Figure 5E), only ~5% of all cells were positive (Figure 5F), indicating the presence of a small population of bipotential stem cells that was interspersed between the mostly negatively staining, more differentiated, cells, which were albumin- and/or α -FP-positive. However, an extremely significant ($P < 0.001$) increase in these numbers was observed in both forms of ribbon structures and sheet arrangements in bioreactor cultures that displayed (Figure 6). Lastly CK-19 staining in E17 livers was limited to either bile ducts or plates being defined for newer bile ducts, and bipotential progenitors (Figure 5, G and H). Although a few duct-like structures were observed in bioreactors, they do not seem to be lined by true biliary epithelial cells as discussed earlier. However, a parallel increase comparable to the number of α -FP- and c-kit-positive cells was observed in bioreactor cultures, which was also statistically significant ($P < 0.001$) (Figure 6). These observations indicate a spontaneous expansion of the bipotential progenitor population within the bioreactors along with maintenance of differentiated hepatocytes.

P450 Enzyme Induction after Long-Term Bioreactor Culture

Selected liver-specific activity was studied in one long-term experiment throughout 5 weeks (Figure 7). Interest-

Figure 5. Proliferation and differentiation in E17 mice livers. **A:** PCNA-positive cells (arrow) constitute ~30% of E17 liver as seen in this representative section. Most other cells are PCNA-negative (arrowhead). A higher magnification inset shows positive (arrow) and negative cells (arrowhead) with greater precision. **B:** A few (<15%) cells in E17 liver were Ki-67-positive (arrow). The predominant cell population was Ki-67-negative (arrowhead). Inset shows positively (arrow) and negatively staining cells (arrowhead) more clearly. **C:** A representative section shows almost all cells to be albumin-positive (arrowhead) in an E17 liver. A higher magnification inset shows a hematopoietic cell staining negative (arrow) as compared to other cells that are albumin-positive (arrowheads). **D:** Approximately half of the cells in E17 livers were α -FP-positive (arrowhead) whereas the remaining cells were negative (arrow). A higher magnification shows α -FP-positive (arrowheads) and -negative (arrows) cells. **E:** A representative section from an E17 liver shows all cells negative for c-kit (arrow). **F:** Another representative section shows a small group of c-kit-positive cells (arrowhead) within predominantly negative cells (arrow). **G:** Left panel represents all cells negative for CK-19 (arrow) in a representative E17 liver section. The right panel represents isolated CK-19 cells (black arrowhead) representing progenitor cell fraction. Although most cells are CK-19-negative (arrow), primitive portal plates (white arrowhead) are CK-19-positive. **H:** Another representative section from E17 liver demonstrates isolated CK-19-positive cells (black arrowhead), portal plates (white arrowhead) among mostly CK-19-negative cells (arrow). Original magnifications: $\times 40$.

Figure 7. Long-term experiment to measure albumin liberation (triangles, left axis) and P450 activity (CYP3A) (bars, right axis). Albumin liberation increased for the first week, then maintained a relatively stable rate for the remaining 4 weeks. Testosterone metabolism to 6β-hydroxytestosterone doubled after pregnenolone carbonitrile induction.

ingly, after opening the 5-week cultures, we still observed several areas that displayed lack of confluency in the cell compartments between the artificial capillary membranes. Albumin liberation increased for the first week, then maintained a relatively stable rate of ~80 μg/hour for the remaining 4 weeks. Testosterone metabolism to 6β-hydroxytestosterone was ~3.47 nmol/minute on day 11 and 3.08 nmol/minute on day 27. We performed a 5-day P450 enzyme induction using pregnenolone carbonitrile during week 5, and the metabolism (CYP3A) doubled to 7.33 nmol/minute.

Discussion

Bioreactors for extracorporeal liver support may offer a therapeutic option for assisting the failing liver until regeneration occurs or until transplantation can be performed. Recent clinical reports describe the use of dialysis/plasma separation cartridges, each containing 50 to 200 g of primary hepatocytes or liver tumor cells and applied singly for 6 to 8 hours per day.⁴ In this type of two-compartment cartridge the cell mass is restricted due to the limitations of oxygen and plasma transfer. Four functional compartments are required to support integrated oxygenation and controlled mass exchange with physiological gradients.² Our four-compartment bioreactor consists of interwoven, independent capillary membrane systems. Two capillary compartments provide medium flow with high-mass exchange efficiency and low solute gradients within the cell compartment. The third compartment provides a well-distributed integral oxygen supply to the cells localized in the extracapillary space between all hollow fibers (fourth, cell compartment). This three-dimensional network of artificial capillaries represents an artificial equivalent to the hepatic vasculature at the lobular level.²⁴ We have already seen that human adult liver parenchymal cells in co-culture with nonparenchymal cells aggregate and immobilize to the capillary surface and spontaneously form parenchyma-like struc-

tures, resulting in a spontaneous reassembly of liver cell structures at the sinusoidal level.²⁵ The present study uses this technology to examine the behavior of the more plastic and proliferative population of mouse fetal hepatocytes.

Cell source issues have been among the major limiting factors for the successful implementation of liver cell-based bioartificial liver development.^{11,27,28} A wide variety of cell types have been examined for their potential use in the bioreactors but with mixed results.¹³ Initial clinical studies, using primary porcine liver cells for extracorporeal liver support, were performed by others and by our group.^{6,11,13} However, the use of primary porcine cells is controversial for various reasons, including the possible transfer of porcine endothelial retroviruses and the risk of immune reactions by the contact with xenogeneic proteins.¹² The use of hepatocyte tumor cell lines is associated with a risk of metastases and different metabolic patterns compared with primary hepatocytes.^{28,29} We examined fetal hepatocytes in a laboratory-scale version of our liver support bioreactor for liver support to characterize their behavior within the four-compartment three-dimensional hollow fiber arrangements providing artificial capillary beds.

Major limitations with the use of adult human primary hepatocytes in bioreactor applications is their low initial viability, decreasing activity, cell death, apoptosis, and limited proliferative capacity. Fetal liver cells with progenitor characteristics may address this problem because they could provide intermediate function as well as replenishing the pool of progenitors to repopulate the lost mass. Several laboratories demonstrate bipotential progenitors in developing livers.^{30–32} We investigated the use of fetal hepatocytes in liver support bioreactors, choosing E17 livers based on functional and molecular parameters at this stage. These cells demonstrate an intermediate function and differentiation as compared to earlier development or later mature stages based on gene array analysis. The progenitor cell characteristics are reflected by high expression of α -FP as compared to its absence in adults. More differentiated characteristics are reflected by high expression of albumin gene as compared to earlier stages. Similarly other genes representing metabolic or synthetic functions such as haptoglobin, cytochrome P450s, and others show an intermediate expression between earlier and adult stages.^{33,34} This analysis reveals an initiation of a trend of gain of hepatocyte function and at the same time a maintenance of a small population of bipotential progenitor cells as seen by c-kit-positive cells (also positive for albumin, α -FP, and CK-19).

On inoculation of these cells into the bioreactors, viability of the cell mass was maintained and the investigated biochemical (see Materials and Methods) and histological parameters indicated a considerable cell growth, which was confirmed after opening and inspecting the bioreactor. An initial increase in lactate dehydrogenase and transaminase release can be interpreted as the result of cell injury due to enzymatic treatment during cell isolation. Isolation injury may also lead to the low onset of activity during the initial culture phase. Despite

these early observations, there was a continuous glucose uptake, lactate production, and albumin synthesis. The observed rising metabolic activities after day 3, with the need to increase the culture medium feed rate, indicated a transition from the adaptation and recovery period into active cell proliferation. The carbon dioxide supply had to be reduced on a daily basis to maintain physiological levels of pH in the bicarbonate-buffered medium. This also contributed to stabilization of the biochemical parameters that were comparable to the time course observed in primary cultures of human hepatocytes.³⁵ The fetal liver cells, however, started to continuously become more active throughout the culture period of 3 weeks, unlike the well-known results using adult hepatocytes. Because adult hepatocyte cultures demonstrate a continuing decline of activity starting after ~3 weeks, we performed a long-term experiment throughout 5 weeks demonstrating relatively stable albumin liberation by the fetal cells. In addition, cytochrome P450 induction in the fifth week resulted in a twofold increase in testosterone metabolism (CYP3A).

Immunohistochemistry revealed significant cell proliferation as detected by a high number of PCNA- and Ki-67-positive cells. Furthermore, cell viability was maintained and ~5% of cells showed apoptotic nuclei (well within the physiological limits). The balanced apoptosis in the growing cell mass is suggestive of ongoing architectural reorganization within the bioreactors.

Fetal hepatocytes from E17 underwent a notable change in their differentiation status in the bioreactor cultures. Initial population of cells was predominantly albumin-positive with a subset of α -FP-positive, and a further subset of c-kit- and CK-19-positive cells. After culture, these cells retained albumin positivity and ~50% of these cells in the peripheral areas of ribbon-like structures also showed concomitant positivity to c-kit, CK-19, and α -FP reflecting an increase in bipotential progenitor numbers. This could be due to spontaneous dedifferentiation of fetal hepatocytes as is seen in primary adult hepatocyte cultures, which can be induced to undergo redifferentiation by addition of extracellular matrix (Matrigel).^{36,37} Alternatively, the existing bipotential progenitors in E17 livers could have undergone expansion secondary to yet unknown cues or microenvironment within the bioreactors. Further investigation, such as co-cultures with labeled cells to track their fate, will be essential to address such questions. However, a maintained pool of expanded progenitors could be an innate cell-replenishing source. Analysis by exclusion demonstrates that the more differentiated cells (only albumin-positive) occupy the central areas of the ribbons, while the progenitors stay peripheral, just inside of the endothelial lining. Again, the mechanism of this arrangement is unclear but might be influenced by the flow dynamics dictating parameters such as relative oxygenation. Also, concurrent positivity of PCNA and Ki-67 in the progenitor cells suggests an ongoing but balanced proliferation, in addition to regulated differentiation in these cells. These two functions have been shown previously to be relatively independent in liver development.³⁸ Other minor arrangement of sheet arrangements were composed of only differentiated (albumin-positive) cells or bipotential progenitors (c-kit, CK-19, α -FP, and albumin-positive). Thus

after long-term culture, we observed a heterogeneous population of differentiated and undifferentiated cells, and while the former enabled function, the latter conveyed longevity to the culture by maintenance of endogenous cell renewal source.

Although, we have demonstrated maintenance of a hepatoblast fraction throughout several weeks, we have to question the effect of procedural or technical limitations. Cell confluency was not fully reached in these experiments and that might explain prevalence of at least part of the progenitor pool that has been reported previously as well.³⁹ One strategy to prevent confluency, if this becomes an issue, would involve isolation of confluent cells by trypsinization and reseeding into additional reactors at lower density. Although speculative at present, this cell passaging might also address the cell expansion issue toward developing progenitor cell-based therapies. However, in the present study cell confluency was not an issue and with the acquired cell density, we demonstrate appropriate hepatocyte functionality as well as progenitor component.

The bioreactor cultures were also examined for other cell types such as biliary epithelial and mesenchymal cells native to the liver. We observed ductal structures that were CK-19-positive but the typical columnar morphology was lacking. Moreover several such cells were simultaneously positive for c-kit, α -FP, and albumin. Flattened endothelial cells, positive for isolectin B4, were observed at the edges of the ribbons or sheets or lining some of the neo-sinusoidal spaces between hepatocyte cords. This might be a consequence of the flow dynamics especially at the tissue-hollow fiber interface. Presence of desmin-positive stellate cells was also evident in the cultures. A few areas also showed α -SMA-positive myofibroblast-like cells indicating stellate cell function as well as existence of smooth muscle cells. Some areas containing vimentin-positive cells indicated the presence of other mesenchymal components as well. It also appears that several cells with hepatocyte-like morphology were positive for mesenchymal markers, indicating epithelial to mesenchymal transition within the neo-tissue formation. This has been demonstrated previously in our cultures and other hepatocyte culture systems such as roller bottle cultures.²¹ Also, from the integrity of the tissue, we believe that E17 fetal cells are sufficient to produce and maintain their own extracellular matrix. This has been demonstrated previously in our and other hepatocyte culture systems such as roller bottle cultures.^{25,40} Thus presence of various normal cell types in the cultures might be assisting in the maintenance of structure and function in the neo-tissue formations.

Bioreactors enabling proliferation and differentiation of liver cells while maintaining a progenitor population may address hepatic cell source issues in regenerative medicine research. Our study revealed an interesting cell source for such study. Further studies are underway to examine the behavior of sorted purified cells from developing livers, alone or in combination. This would be of special relevance in using human cells, to be able to propagate human cells or even generate cell lines or clones for possible bioreactor applications.

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