Background. New bioartificial liver devices are needed to supplement the limited supply of organ donors available for patients with end-stage liver disease. Here, we report the results of a pilot study aimed at developing a humanized porcine liver by transplanting second trimester human fetal hepatocytes (Hfh) co-cultured with fetal stellate cells (Hfsc) into the decellularized matrix of a porcine liver.

Material and Methods. Ischemic livers were removed from 19 Yorkshire swine. Liver decellularization was achieved by an anionic detergent (SDS). The decellularized matrix of three separate porcine liver matrices was seeded with $3.5 \times 10^8$ and $1 \times 10^9$ of Hfsc and Hfh, respectively, and perfused for 3, 7, and 13 d. The metabolic and synthetic activities of the engrafted cells were assessed during and after perfusion.

Results. Immunohistologic examination of the decellularized matrix showed removal of nuclear materials with intact architecture and preserved extracellular matrix (ECM) proteins. During perfusion of the recellularized matrices, measurement of metabolic parameters (i.e., oxygen concentration, glucose consumption, and lactate and urea production) indicated active metabolism. The average human albumin concentration was $29.48 \pm 7.4 \text{ mg/mL}$. Immunohistochemical analysis revealed cell differentiation into mature hepatocytes. Moreover, 40% of the engrafted cells were actively proliferating, and less than 30% of cells were apoptotic.

Conclusion. We showed that our decellularization protocol successfully removed the cellular components of porcine livers while preserving the native architecture and most ECM protein. We also demonstrated the ability of the decellularized matrix to support and induce phenotypic maturation of engrafted Hfh in a continuously perfused system.

Key Words: tissue engineering; liver transplantation; hepatocyte transplantation; bioartificial liver; end-stage liver disease.

INTRODUCTION

Liver transplantation (LT) is the treatment of choice for patients with end-stage liver disease (ESLD); however, the shortage of deceased donor organs limits the success of this approach [1]. Other surgical techniques (e.g., split-liver transplantation, living-related-liver transplantation, domino-liver transplantation, and donation after cardiac death) are available, but modest organ yields with these techniques do not overcome the donor shortage. Xenotransplantation using porcine livers may provide an additional source of organs; however, concerns regarding immunologic reactions, physiologic differences, and zoonotic disease transmission make it unlikely that xenotransplantation will replace or supplement allotransplantation. Therefore, other alternatives are desperately needed.

Hepatocyte transplantation in the form of either isolated cell transplantation or cells incorporated into bioartificial liver bioreactors has been proposed as an alternative method for treating patients with metabolic liver disorders and for supporting patients with acute liver injury until they recover or undergo LT [2, 3]. Although clinical studies have shown the safety and efficacy of hepatocyte transplantation, it is not implemented as a standard therapy in most liver centers [4]. Key issues in hepatocyte transplantation are...
selecting an optimal cell source with unlimited supply and improving engraftment efficiency [5, 6]. Furthermore, the survival of actively proliferating cells implanted in two- or three-dimensional scaffolds is limited by inadequate diffusion of oxygen and nutrients and disposal of waste products [7, 8]. However, researchers have improved the engraftment, survival, and function of hepatocytes transplanted into three-dimensional matrices by using different seeding and perfusion techniques [7, 9, 10].

Of the matrices or devices currently available, none has demonstrated long-term success [7, 8, 11], largely because the scaffolds do not resemble the size and three-dimensional architecture of a human liver that are needed to support the engraftment, proliferation, and survival of a sufficient mass of human liver cells to support patients with ESLD. Theoretically, utilizing porcine livers depleted of their cellular components is an attractive approach because they provide a three-dimensional biologic scaffold that can support the proliferation and survival of engrafted human liver cells in a continuous perfusion system to create a functional, humanized liver graft suitable for transplantation, without the risks associated with xenotransplantation. In 2008, Ott and colleagues [12] created a functional bioartificial heart in mice by using decellularized heart matrix reseeded with cardiac and endothelial cells. Inspired by their work, we developed a new decellularization strategy using porcine livers to prepare a biologic matrix with an intact microvasculature that can be seeded with human fetal hepatocytes (Hfh) and nonparenchymal human fetal stellate cells (Hfsc). In addition, we developed seeding and perfusion techniques to optimize cell engraftment, proliferation, and survival—a first step toward developing a functional liver graft suitable for transplantation in humans.

MATERIALS AND METHODS

Liver Retrieval Procedure

Ischemic livers were removed from 19 pigs (25–35 kg) after cardiac arrest under sterile conditions at the end of study protocols approved by the Texas Heart Institute Institutional Animal Care and Use Committee. All animals were cared for in accordance with the requirements of the Laboratory Animal Welfare Act (P.L.89-544) and its amendments. The abdominal cavity was opened through a long midline incision. The portal vein within the portal triad was skeletonized, dissected to the superior border of the pancreas, ligated proximally, and cannulated. The vena cava was encircled below the level of the liver. A midline sternotomy was performed to expose the suprahepatic vena cava. The liver was then infused with 6 to 10 L of distilled sterile water through the portal vein, and both supra and infrahepatic vena cavae were divided. The liver was excised and infused further on the backbench with sterile distilled water. The posterior segment of the right lobe of the liver was divided. Attached diaphragmatic muscles and connective tissues were removed. The posterior segment was measured and weighed. The hepatic artery and the bile duct were ligated.
cross-linking and stabilization by using 10% formalin. The posterior segment was infused and stored in 2 L of 10% formalin for 48 h at room temperature. This was followed by infusing 40 L of Dulbecco’s phosphate-buffered saline (1 ×) (Invitrogen, Carlsbad, CA). At the end of the procedure, the decellularized matrices were divided into eight sections and submitted for histologic examination. For matrices selected for recellularization, the cut surface was sutured, and any sources of fluid leak were further secured by using 6-0 Prolene stitches. Two vascular catheters were introduced and secured to the supra- and infrahepatic venae cavae. Matrices were stored at 4°C in culture medium with antibiotics (i.e., penicillin, streptomycin) and antimycotic amphotericin until recellularization.

Liver Matrix Perfusion System

The perfusion culture system developed in our laboratory comprises units for housing, perfusion, heating, and gas mixing. The housing unit for the liver matrix was composed of a sterile organ cassette with vented dual lids that was part of a continuous hypothermic machine perfusion system for the preservation and eventual transplantation of kidneys (LifePort Kidney Transporter, Des Plaines, IL). The cassette was modified to allow introduction of a drainage catheter and was placed in a 37°C water bath (Fisher Scientific IsoTemp 210 Water Bath; Pittsburgh, PA). The culture medium was infused into the liver matrix from a 3-L reservoir through oxygenator tubing (Capiox SX 10; Terumo, Somerset, NJ) connected to a gas mixture (air and carbon dioxide). The flow rate of air and carbon dioxide was individually adjusted according to the gas analysis. The culture medium was recirculated from the cassette back to the reservoir by using an infusion pump (Baxter Flo-Gard 6301; Deerfield, IL). To maintain a stable culture condition, fresh medium was infused at a rate that was adjusted according to the glucose and lactate concentrations via an extra pump in-line with the infusion pump. The temperature of the entire perfusion unit was maintained at 37°C by the heating unit, which consists of a water bath and a hotline fluid warmer (Smiths Medical ASD, Inc., Rockland, MD). The perfusion unit was sterilized by using ethylene oxide according to clinical standards.

Human Fetal Liver Cells

Second trimester Hfh and Hfsc (Science Cell Research Laboratories, San Diego, CA) were cryopreserved at secondary culture after purification and were delivered frozen in 1-mL vials. Each vial contained 5 × 10^8 and 1 × 10^9 of Hfsc and Hfh, respectively. The cells were negative for human immunodeficiency virus-1, hepatitis-B virus, hepatitis-C virus, mycoplasma, bacteria, yeast, and fungi. The Hfh culture medium consisted of basal medium containing essential nutrients, amino acids, vitamins, organic and inorganic compounds, trace minerals, and a low concentration of fetal bovine serum (FBS; 5%) supplemented with apo-transferrin (10 µg/mL), insulin (5 mg), EGF (2 ng/mL), FGF-2 (2 ng/mL), IGF-1 (2 ng/mL), retinoic acid (1 × 10^{-7} M/mL), hydrocortisone (1 mg/mL), and 5 mL of penicillin/streptomycin solution (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin). The medium was bicarbonate buffered (pH = 7.4). Hfsc were cultured in a similar medium but with a lower concentration of FBS (2%) and no retinoic acid.

Characterization of Hfh

To characterize Hfh and monitor their development before implantation, one vial of cryopreserved second trimester Hfh (1 × 10^8 cells) was thawed in a 37°C water bath and dispensed into a 75-mm Corning cell culture flask (Sigma-Aldrich, St. Louis, MO) precoated with fibronectin (Sigma-Aldrich). The culture was maintained at 37°C with 5% CO₂, and the culture medium was changed every 2 d. When the cells reached 80% to 90% confluence, they were split by using a solution of trypsin and were serially cultured for 3 wks. At the end of wks 1 and 3, the cells were collected in RPMI solution and centrifuged for 5 min.

The supernatants were decanted, and the cells were centrifuged for another 5 min. Cells were then stained with antibodies against α-fetoprotein (AFP; Ventana Medical Systems Inc., Tucson, AZ), cytokeratin (CK)-18 (Diagnostic BioSystems, Pleasanton, CA), CK-19 (Ventana Medical Systems), and the adult form of the drug metabolism enzyme CYP3A4 (Abnova, Walnut, CA). Glycogen deposition was assessed with periodic acid-Schiff (PAS) stain. Immunofluorescence staining was performed by using rabbit polyclonal antibody against human albumin (Lab Vision, Fremont, CA). To evaluate the synthetic function of Hfh, samples were collected from the culture medium every 2 d, stored at –80°C, and analyzed with the sandwich enzyme-linked immunosorbent assay (ELISA) by using a human albumin quantification kit (Bethyl Laboratories, Inc., Montgomery, TX) according to the manufacturer’s instructions.

Cell Expansion Using Single Layer Culture

Each cryopreserved vial of Hfh and Hfsc was thawed in a 37°C water bath and dispensed into three separate 75-mm Corning cell culture flasks precoated with fibronectin. A total of 12 × 10^8 Hfh and 2 × 10^9 Hfsc were subcultured and maintained at 37°C with 5% CO₂. Once the culture was established, the medium was changed every 2 d. The cells were split at 80% to 90% confluence by using a solution of trypsin (Sigma-Aldrich). Cells were serially cultured for 6 to 8 d. After four passages, they were collected and resuspended in warm culture medium at a density of 5 × 10^6/mL and seeded within the liver matrix immediately.

Recellularization Procedure

The posterior segments of three separate porcine liver matrices were seeded with 3.5 × 10^8 Hfh and 1 × 10^9 Hfsc and Hfh, respectively, and perfused for 3, 7, and 13 d. To test the engraftment of Hfsc, 3.5 × 10^8 Hfsc were seeded separately into a recellularized posterior segment and perfused for 3 d. The liver matrix was placed in the sterile cassette and connected to the perfusion system through the portal vein and the suprahepatic inferior vena cava. The infrahepatic inferior vena cava was closed by using a three-way stopcock. The flow rate of the medium required to meet the oxygen consumption rate for metabolically active cells was estimated to be between 1 and 1.5 mL/min [7]. Hfh culture medium was infused continuously through the portal vein at a rate of 90 mL/h. The air and CO₂ flow rate was set at 50 mL/h and 2 mL/h, respectively, to provide an inflow partial oxygen tension of 160 to 180 mmHg and maintain a neutral pH (7.4-7.5). The medium temperature was maintained at 37°C by the heating unit. During perfusion, both lids of the cassette were kept locked at all times to minimize contamination. After 2 h of perfusion, approximately 3.5 × 10^8 Hfh suspended in 70 mL of warmed Hfh medium were infused into the portal and hepatic veins under gravitational flow to allow the uniform distribution of cells within the hepatic lobules and portal space. During the seeding process, portal perfusion was stopped and resumed at 70 mL/h at the end of cell infusion. The effluent percolated from the surface of the liver, and it was collected by the drainage catheter and recirculated back into the perfusion system. Fresh medium was infused continuously at a basal rate of 20 mL/h and was adjusted to maintain a sufficient supply of nutrients, as described above. After 2 d of continuous perfusion, 1 × 10^8 Hfh suspended in 200 mL of warm hepatocyte medium were infused into the liver matrix via the portal and hepatic veins under gravitational flow in four steps, with 2-h intervals between each step. Engraftment efficiency was determined by Trypan blue exclusion by estimating the difference between the initial number of infused cells and the number of cells in the effluent. The three recellularized liver matrices were perfused continuously with hepatocyte medium at 90 mL/h for 3, 7, and 13 d. The continuous, fresh hepatocyte medium supply was added to the perfusion line as described above. The medium was supplemented every day with hepatocyte growth factor (100 ng/mL) (Sigma-Aldrich). At the end of
the perfusion date, the cassette lids were opened, and the liver was removed and divided into eight sections for histologic analysis.

Assessment of Metabolic Function

The metabolic activity of the cells within the liver matrix was assessed during the perfusion period by using a hand-held analyzer iSTAT (Abbott Laboratories, Princeton, NJ), which measured the pH, partial pressure of oxygen (PO2), partial pressure of carbon dioxide (PCO2), lactate, glucose, and urea nitrogen in the effluent. These values were compared with the values obtained from the perfusate every 6 h. To evaluate the synthetic function of Hfh, samples were collected from the outflow effluent through the drainage catheter daily and stored at –80°C until they were analyzed for human albumin by ELISA according to the manufacturer’s instructions.

Histologic and Immunohistochemical Analyses

Sections from liver matrices that were obtained at the end of each decellularization procedure were formalin-fixed in 10% formalin and embedded in paraffin. Samples were cut into 4-μm sections and stained with hematoxylin and eosin (H&E) to evaluate tissue architecture and organization. Paraffin-embedded samples were stained immunohistochemically for collagen type I (Abcam, Cambridge, MA), collagen type IV (DAKO, Carpinteria, CA), fibronectin (Abcam), and laminin (Sigma-Aldrich).

At the end of each perfusion period (d 3, 7, and 13), the recellularized liver matrices were cut into eight sections and immediately fixed in 10% formalin overnight, and each section was then cut into 4 μm-thick slices and examined for histology and immunohistochemistry. Immunofluorescence staining was performed by using rabbit polyclonal antibody against human albumin. H&E staining was used to examine cell distribution, organization, and viability. Hfh were characterized by immunohistochemistry by using antibodies against albumin and CK-18. Hfh were identified by immunohistochemistry with antibodies against fibronectin and desmin (DAKO). To assess hepatocyte function at the end of each perfusion period, we examined the expression of several hepatic proteins by immunohistochemistry and immunofluorescence by using antibodies against proteins secreted early in the fetal liver, such as AFP and albumin. Hfh maturation was evaluated by immunostaining for the fetal and adult drug metabolism enzymes CYP3A7 and CYP3A4, respectively. To determine whether Hfh have the potential to differentiate into biliary epithelial cells, we examined the expression of CK-19 by immunohistochemistry. The PAS stain was used to detect glycogen in the cultured Hfh.

Cell proliferation was assessed by using polyclonal goat anti-Ki67 (DAKO) antibody. Cell viability was further assessed by terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) staining (Millipore, Billerica, CA), whereby apoptotic nuclei were detected by positive staining. Antibody specificity was determined by using IgG-specific antibodies against known positive and negative control livers.

Assessment of Liver Matrix Vasculature

To evaluate the integrity of the vascular bed after complete decellularization of the liver, we injected radio-opaque contrast solution (Visipaque; GE Healthcare Ireland, Cork, Ireland) separately into the portal and hepatic veins and observed its distribution within the liver vasculature in real-time by using fluoroscopic examination. We further illustrated the intricate vascular bed of both the portal and hepatic venous systems by creating a corrosion cast of decellularized matrix.

In vivo Transplantation of Decellularized Liver Matrix

To examine the behavior of the matrix in vivo and its ability to withstand the shear stress created by normal portal venous flow and pressure, we performed an auxiliary transplantation of the decellularized matrix into a recipient porcine model. The liver was implanted in the infrahepatic space by using the recipient portal vein and infrahepatic inferior vena cava as an inflow and outflow, respectively. Briefly, at the end of study protocols approved by the Texas Heart Institute Institutional Animal Care and Use Committee, the abdominal cavity of a 45-kg Yorkshire swine was opened by a long midline incision. The duodenum was dissected medially. The portal vein within the portal triad was skeletonized, dissected to the superior border of the pancreas, and encircled with a vessel loop. The vena cava was encircled below the level of the liver and above the renal veins. The mean portal venous pressure within the main portal vein of the recipient and the matrix was measured by using a 22G catheter. Clamps were applied to the proximal and distal ends of the portal vein and inferior vena cava. The matrix was brought to the operating field, and standard end-to-side anastomosis was carried out between the recipient and the matrix infrarenal inferior vena cava by using continuous 6-0 prolene sutures. The portal venous anastomosis was performed by using continuous 6-0 prolene sutures in an end-to-side fashion. Upon completion of the two anastomoses, the inferior vena cava clamps were removed, followed by the portal vein clamps, and the matrix was perfused.

Statistical Analysis

Values are presented as the mean ± standard deviation or as median and range. A two-tailed, paired Student t-test was used to compare laboratory results before and after recellularization. A probability (P) value of < 0.05 was considered significant.

RESULTS

Evaluation of the Decellularized Liver Matrix

After 48 h of storage in 0.25% SDS in distilled water, the median time that was needed to decellularize the posterior segment of the right liver lobe was 100 min (range, 45–234 min). By the end of the decellularization procedure, the liver maintained its gross appearance and size but was white and translucent, weighing 20% to 30% less than the original liver. The decellularized liver segments weighed an average of 232 ± 67.5 g and measured an average of 11.7 × 11 × 6.5 cm (Fig. 2A and B).

Fluoroscopic examination of the vascular bed revealed that the injected contrast material had promptly distributed from large vessels into capillaries, with no obvious extravasations, indicating that the vascular system was functional with normal flow from the portal and hepatic veins into the inferior vena cava (Fig. 2C and D). The corrosion cast further confirmed the preservation of the large portal and hepatic veins and their extensive network of microvascular branches (Fig. 2E and F).

Examination of normal (Fig. 3A) and decellularized matrices by H&E staining showed that the decellularized liver architecture was relatively intact; hexagonal-shaped lobules were separated by sheets of connective tissue, with the central vein in the middle and the portal triads at the vertices (Fig. 3B). Furthermore, the delicate honeycomb-shaped network of connective tissue that outlines the eradicated plates of hepatocytes within the lobules was clearly visible.
Only a few residual nuclei were observed within the examined sections.

Immunohistochemical staining for extracellular matrix (ECM) proteins (i.e., collagen I and IV, fibronectin, and laminin) indicated the preservation of type I collagen within the portal triad area (Fig. 3C). Type IV collagen and fibronectin (Fig. 3D and E) were observed mainly within the decellularized lobules, although the staining intensity was more prominent for collagen than for fibronectin. The basement membrane of the vascular structures stained positive for laminin (Fig. 3F).

**Assessment of the Matrix After In Vivo Transplantation**

The mean portal venous pressure within the main portal vein of the recipient was 8 mmHg, and 5 mmHg within the portal vein of the matrix. After reperfusion, the matrix was perfused, filling with blood within 5 min. During 2 h of perfusion, the matrix maintained a soft texture and homogenous color, with no extravasation of blood from the surface of the matrix (Fig. 4). At the end of perfusion, the graft was harvested for histologic analysis. H&E and immunohistochemical staining revealed intact structural and basement membrane components of the ECM (data not shown).

**Immunohistochemical Characterization of Hfh Before Implantation**

Throughout the culture period, we observed two different morphologic types of Hfh by light microscopy. After the first week of culture, the predominant cell type...
was small and large round cells with peripherally located nuclei and granular cytoplasm (Fig. 5A). Several of the cells were attached to each other, forming clusters. At the end of wk 3, the most predominant cell type was normal polygonal-shaped cells that contained a centrally located nucleus (Fig. 5B). The cells were more homogeneous, forming a continuous monolayer.

Adult hepatocytes express CK-18. As the culture period progressed, we observed two different patterns of staining with anti-CK-18 antibodies. At the end of the first week, several small cells expressed CK-18 in the nucleus rather than in the cytoplasm (Fig. 5A), whereas at the end of wk 3, CK-18 expression was homogeneous and confined to the periphery of the cytoplasm with no nuclear staining (Fig. 5B). To determine the functional characteristics of Hfh, the expression of several hepatic proteins was analyzed by immunohistochemistry. Albumin and AFP are proteins secreted early in the fetal liver [13]. More than 90% of cells stained positive for albumin at the end of wk 1 and 3 (Fig. 5C and D, respectively). However, approximately 20% of cells stained positive for AFP in the first week (Fig. 5E), and no Hfh stained positive for AFP in the third week (Fig. 5F).

The drug metabolism enzyme CYP3A4 is considered a late-phase hepatocyte protein. Less than 5% of cells stained positive for CYP3A4 after the first week of culture (Fig. 5G). However, the staining increased to approximately 30% by the end of wk 3 (Fig. 5H). PAS staining revealed increased glycogen deposition from the end of wk 1 to the end of wk 3 (Fig. 5I and J, respectively).
respectively). CK-19 is expressed in the biliary epithelial cells of the adult liver and in bipotential progenitor cells of the fetal liver [14]. At the end of wk 1 and 3, scattered hepatocytes (30%) stained positive for CK-19 without forming any tubular structures (Fig. 5K and L, respectively). There was no difference between the number of cells that stained positive for CK-19 after wk 1 and 3. These morphologic and immunohistochemical analyses indicated that the cells were fetal hepatocytes at a mid- to late stage of development.

Cell Expansion and Serial Passages

After thawing, cell viability determined by Trypan blue exclusion was >95%. With the use of the subculture technique and serial passages for 6 to 8 d, the number of Hfh and Hfsc increased by approximately 80- and 100-fold, respectively. At the end of the fourth passage, the total number of Hfh and Hfsc from each vial increased from $1 \times 10^6$ to 60–85 $\times 10^6$ and from $5 \times 10^5$ to 50–120 $\times 10^6$ cells, respectively. Immediately before implantation, more than 95% of expanded Hfh and Hfsc maintained their viability.

Metabolic and Synthetic Activity of Recellularized Matrix

The oxygen concentration in the culture medium decreased consistently by an average of 13% ± 4% from the inflow to the outflow effluent of the liver matrix during the three periods of perfusion (Fig. 6A). In contrast, glucose consumption and lactate and urea production increased significantly during the 13-d perfusion period (80.4 ± 6.8 mg/dL versus 63 ± 8.9 mg/dL; 1.16 ± 0.76 mmol/L versus 2.72 ± 1.6 mmol/L; and 3.0 mg/dL versus 11.3 ± 0.76 mg/dL, respectively; $P < 0.0001$), indicating active metabolism within the recellularized matrices (Fig. 6B–D). The synthetic activity of the engrafted Hfh was assessed by measuring the concentration of human albumin within the outflow effluent. After the concentration of albumin initially increased during the first 3 d, the rate of excretion decreased toward the end of d 13. During the 13-d perfusion period, the average albumin concentration was 29.48 ± 7.4 mg/mL (Fig. 6E). The average albumin secretion was slightly higher (31.63 ± 7.7 mg/mL) after the 3-wk static hepatocyte culture before implantation than after the 13-d culture within the liver matrix (Fig. 6E). However, this difference was not statistically significant ($P = 0.84$).

Histologic and Immunohistochemical Analysis of Recellularized Matrix

By the end of each perfusion period, recellularized liver matrices maintained their shape and size. At the end of the 13-d perfusion period, small, scattered areas at the proximal sections of the matrices lost their translucent appearance, which was replaced by small, opaque overgrowths (Fig. 7A and B). H&E staining
revealed that these areas contained a higher proportion of engrafted cells than did the distal sections. The engraftment efficiency, determined by Trypan blue exclusion, was $>90\%$ for both Hfh and Hfsc. However, within the matrix, the percentage of cellular engraftment varied at different times of perfusion (Fig. 8). At the end of the 3-d perfusion period, cells were found in and around the portal and hepatic veins. However, at the end of the 1-wk and 13-d perfusion periods, the cells had migrated beyond the large vessels and had populated the surrounding decellularized matrix.

At the end of the 13-d perfusion period, immunohistochemical analysis of cell proliferation showed that approximately 40% of the engrafted cells stained positive with Ki67 antibody (Fig. 9A). The highest percentage of staining was noted in the proximal sections that corresponded to the overgrowth areas ($60\%$ versus $30\%$). Hfh showed a higher percentage of proliferation...
than did Hfsc (40% versus 20%). In conjunction with our metabolic analyses, these results indicated that engrafted cells were actively proliferating. The viability of co-cultured Hfh and Hfsc was maintained throughout the perfusion periods, as shown by H&E staining, and was further confirmed by immunohistochemistry for TUNEL, which revealed that less than 30% of cells were apoptotic by the end of the 13-d perfusion period (Fig. 9B–D). Again, we noticed a greater number of apoptotic cells in the distal sections than in the proximal recellularized sections of the liver matrix.

Approximately 70% of Hfh stained positive for albumin throughout the perfusion periods (Fig. 10A). However, the number of cells that expressed AFP diminished as the perfusion period progressed. At the end of the 3-d period, more than 60% of cells stained positive for AFP, but by the end of the 13-d period, just more than 20% of cells showed positive AFP staining (Fig. 10B). In normal human adult liver, few cells stained positive for AFP (Fig. 10C). At the end of the 13-d perfusion period, the morphologic features of Hfh (i.e., round cells, a high nucleus-to-cytoplasm ratio) and the staining patterns for CK-18 (i.e., occasional CK-18 nuclear staining) were similar to those observed after the 1-wk static culture period (Fig. 10D). In normal human adult liver, all cells showed peripheral staining for CK-18 (Fig. 10E). Staining for CYP3A4 increased steadily during the three periods of perfusion; by the end of the 13-d perfusion period, more than half of hepatocyte-like cells stained positive for anti-CYP3A4 antibody (Fig. 10F). In normal human adult liver, all cells stained positive for CYP3A4 (Fig. 10G). CYP3A7 is expressed in Hfh and disappears in adult hepatocytes. At the end of the 13-d perfusion period, few hepatocytes stained positive for CYP3A7 (Fig. 10H), which was similar to the staining observed in normal human adult liver (Fig. 10I). Glycogen deposition increased during the three periods of perfusion, as shown
by intense PAS staining at the end of the 13-d perfusion period (Fig. 10J); similar staining was observed in normal human adult liver (Fig. 10K). During the three perfusion periods, we observed steady numbers of Hfh that stained positive for CK-19. However, we did not see tubular structures to indicate that these cells formed bile ducts within the matrix (Fig. 10L), which were seen in normal human adult liver (Fig. 10M). Our results indicate that the maturation process for Hfh was not complete after 13 d of perfusion.

In a separate experiment, hepatic lobules that were seeded with isolated Hfsc followed by 3 d of perfusion showed a more prominent staining intensity for fibronectin than did decellularized lobules, indicating that the Hfsc actively produced and secreted fibronectin (Fig. 11).

**DISCUSSION**

In this pilot study, we designed and evaluated a new decellularization strategy in a large animal model that utilizes porcine livers to create a biologic matrix with intact microvasculature suitable for human hepatocyte implantation. In addition, we established a recellularization and perfusion technique for the sufficient engraftment, proliferation, and survival of human hepatocytes in the decellularized porcine liver matrix. Our work provides a foundation for using a large
animal model to engineer humanized livers suitable for transplantation.

To obtain acellular matrix from a porcine liver, we modified the perfusion-decellularization procedures of other investigators [12, 15, 16]. We found that rapid infusion of a large volume of 0.5% SDS under hydrostatic pressure was efficient for removing cells from the native liver, with minimal disruption of the three-dimensional architecture and without significant loss of structural proteins. Post-decellularization angiography and corrosion casting revealed an intact microvascular network with no extravasations of contrast material into the surrounding parenchyma, despite complete removal of the vascular endothelial cells. Histologic examination using H&E and immunohistochemical staining confirmed the complete removal of endothelial cells, as well as the biliary epithelium, and an intact basement membrane, as demonstrated by intense laminin staining of both the microvasculature and bile ducts. The integrity of the basement membrane was essential in maintaining the functionality of the vascular system as a conduit. However, engineering an intact, functional whole-liver graft will require reestablishing the epithelial lining of both the vascular and biliary systems to restore the unique, functional characteristics of the hepatic microvascular circulation and to prevent biliary stricture. H&E staining of sequential sections through the liver matrix confirmed the complete removal of cells and most nuclear material. Although H&E staining is not as sensitive as other methods, such as gel electrophoresis or confocal microscopy to identify DNA in propidium iodide- or DAPI-stained tissues, the presence of trace amounts of DNA has been shown to exert no adverse effects in other commercially available ECM products [16, 17].

The composition and concentration of ECM proteins are important for cell attachment, growth, and differentiation. Immunostaining for four ECM proteins (type I and IV collagen, laminin, and fibronectin) indicated that the structural proteins of the liver lobules and portal space remained relatively intact. However, the staining intensity was weaker for fibronectin than for other ECM proteins, which indicated a relative loss of fibronectin during the decellularization process. Although a few liver lobules collapsed and lost their configurations, most maintained the hexagonal arrangement delineated by connective tissue that is characteristic of porcine livers. During our initial attempts of recellularization and perfusion, we observed considerable shrinkage of the decellularized matrix that resulted from the disintegration of structural proteins (data not shown). However, when we used collagen cross-linking with
10% formalin after the decellularization procedure to maintain the strength and stability of the matrix, we observed preservation of the matrix shape and size without further disintegration during prolonged perfusion. The volume of formalin and the duration of exposure to formalin were chosen to allow sufficient penetration of the formalin through the entire thickness of the matrices. Although chemical cross-linking eliminates the risk of zoonotic transmission [18, 19], the immune response to cross-linked, decellularized xenogenic implants may persist, despite diminished antigen recognition [18]. Future studies are warranted to investigate the immunogenic effects of decellularized xenogenic matrices in humans and to compare different methods of cross-linking, including photo-oxidative cross-linking and the use of Genepin, which is a natural cross-linker for collagen that may be less toxic than aldehyde-based solutions [20, 21].

A key issue in human hepatocyte transplantation is selecting a type of cell that can provide mass that is sufficient to replace or supplement the function of a failing liver. In conventional cell culture, adult hepatocytes have insufficient proliferative capacity and tend to progressively lose their differentiated phenotype and functional characteristics [22, 23]. Although immortalized human hepatocytes have been used to overcome these shortcomings of adult hepatocytes, their safety is a concern [24–26]. Therefore, in this study, we used Hfh because they have a greater proliferative capacity than adult hepatocytes [27, 28] and a lower risk of tumorigenicity than immortalized cells. However, we are not certain about whether the functional maturity and availability of Hfh are suitable for clinical applications [29]. Nevertheless, Lazaro and colleagues [27] showed that Hfh maintained in primary culture for several months did not lose function or major differentiated traits. Moreover, another group induced the spontaneous expansion and maturation of Hfh while maintaining bipotential progenitor cells by using high-density three-dimensional perfusion in a bioreactor culture system for 14 d [14]. Because of the inherent difficulty of retrieving cells from bioreactors, we used a conventional culture system for the initial expansion of cells before implantation. During the expansion period, we were able to increase the initial number of cultured cells by approximately 60 to 100 times in 6 to 8 d using standard culture technique and soluble growth factors.

In contrast to monoculture technique, co-culture techniques that use nonparenchymal cells have been successful in maintaining the viability and function of cultured hepatocytes [30, 31]. We chose Hfsc as a co-culture component because of their established roles in liver homeostasis and regeneration by either direct cell contact or the secretion of soluble growth factors that support hepatocytes and ECM proteins.
[32–35]. Furthermore, Hfsc that are pre-cultured for 1 to 2 d before being co-cultured with hepatocytes exerted beneficial effects on hepatocyte function, structure, and differentiation [33]. Because one stellate cell spans two to three hepatocytes in vivo [36], we seeded Hfsc 2 d before the infusion of Hfh at a ratio

FIG. 10. Immunohistochemical and immunofluorescence staining of recellularized matrix. (A) The majority of cells stained positive for human albumin protein (arrow). (B) At the end of the 13-d perfusion period, more than 20% of cells stained positive for AFP (arrow). (C) Only a few cells stained positive for AFP in normal human adult liver (control). (D) At the end of the 13-d perfusion period, 90% of Hfh stained positive for CK-18 (arrows). Most cells were round with a high nucleus-to-cytoplasm ratio and occasional CK-18 nuclear staining, similar to cells after 1 wk of static culture. (E) All hepatocytes in normal human adult liver stained positive for CK-18. (F) At the end of the 13-d perfusion period, more than 50% of cells stained positive for CYP3A4, whereas (G) all cells stained positive for CYP3A4 in normal human adult liver. Few cells stained positive for fetal drug metabolism enzyme CYP3A7 in (H) the recellularized matrix (arrows) and (I) normal human adult liver. (J) Glycogen deposition (shown by PAS staining) increased at the end of the 13-d perfusion period (arrows) and (K) was similar in normal human adult liver. (L) Steady numbers of Hfh stained positive for CK-19 during the three periods of perfusion, without forming tubular structures (arrows), which were seen in normal human adult liver (M). Original magnification, 20×.
of 1:3 using intermittent infusion via the hepatic and portal veins, resulting in engraftment efficiency >90%. Because of the anatomical structure of the hepatic lobules, hepatic venous infusion allowed a large number of cells to be seeded within the lobules. Compared with decellularized lobules, the hepatic lobules that were seeded with Hfsc showed a more prominent staining intensity for fibronectin, indicating that the Hfsc actively produced and secreted fibronectin, which can support the engraftment of Hfh into the adjacent parenchyma.

At the end of the 13-d perfusion period, the engrafted cells continued to proliferate, and more than 70% remained viable, as shown by the Ki67 proliferation index and TUNEL staining, respectively. Furthermore, the maturation of Hfh during the perfusion period was demonstrated by phenotypic changes and was further confirmed by immunohistochemical analyses that showed decreased expression of AFP and CYP3A7 and increased expression of CYP3A4, CK-18, and PAS. This pattern corroborated the one seen in standard culture before implantation. Although the maturation of Hfh was not complete at the end of the 13-d perfusion, the trend showed promise. Furthermore, the results of these morphologic and immunohistochemical analyses suggest that Hfh co-cultured with Hfsc maintain their proliferative capacity and viability in continuously perfused, recellularized porcine matrices. In future work, additional characterization of hepatocyte function and gene expression will be assessed when using a larger number of seeded cells and longer periods of perfusion, given that robust hepatocyte metabolism and gene transcription may require a longer period of culture to stabilize [37]. In addition, we observed the expression of CK-19, which is expressed exclusively in the biliary epithelium of adult livers, in hepatocyte-like cells during the perfusion periods. However, these cells failed to form duct-like structures within the matrices, indicating that they may represent bipotential liver progenitor cells that could develop into hepatic biliary epithelial cells with longer periods of perfusion [14]. Future studies will examine the transdifferentiation potential of Hfh into various types of liver cells, as the seeding of all cell types may not be necessary.

Human liver is a complex organ with highly sophisticated function. To engineer such an organ is a monumental task. Several generalized issues need to be addressed. First, a recellularization strategy is needed to populate the entire liver with cellular components, including hepatocytes, sinusoidal endothelial cells, stellate cells, biliary epithelial cells, and Kupffer cells. We speculate that restoring the integrity of the vascular endothelium should be done at the end of the seeding process to facilitate migration of the cells into the liver parenchyma. Second, re-epithelialization of the extrahepatic bile duct, portal vein, and hepatic artery must be established to allow for bile duct and vascular reconstruction. Third, different cell sources of human origin need to be further investigated. As we mentioned, Hfh are more advantageous than are adult hepatocytes or immortalized cell lines. However, extensive expansion of Hfh in vitro before implantation may decrease their functionality [29]. In addition, the cost of populating the entire liver with parenchymal and nonparenchymal fetal cells could be prohibitive. In our study, the expense of infusing Hfh and Hfsc during the 13-d perfusion was at least $25K, and the number of hepatocytes we infused accounts for approximately 2.5% of the total number of human hepatocytes that normally occupy a 200-g liver (139 ± 25 million cells per 1 g of liver tissue, or 4 × 10^9 cells per kg of body weight) [38, 39]. Moreover, a liver of the size used in this study would only be sufficient to support or replace a liver in a pediatric patient who weighs 20 to 30 kg. Finally, a preservation technique for the engineered liver will be essential because long preparation time is not ideal for patients with acute liver failure.

We showed that our decellularization protocol successfully removed the cellular components of porcine livers while preserving the vasculature, native architecture, and most extracellular proteins. We also demonstrated the ability of the decellularized matrix to support and maintain engrafted Hfh that were co-cultured with Hfsc in a continuously perfused system. Future work will aim at populating the entire liver segment by increasing the number of implanted Hfh and nonparenchymal Hfsc and improving our perfusion technique to optimize cell proliferation and function, followed by transplantation of the graft into immunosuppressed pigs to evaluate the in vivo
functionality of the engineered liver. Until we successfully achieve the ultimate goal of engineering whole organs for liver transplantation, seeding a segment of liver matrix with hepatocytes may be a useful adjunct to support patients with acute liver failure until their native liver function recovers. Moreover, the technique of decellularization and recellularization can be applied to donor livers that are deemed unsuitable for transplantation, which would further expand the pool of liver grafts available for patients with ESLD.

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