

Isolation of Human Fetal Liver Progenitors and Their Enhanced Proliferation by Three-Dimensional Coculture with Endothelial Cells

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ABSTRACT

Liver progenitor cells, characterized by the coexpression of biliary and hepatocyte lineage markers and the ability to form colonies in culture, were isolated by flow cytometry from primary human fetal livers. These prospectively isolated liver progenitor cells supported hepatitis D virus infection, expressed, and produced albumin and α -fetoprotein, as tracked by albumin- and α -fetoprotein-driven lentiviral promoter reporter constructs and measured by ELISA, respectively. Coculture in three-dimensional (3D) fibrin gel with endothelial cells resulted in the formation of vascular structures by the endothelial cells and increased proliferation of liver progenitors. The enhanced proliferation of liver progenitors that was observed when liver progenitors and endothelial cells were cultured in direct contact was not achieved when liver progenitors and endothelial cells were cultured on adjacent but separate matrices and when they were cultured across transwell membranes. In conclusion, coculture of liver progenitors and endothelial cells in three-dimensional matrix resulted in enhanced liver progenitor proliferation and function. This coculture methodology offers a novel coculture system that could be applied for the development of engineered liver tissues.

INTRODUCTION

BIO-ARTIFICIAL LIVER DEVICES and cellular transplantation are potential alternatives to orthotopic liver transplantation for the treatment of liver diseases. Both efforts are hampered by a shortage of reliable sources of human hepatocytes and by the inability to successfully expand human hepatocytes and maintain their function *in vitro*.^{1,2} Upon plating on plastic in standard two-dimensional (2D)

conditions, adult hepatocytes that have been dissociated from the native hepatic microarchitecture and intrinsic cellular interactions rapidly lose hepatic-specific markers and functions, including the ability to support viral infection, and generally fail to proliferate.³ Coculture of mature hepatocytes with nonparenchymal cells has been used, with limited success, to restore hepatocyte functions.⁴⁻⁸ Human liver progenitor cells (LPCs), which exhibit spontaneous proliferation and the ability to differentiate into hepatocyte

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and biliary duct cells, may serve as an ideal hepatocyte source.^{9–15} The focus of our current study utilizes liver progenitor cells derived from primary human fetal liver tissue.

Antibodies against specific cell surface markers have been successfully used to identify and enrich populations of progenitor cells from a variety of tissue compartments.¹⁶ One challenge in raising the desired antibodies is that the immunodominance of certain epitopes, such as human leukocyte antigen (HLA) antigens, overwhelms the ability to obtain antibodies specific for less highly expressed progenitor cell markers. One solution is the technique of decoy immunization.¹⁷ Monoclonal antibodies isolated in this fashion have enabled the isolation of functional progenitor cells from blood and brain compartments. Using this approach, LPCs were isolated from human fetal liver using monoclonal antibodies.¹⁸ These LPCs express 5E12, a marker originally described to isolate neural stem cells¹⁶ and express low levels of HLA-Class I A,B,C. The HLA^{low} 5E12⁺ cell population is relatively high in forward scatter (termed R2 cells) compared to hematopoietic cell populations. Freshly isolated R2 HLA^{low} 5E12⁺ cells display a bipotent hepatic progenitor phenotype, coexpressing both the biliary marker cytokeratin 19 (CK19) and the hepatic marker protein albumin (ALB). These isolated cells give rise to colonies of ALB⁺ hepatic cells at the clonal level, and linear regression analysis of limit dilution cultures seeded with sorted cells showed that liver stem/progenitor cell activity of fetal liver was significantly enriched in R2 HLA^{low} 5E12⁺ cells by a factor of 16 (α -fetoprotein, AFP) to 2000 (ALB or α -1-antitrypsin, AAT). Transplantation of R2 HLA^{low} 5E12⁺ cells into the liver of immunodeficient mice resulted in long-term engraftment with circulating human ALB or AAT detectable in serum for up to 6 months for newborn or adult recipients.¹⁸

Liver development and liver regeneration are linked not only to the presence of endothelial cells but also to the latter's ability to undergo angiogenesis *in vivo*.^{19–23} Additionally, the implantation of adult hepatocytes into vessel-rich sites, or transplanting scaffold containing hepatocyte and proangiogenic factors, has resulted in increased hepatocyte survival and function.^{24–26} Recent reports demonstrate that transplanting endothelial progenitor cells alone into the injured livers of mice or rats promotes liver regeneration and improves survival after liver injury.^{27,28} The expansion of human adult primary hepatocytes, along with the formation of the vascular structures of endothelial cells, in liver tissue-engineered constructs is still extremely difficult. To overcome this difficulty, we hypothesized that coculture conditions designed to induce *in vitro* angiogenesis would result in improved fetal-derived LPC expansion and function.

We therefore cocultured LPCs with human umbilical vein endothelial cells (HUVECs) in three-dimensional (3D) fibrin gels, a well-described system for inducing *in vitro* capillary-branched formation by endothelial cells.²⁹ These 3D cocultures resulted in improved LPC proliferation compared to 2D cocultures and compared to LPCs cultured

alone. This coculture method may prove useful for antiviral testing and bioartificial liver devices.

MATERIALS AND METHODS

Cells, culture media, and chemicals

HUVECs, human hepatoma cell line Huh7, and 293T, were purchased from ATCC (Rockville, MD); mouse embryo fibroblasts (FFS) were cultured as described.³⁰ LPC growth media was composed of Dulbecco's Modified Eagle's Medium (D-MEM)/F-12 (Gibco/BRL, Rockville, MD), supplemented with insulin, transferrin, selenium (ITS+) premix (BD Pharmingen, San Jose, CA), 10^{-7} M of dexamethasone, 10 mM of nicotinamide, 0.5 mM of ascorbic acid 2-phosphate, 4 mM of L-glutamine, 0.1 mg/mL of heparin, 5% fetal bovine serum, 100 U/mL penicillin G and streptomycin, and 20 ng/mL of epithelial growth factor. All cells were then placed in a humidified, 5% carbon dioxide, 95% air incubator at 37°C. All chemicals and reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise specified.

LPCs isolation

LPCs were provided by Stem Cells, Inc., (Palo Alto, CA). Human fetal livers (14–20 weeks) were purchased from Advanced Biosciences Research (Alameda, CA) in accordance with all state and federal regulations. Each liver was minced, digested with 0.1% collagenase H (Roche Applied Science, Nutley, NJ) for 20 min at 37°C, and then filtered through a 70 μ m nylon mesh. Hematopoietic cells and red blood cells in liver cell suspensions were depleted through incubation with murine monoclonal CD45 and glycophorin A antibodies (Caltag Laboratories, Burlingame, CA), followed by anti-mouse Dynabeads (Dynal Biotech, Oslo, Norway). The LPCs with HLA^{low} 5E12⁺ were enriched by FACS Vantage SE (Becton Dickinson, San Jose, CA) and identified by the coexpression of albumin and CK19 and the secretion of hepatocyte lineage marker proteins (albumin, AAT, and AFP).^{16,31} Highly purified LPC populations used in our experiments were prepared by sorting twice for the HLA^{low} 5E12⁺ population.

Hepatitis delta virus infection

LPCs were plated on FFS feeder cells in slide chambers for immunofluorescence or a six-well plate for Northern blots. Following incubation with inoculums of infectious hepatitis delta virus (HDV) serum, the cells were washed to remove residual inoculums. Total cellular RNA was then extracted with Trizol reagent (Invitrogen, Carlsbad, CA) immediately (day 0), or on days 6 and 12 postinoculation for Northern analysis.

Cells in slide chambers were fixed on day 12 and subjected to immunofluorescence analysis using human anti-

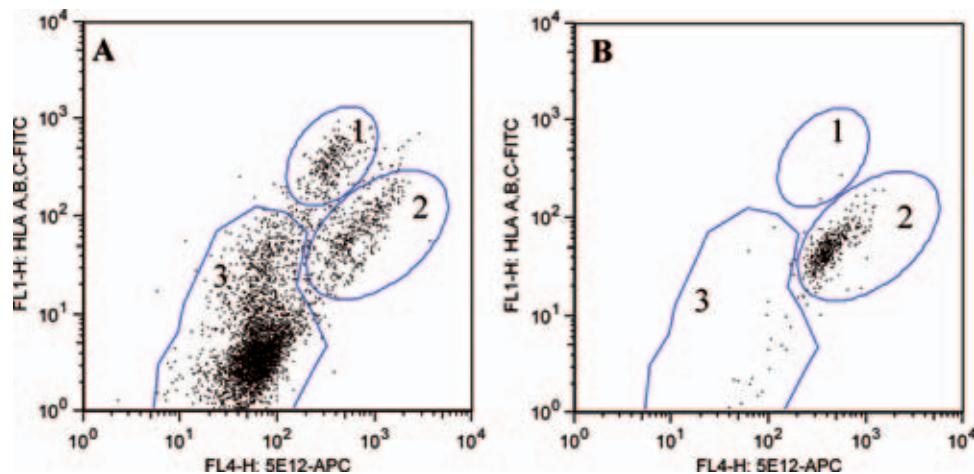


FIG. 1. Flow cytometric analysis and cell sorting of human fetal liver cell suspensions. A representative example of clustered subpopulations after cell surface staining with anti-human HLA A, B, C-FITC and 5E12-APC is shown. Sorting gates used to define each of the three subpopulations (1, HLA^{high} 5E12⁺; 2, HLA^{low} 5E12⁺; 3, HLA^{low} 5E12^{low}) are outlined in blue (A). Resorting the cell suspension shown above in (A) using the region 2 gate resulted in enrichment of HLA^{low} 5E12⁺ cells, referenced below as LPCs (B).

HDV, monoclonal anti-CK19 (Santa Cruz biotechnology, Santa Cruz, CA), and rabbit anti-human albumin primary antibodies (Zymed, South San Francisco, CA), followed by secondary antibodies (BD Biosciences, San Jose, CA). Nuclei were counterstained with Hoechst 33342 stain (Invitrogen). Phase and fluorescent pictures were taken on a Nikon Eclipse TE300 microscope. Staining was pseudocolored using the QED Imaging program (Media Cybernetics, Silver Spring, MD).

Northern blot analysis was essentially as described.³² Briefly, extracted RNA was fractionated on a 1.5% agarose gel. Following capillary transfer to Zeta-Probe (Bio-Rad, Richmond, CA) membrane, the latter was UV-crosslinked using a Stratalinker (Stratagene, La Jolla, CA), and hybridized with a riboprobe labeled with [α -³²P]UTP (3000 Ci/mmol; Amersham, Piscataway, NJ). For detection of amplified genomic HDV RNA, a probe corresponding to the antigenomic HDV sequence was used. After hybridization, blots were washed, dried, and subjected to autoradiography.

Lentivirus

The cloning and hepatic-specific activity of human AFP and albumin promoters, and the preparation of lentiviral particles were determined as described in the supplemental data (available online at www.liebertpub.com).³³⁻³⁵

Coculture of endothelial cells and LPCs in 3D and 2D fibrin gels

Before coculturing, HUVECs were transduced with pRRLsin-phospho-glycerate kinase enhanced green fluorescent protein (PGK-EGFP) at a multiplicity of infection (MOI) of 50:1. The transduced HUVECs and freshly iso-

lated LPCs (cell ratio 2:1, total cells 3×10^4 : 1.5×10^4) were mixed in 0.5 mL of fibrinogen solution (final concentration 4 mg/mL; Calbiochem, La Jolla, CA). About 0.625 U/mL of human thrombin was added to allow polymerization as described.²⁹ The mixture was immediately applied to each well of a 12-well plate to create 3D fibrin gels. After 30 min polymerization at 37°C, LPC media containing 200 U/mL of aprotinin were added to each well to inactivate the thrombin. After 2 h, the media was replaced with fresh LPC media. In order to create a thin and relatively rigid 2D fibrin gel, 50 μ L of fibrinogen solution (4 mg/mL) containing 0.625 U/mL of human thrombin was applied to each well of a 12-well plate and spread evenly by cell scraper. After polymerization, the thrombin was inactivated by aprotinin addition, and cells were added to each well. To maintain HUVECs in coculture, 10 μ g/mL of endothelial cell growth supplement was added

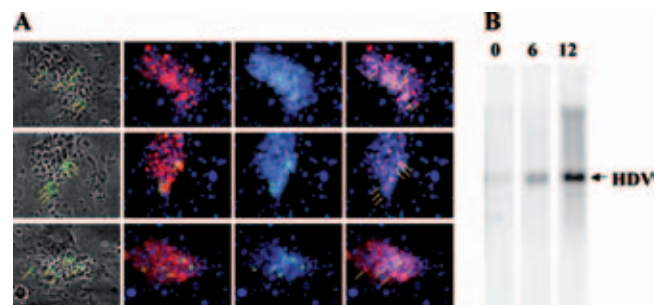


FIG. 2. HDV infection of LPC colonies. LPCs were isolated and grown on FFS feeder cells. Twelve days after inoculation with HDV, infection is demonstrated by the presence of delta antigen (pseudocolored green) in the nuclei of infected cells and increasing genomic RNA in whole cell lysates, as detected by immunofluorescence (A) and Northern blot (B) assays, respectively. Red, staining for CK19; light blue, staining for albumin; royal blue, Hoechst stain of nuclei.

to the LPC media during the first 4 days. Formation of vascular structures of EGFP-labeled endothelial cells, suggestive of angiogenesis,²⁹ was photographed with a digital camera under fluorescence microscopy. The morphology and growth of LPCs were tracked *in situ* by the expression of both EGFP driven by the AFP promoter and DsRed2 driven by the Alb promoter following transduction with both pRRLsin-AFP-EGFP and pRRLsin-Alb-DsRed2 lentiviruses, respectively, at multiplicity of infection (MOI) 50:1 for 24 h at day 3 of coculture.^{34,35} LPC clusters were defined as more than 20 closely adjacent cells expressing both EGFP and DsRed2 and manually counted under fluorescent microscope.

A two-layer culture system or “split 3D” fibrin gel culture system, composed of a bottom layer of HUVECs and a top layer of LPCs, was used to limit the close LPC–HUVEC association. For this, fibrin gel (0.25 mL) containing 3×10^4 of HUVECs was first polymerized and then covered by 0.25 mL of fibrin gel containing 1.5×10^4 of LPCs.

In one of the LPC–endothelial cell coculture experiments, a transwell culture was performed in order to prevent direct LPC–HUVEC contact while allowing exposure of both cell populations to secreted factors in the media. In those transwell experiments, triplicate wells were seeded in the following fashion. About 1.5×10^4 of LPCs in 0.5 mL of media were seeded on the bottom of the 12-well plate coated with a thin and relatively rigid fibrin, whereas 3×10^4 of endothelial cells were added on top of the insert membrane with 0.5 mL of media.

ELISA for human albumin and AFP

Supernatants were collected every 2 days and placed at -80°C until processing. The *de novo* production of albumin and AFP in culture media was measured by standard sandwich enzyme-linked immunosorbent assay (ELISA), using a human albumin ELISA kit (Bethyl, Montgomery, TX) and a human AFP ELISA kit (DAKO, Carpinteria, CA), respectively.

Immunohistochemistry and BrdU labeling

After cells were exposed to $10 \mu\text{M}$ of BrdU for 18 h, fibrin gels were rinsed with phosphate-buffered saline and fixed in 4% fresh paraformaldehyde with 0.15% picric acid. The gels were balanced with 10–20% of sucrose at 4°C overnight, detached, and embedded in optimal cutting temperature (OCT) medium (Sakura, Torrance, CA). About 10–20 μm frozen sections were cut on a Microtome (Microm, Walldorf, Germany). After blocking in tris buffered saline (TBS) containing 10% goat serum, 1% bovine serum albumin, and 0.05% of Tween 20, slides were stained using mouse anti-human albumin antibody (R&D systems, Minneapolis, MN) for 45 min, and Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for an additional 45 min. BrdU was stained using fluorescein isothiocyanate (FITC)-conjugated BrdU antibody (BD Pharmingen) after DNA denaturation in 2 N HCl for 30 min.

Nuclei were counterstained with Hoechst 33258 (Invitrogen) and mounted prior to microscopic examination.

To verify the association of LPCs and HUVECs in 3D, cocultures of LPCs and HUVECs at day 18 were processed as above. LPCs were stained with mouse anti-human albumin antibody (Sigma) for 45 min, and Alexa 555-conjugated goat anti-mouse IgG2a (Invitrogen) for an additional 45 min. HUVECs were stained using FITC-conjugated CD31 antibody (BD Pharmingen), while nuclei were counterstained with 4',6-Diamidino-2-phenylindole (DAPI) (Invitrogen) and followed by mounting prior to examination under Zeiss LSM 510 confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY).

To score the number of albumin-positive cells in each sample, the total number of cells released after enzymatic digestion of the fibrin gels was first counted with a hemacytometer. Aliquots of cells were then smeared onto slides for albumin cytospin staining. After blocking endogenous peroxidases with 3% hydrogen peroxide, slides were stained with monoclonal mouse anti-human albumin antibody (R&D systems) and horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma). The numbers of albumin-positive and albumin-negative cells were counted at $40\times$ magnification. At least 1000 albumin-positive cells were counted in each sample. The number of albumin-positive cells was determined by multiplying the percentage of albumin-positive cells in the sample by the total number of released cells.

Data analysis

Data presented in the graphs are expressed as mean \pm standard deviation (SD) of triplicate wells. Each graph corresponds to a representative isolation from an individual donor. Data from multiple isolations are also presented in bar graphs, where for each isolation, the peak albumin or AFP production, expressed as a percentage of that produced in 3D coculture, was determined. The two-way ANOVA was conducted. Differences were considered statistically significant at a *p*-value less than 0.05.

RESULTS

LPC isolation

Based on the hypothesis that progenitor cells of different tissues may share some common surface markers, a monoclonal antibody—termed 5E12—isolated by decoy immunization and successfully used to enrich for neuroprogenitor cells¹⁶ was employed to help fractionate cells from human fetal liver. Flow cytometric analysis of human fetal liver cells stained with both the HLA and 5E12 antibodies revealed three distinct subpopulations (Fig. 1A). Each subpopulation was re-sorted to initiate replating by limiting-dilution in *in vitro* cultures ranging from 1 to 1000 cells/well onto murine fibroblast feeder cells.⁹ The conditioned media were

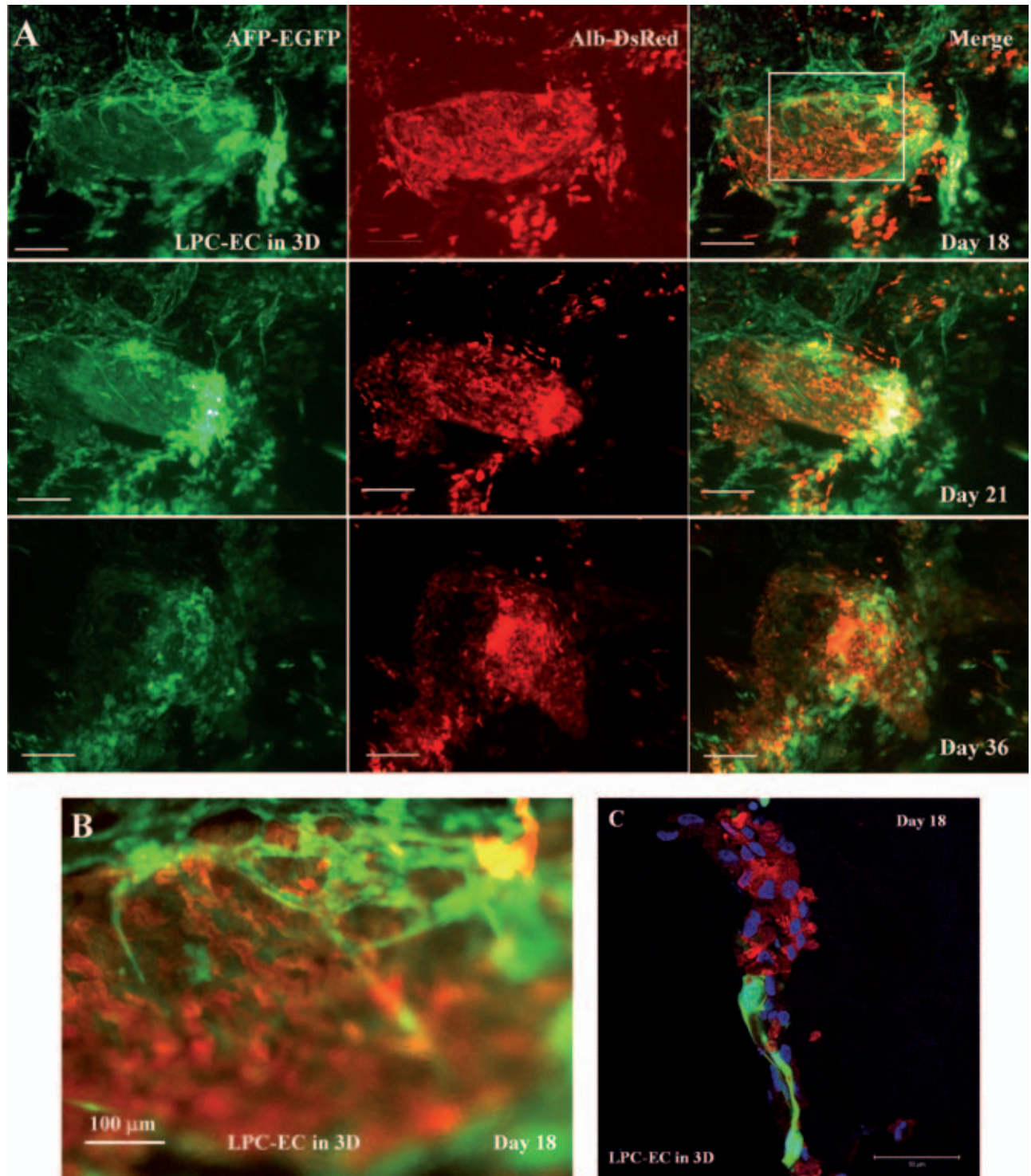


FIG. 3. LPCs cultured with HUVECs in 3D fibrin gel. Representative examples of LPCs transduced by both pRRLsin-AFP-EGFP and pRRLsin-Alb-DsRed2 lentiviral promoter–reporter vectors cocultured with HUVECs transduced by pRRLsin-PGK-EGFP in 3D fibrin gel. LPCs in cultures were tracked by both AFP-driven EGFP (left panel) and albumin-driven DsRed2 expression (middle panel) at the indicated time points (A). In the right panel is the merged graphics of EGFP- and DsRed2-based images using the Adobe Photoshop program. Vascular structures of HUVECs were identified by EGFP only (no DsRed2) expression. A magnified view white-lined box in upper right panel of A, boshowed the relationship between vascular structures and an LPC cluster at day 18 (B). In a separate coculture experiment, the association of LPCs and HUVECs in 3D fibrin at day 18 was further examined under confocal microscope (C). The LPC marker albumin was visualized with Alexa 555–conjugated secondary antibody (red), HUVEC was detected with FITC–conjugated CD31 antibody (green), and nuclei were counterstained with DAPI (blue). The white scale bar is 100 μm in (A, B) and 50 μm in (C).

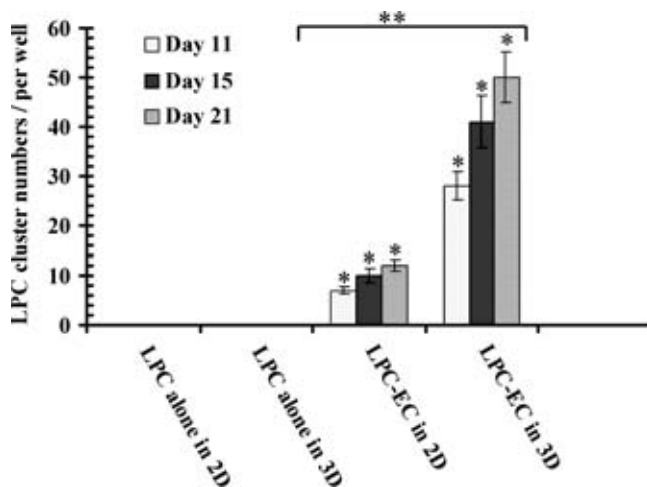


FIG. 4. Cluster numbers are greatest when LPCs are cultured with HUVECs in 3D fibrin gel. LPC cluster (as detected by lentiviral albumin and AFP promoter reporters in groups of more than 20 cells) numbers in each well were counted under a fluorescent microscope (40 \times) at the indicated time points. There were no clusters found in LPC-alone cultures either in 2D or 3D fibrin gels as indicated in the left columns of the bar graph. The cluster numbers at the indicated time points for the LPC–HUVEC (LPC-EC) cocultures in 2D or 3D fibrin gels, respectively, are shown. Data represent the mean \pm SD (error bar) of triplicate wells from a representative batch. The two-way ANOVA for cluster numbers reveals statistically significant differences between LPC-alone and coculture of LPCs and HUVECs ($*p < 0.001$) and between the 3D coculture and the 2D coculture ($**p < 0.005$) at each indicated time point.

serially assayed by ELISA for the secretion of hepatocyte lineage marker proteins (albumin, AAT, and AFP) to enable identification of subpopulations enriched for hepatic lineage progenitors. Accordingly, the isolated HLA^{low} 5E12⁺ cells (purity > 90%) were found to represent a highly enriched subpopulation of bipotential LPCs (Fig. 1B).³¹

HDV infection of LPC cultures

Colonies arising from LPCs could be identified by coimmunofluorescence with antibodies against albumin, AFP, and CK19.³¹ These colonies could be infected with HDV (Fig. 2). As shown in Figure 2A, individually infected cells could be readily identified by the characteristic nuclear staining pattern of HDV delta antigen.³² Amplification of viral RNA in cultures containing LPC colonies, indication of active replication, could be detected by Northern blots (Fig. 2B) of total cellular RNA extracted at various times after inoculation and subsequently probed for HDV genomic RNA.

Cocultures of LPCs and HUVECs

The above immunohistologic characterizations required that the cells be fixed. To follow the expression profile and

growth of LPCs over time in live cells, LPCs were transduced with lentiviral promoter–reporter vectors pRRLsin-AFP-EGFP and pRRLsin-Alb-DsRed2. These drive the expression of EGFP and DsRed in cells with the capacity to actively transcribe endogenous AFP and albumin genes, respectively (see Supplemental Fig. 1, available online at www.liebertpub.com). Both fluorescent markers could be detected in cells by day 6 to 8 posttransduction. Similarly, HUVECs stably transduced by pRRLsin-PGK-EGFP, in which EGFP expression is driven by a nonspecific housekeeping promoter PGK, were employed. Although green cells could formally represent LPCs or HUVECs, their distinct morphology usually allowed the HUVECs to be readily distinguished.

Coculture of LPCs and HUVECs in 3D fibrin gels led to the appearance of LPC clusters (Fig. 3A), defined as more than 20 closely adjacent cells expressing both EGFP and DsRed2. LPCs alone, whether in 2D or 3D fibrin gels, did not efficiently form clusters (Fig. 4 and Supplemental Fig. 2B, C, available online at www.liebertpub.com). Vascular structures of EGFP-expressing endothelial cells also became apparent after around 2 weeks in 3D coculture (Fig. 3A). Approximately 30 LPC clusters/well (12-well plate) were detectable in the 3D cocultures by day 11, and this increased to 50 clusters/well by day 21 (Fig. 4). LPC clusters started to become confluent after 4 weeks of coculture, and it was difficult to distinguish individual clusters thereafter. In contrast, in the 2D cocultures, clusters only formed close to the edges of the culture plate where there were some branched structures of HUVECs (Supplemental Fig. 2). HUVECs cultured alone in either 2D or 3D fibrin gel, even in the presence of LPC culture media, did not form vascular structure.

As shown in Figure 3A, the LPC clusters spanned up to 1000 μ m in length and 400 μ m in width by day 18. Vascular structures expressing EGFP-only HUVECs were noted in close association with the DsRed2- and EGFP-labeled LPC clusters (Fig. 3B). This close association was further confirmed by immunofluorescent staining for the CD31-positive HUVECs and the albumin-positive LPCs (Fig. 3C) under confocal microscope examination. By day 36, vascular structures began to regress and were almost undetectable by fluorescent microscopy at day 48. At approximately 5 weeks of coculture, the 3D fibrin gel began to undergo degradation with visible small open holes in the gel, suggestive of cell-induced degradation. After 8 weeks of coculture, this fibrin gel began to contract into an uneven stack of cells and extracellular matrix.

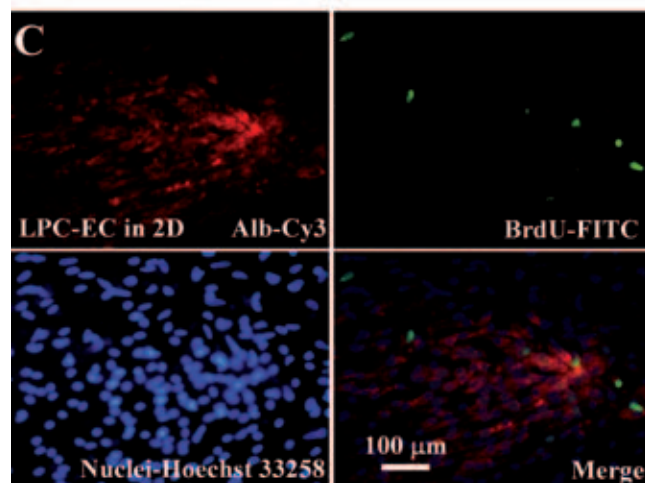
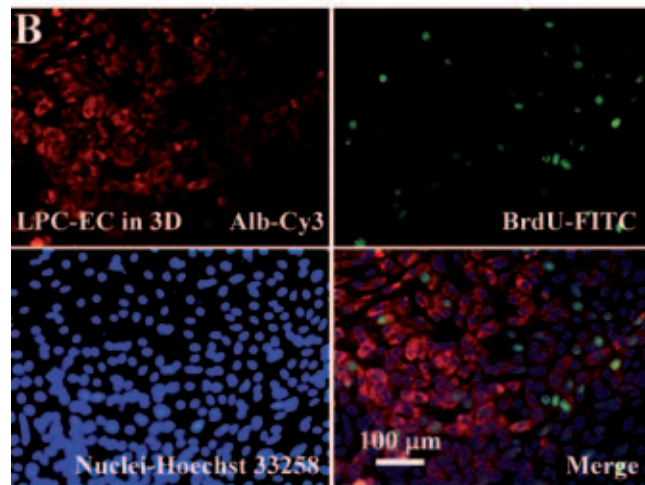
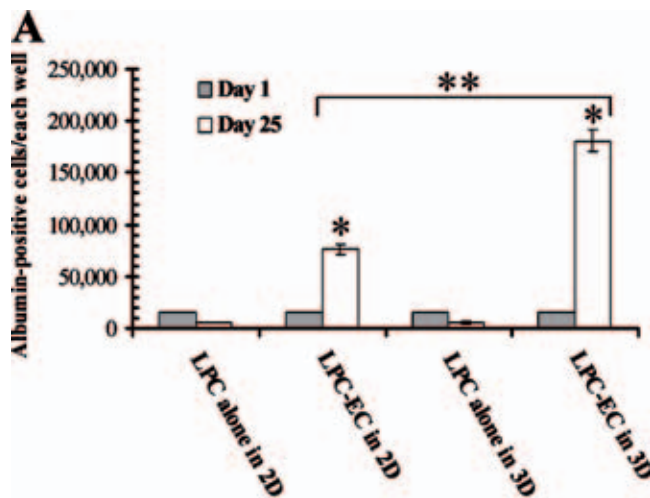
Albumin immunostaining analysis of cells extracted after 25 days of 3D coculture revealed a 12-fold increase in LPCs above the number extracted on day 1; in contrast, there was only a fivefold increase in the number of LPCs in 2D cocultures (Fig. 5A). There were less albumin-positive cells when LPCs were cultured alone in either 2D or 3D fibrin gels (Fig. 5A). As demonstrated by BrdU labeling of albumin-positive cells, LPCs proliferated when cocultured with

HUVECs in 3D (Fig. 5B) or 2D (Fig. 5C) fibrin gel. LPCs alone in 2D and 3D did not uptake BrdU at the same time point (data not shown). These data indicated that coculture of LPCs and HUVECs resulted in active proliferation of LPCs; further, LPCs demonstrated enhanced proliferation in

3D coculture conditions compared to LPCs cocultured in 2D gel or LPC-alone cultures.

Increased LPC albumin and AFP production in 3D coculture

To confirm that the lentiviral promoter reporters for albumin and AFP did indeed reflect increases in albumin and AFP production, ELISA for the latter was performed on media samples collected serially over time. For purposes of comparison, representative results shown in Figures 3A, 3B, 4, 5A, and 6 were from the same batch of LPCs. Compared to LPCs cultured alone, LPCs cocultured with HUVECs in either 3D or 2D gels secreted and sustained higher albumin levels for up to 8 weeks. This effect was more prominent in 3D cocultures than in 2D cocultures (Fig. 6A, C). After 1 week of culture, 3D coculture demonstrated increased AFP production above that observed with LPC-alone cultures (2D or 3D) or 2D coculture (Fig. 6B, C). High levels of AFP production were maintained for up to 38 days in 3D cocultures but were markedly diminished by 26 days in 2D coculture. There was no increase in AFP or albumin production in LPCs alone in 2D or 3D fibrin gel (Fig. 6A–C). HUVECs in either 2D or 3D fibrin gel did not produce albumin or AFP.



Coculture enhancement of albumin production requires a close association

To determine whether close association between the LPCs and HUVECs was essential to the enhanced LPC albumin production in 3D coculture, we cocultured LPCs with HUVECs in each of two layers of 3D fibrin gels in the

FIG. 5. LPC proliferation and BrdU incorporation in cocultures of LPCs and HUVECs. LPCs (1.5×10^4) were cultured alone or with HUVECs (3×10^4) in 2D or 3D fibrin gels, respectively. After 25 days of culture, cells in 2D or 3D fibrin gel were released, counted, and stained for albumin. Total albumin-positive cells in each sample were calculated. Coculturing LPCs with HUVECs in 3D resulted in more albumin-positive cells than that in 2D coculture or LPCs cultured alone (A). Data presented in the bar graph are expressed as mean \pm SD of triplicate wells from a representative batch. The two-way ANOVA reveals statistically significant differences between the number of albumin-positive cells in LPC-alone cultures versus that in LPC–HUVEC cocultures ($*p < 0.001$) and in the number of albumin-positive cells in 3D cocultures versus 2D cocultures ($**p < 0.005$). BrdU incorporation in LPC–HUVEC cocultures in 3D (B) or 2D (C) at day 18 further indicated that LPCs proliferated. The LPC marker albumin was visualized with a Cy3-conjugated secondary antibody (red, Albumin-Cy3), BrdU was detected with FITC-conjugated anti-BrdU antibody (green, BrdU-FITC), and nuclei were counterstained with Hoechst 33258 (blue, Nuclei-Hoechst 33258). Merged graphics were generated by the Adobe Photoshop program using the Albumin-Cy3, BrdU-FITC, and Nuclei-Hoechst 33258 images. Scale bar is 100 μ m.

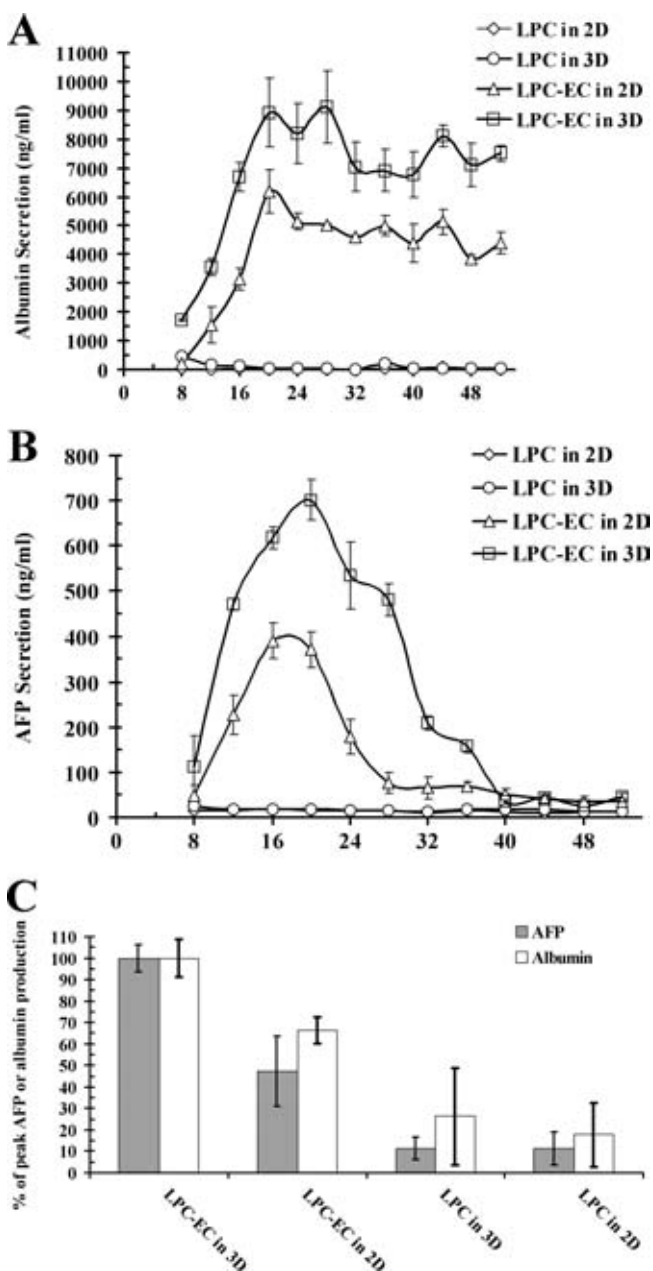


FIG. 6. The increased secretion of albumin and AFP in cocultures of LPCs and HUVECs. Supernatants were collected every 2 days from LPC-alone cultures or cocultures of LPCs and HUVECs. Albumin (A) and AFP (B) concentrations in the supernatants were measured by ELISA. Coculturing of LPCs and HUVECs resulted in higher levels of secreted albumin and AFP than culturing LPCs alone. LPCs cocultured with HUVECs in a 3D fibrin gel exhibited greater elevation and sustained levels of secreted albumin and AFP when compared to a 2D fibrin gel. Data presented in the line graphs are expressed as mean \pm SD of triplicate wells from a representative batch. Data from multiple isolations are also presented in bar graphs where for each isolation, the peak albumin or AFP production, expressed as a percentage of that produced in 3D coculture, was determined (C).

same dish with the same culture media. The separation of LPCs and HUVECs in this coculture method, which limited initial LPC–HUVEC association, resulted in abolishment of vascular structures, and was accompanied by the lowest production of albumin (Fig. 7A, C) and of AFP (Fig. 7B, C).

To further confirm this close LPC–HUVEC association, means a LPC–HUVEC contact, the LPC–HUVEC transwell culture was performed. HUVECs, seeded onto the collagen-coated transwell insert, reached confluence in 3 to 4 days. The levels of the secreted AFP and albumin in the supernatants of transwell culture are as low as those in the supernatants of LPCs alone, and much lower than those in the supernatants of LPC–HUVEC coculture (Fig. 7D, E).

DISCUSSION

In this paper, we evaluated culture conditions to optimize the proliferation of human LPCs that were prospectively isolated by cell surface markers using FACS sorting.^{18,36} Although the identity of the antigen recognized by the 5E12 monoclonal antibody is not yet known, it has been shown to be a relevant marker for progenitor cells in at least the neural lineage.^{16,36} This antibody enabled the enrichment of LPCs from human fetal liver (Fig. 1). These LPCs cultured on feeder layers of FFS were capable of giving rise to colonies of bipotential phenotype including both albumin and CK19-expressing cell characteristic of hepatocyte and biliary cells, respectively.^{18,36} Among the markers of advanced differentiation arising in these colonies are the receptors for infection by hepatitis D virus, as demonstrated in Figure 2.

When these highly purified LPCs were cultured alone, under standard 2D conditions, there was minimal *in vitro* expansion of these cells. Since others have reported that human fetal liver cells or partially purified fetal liver progenitors^{10–12,37} are able to expand when cultured under standard 2D conditions or cocultured with stromal cells such as FFS,⁹ we postulated that the highly purified LPC population excludes some cells that may support progenitor cell proliferation *in vitro*.

We further postulated that these supporting cells may be endothelial cells based on the findings of others that liver development and regeneration have been demonstrated to be closely linked to angiogenic endothelial cells *in vivo*.^{19,24,26,27,38} To test this hypothesis, we cocultured highly purified LPCs with nonparenchymal endothelial cells in 3D fibrin gel in order to allow LPCs to utilize natural *in vivo* mechanisms to facilitate their expansion and function *in vitro*. We report that, compared to 2D culture conditions and to cultures of LPCs alone in 3D, LPCs cocultured with EC under 3D conditions exhibited increased expansion and increased and sustained production of albumin and AFP.

Our data supported our hypothesis and demonstrated that coculturing LPCs with endothelial cells in 3D fibrin gel enhanced the LPC expansion along with the formation of

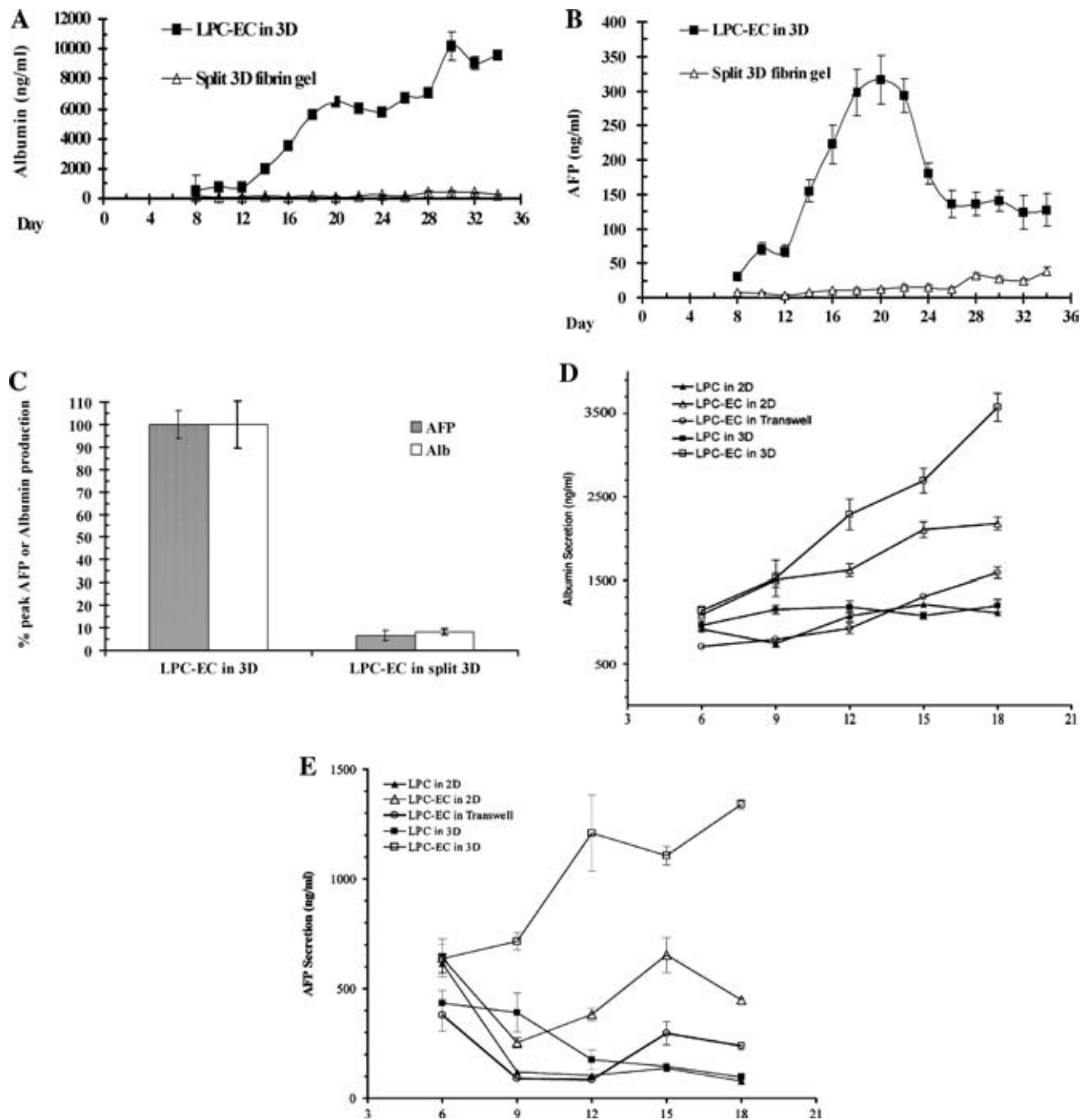


FIG. 7. Albumin and AFP secretion from LPC–HUVEC cocultures grown in split 3D fibrin gels and transwell culture. The secretion of albumin (A) and AFP (B) in “split 3D” fibrin gel culture, compared to the control cocultures with the same number of cells grown together in a single 3D fibrin gel, was measured by ELISA. Data presented in the line graphs are expressed as mean \pm SD of triplicate wells from a representative batch. Data from multiple isolations are also presented in bar graphs where for each isolation, the peak albumin or AFP production, expressed as a percentage of that produced in 3D coculture, was determined (C). Albumin (D) and AFP (E) production, measured by ELISA, in the supernatants of triplicate wells in transwell cocultures (open circles) are compared to the secretion of aforementioned proteins in cocultures with the same number of cells grown together in a single 2D or 3D fibrin gel and are compared to LPC-alone cultures.

vascular structures. The LPCs had the capacity for further growth and expansion in 3D coculture, as demonstrated by the BrdU labeling (Fig. 5). LPC proliferation and production of albumin and AFP both were enhanced by coculture with

HUVECs in 3D fibrin gel (Figs. 5 and 6). The split-3D fibrin gel and transwell culture of LPCs and HUVECs indicated that the LPC–HUVEC direct contact is essential for LPC function (Fig. 7). Less frequently, some clusters of LPCs in

these 3D cocultures were observed to not be directly associated with HUVECs, when examined *in situ* by fluorescent or confocal microscopy. These findings of EC-dependent growth, even without continued direct contact, may be explained by a recent report that direct contact between hepatocytes and supportive stromal cells—fibroblasts, followed by a sustained short-range soluble signal, is enough for maintenance of the hepatocellular phenotype in coculture.³⁹ The ability of both cells to survive and expand to the late time points, as described in our results, implies that LPCs and HUVECs had a reciprocal effect on each other's growth (Fig. 4). The vascular structures described in the 3D culture model in this paper, which occurred in a period of 1 to 2 weeks, are consistent with *in vitro* angiogenesis model systems described by others.^{40–42} This structure, however, began to regress at day 36 and was almost undetectable by fluorescent microscopy at day 48. Nahmias *et al.*⁸ described a coculture system containing mature hepatocytes and endothelial cells on matrigel, in which mature hepatocytes were recruited to endothelial vascular structures by endothelial cell–derived hepatocyte growth factor and then formed a sinusoid-like structure. This sinusoid-like structure retained hepatic function up to 2 months.⁸ Interestingly, the stability of the sinusoid-like structures was dependent on the presence of dermal fibroblasts in culture.⁸ It remains to be addressed whether LPCs in our coculture system were contaminated by fetal liver–derived fibroblast-like cells and whether, if contaminated, those fibroblast-like cells have a similar effect on vascular structures as dermal fibroblasts had.

We did not address in this paper whether these vascular structures demonstrated functional characteristics of blood vessels, and limited ourselves to correlating the differences in LPC function with the structural events noted to occur with these endothelial cells. We did, however, confirm that these resulting vascular structures arose from EGFP-expressing HUVECs. We do not believe that the findings were specific to HUVECs in that freshly isolated endothelial cells from human fetal liver also enhanced the secretion of albumin and AFP when cocultured with LPCs (data not shown). How cells, including endothelial cells, LPCs, and maybe other types of cells, extracellular matrix, and cytokines/growth factors in 3D fibrin gel enhanced LPC proliferation and how the initiation, development, and regression of vascular structures were affected by LPC expansion and manipulations to optimize *in vitro* expansion in this model are the subjects of ongoing studies.

We selected fibrin gel as a polymer because it is a natural extracellular matrix and hydrogel and has already been used in clinical applications. Fibrin gel is derived from fibrinogen and does not contain other extracellular matrix and growth factors as matrigel does. The 3D fibrin gel has been proven to be suitable for an *in vitro* angiogenesis model,²⁹ and it is biocompatible and biodegradable, desirable characteristics for tissue engineering purposes.⁴³ During preparation of 3D fibrin gels containing LPCs and endothelial cells, polymerization just requires a very mild enzymatic reaction condi-

tion and thus has no adverse effects on the cells. The firmness of fibrin gel is adjustable through changing fibrinogen concentration, and cell density in 3D fibrin is also changeable. The fibrin gel–containing cocultures, however, underwent obvious degradation beginning at the fourth week and eventually became degraded into an uneven stack of cells and extracellular matrix. The secretion of matrix metalloproteinases and plasmin in a coculture was the likely cause for fibrin gel degradation. The degradation of fibrin may affect long-term maintenance of hepatocyte culture *in vitro* but is an attractive characteristic of a cell delivery vehicle for implants *in vivo*.^{44–46}

Though there were some clusters formed in the 2D cocultures, these clusters were primarily located on the edges of the culture wells, which differ from the distribution of LPC clusters in the 3D coculture system (which were located throughout). The fibrin gel was relatively thicker at the edges of the 2D fibrin cultures than in the central counterpart. We therefore postulate that the thicker edges of the 2D fibrin gel allow for both formation of vascular structure similar to the 3D gels and the secondary effects of HUVECs on LPC growth and function.

In summary, we have successfully tested the hypothesis that isolated bipotential liver progenitor cells from human fetal liver, when cultured under 3D angiogenic conditions, demonstrate the enhanced proliferation in culture. We propose that these culture conditions also favor the development of an optimal microenvironment for LPC expansion including LPC–HUVEC contact, growth factors, and extracellular matrix. Our novel description of the LPC expansion along with the formation of vascular structures of endothelial cells in this 3D model might provide a unique advantage for engineered liver devices and antiviral screening.

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