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Monolayer and Spheroid Culture of Human Liver Hepatocellular Carcinoma Cell Line Cells Demonstrate Distinct Global Gene Expression Patterns and Functional Phenotypes

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Understanding cell biology of three-dimensional (3D) biological structures is important for more complete appreciation of *in vivo* tissue function and advancing *ex vivo* organ engineering efforts. To elucidate how 3D structure may affect hepatocyte cellular responses, we compared global gene expression of human liver hepatocellular carcinoma cell line (HepG2) cells cultured as monolayers on tissue culture dishes (TCDs) or as spheroids within rotating wall vessel (RWV) bioreactors. HepG2 cells grown in RWVs form spheroids up to 100 μm in diameter within 72 h and up to 1 mm with long-term culture. The actin cytoskeleton in monolayer cells show stress fiber formation while spheroids have cortical actin organization. Global gene expression analysis demonstrates upregulation of structural genes such as extracellular matrix, cytoskeletal, and adhesion molecules in monolayers, whereas RWV spheroids show upregulation of metabolic and synthetic genes, suggesting functional differences. Indeed, liver-specific functions of cytochrome P450 activity and albumin production are higher in the spheroids. Enhanced liver functions require maintenance of 3D structure and environment, because transfer of spheroids to a TCD results in spheroid disintegration and subsequent loss of function. These findings illustrate the importance of physical environment on cellular organization and its effects on hepatocyte processes.

Introduction

Liver transplantation is currently the only cure for patients with end-stage liver disease, but availability of donor organs is a major limitation.[1] Tissue engineering is a burgeoning field of investigation and explores the possibility of "building" a liver *ex vivo* for therapeutic replacement.[2]

A number of challenges exist for constructing a functioning complex solid organ such as the liver. One of the first requirements is to provide a three-dimensional (3D) environment for the cells to form tissues. A variety of strategies have been used to provide a 3D structure for culturing primary hepatocytes and hepatic cell lines. A major approach is to use biodegradable scaffolds.[3–14] However, hepatocytes can also self-assemble into spheroids without scaffolding. This can be achieved by culturing in spinner flasks[15] or on specially treated surfaces.[16,17] Spheroids formed using these methods range from 50 to 100 μm in size. Yoffe *et al.*[18,19] first described one efficient culture technique for generating larger hepatic spheroids using the rotating wall vessel (RWV) bioreactor. The RWV is a disc-like vessel completely filled with medium that rotates around a horizontal axis during culture. RWVs provide a low-turbulence, low-shear-force environment with 3D spatial freedom for the cells to aggregate and grow.[20,21] In our research, we use spheroids formed in the RWV to study the biology of hepatocyte 3D culture without confounding interactions with scaffolding or substratum materials.

Some studies have reported better liver-specific functions in hepatocyte 3D cultures than in conventional two-dimensional (2D) monolayers,[3,17,22] however, the mechanisms of the functional improvement remain unclear. There is a growing body of evidence that mechanical stress mediated by adhesion to extracellular matrix (ECM) or other cells modulates signal transduction and gene transcription in a variety of cell types.[23,24] In this study, we demonstrate that human liver hepatocellular carcinoma cell line (HepG2) cells respond to differing physical environments of 2D and 3D culture with altered actin cytoskeleton structure and cell shape. Through global gene expression analysis, we find that distinct genetic programs are initiated depending on the physical structure of the cells. Monolayers express high levels of ECM, cytoskeleton, and adhesion molecules. These transcripts are downregulated in the spheroids while metabolic and synthetic functional genes are upregulated. The differences in gene

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expression reflect the greater cytochrome P450 activity and albumin production in spheroids. Enhanced liver-specific functions are dependent on maintenance of 3D structure because they are lost after transfer of spheroids to a tissue culture dish (TCD). Together, these results illustrate the importance of the physical environment on hepatocyte cellular function and inform future efforts in liver tissue engineering.

Material and Methods

Cell culture

HepG2 Cells (ATCC, Manassas, VA) were maintained in T75 culture flasks in 10% fetal calf serum (Hyclone, Logan, UT) in Eagle's minimum essential medium supplemented with glutamine, antibiotics and pyruvate (Fisher, Philadelphia, PA). For experiments, cells were placed in 6 cm TCDs or 10 mL RWVs with a diameter of 6 cm (high-aspect-ratio vessels, HARVs; Synthecon, Houston, TX). Cells were cultured in 10 mL of medium at a cell density of 5×10^4 cells/mL for TCDs and RWVs. For short-term cultures up to 7 days, no medium was exchanged in TCD or RWV cultures and 10 mL HARVs were rotated at 16 rpm with the RCCS-4 culture system (Synthecon). Cell densities in TCDs and RWVs were approximately 3×10^5/mL at day 3 of culture and 4×10^5 to 5×10^5/mL at day 7 of culture. For long-term culture (6–10 weeks), 50 mL HARVs were rotated at 16 rpm for the first 7 days and then at 20 rpm thereafter to keep spheroids in the center of the rotational axis. Medium was changed weekly in long-term cultures.

Light and fluorescence microscopy

Phase contrast photos were taken using a Canon PowerShot A540 (Canon USA, Lake Success, NY) adapted to the microscope eyepiece. Cell sizes were measured using a stage micrometer. For fluorescence microscopy, cells were stained with rhodamine phalloidin (1:50 dilution; Invitrogen, Carlsbad, CA) and Hoechst dye (2 μg/mL; CalBiochem, San Diego, CA) in 1% bovine serum albumin/phosphate buffered saline for 30 min at room temperature. Cells were then washed and mounted on slides with Flouromount (SouthernBiotech, Birmingham, AL). Spheroids were mounted as a wet preparation without a coverslip to preserve the natural 3D architecture. Fluorescent images were taken using a Zeiss Axioscope fluorescent microscope (Carl Zeiss, Oberkochen, Germany) and Orca ER CCD digital camera (Hamamatsu, Bridgewater, NJ)

Microarray sample preparation and analysis

RNA from day 3 cultures of monolayers and spheroids were amplified and biotinylated using the MessageAmp II-Biotin Enhanced Kit per the manufacturer's instructions (Ambion, Austin, TX). Initial RNA integrity was verified and final fragmented amplified RNA (aRNA) analyzed using the Agilent 2100 Bioanalyzer (Santa Clara, CA). Fifteen μg of biotinylated aRNA were hybridized onto the Human U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA). Microarray data were analyzed using GeneSpring 7.3 software (Silicon Genetics, Redwood City, CA). Expression was normalized to median and raw expression data cut off at 50 to eliminate non-specific background. Significant genes were flagged with present calls and demonstrated at least 2-fold changes in expression between monolayers and spheroids.

Quantitative real-time reverse transcription polymerase chain reaction

Two-step quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed on RNA from day 3 monolayer and spheroid cultures as previously described.[25] Selected genes were also tested on day 7 cultures (3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGCR), low-density lipoprotein receptor (LDLR), albumin (ALB), and cytochrome P450 1A1 (CYP1A1)). The thermal profile was 50°C for 2 min, then 95°C for 10 min, followed by 40 amplification cycles consisting of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. Relative quantification of genes was calculated using the $2^{-(Ct\ gene\ 1 - Ct\ CPHI\ 1)-(Ct\ gene\ 2 - Ct\ CPHI\ 2)}$ equation, where "C_t gene 1" represented the calculated threshold cycle (C_t) of the target gene in culture condition 1 (2D or 3D). "C_t gene 2" was the C_t of the target gene in the other culture condition. "C_t CPHI 1" and "C_t CPHI 2" were for the cyclophilin housekeeping gene in each of the respective conditions. Cyclophilin expression levels remained constant between TCD and RWV conditions and between all time points tested.

Primers were designed using Oligo 6.0 software to span introns and custom made by Operon (Huntsville, AL). See Table 1 for primer sequences.

Cyquant assay

Cell aliquots were taken for Cyquant Assay following the manufacturer's instructions (Invitrogen).

Albumin enzyme-linked immunosorbent assay

Cell culture supernatants were collected and albumin concentration determined by a human albumin enzyme-linked immunosorbent assay (ELISA) kit per the manufacturer's instructions (Bethyl, Montgomery, TX).

7-ethoxyresorufin-o-dealkylase assay for CYP1A1activity

7-ethoxyresorufin-o-dealkylase (EROD) assays were performed as previously described.[7,26]

Statistical analysis

Statistical analyses were performed with InStat 3.0 software using the two-tailed Student *t*-test. Significance is considered when $p < 0.05$.

Results

HepG2 cells cultured in the 3D environment of RWVs form cellular spheroids with cortical actin organization up to 500 μm to 1 mm in diameter

HepG2 cells cultured on TCDs proliferated as 2D clusters and eventually became confluent as a monolayer (Fig. 1A, C). In contrast, within the 3D environment of the RWV, HepG2 cells formed 3D aggregates and spheroids. By day 3 of culture, visible spheroids up to 100 μm in diameter were formed within the RWV (Fig. 1B). Whereas cells grown in TCDs were spread and flat, cells in the spheroids were round

Table 1. Primers for qRT-PCR

Primer Name	*Primer Sequence (5′ to 3′)*
CYP1A1	F- ggA gCT AgA CAC AgT gAT Tgg C
	R- ggT gAA ggg gAC gAA ggA
AKR1C1	F- AgA CAT TgT TCT ggT TgC CTA T
	R- AAg ggT CAA ATA TCg CAC AT
EPHX1	F- CCA gAA gCA TgA gCg gAT gA
	R- CgA CAg gAA CTT gCg gAT gT
LTB4DH	F- ACC TTg AAg AAA gCg TCT CC
	R- TCC TTg CCA gCg gTA gA
LDLR	F- CAA TgT CTC ACC AAG CTC T
	R- TCT gTC TCg Agg ggT AgC T
HMGCR	F- TAC CAT gTC Agg ggT ACg T
	R- CAA gCC Tag AgA CAT AAT CAT
GSTA1	F- AgA gCC CTg ATT gAT ATg TA
	R- gTT gCC AAC AAg gTA gTC TT
GCLM	F- ATC AgT ggg CAC Agg TAA AA
	R- TgA CCg AAT ACC gCA gTA g
ALB	F- CgT TCC CAA AgA gTT TAA TgC
	R- AAg CTg CgA AAT CAT CCA TAA C
ATP5I	F- CAg gTC TCT CCg CTC ATC
	R- TTC TCT ggC AAT CCg TTT CA
NDUFA3	F- Tgg TCg TgT CCT TCg TCg TC
	R- ggg CAC Tgg gTA gTT gTA g
COL1A1	F- gTC CCC CTg gCT CTg CTg gTT
	R- Tgg gTA gAA ggA gAg TTT ggT A
CSPG2	F- gTg TgC CAg gAT ACA gC
	R- TTg TgC CAg CCA TAg TCA
CDH1	F- gCC CTg CCA ATC CCg ATg AA
	R- CgC CgC CTC CgT ACA T
CLDN6	F- CAT Cgg CAA CAg CAT CgT
	R- CgC CAg CAg TgA gTC gT
RAB3B	F- ggT ggg gAA CAA gTg TgA CAT
	R- ggg TgT CCg AgA gAC gC
AXL	F- TTT CCT gAg TgA AgC ggT CT
	R- gCA TCT gAg Tgg gCA ggT ACA C
CPHI	F- TgA CTT CAC ACg CCA TAA Tg
	R- CAC ATg CTT gCC ATC CAA CCA C

F, forward primer; R, reverse primer.

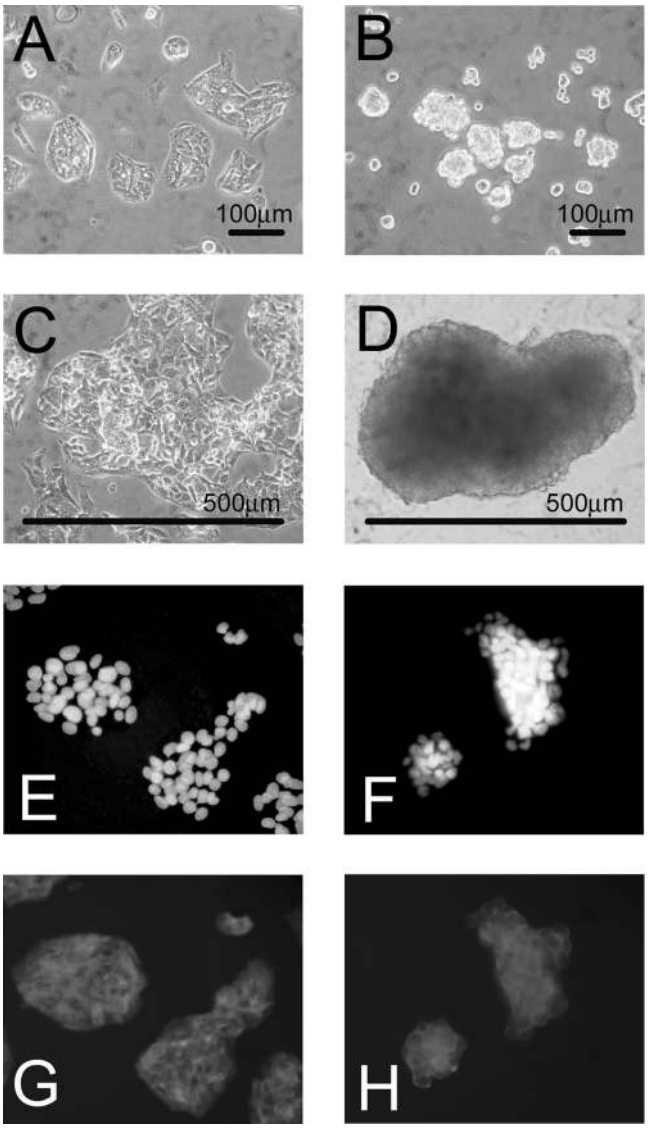

FIG. 1. Human liver hepatocellular carcinoma cell line (HepG2) cells form cellular spheroids with cortical actin organization when cultured in the three-dimensional environment of rotating wall vessels (RWVs). HepG2 cells were cultured on tissue culture dishes (TCDs) (**A, C, E, G**) or in RWVs (**B, D, F, H**). Light-field microscopy was performed after 3 days (**A, B**) or 7 days (**C, D**). Cells grown on TCDs had spread cytoplasm and formed a monolayer by day 7. Spheroids in RWVs were up to 100 μm in diameter by 3 days and 500 μm by 7 days. Cells were stained with Hoechst dye for nuclei (**E, F**) and rhodamine phalloidin for actin (**G, H**) at day 3 of culture and visualized using fluorescence microscopy. Whereas cells in monolayers formed F-actin stress fibers, cells in spheroids had cortical actin organization. Magnification 30×.

and had more compact cytoplasm. The spheroids increased in size up to 500 μm by day 7 (Fig. 1D), and long-term culture created large spheroids up to 1 mm in diameter (Fig. 2B). Hoechst staining of nuclei demonstrated that cells were spread out two-dimensionally on the TCD, but clustered tightly three-dimensionally in the RWV spheroids (Fig. 1E, F). Moreover, the actin cytoskeleton of cells in monolayers and spheroids were remarkably different. Cells in monolayers showed mainly F-actin stress fibers for attachment to the surface. In contrast, cells in spheroids demonstrated cortical actin organization outlining the cells (Fig. 1G, H).

Global gene expression analysis reveals differential upregulation of structural genes in monolayers and metabolic functional genes in spheroids

To investigate the underlying cellular and molecular differences between monolayers and spheroids, we performed a global gene expression analysis using the whole human genome microarray from Affymetrix. HepG2 cells were subcultured on TCDs or in RWVs. Three days later, when 100-μm spheroids were first being formed in the 3D cultures, RNA from each condition was purified for microarray analysis.

Significant genes were filtered according to background expression (raw expression greater than 50), presence of flags, and at least 2-fold expression differences between the culture conditions. Using these criteria, 250 genes were identified to be upregulated in the monolayers. A distinct set of 210 genes were upregulated in the spheroids. Close examination of the biological functions of these differentially expressed genes

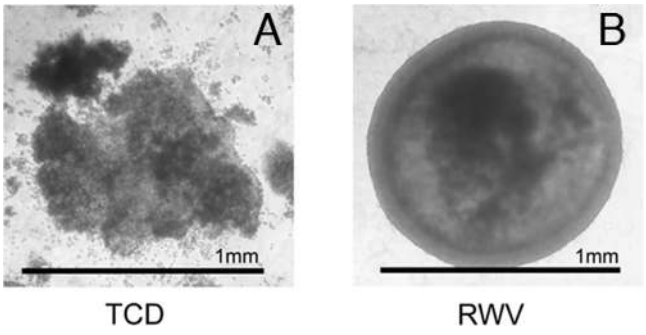

FIG. 2. Spheroid cellular architecture is dependent on continued culture in rotating wall vessels (RWVs). Human liver hepatocellular liver carcinoma cell line cells were cultured for 6 weeks in the three-dimensional (3D) environment of the RWV bioreactor. Formed cellular spheroids were subcultured in tissue culture dishes (TCDs) or RWVs for 7 days. Light microscopy showed disintegration of the spheroids in TCDs (**A**), compared with intact spheroids that remained in the 3D environment of RWVs (**B**). Magnification 20×.

revealed that significantly more genes related to ECM, cytoskeleton, and cell adhesion were expressed in monolayer cells (Table 2). On the other hand, genes involved in liver-specific functions of xenobiotic and lipid metabolism were upregulated in spheroids. In addition, more genes involved in cell cycle and regulation of growth and proliferation were upregulated in monolayers. There were also functional categories such as apoptosis, signal transduction, and transcription in which the number of genes differentially regulated were not significantly different between the two conditions.

Representative genes identified using the microarray analysis were verified with qRT-PCR. Genes upregulated in spheroids (Fig. 3A) could be broadly categorized into those involved in metabolic or synthetic pathways. In particular, expression of genes involved in xenobiotic metabolism was markedly upregulated compared to monolayers, including cytochrome P450 1A1 (CYP1A1) (10.6 ± 2.8 fold), aldo-keto reductase 1C1 (5.2 ± 2.3 fold), and epoxide hydrolase 1 (2.8 ± 0.7 fold). Expression of leukotriene B_4 12-hydroxydehydrogenase, important for metabolic inactivation of leukotriene B_4, was also greater (3.5 ± 1.3 fold). For synthetic pathways, two critical genes for glutathione synthesis, glutathione S-transferase A1 (5.0 ± 2.0 fold) and glutamate-cysteine ligase (2.7 ± 0.5 fold), were highly induced in spheroids. Transcription of albumin, an important liver-specific protein product, was greater (1.4 ± 0.3 fold). There was also a modest but significant increase in the expression of genes necessary for adenosine triphosphate (ATP) production (ATP synthase (1.4 ± 0.2 fold) and nicotinamide adenine dinucleotide dehydrogenase (1.3 ± 0.2 fold)).

The expression of metabolic and synthetic functional genes within spheroids changed differently with time. Expression of key genes for cholesterol metabolism, low-density lipoprotein receptor (LDLR) and HMG-CoA reductase (HMGCR), was comparable to that of controls in day 3 spheroids (Fig. 3) and significantly greater by day 7 (LDLR, 1.4 ± 0.2 fold, and HMGCR, 2.0 ± 0.4 fold, $p < 0.001$). Expression of albumin transcripts, significantly higher in day 3 spheroids, was not different from that of controls by day 7 (0.9 ± 0.3 fold, not statistically significant). Finally, the marked 10-fold increase in CYP1A1 expression in spheroids on day 3 was maintained in day 7 spheroids (10.1 ± 1.0 fold, $p < 0.005$).

There were also genes that were significantly up regulated in monolayers. Specifically, levels of ECM genes were dramatically higher compared to spheroids (Fig. 3B). Expression of collagen type I, alpha 1 and chondroitin sulfate proteoglycan 2 (versican) was higher in monolayers by 69.2 ± 7.5 fold and 11.4 ± 1.6 fold, respectively, compared to spheroids. Genes important for cell-to-cell adhesion, such as E-cadherin (CDH1, 3.2 ± 0.4 fold) and claudin 6 (component of tight junctions; CLDN6, 3.5 ± 0.8 fold), were also expressed significantly higher in monolayers. In addition to structural genes, expression of RAB3B (7.5 ± 1.2 fold) and AXL (6.0 ± 1.7 fold), two genes likely important in regulating cell differentiation and proliferation, were also increased in monolayers.

Using qRT-PCR, we compared the expression of several liver-enriched transcription factors, including forkhead box protein A1 (FOXA1), FOXA2, FOXA3, FOXM1, hepatic nuclear factor 1 alpha (HNF1α), HNF4α, HNF6, and CCAAT-enhancer-binding protein beta. None of these transcription factors showed significant differential expression between monolayers and spheroids (data not shown).

Table 2. Number of Genes Upregulated by at Least 2 Fold in Monolayers or Spheroids as Determined by Microarray Analysis

	Number of Genes	
Category	*Monolayers*	*Spheroids*
Total	250	210
Extracellular Matrix	10	0
Cytoskeleton	10	5
Cell Adhesion	21	4
Cell Cycle	13	7
Growth/Proliferation	25	10
Xenobiotic Metabolism	0	6
Lipid Metabolism	4	11
Apoptosis/Cell Death	11	12
Signal Transduction	26	20
Transcription	20	21

Overall proliferation is comparable between monolayer and spheroid cultures, but liver metabolic and synthetic functions are enhanced in spheroids

We tested whether differential gene expression between 2D and 3D cultures was reflected in the functional phenotypes of HepG2 cells and compared their proliferation cultured as monolayers or as spheroids. At 6 h, 24 h, 72 h, and 6 days after subculture in the two conditions, cell numbers were determined using the Cyquant cell proliferation assay (Fig. 4). More cell cycle and proliferation genes were upregulated in monolayers (e.g., AXL confirmed using qRT-PCR) at 72 h, correlating with the greater cell proliferation in monolayers at this time point. However, there were not consistent significant differences in overall proliferation between 2D and 3D cultures. Cell numbers were higher in the spheroid cultures at early time points but became lower and equivalent to those in monolayers by 72 h and 6 days, respectively.

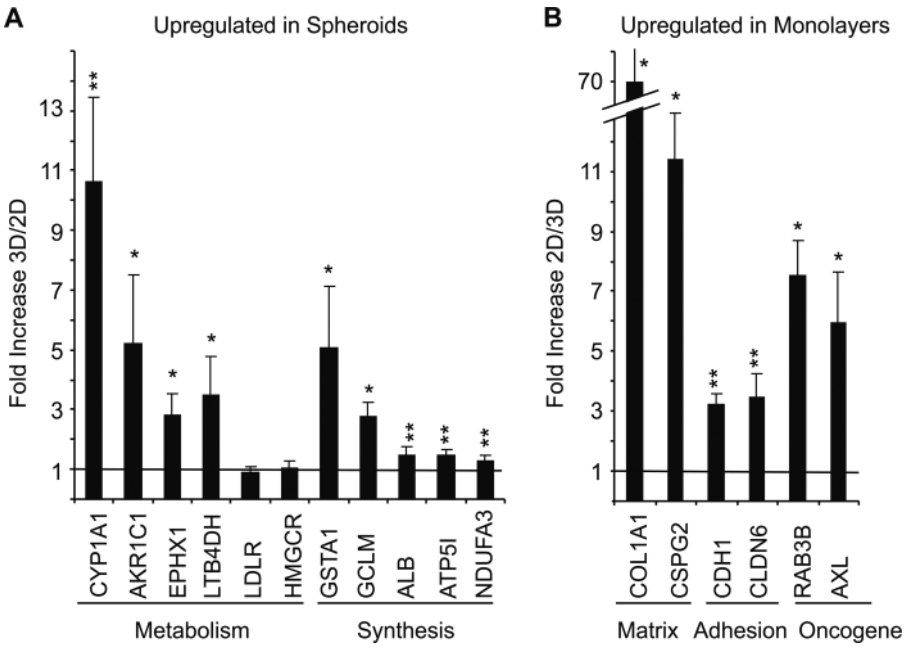

FIG. 3. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) of selected genes shows that metabolic and synthetic genes are upregulated in spheroids, whereas structural genes and certain oncogenes are upregulated in monolayers. qRT-PCR was performed on day 3 cultures of monolayers and spheroids. Gene expression was normalized to the housekeeping gene cyclophilin, and fold increase in one culture condition was calculated relative to the other condition. Figure represents pooled data from three independent experiments with three independent biological samples for each condition ($n = 9$). Gene symbols: CYP1A1, cytochrome P450 1A1; AKR1C1, aldo-keto reductase 1C1; EPHX1, epoxide hydrolase 1; LTB4DH, leukotriene B_4 12-hydroxydehydrogenase; LDLR, low-density lipoprotein receptor; HMGCR, 3-hydroxy-3-methyl-glutaryl coenzyme A reductase; GSTA1, glutathione S-transferase A1; GCLM, glutamate-cysteine ligase; ALB, albumin; ATP5I, adenosine triphosphate synthase; NDUFA3, nicotinamide adenine dinucleotide dehydrogenase; COL1A1 – collagen type I, alpha 1; CSPG2, chondroitin sulfate proteoglycan 2 (versican); CDH1, E-cadherin; CLDN6, claudin 6. $^*p < 0.001$; $^{**}p < 0.005$.

Liver-specific metabolic and synthetic functions were significantly higher in spheroids compared to monolayers. 7-ethoxyresorufin-o-dealkylase assays were performed in 7-day monolayers and spheroids to compare CYP1A1 activity between 2D and 3D cultures. Cell numbers were determined using the Cyquant assay to calculate the level of CYP1A1 activity (i.e. amount of resorufin product formed) per cell. HepG2 cells within the spheroids exhibited significantly greater CYP1A1 metabolic activity than in monolayers (Fig. 5A). In addition, culture supernatants were collected and albumin content measured using ELISA assay. Amount of albumin was divided by cell number to determine the synthetic function per cell. HepG2 cells in spheroids produced significantly more albumin than in monolayers (Fig. 5B).

Spheroid morphology and enhanced liver functions are dependent upon continuous culture within the RWV bioreactor

To determine whether continuous culture within the RWV was necessary to maintain spheroid architecture and enhanced liver-specific functions, we created long-term spheroid cultures for 6 to 10 weeks. Spheroids up to 1mm in size were formed and then subcultured in TCDs or continued in the 3D environment of RWVs. After 7 days, spheroids in TCDs were completely dissociated without external disruption, and some cells appeared to be attaching to the surface (Fig. 2A). In contrast, spheroids continued in the RWV maintained their spheroid morphology (Fig. 2B).

Not only was continued culture within the RWV necessary to maintain spheroid morphology, it was also important for maintaining the enhanced liver-specific functions. After a 7-day subculture of the spheroids in TCDs or RWVs, CYP1A1 activity and albumin production were measured. Spheroids maintained a high level of cytochrome P450 metabolism and albumin synthesis when cultured continuously in the RWV. These functions were significantly lower in the cells of spheroids transferred into TCDs (Fig. 6).

Discussion

Understanding cell biology in 3D culture is an important step toward tissue engineering solid organs. We compared the morphology, global gene expression, and function of

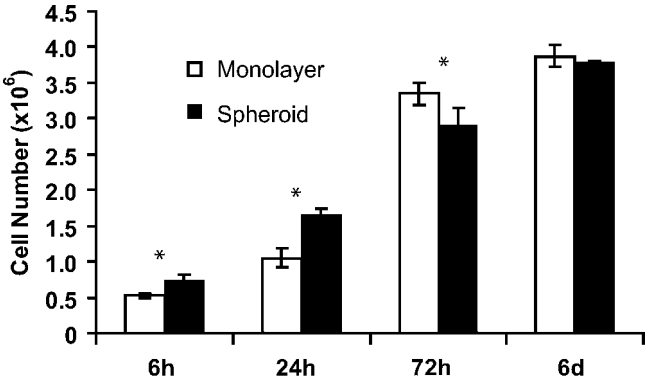

FIG. 4. Overall proliferation is comparable between human liver hepatocellular carcinoma cell line (HepG2) cells cultured as monolayers and spheroids. HepG2 cells were grown in tissue culture dishes or rotating wall vessels for 6 h, 24 h, 72 h, or 6 days. At each time point, Cyquant DNA quantification assays were performed to determine cell number. Data are representative of three independent experiments. $^*p < 0.05$.

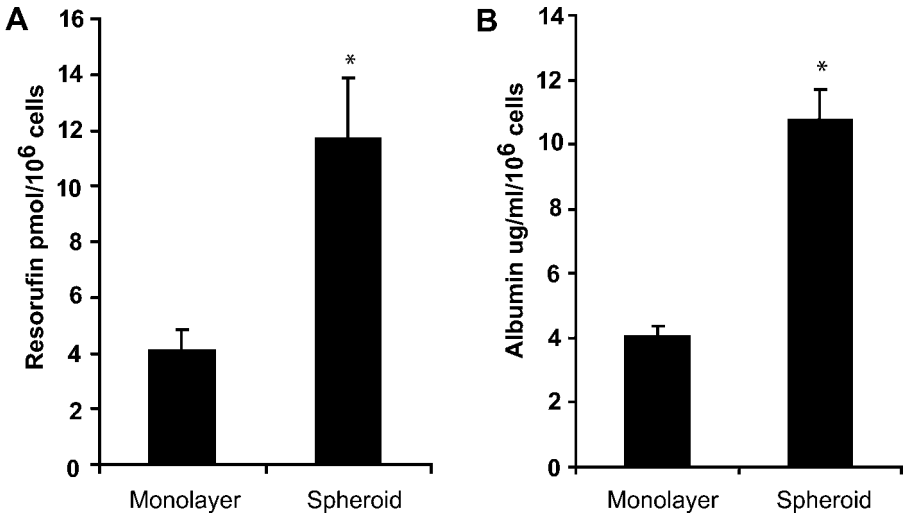

FIG. 5. Human liver hepatocellular carcinoma cell line spheroids cultured in rotating wall vessels demonstrate better liver-specific metabolic and synthetic functions than in monolayers. (**A**) Cytochrome P450 activity is greater in spheroids. After 7 days of culture as monolayers or spheroids, 7-ethoxyresorufin-o-dealkylase assays were performed to measure cytochrome P450 1A1 activity. Cyquant assays determined cell numbers in order to present data as amount of resorufin product per million cells. (**B**) Albumin production was greater in spheroids. After 7 days of culture, culture supernatants were collected and albumin quantified according to enzyme-linked immunosorbent assay. Cyquant assays were performed to determine cell numbers. Data represent concentration of albumin in culture supernatant per million cells. Data are representative of three independent experiments. $^{*}p < 0.001$.

HepG2 monolayers and spheroids to determine the distinct cellular and molecular responses to 2D and 3D environments. We showed that culturing within RWV bioreactors is an efficient method for generating large functioning hepatic spheroids. Structural genes encoding ECM, cytoskeletal, and adhesion proteins are upregulated in monolayers, whereas metabolic and synthetic genes are upregulated in spheroids.

Hepatic spheroids formed in the RWV are larger than self-assembled spheroids formed using other methods and are not confined within the lattices of scaffolds. In RWV bioreactors, spheroids are 100 μm in diameter by day 3 of culture and grow in size up to 1 mm with long-term culture, 10-fold larger than the spheroids formed using other culture techniques.[5,15–17] RWV bioreactors can form larger spheroids because cells are free to aggregate and grow three-dimensionally without the constraints of surfaces and scaffolds. Only diffusion of nutrients to the center and the increasing shear force experienced by larger spheroids limit spheroid size.[20,21] Previous studies showed that cell aggregates in RWVs may reach 1 to 3 mm in length, and there are no apoptotic centers in spheroids smaller than 1 mm in diameter.[19] The lack of necrosis in our spheroids was verified when they were transferred into tissue culture dishes, because all cells were viable when the spheroids self-disassembled.

In this study, we examined 3D cell biology of the spheroids formed in RWV bioreactors without confounding interactions of scaffolding or surface materials. Through global gene expression analysis and subsequent verification with qRT-PCR, we identified several metabolic pathways that are upregulated in spheroids. Genes involved in xenobiotic metabolism are most markedly upregulated and expression of genes involved in leukotriene metabolism, cholesterol metabolism, glutathione synthesis, albumin synthesis, and ATP synthesis are all significantly greater. This suggests that spheroids are overall more metabolically active than monolayers. On the other hand, monolayers express significantly higher levels of ECM genes, specifically collagen type I and versican. Other cytoskeletal and adhesion genes, including E-cadherin and claudin 6 (tight junction), are also significantly upregulated in monolayers.

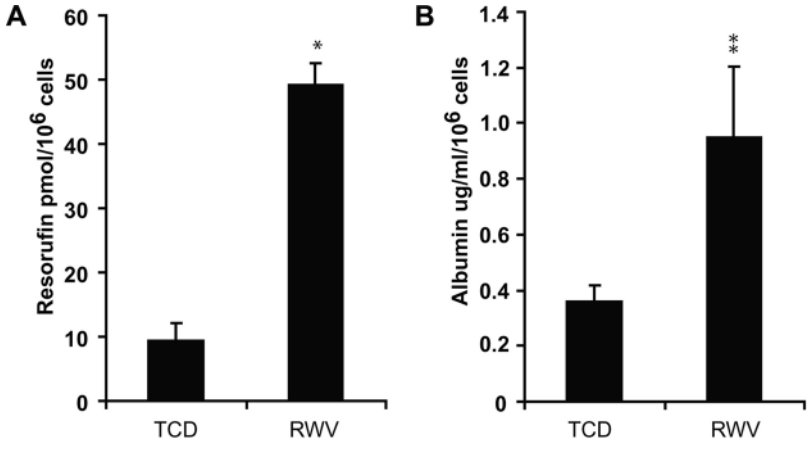

FIG. 6. Spheroids lose liver-specific functions when placed in tissue culture dishes (TCDs). Human hepatocellular liver carcinoma cell line cells were cultured for 6 to 10 weeks in the rotating wall vessels (RWVs). Formed cellular spheroids were subcultured in TCDs or RWVs for 7 days. Cytochrome P450 activity as measured according to resorufin formation (**A**) and albumin production (**B**) was determined. Data were adjusted per million cells as determined using the Cyquant assay. $^{*}p < 0.005$, $^{**}p < 0.001$.

We found that expression of various functional genes in spheroids changed with time. Expression of LDLR and HMG-CoA mRNA increased in spheroids from day 3 to 7. CYP1A1 remained 10-fold higher in spheroids than in monolayers during this period. Albumin gene expression was higher in spheroids on day 3 and was the same as in controls by day 7. The accumulated albumin protein in culture supernatants over 7 days was significantly (2.5 fold) higher in spheroid cultures than in monolayers. Albumin is a stable protein and has a serum half life of 20 days.[27] Albumin protein synthesis is mostly regulated on the transcriptional level,[28–30] and some post-transcriptional events also play a role.[31] The accumulated greater albumin protein detected in day 7 spheroid cultures reflected the continual expression of albumin messenger RNA throughout the 7-day culture period. Additional post-transcriptional regulation of albumin synthesis may be involved and may be investigated in future studies. Although metabolic and synthetic functions were higher in RWVs for short-term (7 days) and long-term culture (6–10 weeks), P450 metabolic activity per cell within spheroids increased with culture time length, and albumin production per cell decreased (compare Fig. 5 and 6). This is consistent with the observation that albumin transcript levels in spheroids decreased with time while cytochrome P450 1A1 transcripts remained markedly higher compared to monolayers. It suggests that culture conditions that support long-term metabolic functions may be different from those that support synthetic functions. RWV culture may be more efficient at sustaining P450 activity than albumin synthesis.

It is interesting to compare our findings in RWV spheroids with other models of 3D hepatocyte culture. HepG2 cells encapsulated within alginate demonstrated greater synthesis of albumin and fibrinogen as well as higher cytochrome P450 and steroid metabolism.[3] This supports our finding that 3D culture enhances synthetic and metabolic functions. In contrast, alginate-encapsulated HepG2 showed greater proliferation when cultured in a rotary culture system[4] and greater surrounding ECM as measured using immunohistochemistry than when cultured in monolayers.[32] In our system, proliferation of cells cultured as spheroids was greater at early time points, less by 72 h, and was comparable to that of monolayers by 6 days. In addition, RWV spheroids had significantly lower expression of ECM (collagen I and versican) transcripts according to qRT-PCR than monolayers. It is likely that these differences between RWV spheroids and alginate encapsulation beads reflect the differing physical culture environments of the two systems. Alginate-encapsulated HepG2 cells have significant interactions with the surrounding alginate. This cell–scaffold interaction may have characteristics analogous to 2D culture in which cells adhere to an underlying surface. Spheroids in RWVs, on the other hand, are cell aggregates that have no exogenous scaffolding. RWV spheroids have only cell–cell interactions, while alginate encapsulation beads have cell–scaffold interactions that most certainly have additional effects on cell shape and stretch that influence cell function.

A number of studies have shown that tension forces and cell shape can determine cell fate in a variety of cell types.[33–36] Markers of terminal differentiation are induced in keratinocytes forced into a spherical shape, as opposed to cells spread out on a substratum.[35] Human mesenchymal stem cells differentiate into osteocytes when flat and spread out, whereas round cells with limited surface contact undergo adipogenesis.[33] Cell stretch, in particular, appears to be an important signal for proliferation in endothelial and smooth muscle cells.[37,38] The cytoskeleton and cell shape of HepG2 cells may play a role in determining their distinct global gene expression patterns as monolayers or spheroids. Cells adherent to a surface may be stimulated to express structural genes such as ECM and adhesion molecules, whereas RWV spheroids switch off expression of these structural genes and acquire more metabolic functions.

In addition to greater metabolic functions, 3D organization of hepatocytes also induced development of more-differentiated cell morphologies. HepG2 cells within alginate[3] or porous polystyrene scaffolds[39] expressed large numbers of microvilli and formed structures resembling canaliculi. Similarly, previous studies of HepG2 cells and primary hepatocytes cultured in RWVs showed that cells within spheroids formed bile canaliculi with multiple tight junctions.[19] These results demonstrate that more-complex cellular organization can be achieved using various 3D culture strategies.

Through microarray analysis, we identified potentially novel pathways that may be important in determining cell function in 2D and 3D culture. RAB3B is a Ras-family member small GTPase that is highly expressed in monolayers and downregulated in spheroids. It was recently identified as one of the key genes regulating mesenchymal stem cell differentiation[40] and a specific marker of liver tissue–based stem cells (oval cells).[41,42] In addition, AXL, a tyrosine kinase receptor and oncogene that mediates cell proliferation and survival signals,[43] is also differentially upregulated in monolayers compared to spheroids. The absence of consistent significant differences in proliferation between monolayers and spheroids through the various time points tested, however, may reflect the redundancy of proliferative signals in HepG2 cells.

RWV spheroids demonstrated enhanced cytochrome P450 activity and albumin synthesis even after prolonged culture of 6 to 10 weeks. Moreover, the functional phenotype of spheroids requires continued culture within a 3D environment. Enhanced metabolic functions of spheroids are lost when spheroids are placed into a TCD, suggesting that cells respond to their physical environment and modify their gene expression accordingly. This has important implications for future tissue engineering efforts of 3D solid organs. In addition to biochemical signals, physical signals can be manipulated to achieve the desired cellular functions and responses. Our data suggest that hepatocytes stretched against bioscaffolds may produce ECM, whereas unstretched cells in the interior are likely to be more metabolically active. Within a 3D structure, cells may demonstrate different functional phenotypes depending on the immediate physical environment. Cell biology in unrestricted 3D environments is an exciting area of research important for tissue engineering and for understanding normal cellular processes *in vivo*.

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