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Rat hepatocyte spheroids formed by rocked technique maintain differentiated hepatocyte gene expression and function

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Abstract

The culture of primary hepatocytes as spheroids creates an efficient 3-dimensional tissue construct for hepatic studies *in vitro*. Spheroids possess structural polarity and functional bile canaliculi with normal differentiated function. Thus, hepatocyte spheroids have been proposed as the cell source in a variety of diagnostic, discovery, and therapeutic applications, such as a bioartificial liver. Using a novel rocking technique to induce spheroid formation, kinetics of spheroid formation, cell-cell adhesion, gene expression and biochemical activities of rat hepatocyte spheroids were tested over 14 days of culture. Evidence was provided that the formation of spheroids occurred faster and with fewer non-adherent hepatocytes in rocked suspension culture compared to a traditional rotational system. Hepatocyte spheroids in rocked culture showed stable expression of over 80% of 242 liver-related genes including those of albumin synthesis, urea cycle, phase I and II metabolic enzymes, and clotting factors. Biochemical activity of rocked spheroid hepatocytes was superior to monolayer culture of hepatocytes on tissue culture plastic and collagen. In conclusion, spheroid formation by rocker technique was more rapid and more efficient than rotational technique. Rocker formed spheroids appear suitable for application in a bioartificial liver or as an *in vitro* liver tissue construct.

Keywords

liver tissue construct; bioartificial liver; custom microarray; drug metabolism; spheroid

Novel systems are needed to facilitate short and long-term culture of hepatocytes for diagnostic, discovery, and therapeutic applications (1). Traditional monolayer culture of primary hepatocytes on tissue culture plastic is problematic and has been associated with a rapid loss of differentiated function (2). While single and double layer surfaces of collagen or other biomatrix materials are associated with improved differentiated functions *in vitro* (3), biological surfaces can pose manufacturing hurdles and do not support high cell density culture of primary hepatocytes (exceeding 1×10^7 cell/mL). In contrast, spheroids, non-adherent multi-cell aggregates of greater than 40 μ m diameter, provide a 3-dimensional tissue construct which form spontaneously and allow suspension culture of primary hepatocytes at high cell density under oxygenated bioreactor conditions (4). Spheroid

formation allows recapitulation of the cuboidal geometry of primary hepatocytes with relatively stable long-term differentiated function (5,6). Reports of structural polarity and bile canaliculi formation by primary rat hepatocytes in spheroid aggregates provide further evidence that hepatic spheroids mimic the hepatocellular microanatomy of the liver (7).

Original observations of tissue-like aggregate formation from isolated cells was reported by Moscona in 1961 using fetal liver cells and a rotational technique (8). The descriptive term “spheroid” was coined years later by Landry in 1985 when multi-cellular aggregates were formed from isolated rat hepatocytes after 3–5 days of culture on non-adherent plastic surfaces (9). Later, spheroids were observed from primary hepatocytes after 24 hours of rotational culture with 56% of the hepatocyte inoculum forming multi-cellular aggregates of greater than 40µm in diameter (10). More recently, we reported preliminary observations that hepatocytes formed spheroids when suspended in rocked (oscillatory) motion (11). Rocking promoted mixing, oxygenation, and increased frequency of collisions between freshly isolated hepatocytes, which in turn accelerated their aggregation into clusters and the formation of spheroids.

Our preliminary report of spheroid formation under rocked conditions was limited to a 5 day culture interval using porcine hepatocytes (11). The goals of the current study were to compare the spheroid formation kinetics of rat hepatocytes in rocked vs. rotational culture and to characterize the gene expression and biochemical activity of rocker-formed rat hepatocyte spheroids over extended (14 days) duration. Spheroid formation was measured by direct observation and automated Coulter counter. In order to expand the evaluation of gene expression by hepatocyte spheroids, we utilized a custom microarray consisting of 242 liver-related genes in a wide array of cellular functions: cell adhesion, cell respiration, phase I and II metabolism, ureagenesis, glycolysis, stress response, and assorted transcription factors. Functionality of spheroids was compared to monolayer controls (tissue culture plastic, TCP; collagen monolayer, CM) using clinically relevant liver-specific biochemical assays: albumin production, ureagenesis, and cytochrome P450 activities. All studies were conducted using rat hepatocytes inoculated at an intermediate cell density (1×10^6 cell/mL) to allow comparisons of monolayer and spheroid geometries formed by static, rocked or rotational technique. Our results indicate that the rocked technique is a simple yet rapid and highly efficient means of forming three-dimensional, suspension-stable, hepatic tissue constructs.

Experimental Procedures

Animals were provided ad lib access to water and standard chow. All animal procedures were performed under the guidelines set forth by the Mayo Foundation Animal Care and Use Committee and are in accordance with those set forth by the National Institutes of Health. All compounds were obtained from Sigma (St. Louis, MO) unless noted otherwise.

Rat hepatocyte isolation

Hepatocytes were isolated from 250–300 g male Sprague Dawley rats (Harlan, Indianapolis, IN) by a two-step perfusion method as previously described (12). All harvests yielded hepatocytes with viability exceeding 90% by trypan-blue dye exclusion.

Spheroid and monolayer culture

Freshly isolated hepatocytes were suspended in culture medium [Williams-E medium with 10% fetal bovine serum (FBS; Mediatech, Herndon, VA), 10 U/mL penicillin G, 100 µg/mL streptomycin, 10 µg/mL insulin, 5.5 µg/mL transferrin, 5 ng/mL sodium selenite] at 1×10^6 cells/mL. Suspended hepatocytes were inoculated into four different culture vessels:

spheroid rocker dishes (10×8×2cm); spheroid rotational flasks (6cm diameter); monolayer plates of tissue culture plastic (60mm; Corning, Corning, NY); monolayer plates of type I collagen-coated plastic (60mm Biocoat; BD Labware, Franklin Lakes, NJ). Cell density (1×10^6 cells/mL) and plating density (1.8×10^5 cells/cm²) were standardized by inoculating cell suspensions of 20mL per spheroid vessel and 5mL per monolayer plate. Spheroid dishes were rocked continuously at 0.25Hz while spheroid flasks were rotated on an orbital spinner. Monolayer plates were placed on a stable rack. All cultures were incubated at 37°C and 5% CO₂.

Media changes

Medium was first changed at 24 hours and subsequently at weekly intervals. Dexamethasone (100ng/mL) was added on day 1 and thereafter. One day prior to sampling, cultures received 10µM β-naphthoflavone (βNF), 8µM diazepam, and 5% v/v heavy deuterium-enriched ammonia gas (2.23mM; Cambridge Isotope Laboratories, Inc, Andover, MA) to test the hepatic clearance ability of these compounds. βNF was omitted from non-induced cultures. Samples were stored at -80°C.

Morphometrics of hepatocyte spheroid formation

Rocked and rotational cultures were sampled (1mL) from 4 to 48 hours. Spheroid diameter, spheroid number and free hepatocyte number were measured by phase contrast microscopy (Axiocam; Carl Zeiss, Inc., Thornwood, NY) and by automated technique using a Multisizer3 (560µm aperture; Beckman Coulter, Fullerton, CA).

Immunofluorescence microscopy

Hepatocytes were fixed in 4% paraformaldehyde, rinsed with PBS and prehybridized with 0.1% Triton and 1% goat serum. Cells were incubated in mouse anti-rat E-cadherin antibody (1:500; #610181, BD Biosciences, San Jose, CA) and secondary antibody - fluorescein-5-isothiocyanate (FITC)-labeled goat anti-mouse IgG (1:1000; Molecular Probes, Invitrogen, Carlsbad, CA). Immunofluorescence was imaged by confocal microscopy (LSM510, Carl Zeiss, Inc.).

Electron microscopy

Spheroids were fixed in Truumps solution, sectioned, and imaged by transmission electron microscopy in the Microscopy Core Facility (Mayo Clinic, Rochester, MN).

Hepatocyte viability

Spheroid samples (300µL) were resuspended in a 50µL mixture of acridine orange and ethidium bromide stain (FluoroQuench™; One Lambda, Canoga Park, CA). Cells were imaged using fluorescent microscopy using FITC filters and AttoArc HBO 100 W excitation. Fluorescence was quantified using ImageProPlus6.0 (Media Cybernetics, Silver Springs, MD).

Calcium influence on spheroid formation

Hepatocytes were cultured in medium with or without EGTA supplementation - equimolar (31.4 mM) to calcium concentration. Hepatocytes were viewed by phase contrast microscopy after 48hrs of rocking. EGTA was also added after spheroid formation (48hrs) to assess the influence of calcium depletion on formed spheroids.

Extraction of DNA and RNA

Samples of spheroids (5mL) and hepatocytes scraped from plates were pelleted and aliquoted for DNA or RNA analysis. DNA quantification was performed using DNeasy kit (Qiagen, Valencia, CA). Total RNA was extracted immediately and stored at -80°C in RNeasy Lysis Reagent (Qiagen, Valencia, CA) until quantification.

Custom microarray analysis

A custom microarray of 242 liver-related genes with rat-specific oligonucleotide sequences was created and analyzed by the Advanced Genomic Technology Center (Mayo Clinic, Rochester, MN). Microarray analysis was performed on spheroid samples obtained on days 2, 7, 14 and compared to fresh hepatocytes (day 0). Each sample not induced with βNF was compared to the baseline (day 0) sample, and the βNF -induced samples were compared to non-induced samples from the same day. Ten comparisons were performed for each gene sequence. Gene expression was reported as the \log_2 fold-change of signal intensity between compared samples.

Albumin gene expression and production of rat albumin

Rat albumin mRNA copy number was measured by a standardized quantitative competitive-reverse transcription-polymerase chain reaction (QC-RT-PCR) with capillary electrophoresis detection in the Mayo Immune Monitoring Lab Core Services as previously described (13). Concentration of rat albumin in culture media was determined by ELISA kit (Bethyl Laboratories, Montgomery, TX) using a sheep anti-rat albumin antibody.

qPCR analyses of hepatocyte gene expression

Expression of *Cdh1*, *Nags*, *Ass*, *Asl*, *Otc*, *Arg1*, *Cps1*, *Cyp1a1*, *Cyo1a2*, *Aldh3a1*, *Nqo1*, *Gsta2*, *Chst10*, *Cyp2c*, *Cyp2a1* and *Hmox1* genes was determined using real time RT-PCR (qPCR). Briefly, 8 or 16 ng total RNA and 50ng of primers were added to a 10ul reaction containing Quantifast SYBR green RT-PCR master mix (Qiagen). qPCR reactions were performed in 384-Well Clear Optical reaction plates (Applied Biosystems) and detection was determined using an ABI 7900 HT Fast Real Time PCR system (Applied Biosystems). Cycle Threshold (CT) values were determined using Applied Biosystems SDS 2.0 software. Fold changes in gene expression were calculating using the $\Delta\Delta\text{CT}$ method. Results were based on RNA obtained from four independent experiments.

Quantification of ammonia and isotopes of urea

Rates of ureagenesis were determined by the conversion of heavy ammonia ($^{15}\text{ND}_3$) to native and deuterium-enriched urea. Isotopes of urea were quantified with capillary gas chromatography/mass spectrometry as previously reported (14).

CYP1A activity

Cytochrome P450 1A (CYP1A) activity was assessed by the conversion of 7-ethoxyresorufin to resorufin as previously described (15). Resorufin was detected at excitation and emission wavelengths of 530 and 590 nm, respectively, using a FL600 microplate fluorescence reader (Bio-Tek Instruments, Inc, Winooski, VT).

CYP3A and CYP2C11 activity

CYP3A and CYP2C11 activities were determined from media samples supplemented with diazepam as previously described (11). Data acquisition was completed by Chrom Perfect® software (Denville, NJ).

Statistical methods

Two-way ANOVA and student's t-test were used to assess statistical significance of differences in biochemical activity and gene expression between culture conditions and time points. A Mann-Whitney rank sum test was used to compare diazepam metabolism values. A non-parametric Spearman correlation was used to compare albumin QC-RT-PCR and albumin protein data. Results were expressed as mean values \pm SEM.

Gene expression was compared using \log_2 transforms of the median signal intensity. Data was normalized by a semi-parametric approach to correct for intensity-dependent effects (16). We used SAS© Version 9 statistical software (SAS Institute Inc., Cary, NC) for these analyses.

Results

Total hepatocyte number, hepatocyte viability, and incorporation into spheroids

The kinetics of spheroid formation was compared under rocked and rotational conditions as summarized Fig. 1, upper panel. Measurements of spheroid size, spheroid number, hepatocyte number, and percent incorporation of hepatocytes into spheroids were obtained at the time of inoculation through 48 hours. These measurements were made in triplicate. Aggregation of hepatocytes was observed under both rocked and rotational conditions by 4 hours; however, hepatocyte aggregates were larger and more numerous in rocked cultures at all time points (Fig. 1, lower panel). Well-formed spheroids were observed throughout the rocked spheroid cultures at 12 hours. By 24 hours of rocking, 85% of inoculated hepatocytes had incorporated into well-formed spheroids of greater than 40 μ m diameter (Table 1). At 48 hours, only 13% of cell particles measured less than 40 μ m diameter by Coulter counter (Fig. 2). The majority of rocker-formed aggregates (84%) ranged in diameter of 75–200 μ m (Fig. 3, day 2). In contrast, only 58% of hepatocytes incorporated into spheroids under rotational conditions at 24 hours. Most unattached hepatocytes appeared dead. Rotational formed spheroids were less spherical at earlier time points.

Spheroid number and cell number remained relatively stable after day 2 during the first week of rocked culture. In contrast, average spheroid size decreased from day 2 (125–150 μ m) to day 7 (75–100 μ m) consistent with formation of mature spheroids during the first week of rocked culture (Fig. 3). Spheroid formation provided a protective benefit as over 95% of hepatocytes present in spheroids were viable at each time point. A decline in spheroid number and cell number was observed during the second week of culture. Approximately 30% of hepatocytes inoculated into rocked dishes remained in spheroids on day 14. In contrast, less than 10% of hepatocytes inoculated into rotational flasks remained in spheroids on day 14. Therefore, spheroids formed by rocker technique were used in subsequent studies.

Expression of liver-related genes in spheroids by custom microarray

We profiled gene expression of rat hepatocytes over 14 days of rocked spheroid culture. A custom microarray of 242 liver-related genes was developed for this task. We observed that the average expression of 85% (206) genes remained stable - less than 2-fold increase or 50% decrease relative to baseline - over 14 days in culture (Fig. 4). Expression increased 2-fold or greater in 11 (5%) genes and decreased 50% or more in 25 (10%) genes. A complete listing of custom microarray data can be found at http://mayoresearch.mayo.edu/mayo/research/nyberg_lab/.

Hepatocyte cell adhesion

The expression of several cell adhesion genes including E-cadherin (*Cdh1*), intracellular adhesion molecule 1 (*Icam1*), and fibronectin (*Fn1*) remained stable in rocked spheroid culture (Fig. 5). Expression of N-cadherin (*Cdh2*), selectin (*Sele*), laminin gamma 1 (*Lamc1*) and six other integrin-related genes (*Itga1*, *Itgae*, *Itgam*, *Itgb1*, *Itgb3*, *Itgb4*) also remained stable. The pattern of expression of *Cdh1* was validated by qPCR.

Because of the known importance of E-cadherin as an adhesive protein between epithelial cells, we studied the role of E-cadherin during spheroid formation by rocked technique. Cell samples were examined by immunohistochemical staining during initial hours of spheroid formation in culture medium and culture medium supplemented with EGTA to remove free calcium. We observed rapid spheroid formation and strong staining of E-cadherin on the surface of cells formed in the presence of free calcium (Fig. 6a-f). However, spheroids failed to form in medium supplemented with EGTA (Fig. 6g). Furthermore, formed spheroids quickly fragmented after addition of EGTA on day 2 (Fig. 6h,i). Most unattached hepatocytes appeared dead after 24 hours in calcium-free conditions confirming the importance of calcium-dependent adhesion in the formation and viability of liver tissue constructs such as spheroids. These studies suggest an essential role for E-cadherin in the prevention of cell death after separation of hepatocytes from their extracellular matrix. This form of cell death, termed *anoikis*, has been observed after separation of other epithelial cell types from their surrounding matrix (17).

Gene expression and albumin production

Custom gene microarray, QC-RT-PCR, and ELISA were used to assess gene expression and production of albumin under rocked spheroid and monolayer culture conditions. By custom microarray, albumin gene expression in spheroid cultures remained at 86% of baseline on day 2 and declined to 57% on day 14. This pattern of albumin gene expression was confirmed by QC-RT-PCR (Fig. 7a). Copy number of the albumin gene was significantly higher in spheroid culture compared to tissue culture plastic or collagen monolayer at all time points ($p < 0.05$). Production of albumin protein was also significantly higher in spheroid culture compared to monolayer control cultures at all time points (Fig. 7b, $p < 0.05$). A correlation was suggested between gene copy number and production rate of albumin by non-parametric comparison (Spearman correlation coefficient=0.56; $p=0.09$).

Urea cycle

Six genes of the urea cycle were included on the custom array (Fig. 8a): n-acetylglutamate synthase (*Nags*), arginino succinate synthase (*Ass*), argino succinate lyase (*Asl*), arginase 1 (*Arg1*), carbamoyl-phosphate synthetase 1 (*Cps1*), ornithine transcarbamolase (*Otc*). *Cps1* and *Otc* encode for proteins which catalyze the initial steps of the urea cycle within the inner membrane of the mitochondria. *Nags* encodes for an inner mitochondrial protein. *Arg*, *Ass*, *Asl* express cytoplasmic proteins of the urea cycle. The expression of three urea cycle genes (*Ass*, *Asl*, *Nags*) remained stable during 14 days of rocked spheroid culture; *Arg1* was up-regulated to 250% at day 2 and declined gradually to 89% of baseline on day 14; *Cps1* and *Otc* showed a mean reduction of 55% and 36% of baseline, respectively. Expression of these six genes was also measured by qPCR. Patterns of expression of all six genes were similar by microarray and qPCR. However, greater reductions in expression of *Cps1* and *Otc* were determined by qPCR (8-fold and 25-fold decrease on day 14, respectively).

Rocker-formed spheroids demonstrated significant levels of ureagenesis at all time points based on conversion of heavy ammonia to heavy urea (Fig. 8b). Total ureagenesis was greater in rocked spheroid cultures than monolayer control conditions at all time points. The ratio of heavy urea to total urea produced by spheroid hepatocytes was also greater than

monolayer controls at all time points.. The decline in ureagenesis over time paralleled the decline in expression of *Cps1* and *Otc*.

Phase I Metabolism

Thirty-nine genes involved in phase I metabolism were assessed by microarray in rocked spheroid culture. These genes showed the greatest variability in expression of any group tested (Figure 4). Average expression of 19 genes (49%) remained stable. One phase I gene [*Cyp1a1* (Fig. 9)] was up-regulated over time (without addition of β NF) by both custom microarray and qPCR, while the gene expression of *Cyp1a2* declined; eighteen other phase I genes were down-regulated less than 50% of baseline including 3 isoforms of *Cyp3a*, 5 isoforms of *Cyp2c*, 4 isoforms of *Cyp2d*, and *Cyp2e1*. *Cyp1a2*, *Cyp2a1*, *Cyp2a2*, and *Cyp2c11* were also down regulated by qPCR.

Biochemical activities of multiple CYP450 enzymes were determined by addition of test substrates to hepatocyte cultures. 7-ethoxyresorufin conversion to resorufin (CYP1A1, CYP1A2) was highest in spheroids compared to monolayer conditions on all days ($p < 0.05$, Fig. 9). Collective activity of CYP3A and CYP2C11 was determined by biotransformation of diazepam to oxazepam. Oxazepam concentrations were significantly higher in spheroids ($4.35 \pm 0.98 \mu\text{M}$) compared to both monolayer culture conditions after day 2 (TCP: $1.42 \pm 0.35 \mu\text{M}$; CM: $1.21 \pm 0.34 \mu\text{M}$; $p < 0.001$) on day 1. Production of oxazepam peaked in spheroid culture on day 7. This trend paralleled the expression of *Cyp3a* genes determined by microarray.

Phase II Metabolism/Conjugation

Expression of 14 of 16 (88%) phase II metabolism genes, including six sulfotransferases and all five UDP-glucuronosyl transferases (*Udpgt*), remained stable during 14 days of spheroid culture. Sulfotransferase 1B (*Sult1b*) was up-regulated while sulfotransferase-estrogen-preferring (*Ste*) was down-regulated.

Gene Induction by β NF

The influence of β NF on gene expression of spheroid hepatocytes was quantified by microarray data at each time point. Nine genes summarized in Table 2 were induced by β NF. The expression of most inducible genes remained high throughout the culture period. Six genes (*Cyp1a1*, *Cyp1a2*, *Cyp2a*, *Aldh3a1*, *Nqo1*, *Hmox*) were also examined by qPCR which confirmed their induction by β NF.

Antioxidants

Expression of 15 of 18 antioxidant genes (83%) remained stable during 14 days of culture. Two antioxidant genes, glutathione synthetase (*Gss*) and glutathione-S-transferase Mu 1 (*Gstm1*), showed increased expression by an average greater than 2-fold over 14 days. Glutathione reductase (*Gsr*), glutathione-S-transferase theta 2 (*Gstt2*), and microsomal glutathione S-transferase 1 (*Mgst1*) showed a decline in expression.

Coagulation factors

Expression of 5 of 7 (71%) clotting factor genes remained stable during 14 days of culture. Thrombin and factor X showed an average 2-fold decrease from baseline over 14 days.

Discussion

The most important observation from the current study is that spheroid formation occurs rapidly and most efficiently by rocker technique. Rapid aggregation of hepatocytes into

spheroids by rocked technique was associated with fewer dead cells and improved spheroid morphology compared to the traditional rotational technique. These observations are significant and predict that a higher dose of primary hepatocytes is possible in a spheroid reservoir configuration compared to prior designs of bioartificial livers (18,19)]. Rocker-formed spheroids may also prove to be an alternative method for preparation and maintenance of hepatocytes prior to cellular transplantation (20). Transplantation of hepatocyte spheroids has been reported previously (21). Studies are planned to test whether rocker-formed spheroids are suitable for therapeutic purposes such as hepatocyte transplantation.

The influence of spheroid size on hepatocyte function was previously studied by Glickis et al (22), who predicted that the diffusion of oxygen would not be rate limiting and central hepatocytes would not become hypoxic if spheroid diameter was on the order of 100 μ m. Our observations support those of Glickis in that we found optimal spheroid size to be approximately 100–150 μ m based on viability and mitochondrial membrane potential of rocked spheroid hepatocytes (unpublished observations). We determined mitochondrial membrane potential of hepatocytes by tetramethylrhodamine ethyl ester perchlorate (TMRE) staining (23).

The formation of spheroids by rocker technique draws focus on the anchorage dependency of epithelial cells such as hepatocytes and the importance of cell-cell contact and adhesion to extracellular matrix in maintaining differentiated phenotype. In turn, hepatocyte spheroids formed by rocker technique preserved differentiated function better than classic monolayer culture techniques. Alternative techniques exist for culture of hepatocytes and creation of in vitro liver tissue constructs, including collagen sandwich (24) and hepatocyte co-culture (6,25). Comparisons to these more elaborate, labor intensive techniques will be helpful in establishing the role of the rocked spheroid technique in experimental biology of liver tissue constructs.

Further optimization of the rocked hepatocyte culture system may also be required as a progressive decline in cell number was observed over the 2-week study interval. The decline in both metabolic activity and protein production paralleled the decline in cell number, despite stable gene expression on a viable cell basis. The reason for cell loss from the spheroid system has not yet been determined. Some of the spheroid losses occurred during changes of media, while other losses occurred by adhesion of spheroids to the walls of the bioreactor and along the liquid-air interface where surface tension was greatest. Cell death between time points was another possibility because hepatocytes appeared viable in intact spheroids when examined by vital staining and transmission electron microscopy (data not shown) during the 14 day culture period.

In conclusion, we offer a thorough analysis of the biochemical functions and liver-specific gene expression of rat hepatocyte spheroids formed by rocker technique over 14 days in culture. Over 85% of hepatocytes were incorporated into spheroids by rocker technique after 24 hours compared to 58% by conventional rotational technique. Cell loss occurred in all culture systems studied; however, the rate of cell loss was lower and biochemical activity was significantly greater at all time points in rocked spheroid cultures compared to control conditions. Custom microarray studies confirmed stable and inducible expression of many liver-related genes in spheroids formed by rocked technique. Further optimization of this system for application as a bioartificial liver is warranted.

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Abbreviations

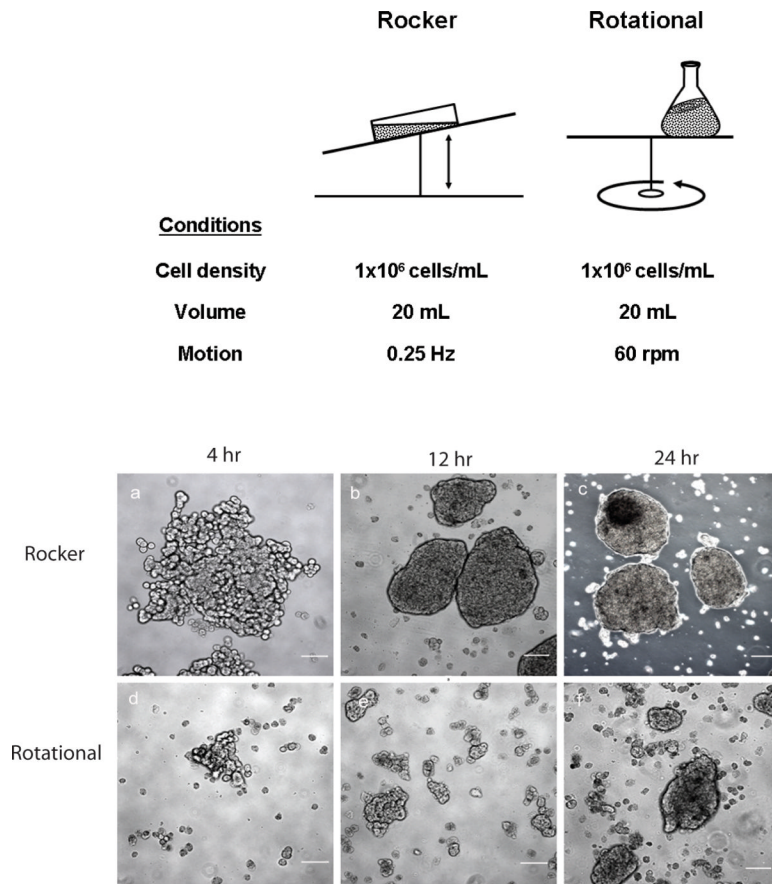
Aldh3a1	aldehyde dehydrogenase 3A1
Arg1	arginase 1
Asl	arginino succinate lyase
Ass	arginino succinate synthase
Ahr	aryl hydrocarbon receptor
Arnt	aryl hydrocarbon receptor nuclear translocator
βNF	β -naphthoflavone
Cps1	carbamoylphosphate-synthetase 1
Chst10	carbohydrate sulfotransferase 10
CM	collagen monolayer
Cdk4	cyclin dependent kinase 4
Ccnd1	cyclin D1
CYP1a1	cytochrome P450 1A1
ELISA	enzyme-linked immunosorbent assay
Fn1	fibronectin 1
Gsta2	glutathione S transferase A2
Hmox1	hemoxygenase-1
Icam1	intracellular adhesion molecule 1
Itga1	integrin alpha 1
Lamc1	laminin gamma 1
Nags	N-acetylglutamate synthase
Nqo1	NADPH quinone oxidoreductase 1
Nrf2	nuclear factor erythroid 2-related factor
Otc	ornithine transcarbamolase
QC-RT-PCR	Quantitative Competitive-Reverse Transcriptase-Polymerase Chain Reaction
Sele	selectin (endothelial cell)
TCP	tissue culture plastic
Udpgt	UDP-glucuronosyltransferase

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**Figure 1.**

Comparison of rocked vs. rotational conditions (upper panel) along with rat hepatocyte spheroids viewed by phase microscopy during spheroid formation (lower panel). Rat hepatocytes (1×10^6 cells/mL) were rocked at 0.25 Hz or spun at 60 rpm to induce spheroid formation. Representative images of spheroid formation by rocked technique are provided at 4hr (a), 12hr (b), and 24hr (c) and by rotational technique at 4hr (d), 12hr (e), and 24hr (f). Scale bars equal 50 μ m.

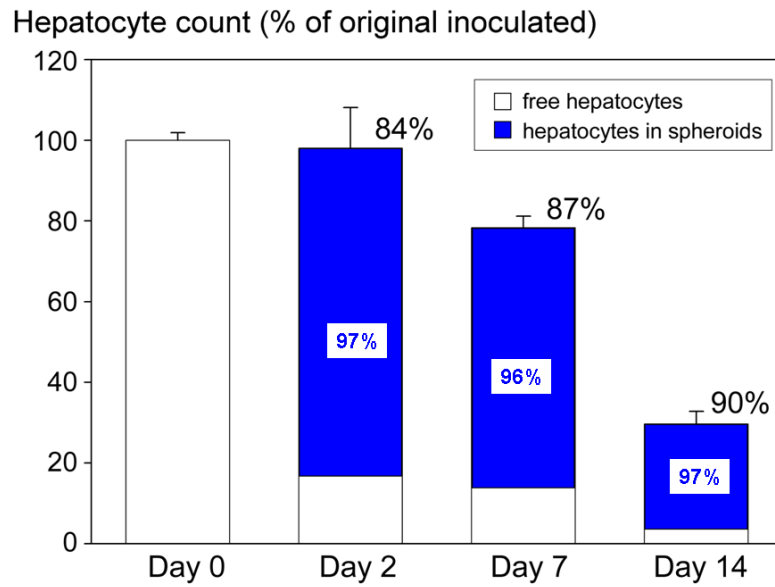


Figure 2.

Hepatocyte count in suspension and incorporated into spheroids by rocker technique. Cell counts were measured by Multisizer 3 Beckman Coulter counter. Cells were inoculated (day 0) at 1×10^6 cells/mL. Bars indicate percent of free hepatocytes (white) vs. hepatocytes in spheroids (blue, $>75 \mu\text{m}$ diameter) of original cells inoculated. Percent values listed above each bar in black indicate proportion of cells present in spheroids, while the percent viability of cells in spheroids is listed inside of each blue bar. Values are averages based on three independent experiments.

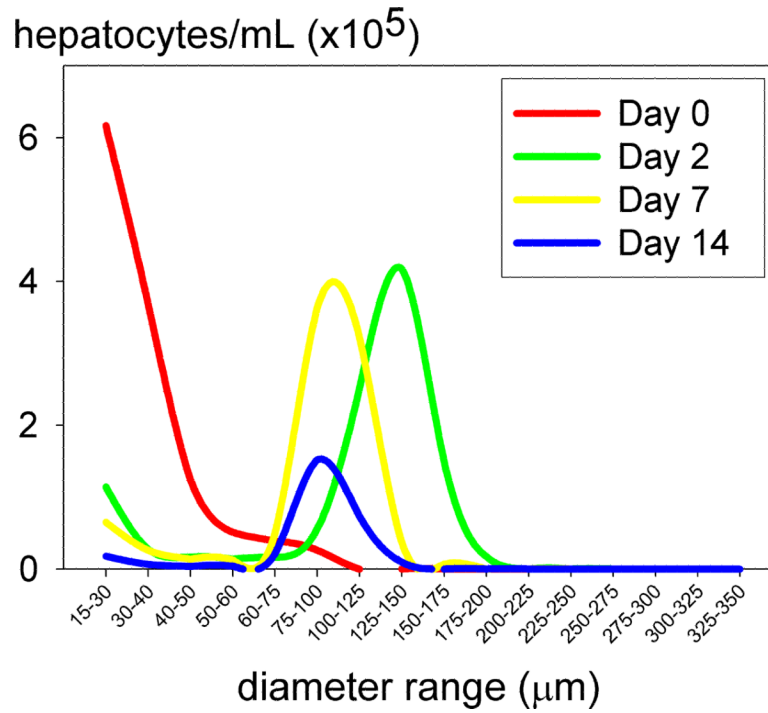


Figure 3. Time course of spheroid size and total cells under rocked conditions. Isolated hepatocytes were originally plated at 1×10^6 cells/mL (equal to the area under the curve on day 0, red line). Measurements were completed by Multisizer 3 Beckman Coulter counter. Repeated in triplicate.

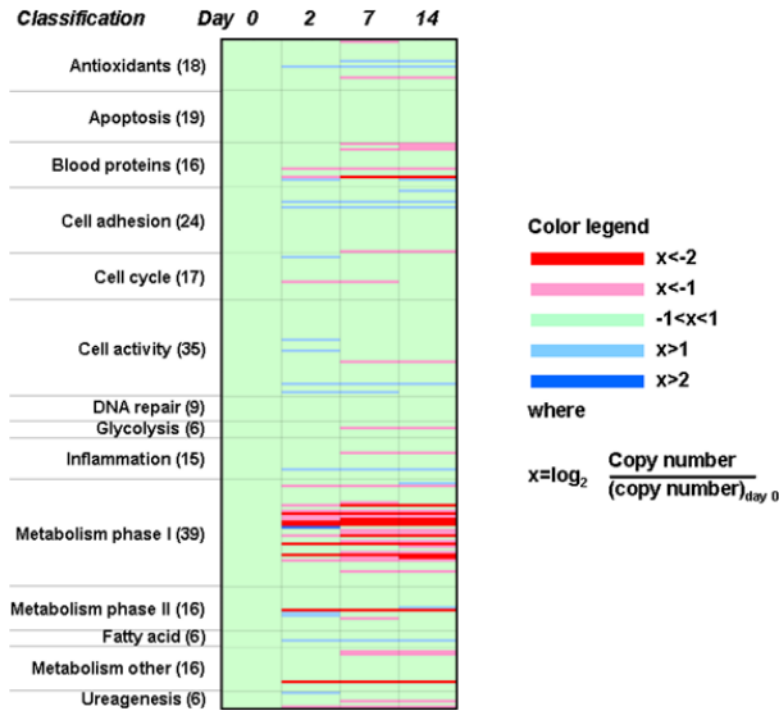


Figure 4. Expression of 242 liver specific genes by custom microarray in rocked spheroid culture over 14 days duration. Average expression of over 80% of these genes was stable over time (green color). A reduction in gene expression from baseline (freshly isolated rat hepatocytes) is indicated in pink or red, while an increase in expression is labeled blue. Genes of phase I metabolism showed the greatest level of fluctuation in rocked spheroid culture.

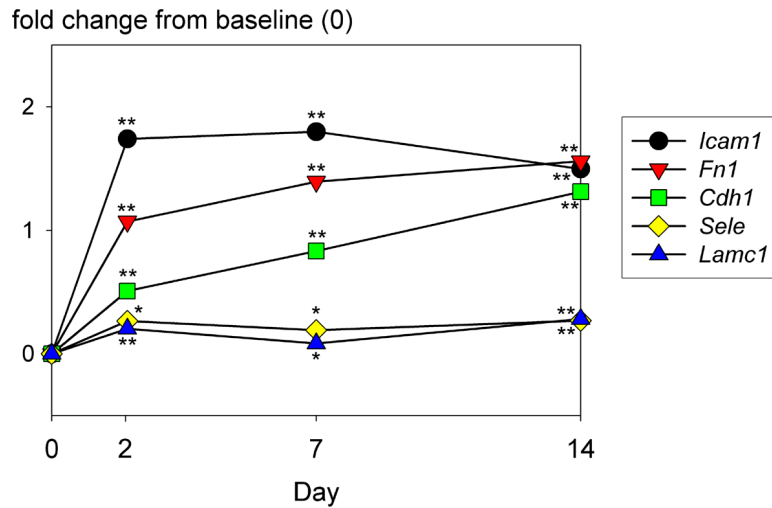


Figure 5. Trends in the expression of cell adhesion genes during formation and extended culture of rat hepatocyte spheroids under rocker conditions. Multiple structural genes important in cell adhesion were stable or up-regulated over time. Intracellular adhesion molecule (*Icam1*); fibronectin (*Fn1*); E-cadherin (*Cdh1*); endothelial cell selectin (*Sele*); laminin gamma 1 (*Lamc1*). The y axis signifies the log base 2-fold change of the gene expression ratio intensity values. ** $p < 0.001$, * $p < 0.05$ vs. baseline (isolated hepatocytes).

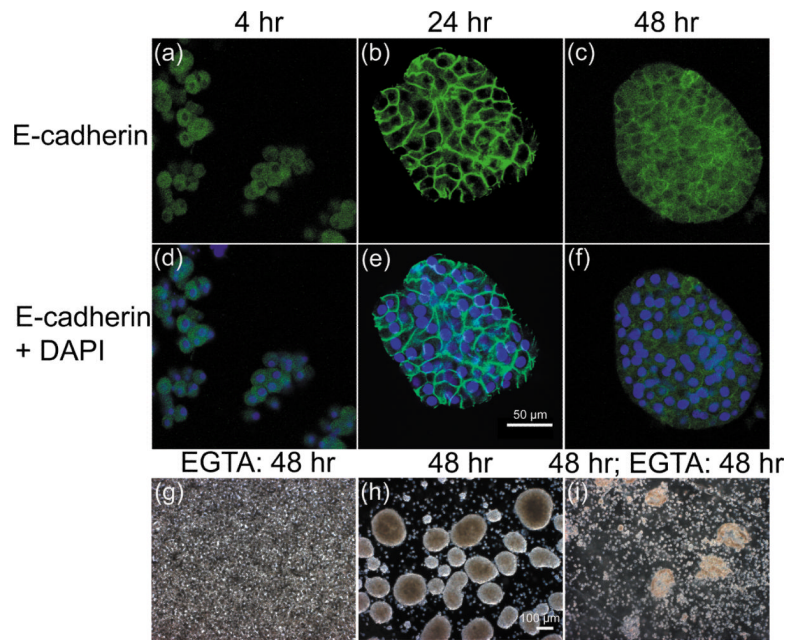


Figure 6.

Importance of calcium-dependent E-cadherin in spheroid formation of primary rat hepatocytes. (a-f) E-cadherin was strongly expressed on the surface of hepatocytes during spheroid formation and its expression continued over 14 days as determined by microarray analysis (see Fig. 4). (a,d,g) Hepatocytes at 4 hours, (b,e,h) spheroids at 24 hours, (c,f,i) spheroids at 48 hours. Scale bar in (e) equals 50 μm and applies to all immunofluorescence images. (g-i) The importance of calcium in spheroid formation is illustrated by phase contrast microscopy. (g) No spheroids form in medium containing EGTA compared to spheroids in normal media condition (h) at 48 hours. (i) EGTA supplementation caused rapid disruption of formed spheroids within an additional 48 hours. Scale bar in (h) equals 100 μm and applies to all contrast microscopy images.

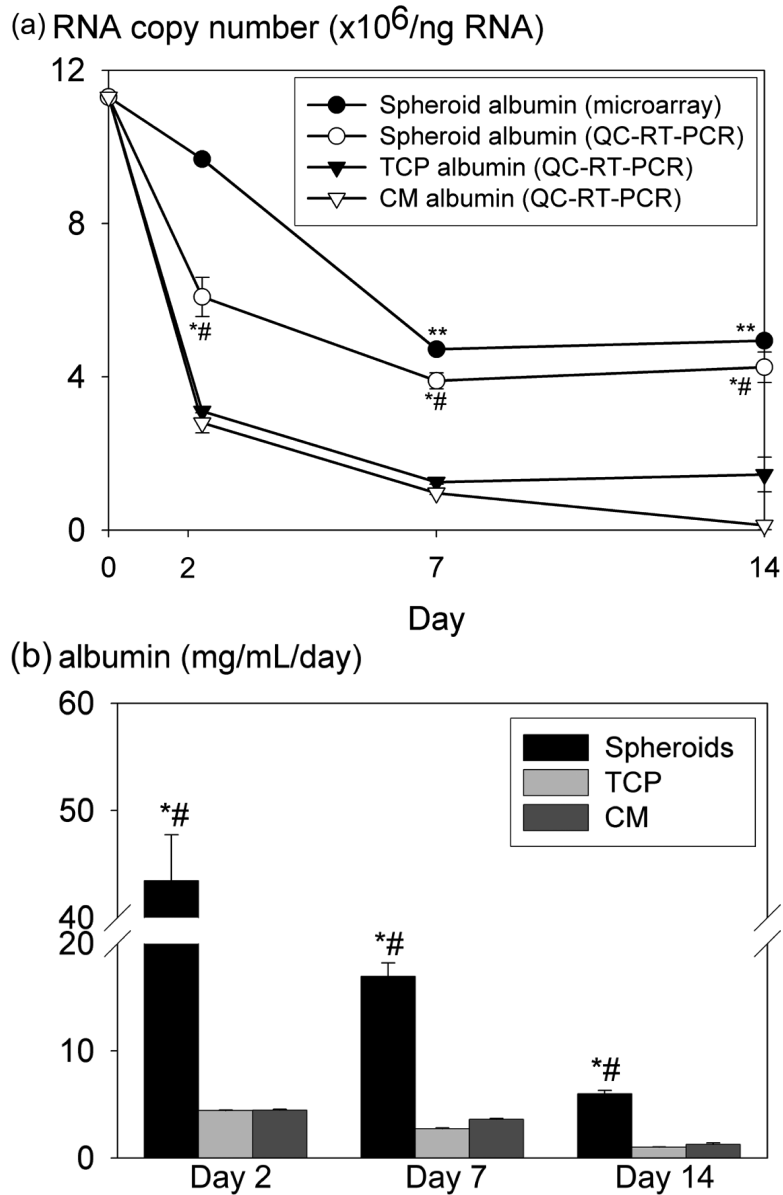


Figure 7. Albumin protein and mRNA measurements of rat hepatocytes. (a) Microarray and quantitative competitive reverse transcriptase-polymerase chain reaction (QC-RT-PCR) values. RT-PCR baseline value indicates RNA measurements taken from individual rat hepatocytes at day 0 (before inoculation). * $p < 0.05$ Spheroids vs. TCP, # $p < 0.05$ Spheroids vs. CM. ** $p < 0.0001$ vs. day 0 hepatocytes (microarray). Spheroid microarray vs. QC-RT-PCR: Spearman correlation coefficient=0.804; $p < 0.001$. (b) ELISA albumin protein measurements. $n = 10$ samples analyzed per data point. * $p < 0.05$ Spheroids vs. TCP, # $p < 0.05$ Spheroids vs. CM.

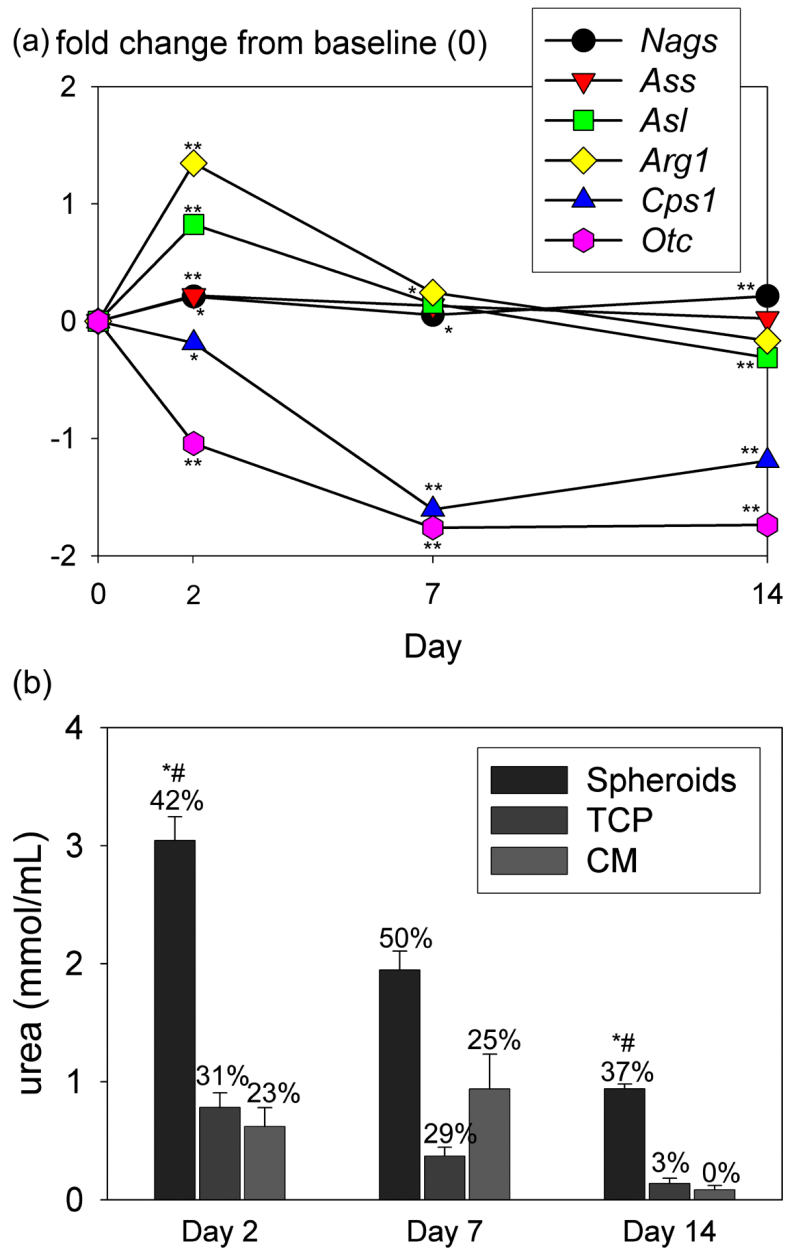


Figure 8.

Ureagenesis measurements in rat hepatocytes. (a) Gene expression measurements of urea cycle enzymes by custom microarray. The y axis signifies the log base 2-fold change of the gene expression ratio intensity values. ** $p < 0.001$, * $p < 0.05$ vs. baseline (isolated hepatocytes). (b) Heavy ammonia (0.5% v/v) was added to culture media and levels of deuterium-enriched urea were determined by GC-MS on samples collected at 24 hours. Percentages indicate proportion of total urea labeled with deuterium (sum of all isotopes) in each sample. $n = 8$ samples analyzed per data point. * $p < 0.0001$ rocked spheroids vs. TCP, # $p < 0.0001$ rocked spheroids vs. CM.

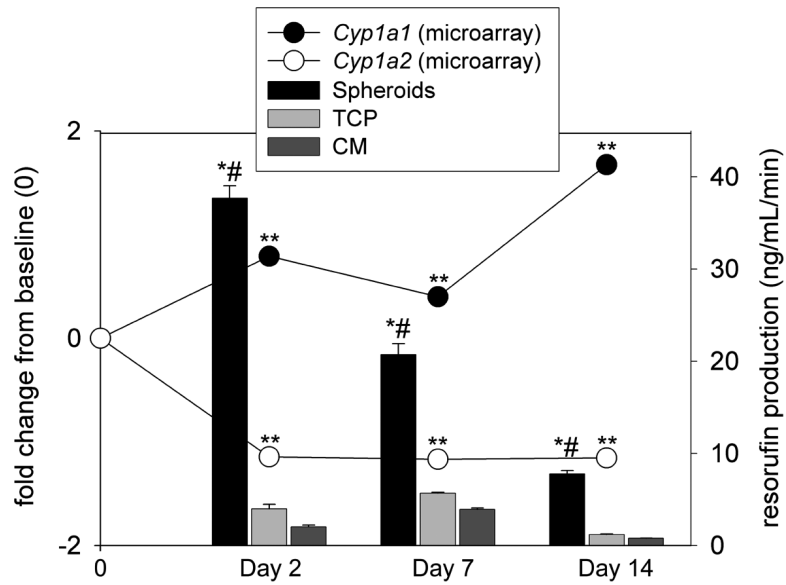


Figure 9.

Cyp1a1 and *Cyp1a2* gene expression were measured in cultures of rocked spheroids and monolayer conditions by custom microarray (line graph, left axis). ** $p < 0.0001$ vs. day 0 hepatocytes. CYP1A1/1A2 activities were measured by the appearance of resorufin in cultures of rat hepatocytes over 14 days (bar graph, right axis). Cultures were exposed to 7-ethoxyresorufin for 60 min after 24 hour induction with β -naphthoflavone. Production of resorufin was fluorescently detected at excitation and emission wavelengths of 530 and 590 nm, respectively. $n=24$. * $p < 0.0001$ rocked spheroids vs. TCP, # $p < 0.0001$ rocked spheroids vs. CM.

Table 1

Spheroid Formation after 24 hours - Rotational vs. Rocked Method

	<u>Rotational (60 rpm)</u>	<u>Rocked (0.25Hz)</u>
Diameter (mean±SEM)	90±36 µm	145±33 µm **
Percent of inoculum incorporated into spheroids		
>40 µm diameter	58%	85% **
>75 µm diameter	52%	80% **

** p<0.001 vs. rotational culture (based on 1000 measurements per group, repeated in triplicate)

Table 2

Rat genes induced by β -naphthoflavone (β NF) in rocked spheroid conditions.

	βNF induction ratio^a		
	Day 2	Day 7	Day 14
<i>Cyp1a1</i>	13.2	15.1	13.0
<i>Aldh3a1</i>	4.0	6.9	10.2
<i>Nqo1</i>	3.0	4.9	4.8
<i>Gsta2</i>	4.6	4.6	3.0
<i>Chst10</i>	2.2	3.6	3.2
<i>Cyp2c</i>	1.4	2.1	2.7
<i>Cyp2a1</i>	1.8	1.7	1.9
<i>Cyp1a2</i>	2.5	1.9	1.6
<i>Hmox1</i>	*1.1	1.5	1.9

^aInduction ratio is the level of expression of induced vs. non-induced spheroid cultures at each time point. The induction ratio was significant at a level of $p < 0.0001$.

* indicates induction ratio was not significant, $p > 0.05$