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Long-Term Culture and Coculture of Primary Rat and Human Hepatocytes

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Abstract

The liver is the largest internal organ in mammals, serving a wide spectrum of vital functions. Loss of liver function due to drug toxicity or viral infection is a major cause of death in the United States. The development of Bioartificial Liver (BAL) devices and the demand for pharmaceutical and cosmetic toxicity screening require the development of long-term hepatocyte culture techniques. However, primary hepatocytes rapidly lose their cuboidal morphology and liver-specific functions over a few days in culture. Accumulation of stress fibers, loss of metabolic function, and cell death are known phenomena. In recent years, several techniques were developed that can support high levels of liver-specific gene expression, metabolic and synthetic function for several weeks in culture. These include the collagen double-gel configuration, hepatocyte spheroids, coculture with endothelial cells, and micropatterned cocultures with 3T3-J2 fibroblasts. This chapter covers the current status of hepatocyte culture techniques, including: hepatocyte isolation, media formulation, oxygen supply, heterotypic cell–cell interactions, and basic functional assays.

Keywords

Liver; Hepatocytes; Metabolism; Oxygen; Coculture; Culture medium; Non-parenchymal cells

1. Introduction

The liver is the largest internal organ in the human body, ascribed with over 500 functions. Among those functions are embryonic hemopoiesis, protein synthesis (albumin, fibrinogen), bile acid production, glycogen storage, and xenoboitic metabolism, as well as metabolic homeostasis by regulating carbohydrate, lipid, and amino acids levels. One of the most unique features of the liver is its capability to fully regenerate even after 80% mass loss (1). In spite of its ability to regenerate from various insults, loss of liver function due to drug toxicity or viral infection is a major cause of death in the United States, reaching over 27,000 individuals in 2006 (2). Despite recent advances in split graft transplantation, there is an extreme shortage of human organs, resulting in high prices and low availability of human hepatocytes for clinical, research, or pharmaceutical application. This shortage is aggravated by the inability to expand primary hepatocytes in vitro (3). For these reasons, most human hepatocytes are isolated from marginal livers (fatty, fibrotic) or those rejected for transplantation. Several companies sell freshly isolated human hepatocytes or cryopreserved cells that can be plated for a variety of research, clinical, or pharmaceutical applications.

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Notes

[5]Phenol red is known to be a weak estrogen (43). Change to phenol red-free basal medium is this is an experimental concern.

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One such research direction is the development of a Bioartificial Liver (BAL) system, an extracorporeal assist device that could extend the life of those waiting for transplantation by providing critical metabolic and synthetic functions (4). Pharmaceutical drug screening is another major field. Drug development is currently estimated at $400 million/drug with the majority spent on preclinical screening (5). However, as animal studies are inadequate to evaluate drug toxicity because of species-specific variations (6), liver drug metabolism models use primary human hepatocytes for ADME/Tox (absorption, distribution, metabolism, excretion and toxicity) screening. Hepatocyte cultures are also used in the study of metabolism (7, 8), liver development (9), regeneration (10), viral infection (11), and inflammation (12).

Reliable culture techniques have become available over the last two decades (13, 14). The main problem with the culture of liver cells is that the cells rapidly lose their cuboidal morphology and liver specific functions during standard culture. The cells accumulate actin stress fibers, described as becoming “fibroblast-like,” lack bile canaliculi and die within a few days. Recent developments allow preserving cell function and structure for several months in culture under several widely different configurations. Culture in sandwich configuration or spheroids relies on the assembly of 3D-like structures, while coculture with endothelial cells or 3T3-J2 fibroblasts depends on heterotypic interactions (15). Finally, oxygen supply and medium formulation are critical aspects in the design of the optimal hepatocyte microenvironment (16).

This chapter covers the current status of hepatocyte culture, problems and limitations, and the most common culture techniques. We describe, primary hepatocyte isolation (see Subheading 3.1), hepatocyte culture media (see Subheading 3.2), and hepatocyte culture techniques (see Subheading 3.3). We also cover heterotypic interactions (see Subheading 3.4), and the importance of oxygen supply (see Subheading 3.6). Finally, we describe standard albumin and urea quantification methods (see Subheading 3.7) to characterize liver-specific function.

2. Materials

2.1. Rat Hepatocyte Isolation

1. $CaCl_2$ solution: Add 2.7 g $CaCl_2 \cdot H_2O$ to 196 mL H_2O. Sterilize solution using a 0.22 μm filter and store at 4°C.
2. Krebs Ringer Buffer (KRB): Add to 4 L Ultrapure water 28.54 g NaCl, 3.98 g D-Glucose, 8.40 g $NaHCO_3$, 1.68 g KCl, and 19.06 g HEPES stored at 4°C, pH 7.2–7.4, filter through 0.22 μm filter.
3. KRB with EDTA: Add 1.7 mL of 0.5 M EDTA to 500 mL of KRB buffer on the day of the procedure. Store at room temperature.
4. Collagenase solution: Add 0.105 g collagenase type IV from *clostridium histolyticum* (Sigma-Aldrich®) to 150 mL of KRB (see Notes 1 and [2]). Add 9 mL $CaCl_2$ (see Subheading 2.1, item 1) to activate the enzyme and sterile filter. Use within minutes to a few hours after preparation.
5. Percoll® solution (Sigma-Aldrich®).
6. Ketamine 100 mg/mL, Xylazine 20 mg/mL.

[1]Adjust collagenase concentration to the specific activity of collagenase per batch.
[2]Collagenase activity is temperature dependent and fails rapidly below 37°C.

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7. Instruments: Scalpel blade, blunt-tipped fine forceps, scissors, hemostat, two cotton buds and 2–3 sets of sterile 2 × 2 pads.
8. 250 and 60 μm Pore mesh nylon filter.
9. Peristaltic pump.
10. 70% (v/v) Ethanol.
11. 3.0 or 5.0 Sutures.
12. 18 or 20 G Catheter.
13. 10 cm Dish.
14. 50 mL Conical tubes.

2.2. Culture Media

1. Thaw Fetal Bovine Serum (FBS): Heat-inactivate the FBS by placing the thawed aliquot in water bath set at 60°C for 30 min.
2. L-Glutamine, 200 mM (see Note 3).
3. Epidermal Growth Factor (EGF) from murine submaxillary gland 0.1 mg (Sigma-Aldrich®): Prepare stock solution by adding 1.0 mL Ultrapure water in sterile conditions, aliquot to 100 μL, and store at −20°C.
4. Penicillin–Streptomycin (10,000 units penicillin and 10 mg streptomycin/mL, Sigma-Aldrich®). Aliquot to 5 mL and store at −20°C.
5. Hydrocortisone sodium succinate for injection, USP, 100 mg (Solu–Cortef®, Pfizer): Prepare stock solution by adding 2 mL Ultrapure water in sterile conditions. Store at 4°C.
6. Insulin, Transferrin, Selenium (ITS) Liquid Media Supplement (Sigma-Aldrich®): 100× stock.
7. Hepatocyte culture medium: Under sterile conditions add 0.2 mM of L-glutamine, 100 μL of EGF stock solution, 75 μL hydrocortisone, 5 mL of liquid ITS, 5 mL of penicillin– streptomycin solution, and 50 mL of FBS, to 440 mL of high glucose Dulbecco's Modified Eagle's Medium (DMEM). Hepatocyte culture medium should be stored at 4°C and used within a few weeks (see Notes 4–[6]).
8. Rat tail collagen type I (BD™) store at 4°C.
9. 10× PBS: Add 80.0 g NaCl, 2.0 g KCl, and 14.4 g Na_2HPO_4 to 1 L Ultrapure water.
10. 1N NaOH: Add 4.0 g NaOH to 10 mL Ultrapure water.

2.3. Albumin and Urea Quantification

1. Purified rat albumin (MP Biomedicals): Prepare 10 mL of 50 μg/mL purified rat albumin in PBS. Store aliquots at −20°C.
2. HRP conjugated Rabbit anti-rat albumin antibody (MP Biomedicals).

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[3] L-glutamine rapidly degrades at temperatures above 4°C and should be added before use

[4] Human cells require ascorbic acid, absent from the above formulation. Add ascorbic acid if using human hepatocytes. Prepare stock solution (100 mM) by adding 1.76 g ascorbic acid to 100 mL Ultrapure water, aliquot, and store at −20°C. Add 5 mL stock solution to 500 mL hepatocyte culture medium.

[6] EGF is not required if hepatocytes are cocultured with 3T3-J2 fibroblasts which are known to secrete this growth factor.

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3. Phenylenediamine dihydrochloride (OPD) pill, 10 mg.
4. PBS-Tween: Add to 975 mL Ultrapure water, 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, and 2.89 Na_2PO_4.
5. 30% H_2O_2.
6. Blood urea nitrogen (BUN) kit (Stanbio Labs™).
7. 96-well plates.
8. Citrate-phosphate buffer: Dissolve 5.1 g Citric Acid Mono-Hydrate and 13.78 g $Na_2HPO_4 \cdot 7H_2O$ in 975 mL Ultrapure water.
9. 8N H_2SO_4: Mix 22.2 mL of concentrated sulfuric acid with 77.8 mL Ultrapure water.

3. Methods

3.1. Primary Hepatocyte Isolation

Primary hepatocytes do not proliferate in vitro and therefore need to be freshly isolated for each experiment. Rats are common source, with 100 to 400 million cells routinely isolated from each animal with >90% viability. The two-step enzymatic digestion technique was first established by Seglen in 1976 (17), and was slightly modified (18). It allows for the isolation of a relatively pure population with high viability. All animals need to be maintained in accordance with National Research Council guidelines and experimental protocols approved by the appropriate institutional Animal Care Committee.

3.1.1. Preparation

1. Open a set of sterilized instruments in a laminar flow hood.
2. Place KRB with EDTA buffer (see Subheading 2.1, item 3) and collagenase solution (see Subheading 2.1, item 4) in water bath and connect to a peristaltic pump. Perfuse at a speed of 17 mL/min.
3. Set a bubble trap and ensure that the line is free of bubbles (see Note 7).
4. Turn on water bath to 40°C.

3.1.2. Surgical Procedure—Rats should normally be 140–180 g body weight. Anesthesia should be administered using an approved protocol and closely monitored. Commonly anesthesia is administered as an intraperitoneal injection of ketamine and xylazine (50–80 mg/kg and 5–10 mg/kg respectively).

1. Once the animal is anesthetized, place and fixate the animal on a surgical table in a dorsal position. Make surgery preparation in a sterile fashion by shaving the abdominal area from the pubis to the xiphoid process and wiping the shaved area with 70% ethanol.
2. Make a median abdominal skin incision. Extend the abdominal incision through the linea alba into the abdominal cavity.
3. Extend the cut to the inferior edge of the xiphoid process. Make two incisions laterally from the midpoint of the abdomen and fixate the flaps with a hemostat.

[7]Reusable glass bubble traps can be obtained through a commercial vendor (i.e., Radnoti Glass Technology), while single use plastic traps can be fitted from an IV line.

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4. Deflect the intestines to the rat's left. Identify the portal vein, pancreatic and bile ducts, abdominal aorta, and inferior vena cava.
5. Divide the falciform ligament on the ventral and anterior aspects of the liver.
6. Identify the esophagus and the esophageal–gastric junction.
7. The accessory lobes of the rat liver lie posterior to the stomach, nestled in the lesser curvature of the stomach and inferior to the esophageal–gastric junction. Pick the lesser omentum and divide it to fully expose the ventral surface of the accessory lobes. Displace the accessory lobes upwards and identify any posterior ligaments attaching the accessory lobe to the stomach. Divide these. Using a gentle rolling motion, retract these lobes through the window within the lesser omentum.
8. Place two ligatures around the portal vein.
    a. Proximal ligature: Use a blunt-tipped fine forceps to create a window in the omental triangle formed by the portal vein, the pancreatic bile duct, and the splenic vein. Grasp the omentum adjacent to the portal vein and retract gently upwards. Pass a suture through this window, position it immediately superior to the splenic vein, and ligate loosely with a half-hitch.
    b. Distal ligature: Create a second window between the mesenteric (cranial (superior) and caudal (inferior)) veins, approximately 1.5 cm distal to the proximal ligature. Identify the posterior vessels. Pass a suture through this window and ligate loosely with a half-hitch.
9. Measure an 18 or 20 G catheter lead to the approximate location of the cannula inlet.
10. Grasp the omentum about 1 cm or less, inferior to the distal ligature. Retract gently ventrally and inferiorly, and insert the catheter into the cranial (superior) mesenteric vein, 5 mm inferior to the distal ligature retracting the needle immediately.
11. Advance the catheter until it is 5 mm superior to the proximal ligation. Draw the ligations securely (distal before proximal). Blood should immediately fill the catheter and begin to drip out.
12. Attach the catheter lead to the cannula, and immediately divide the inferior vena cava using the scalpel. The success of the perfusion should be immediately apparent. Blood will drain from the liver, replaced by perfusate, so the color of the liver will change from red to light brown.
13. Make a horizontal incision in the diaphragm ventral to the vena caval foramen. Cut the superior vena cava.
14. Tie the proximal suture, and then the distal suture.
15. Using small scissors cut the ligaments that anchor the liver to the surrounding tissue and the bile duct.
16. Remove the liver and place it in a 10 cm dish for the duration of the perfusion.

3.1.3. Perfusion-Digestion

1. KRB with EDTA solution (see Subheading 2.1, item 3) should be perfused in a single pass, until the liver becomes homogenously light brown.

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2. Add the entire collagenase solution (see Subheading 2.1, item 4) in a manner that ensures that no bubbles are allowed into the line.
3. Collagenase digestion will vary with each isolation and batch. Successful enzymatic digestion will be marked by: swelling, lobes "bubbling," and the emergence of cellular debris.
4. Once digestion has been complete, disconnect the perfusion system and immediately place the liver in a petri dish containing 5 mL of ice-cold KRB.

3.1.4. Cell Purification

1. Move the tissue to a sterile biological safety cabinet. The metabolic activity of primary hepatocytes demands that all following steps be carried out on ice to prevent hypoxia.
2. Add ice-cold KRB to the dish, grasp the portal system using forceps, disrupt the capsule and shake the liver to remove the cells. Add additional KRB buffer as required to wash the cells from the liver.
3. Using a plastic pipette, aspirate the cell suspension slowly and pass it through a 250 μm pore mesh filter to remove tissue fragments.
4. Aspirate the cell suspension several times and pour it slowly through a 60 μm pore mesh filter to attain a single cell suspension.
5. Divide the suspension into several 50 mL conical tubes and centrifuge at 40 × *g* for 5 min. Centrifuge should be set at 4°C.
6. Re-suspend each cell pellet with 10 mL KRB by gently inverting the tube. Combine cell suspensions to 25 mL volume in 50 mL conical tubes and add additional 25 mL of salt-balanced Percoll solution. Mix by gently inverting the tubes and centrifuge at 500 × *g* for 5 min.
7. Re-suspend each cell pellet with culture medium (see Subheading 2.2, item 7). Combine all fractions into 50 mL tube and centrifuge at 40 × *g* for 5 min. Re-suspend cells and assess viability using hemocytometer.

3.2. Hepatocyte Culture Medium

While primary hepatocytes can be maintained in several types of culture medium, there is a clear distinction between serum-free and serum-containing medium formulations. Serum-free formulations are often based on Williams' Medium E and are ideal for short-term cultures, up to 10 days. One such formulation that yields excellent results is Hepatocyte Culture Medium, available commercially from Lonza. Serum-containing formulations were developed for long-term cultures (several weeks) and require an adaptation period (19, 20). One such formulation is detailed above (see Subheading 2.2).

3.3. Hepatocyte Culture Techniques

One common technique to culture primary hepatocytes is to seed the cells on a single layer of collagen gel. Under these conditions, hepatocytes secrete albumin and urea, and show minimal cytochrome P450 (CYP450) activity. Unfortunately, these liver specific functions decline within the first week, suggesting that significant survival factors are missing (see Fig. 1). In 1989, Dunn et al., suggested culturing hepatocytes in a collagen sandwich configuration (20). Adding the second collagen layer induces the formation of distinct apical and lateral membranes. Cellular cuboidal morphology was maintained for 42 days, with albumin and urea secretion slowly rising and stabilizing after 10 days in culture. Interestingly, cells that were cultured on a single layer of collagen for 1 week and after

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which a second layer of collagen was added, resulted in recovery of albumin secretion levels.

Hepatocytes can also be cultured in the form of hepatic spheroids. When cultured on soft or non-adhesive extracellular matrix, cells form spherical aggregates during the first 48 h. Cells in these aggregates maintain their morphology and liver specific functions for over a month (see Fig. 1). The disadvantages of this technique include lack of control over spheroid size, and thus, variation in the transport of metabolites. Another disadvantage is the formation of a necrotic core in big aggregates (1).

Cell morphology in collagen sandwich and spheroid culture configurations is different (see Fig. 2). While hepatocytes entrapped in collagen matrix show a cuboidal morphology (21), spheroids are round, and form closely associated aggregates, which are not found in the mature liver. One assumption is that spheroids resemble the organization of liver during regeneration (22, 23). Unfortunately, hepatocytes cultured in either configuration cannot generate a large quantity of cells, and show little to no proliferation capacity (24) (see Fig 3).

3.3.1. Hepatocyte Sandwich Technique

1. All solutions must be ice-cold.
2. Determine the final volume of collagen solution needed: for a 10 cm^2 plate, use 0.5 mL collagen solution
3. Determine volume of 10× PBS (see Subheading 2.4) by dividing the final volume calculated by 10.
4. Calculate the volume of collagen needed by dividing the final volume by collagen concentration in the bottle (see specification sheet)
5. The volume of 1N NaOH (see Subheading 2.5) is given by the formula: Volume of collagen × 0.023 mL = mL NaOH
6. Calculate the volume of Ultrapure water needed, by subtracting from the final volume calculated in step 1 the volume of 10× PBS (step 2), collagen (step 3), and 1N NaOH (step 4)
7. In sterile conditions, on ice, add the calculated volume of 1N NaOH to the calculated volume of PBS, and water.
8. Add the calculated volume of collagen and mix gently.
9. Add 0.5 mL mixture into each 10 cm^2 well, and distribute evenly.
10. Incubate at 37°C for 30–40 min.
11. Seed 100,000 cells/cm^2 in a final volume of 1 mL/10 cm^2. This roughly translates to 1×10^6 hepatocyte in each well of a 6-well plate.
12. Incubate cells overnight at 37°C and 5% CO_2.
13. Prepare fresh collagen solution (steps 1–7).
14. Aspirate culture medium and add 0.5 mL of collagen mixture into each well
15. Incubate for 30–40 min.
16. Add 1 mL of hepatocyte culture medium to each well.

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3.4. Heterotypic Interactions

Cellular functions are influenced by a sum of extracellular factors, which include neighboring cells, extracellular matrix, soluble factors, and physical forces (25). Neighboring non-parenchymal liver cells play a major role in the regulation of hepatocyte function (26). To mimic these cell–cell interactions, several groups cocultured hepatocytes with non-parenchymal cells, demonstrating improved maintenance of hepatic function (27). The most successful configurations to date include the coculture of primary hepatocytes with microvascular endothelial cells or 3T3-J2 mouse fibroblasts (19, 28). Other work showed that small hepatocytes can marginally proliferate in culture and form large aggregates, in the presence of epithelial cells and fibroblasts (29, 30). In a later study, those aggregates were formed on a collagen mesh and showed liver-specific structure and function (31). When hepatocytes and non-parenchymal cells are cultured in roller bottles, they self-organize and form simple epithelial structures consisting of a superficial layer from biliary epithelial cells, middle layer of hepatocyte and connective tissue, and an inner layer of endothelial cells (32).

In 1997, Bhatia and colleagues created micropatterns of hepatocytes and 3T3-J2 mouse fibroblasts (see Table 1) using lithographic techniques. It was demonstrated that the fibroblasts maintain urea and albumin secretion through a combination of cell contact and short acting diffusible substances. Only hepatocytes that were in close contact to the 3T3-J2 fibroblasts maintained albumin expression, while those distant from the fibroblasts quickly lost function. In a more recent report, Khetani and Bhatia used a polydimethylsiloxane (PDMS) stencil to pattern islands of collagen. Selective human hepatocyte adhesion to these domains yields islands that were subsequently surrounded by 3T3-J2 mouse fibroblasts. Liver specific functions stabilized within 7–10 days and the cells attained synthetic function, mRNA expression, and CYP450 activity, which were similar to in vivo. The authors report optimal function for the configuration of 500 μm islands with 1,200 μm center-to-center spacing. Cell morphology and gene expression were preserved for 4–6 weeks (28).

Endothelial cells were also shown to have a similar supportive function. Early work by Morin and Normand showed that liver sinusoidal endothelial cells (LSEC) stabilize hepatic urea and albumin secretion for up to a month in vitro (33, 34). Nahmias and colleagues demonstrated that micropattering LSEC using laser guided direct writing into capillaries, allows hepatocytes to assemble into sinusoid-like structures which maintained liver specific function for over a month in vitro (35). Later, the same group demonstrated that LSEC induced hepatic expression of basal receptors for low density lipoproteins (LDL) and epidermal growth factor (EGF) as well as the uptake of HCV-like particles (11). LDL uptake is an important function of hepatocytes and was dramatically elevated following coculture of hepatocytes with LSEC. More recent work from our group demonstrated that under the culture medium and oxygen conditions, primary human hepatocytes cocultured with endothelial cells attain synthetic function, mRNA expression, CYP450 activity and drug clearance equivalent to in vivo (19).

Finally, Kupffer cells, the liver's resident macrophages are known to become activated in response to a foreign stimulus and secrete reactive oxygen and nitrogen species (ROS/RNS), which causes hepatic damage. Cocultures of hepatocytes with Kupffer cells have been shown to mimic in vivo damage due to ischemia or drug toxicity (36). While LSEC and Kupffer cells can be difficult to isolate and do not proliferate in vitro, there are several commercially available sources of cells. Non-parenchymal cell sources and culture specifications are listed in Table 1.

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3.5. Dynamic Flow Cultures

The culture of hepatocytes under flow is thought to mimic certain aspects of the physiological environment found in vivo. For example, the flow of hormones, nutrients and oxygen across a flat plate bioreactor readily forms gradients due to cellular uptake, such gradients are thought to induce metabolic zonation in vivo. In fact, one group suggested that oxygen gradients formed across the reactor induce metabolic zonation (37), with albumin production high at the entrance (periportal) and CYP450 activity higher at the exit (perivenous). However, it is possible that the loss of synthetic function and gain of CYP450 activity were the result of hypoxia, as oxygen content in the bioreactor was much lower than in vivo. Other groups, however, used similar designs to demonstrate the negative effects of shear forces >5 dyn/cm^2 on hepatocyte function (38), and a higher rate of drug metabolism in perfused cells due to enhanced mass transfer (39).

3.6. Oxygen Supply

Oxygen is an important component in the hepatic microenvironment, which could be derived from the fact that a single hepatocyte contains over 1,500 mitochondria. The oxygen consumption rate of hepatocytes ranges from 0.3 to 0.9 nmol/s/10^6 cells. In vivo, in order to supply cells with this amount of oxygen, the liver is connected to a highly oxygenated arterial network, in addition to the portal circulation, delivering close to 1.29 nmol/s/10^6 cells. Solubility of oxygen in aqueous media is low. That is compensated in vivo by the presence of oxygen-binding hemoglobin (40). In vitro, oxygen is supplied by diffusion from the air-liquid interface, severely limiting the cell density that can be seeded in a given culture area (24).

Several groups suggested that high oxygen tensions stimulate production of free radicals possibly damaging cultured cells (41, 42). However, our group demonstrated that hepatic damage at high oxygen tensions is a result of serum adaptation at the early stages of culture. Removing serum completely from the hepatocyte culture medium, while increasing oxygen tension to 95%, caused a dramatic threefold increase in albumin synthesis and 74% increase in CYP450 activity (19). Gene expression, functional polarization, and drug metabolism were similarly enhanced in both rat and human primary hepatocytes. Remarkably, these oxygenated cocultures showed an ability to predict in vivo hepatic clearance rates of both rapid and slow clearing drugs, such as carbamazepine and antipyrine, with R^2 of 0.92.

3.7. Albumin and Urea Quantification

Albumin synthesis and urea production are classical assays for the study of liver-specific function. Albumin is quantified using enzyme-linked immunosorbent assay (ELISA) and several kits for the quantification of human albumin are available commercially (Bethyl Labs). Standard blood urea nitrogen (BUN) kit for all species is available (Stanbio Labs™).

3.7.1. Rat Albumin ELISA

1. Prepare 10 mL purified rat albumin in PBS just before use (see Subheading 2.6.1). Add 100 μL albumin solution to each well of a 96-well plate and cover with adhesive plate sealer. Incubate at 4°C overnight. Coated-plates can be stored at 4°C for several days.
2. Using serial dilution, prepare the following rat albumin standards in hepatocyte culture media: 200, 100, 50, 25, 12.5, and 0 μg/mL.
3. Thaw 5 μL of HRP conjugated rabbit anti-rat albumin antibody (see Subheading 2.3, item 2) and dilute 1 to 10,000 in PBS-Tween.
4. Wash albumin-coated plate four times with 100 μL of PBS-Tween

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5. Add 50 μL of sample or standard to each well in triplicate, and *50* μL of antibody solution to each well. Incubate overnight at 4°C or alternatively for 3 h at 37°C.
6. Dissolve one 10 mg *0*-Phenylenediamine dihydrochloride (OPD) pill in 25 mL citrate-phosphate buffer (see Subheading 2.3, item 8) at room temperature.
7. Wash plate four times with 100 μL of PBS-Tween.
8. Incubate overnight at 4°C or alternatively for 3 h at 37°C. Wash plate four times with 100 μL of PBS-Tween.
9. Add 10 μL of 30% H_2O_2 to OPD solution.
10. Add 100 μL/well of OPD solution using multi-well pipettor. Incubate for 5 min at room temperature and add 50 μL/well of 8N H_2SO_4 using a multi-well pipettor at the same rate as above.
11. Read OPD absorbance on a plate reader at 405 nm or 450 nm.

3.7.2. Urea Quantification

1. Using serial dilution, prepare the following urea/nitrogen standards in hepatocyte culture media: 200, 100, 50, 25, 12.5, and 0 μg/mL.
2. Add 10 μL of standard or sample to each well of a 96-well plate.
3. Mix 5 mL of BUN color reagent with 10 mL of BUN acid reagent (see Subheading 2.6.6).
4. Add 150 μL of the BUN reagent mixture into each well.
5. Cover with adhesive plate sealer and incubate at 60°C for 90 min.
6. Cool plate on ice for 5–10 min before reading.
7. Read plate absorbance at 520 nm.

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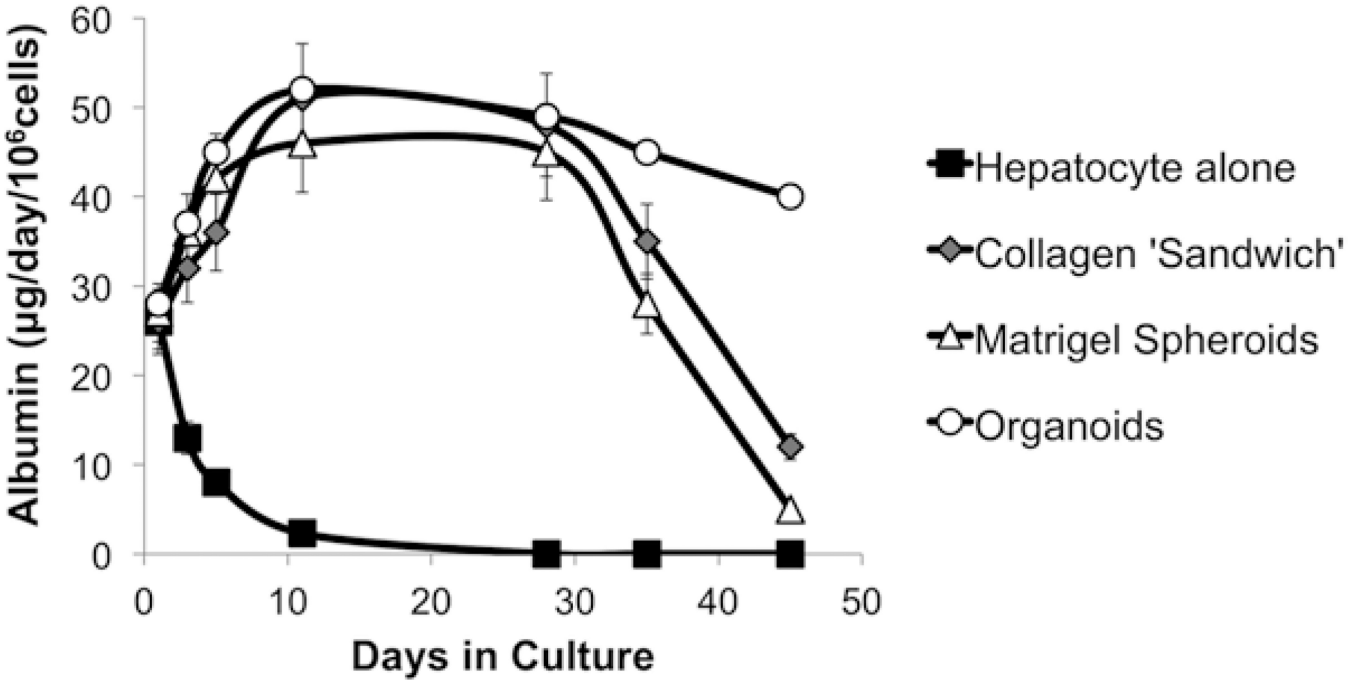

Fig. 1.

Long-term albumin production in cultures of primary rat hepatocytes. Primary hepatocytes cultured alone (Hepatocytes alone) rapidly lose albumin secretion in vitro. Cells seeded on Matrigel™ (Matrigel Spheroids), or in a collagen sandwich configuration (Collagen Sandwich) stabilize over 7–10 days and maintain liver specific function for 28 days. Self-assembled hepatic organoids (Organoids) maintain albumin secretion for over 50 days in culture.

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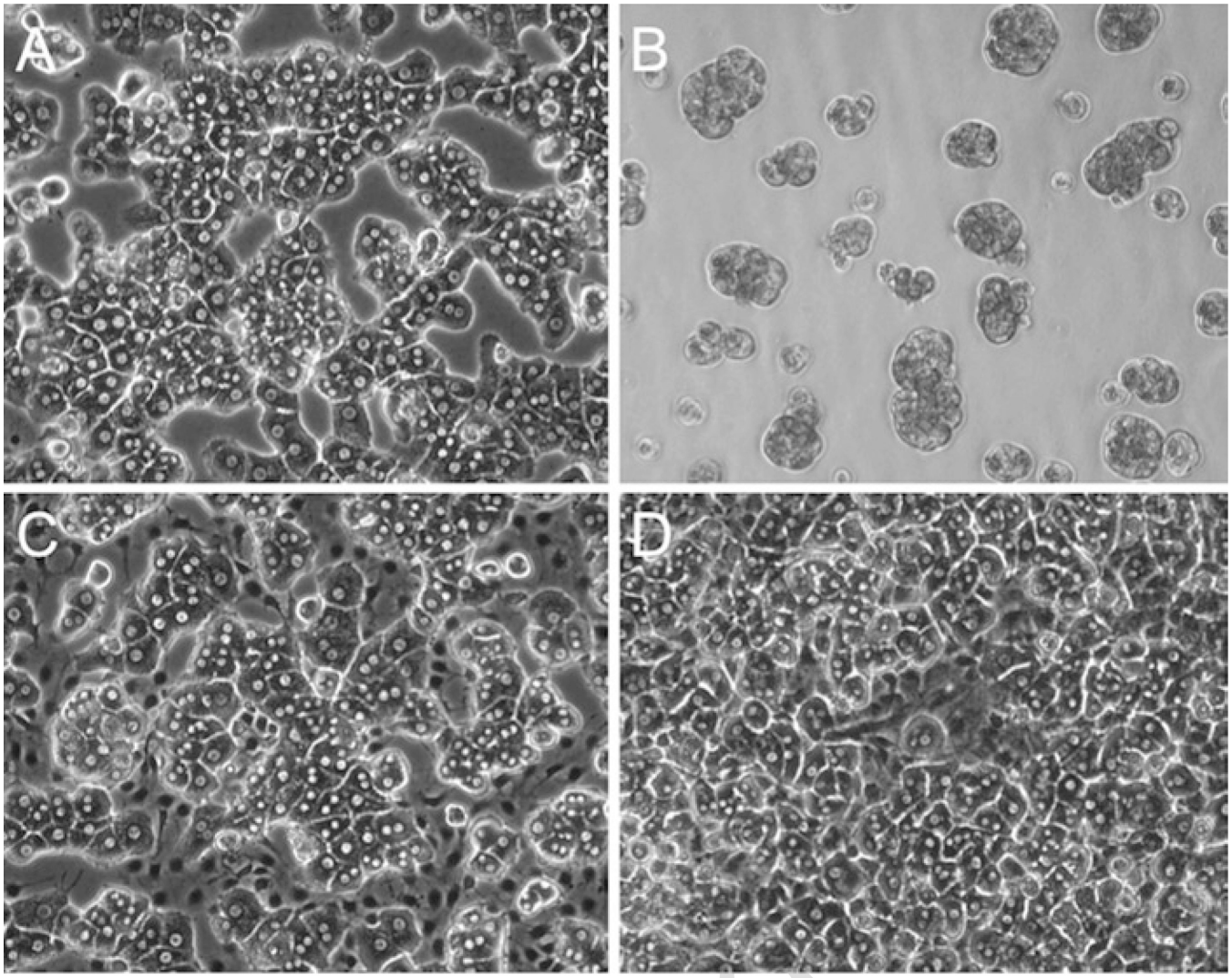

Fig. 2.

Phase images of hepatocyte morphology in culture and coculture. (**a**) Hepatocytes cultured in a collagen double gel configuration form plate-like structures and stabilize synthetic and enzymatic activity a week after isolation. Cells in double gel were shown to exhibit native cell–cell contacts such as E-cadherin and bile canaliculi (45), but do not express sinusoidal receptors such as EGF-R (45) and LDL-R (11). (**b**) Hepatocyte spheroids formed on Matrigel exhibit significant synthetic and enzymatic activity. Spheroids were shown to express E-cadherin (45), form extensive bile canaliculi (46), and show sinusoidal surface markers at the interface between cells in the spheroid (46). (**c**) Hepatocytes cocultured with LSEC show both traditional polarity markers (33, 34), and express a high level of sinusoidal receptors (EGF-R, LDL-R) at the interface between the hepatocytes and the LSEC. At least part of the interaction between hepatocytes and LSEC has been shown to be mediated by growth factors (47, 48). (**d**) Hepatocytes cocultured with 3T3 fibroblasts grow in distinct clusters and exhibit hepatic cell–cell contacts such as connexin-32 (49) and bile canaliculi (50), but also do not express the EGF-R and LDL-R. At least part of the interaction between hepatocytes and 3T3 fibroblasts was shown to be mediated by N-cadherin and decorin (51).

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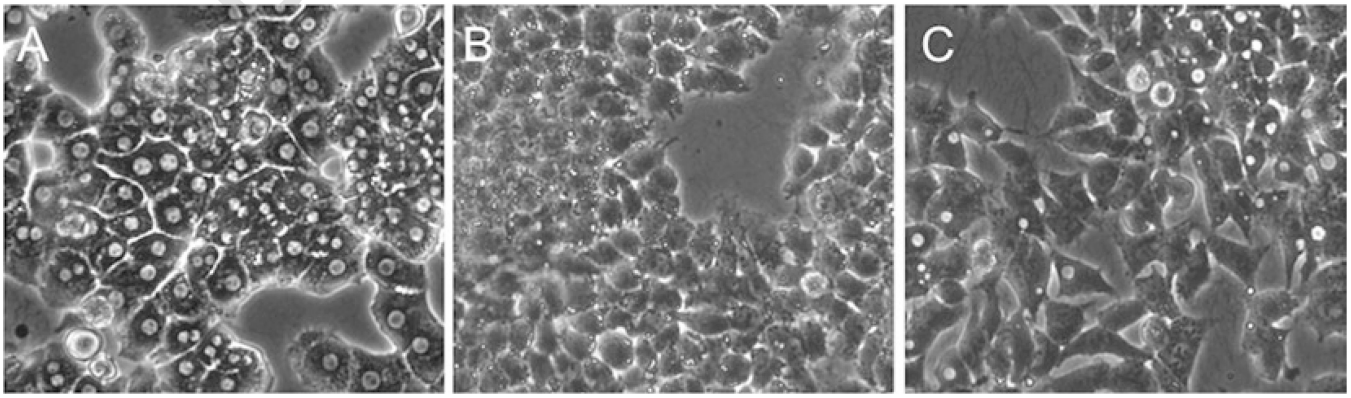

Fig. 3.

Morphological differences between primary hepatocytes and hepatocellular carcinoma cell lines. (**a**) Primary rat hepatocytes, (**b**) Huh7 cells (P53 mutant), (**c**) HepG2 cells (P53 wt).

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Table 1

Non-parenchymal cell source and cell culture

Endothelial cells
Liver sinusoids are composed of a highly specialized type of microvascular endothelial cells, which play an important role in lipid metabolism, coagulation, cellular growth, differentiation, immune, and inflammatory response. Liver sinusoidal endothelial cells (LSEC) are usually purified from the nonparenchymal fraction of the liver by a two-step Percoll gradient separation (44). Alternative for LSEC include rat heart microvaslcular endothelial cells (Vec Technologies®) for coculture with rat hepatocytes, and human lung microvascular endothelial cells (Lonza) for coculture with human hepatocytes. In all three cases the endothelial cells are cultured in EGM2mv (Lonza). Add 5 ng/mL of VEGF to hepatocyte culture medium if coculturing with endothelial cells
Kupffer cells
Kupffer cells are the liver resident macrophage, thought to originate from the bone marrow. Kuppfer cells ingest and degrade old erythrocytes, bacteria, various endotoxins, and play an important role in iron metabolism. Due to their similar density and size it is difficult to separate Kupffer cells from LSEC. However, relatively pure populations can be purified using centrifugal elutriation. The ED2 antibody is specific for Kupffer cells. Kupffer cells do not proliferate and rapidly activate in culture. A mouse Kupffer cell line is available (KC13-2)
Fibroblasts
Stellate Cells (Ito cells, fat-storing cells) are vitamin A storing pericytes (fibroblasts), which decorate the liver's sinusoids. Stellate cells are the main matrix-producing cell in the liver and play an important role in regeneration, differentiation, and inflammation. Stellate cells are thought to become activated during liver fibrosis, increasing collagen-synthesis and acquiring a fibroblast-like phenotype. Low density of the cells allows for simple purification using density centrifugation. CD95 and Desmin II are specific markers for stellate cells. HSC become rapidly activated in culture and readily proliferate. An immortalized rat liver stellate cell line (HSC-T6) is available for coculture. One popular cell line shown to support long-term liver specific function is the 3T3-J2 mouse fibroblast cell line available from Prof. Howard Green (Harvard Medical School). 3T3-J2 readily proliferate when cultured in DMEM culture medium supplemented with 10% fetal bovine serum