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Tissue Engineering of the Liver

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1. BACKGROUND

1.1. Liver Disease and Cell-Based Therapies

Liver failure is a significant clinical problem, representing the cause of death of over 30,000 patients in the United States every year and over 2 million patients worldwide. Organ transplantation is the only therapy to date shown to alter mortality, although the utility of organ transplantation is restricted because of the scarcity of donor organs. Surgical advances such as partial liver transplants from cadaveric or living donors have been demonstrated to be effective treatments and a means to increase the supply of donor organs [Ghobrial et al., 2000; Hashikura et al., 1994; Raia et al., 1989; Schiano et al., 2001]. These approaches take advantage of the body's role in the regulation of liver mass and the significant capacity for regeneration exhibited by the mammalian liver. This regenerative process has been extensively examined with experiments in rodent models, which demonstrate that partial hepatectomy or chemical injury induces the proliferation of the existing mature cell populations within the liver including hepatocytes, bile duct epithelial cells, and others, resulting in the replacement of lost liver mass. However, liver regeneration is not a component of all disease settings (e.g., cirrhosis) and is difficult to control clinically. Furthermore, despite surgical advances such as split liver and living donor transplantation, there is an increasing divergence between the number of patients awaiting transplantation and the number of available organs [Harper et al., 2001], suggesting that it is unlikely that liver transplantation procedures alone will meet the increasing demand. Consequently, alternative approaches are needed and are actively being pursued. These approaches include several nonbiological extracorporeal support systems, such as plasma exchange, plasmapheresis, hemodialysis, or hemoperfusion over charcoal or various resins, although these systems have achieved limited success [Allen et al., 2001; Strain and Neuberger, 2002; Yarmush et al., 1992]. It has been suggested that the inadequate effectiveness of these nonbiological schemes is due to the limited functionality of these devices. The liver exhibits a complex array of over 500 functions, including detoxification, synthetic, and metabolic processes. Thus recapitulation of a substantial number of liver functions will be required to provide sufficient liver support. To provide the myriad of known as well as currently unidentified liver functions, cell-based therapies have been proposed as an alternate approach to both organ transplantation and the use of strictly nonbiological systems. Potential cell-based therapies include the transplantation of hepatocytes, perfusion of blood through an extracorporeal device containing hepatocytes, transgenic xenografts [Costa et al., 1999; Fodor et al., 1994; Schmoeckel et al., 1997], or the implantation of hepatocellular constructs (Fig. 15.1).

1.2. Cell Sources for Liver Cell-Based Therapies

Immortalized hepatocyte cell lines such as HepG2 (human hepatoblastoma) or HepLiu (SV40 immortalized) have been utilized as readily available surrogates for hepatic tissue. However, it has been documented that these cells display an abnormal assortment of differentiated functions [Cederbaum et al., 2001; Fukaya

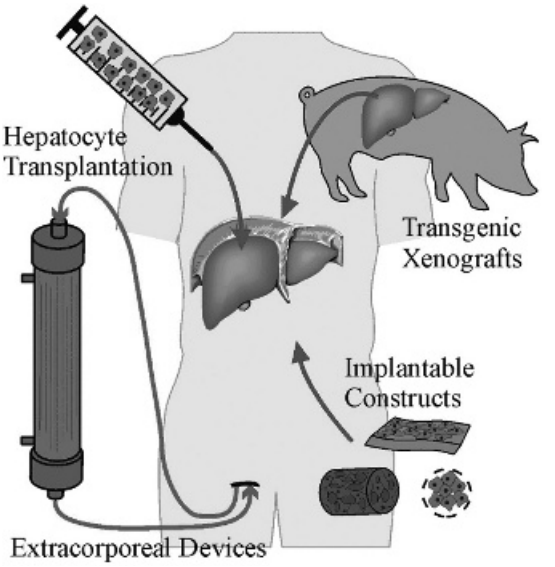

Figure 15.1. Cell-based therapies for liver disease. Extracorporeal devices perfuse patient's blood or plasma through bioreactors containing hepatocytes. Hepatocytes are transplanted directly or implanted on scaffolds. Transgenic animals are being raised in order to reduce complement-mediated damage of the endothelium. From Allen et al. [2001] (See Color Plate 10A.).

et al., 2001; Liu et al., 1999], and for clinical applications there is a risk that oncogenic factors could be transmitted to the patient. Thus the use of primary hepatocyte-based systems would eliminate these deficiencies, providing the appropriate collection of liver functions. The development of primary hepatocyte-based approaches is the focus of substantial ongoing research, yet progress has been hampered by the loss of liver-specific functions exhibited by isolated hepatocytes in vitro. Furthermore, particularly for human hepatocytes, despite the significant proliferative capacity during regenerative responses in vivo, mature hepatocyte proliferation in culture is limited. As a result, alternative primary cell sources for liver cell-based therapies are also being investigated [Allen and Bhatia, 2002], such as various stem cell populations (embryonic and adult), which retain significant proliferative ability in vitro and exhibit pluripotency (embryonic) or multipotency (adult), thereby providing a potential source of hepatocytes as well as other liver cell types. This prospective capacity to generate concurrently additional liver cell types, for example, bile duct epithelial cells from a single proliferative precursor, represents a potential means for enhancing liver-specific function of a tissue-engineered construct. However, this process may require complex differentiation cues, and many challenges remain, including the ability to control the microenvironment and organize resultant structures, strategies for delivery, etc., before stem cells can be utilized as sources of large numbers of hepatocytes or other liver cell types, particularly within multicellular systems. In addition, regardless of the cell source, the stabilization of hepatocyte functions remains a fundamental issue.

1.3. Approaches for the In Vitro Stabilization of Primary Hepatocytes

Several distinct methods have been utilized to promote hepatocyte stabilization in vitro, with a broad goal of mimicking physiologically relevant extracellular

cues that are absent from standard culture models. For example, modifications in culture medium such as hormonally defined preparations with low concentrations of hormones, corticosteroids, cytokines, vitamins, or amino acids, or the addition of low levels of dimethyl sulfoxide or dexamethasone, have been shown to help promote a stabilized hepatocyte phenotype [Baribault and Marceau, 1986; Block et al., 1996; Dich et al., 1988; Isom et al., 1985; Kubota and Reid, 2000]. In addition, extracellular matrix of various compositions is also known to exhibit positive effects on hepatocyte function. These approaches include the sandwich culture of hepatocytes within collagen gel or culture on the tumor-derived basement membrane preparation Matrigel [Bissell et al., 1987; Dunn et al., 1991; Rojkind et al., 1980]. Interestingly, hepatocyte culture on Matrigel results in the formation of spheroid cellular structures [Kang et al., 2004], suggesting that cell-cell communication is a likely component of the stabilization observed in this model system. Consistent with the importance of homotypic (hepatocyte-hepatocyte) interactions, spheroidal aggregates have been shown to promote the formation of bile canaliculi, gap junctions, and tight junctions and the expression of E-cadherins and several differentiated functions [Chang, 1992; Landry et al., 1985; Saito et al., 1992].

Similar to homotypic communication, heterotypic (hepatocyte-nonparenchymal) interactions have also been shown to improve viability and differentiated function. This stabilization of hepatocyte functions has been reported for hepatocyte cocultures with both liver- and non-liver-derived cell types, and, furthermore, beneficial effects of cross-species coculture systems have also been observed [Bhatia et al., 1999]. Together, these findings suggest a highly conserved mechanism by which cocultures enhance the liver-specific function of hepatocytes. However, the precise roles of the potential regulatory factors, such as secreted signals (i.e., cytokines) or cell-associated signals (i.e., insoluble extracellular matrix or membrane-bound molecules), in the "coculture effect" have not yet been clearly defined. An understanding of the mechanisms mediating the stabilization of hepatocytes in coculture would have broad implications in liver cell biology and, specifically, would aid in the development of functional hepatic tissue constructs.

In addition to static culture systems, several bioreactor designs have also been developed as in vitro hepatocyte culture models. Perfusion systems would facilitate enhanced nutrient delivery to hepatocytes, which are highly metabolic, and additionally, in contrast to a batch process, would enable the continuous processing of blood or serum, potentially useful for extracorporeal devices. Overall, the bioreactor systems developed to date fall into the following categories: flat plate, hollow fiber, perfusion scaffolds, and packed beds, each with accompanying advantages and disadvantages [Allen et al., 2001] (Fig. 15.2). In particular, the combination of a flat plate reactor system with sandwich culture or coculture has been illustrated to improve hepatocyte stability for long-term analysis [Bader et al., 1995; Tilles et al., 2001]. Additionally, a recent three-dimensional perfusion bioreactor system has been developed based on the morphogenesis of hepatocytes into three-dimensional structures in an array of channels [Powers et al., 2002a,b]. Similar to static culture

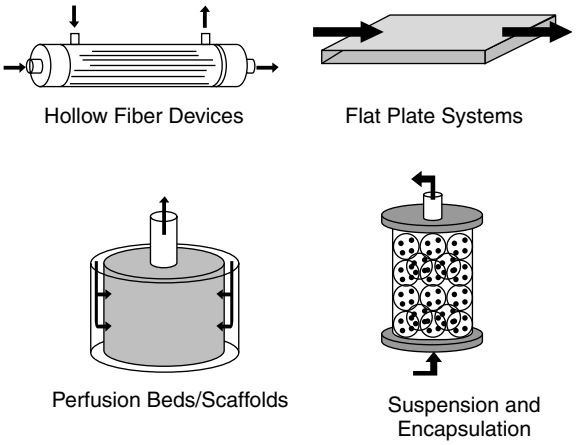

Figure 15.2. Schematics of cell-based bioreactor designs. The majority of liver cell-based bioreactor designs fall into these four general categories, each with inherent advantages and disadvantages, reviewed extensively in Allen et al. [2001].

models, preaggregation of hepatocytes into spheroids enhanced the functionality of hepatocytes in this design.

Overall, the components and characteristics of primary hepatocyte culture models have underscored the importance of microenvironmental signals, including soluble mediators, cell-extracellular matrix interactions, and cell-cell interactions, in the regulation of hepatocyte processes. Accordingly, the development of robust hepatocyte-based tissue engineering platforms will be predicated upon a fundamental knowledge and controlled reconstitution of these environmental factors.

1.4. Regulation of the Hepatocyte Microenvironment Toward the Development of Engineered Liver Tissue

The importance of extracellular signals in hepatocyte culture in vitro correlates with the significant role of the microenvironment in the liver in vivo. It is well established that the proper function of liver cells in vivo is maintained by a complex combination of extracellular cues, including tightly controlled cell-cell interactions and distribution of extracellular matrix [Gebhardt and Mecke, 1983; Michalopoulos and DeFrances, 1997; Olson et al., 1990; Reid et al., 1992]. Furthermore, alterations in the liver microenvironment are important aspects of liver pathologies [Giannelli et al., 2003] such as fibrosis and have additionally been implicated in repair processes [Kim et al., 1997; Michalopoulos and DeFrances, 1997; Rudolph et al., 1999]. Therefore, an understanding of the microenvironmental mechanisms regulating hepatocyte function represents a crucial issue for not only the improvement of in vitro culture models and extracorporeal liver devices, but also the manufacture of implantable hepatocyte constructs. Notably, in addition to tissue engineering applications, functional in vitro hepatic models would

also represent ideal platforms for ADMET (adsorption, distribution, metabolism, excretion, and toxicity) testing of novel drug candidates.

Consequently, our laboratory's strategy toward the fabrication of engineered liver tissue is to develop (1) enabling platforms for the controlled recapitulation of these critical extracellular cues and (2) in vitro models to study the complexities of environmental interactions. Furthermore, current studies in our laboratory are addressing the potential utility of alternative cell sources, such as embryonic stem cells and various adult stem cell populations, for liver tissue engineering. Overall, important criteria for the development of a particular system include (1) the desired application (in vitro model, basic science, or therapeutic) and (2) the important liver properties that must be established for that certain application, such as stabilized phenotype, structural complexity (zonation), directional fluid flow, and in some cases 3-D organization. Our laboratory has developed several distinct model systems that recapitulate critical structural and/or functional aspects of the liver.

Specifically, as a means to investigate systematically the role of heterotypic contact in hepatocyte cocultures, our laboratory has developed and characterized a versatile micropatterning method, which has enabled the quantitative control of the spatial relationship of hepatocytes and fibroblasts [Bhatia, 1997]. This method is one in a repertoire of techniques that we have developed to control cell-cell interactions. These processes enable an examination of the mechanisms of interaction, including the potential importance of numerous aspects such as continuous signaling, gap junctions, and soluble factors. Furthermore, the micropatterning method described in this chapter also represents a robust platform for the in vitro functional stabilization of hepatocytes.

Moreover, to gain another level of control of environmental influences and further enhance the physiologically relevant function of hepatocytes in vitro, we have also developed additional approaches. First, we have recently described the construction of a perfusion bioreactor system that has enabled the formation of steady-state oxygen gradients, resulting in the presence of zonated hepatocyte characteristics [Allen and Bhatia, 2003), which is an important feature of the liver in vivo. For example, numerous drugs exhibit zonal toxicity due to compartmentalization of drug-metabolizing enzymes [Lindros, 1997]. As a result, the in vitro model of zonation described here represents a unique system to investigate, among many aspects, the mechanisms of zonal hepatotoxicity.

Each of these approaches (micropatterning and bioreactor systems) recapitulates two-dimensionally certain aspects of hepatocyte function. In many instances, 2-D systems are ideal because they minimize complexities in aspects such as imaging and molecular, histologic, or immunohistochemical characterization, often introduced in 3-D culture models. In contrast, for implantable systems, the development of 3-D constructs is required. Numerous reports have examined the seeding or recruitment of cells to prefabricated 3-D scaffolds [Badylak et al., 2001; Langer and Vacanti, 1993; Lee et al., 2000; Yang et al., 2001]. However, particularly for hepatocytes, which do not grow or migrate well in vitro, the development of 3-D systems would require the formation of thick constructs containing a homogeneous

distribution of cells. By combining hydrogel polymerization with photolithography techniques, in which UV cross-linking is performed through a mask, we have demonstrated the generation of patterned three-dimensional hydrogels containing living cells [Liu and Bhatia, 2002]. This process is highly versatile and can be adapted to different hydrogel chemistries as well as various cell types. Consequently, this approach provides the foundation for regulating cellular orientations, including cell-cell and cell-matrix interactions, in three dimensions, important for the tissue engineering of spatially complex organs such as the liver.

In this chapter, we have assembled our expertise in several aspects of liver tissue engineering. Included are fundamental techniques such as the isolation of primary rat hepatocytes and assays of hepatocyte function, as well as novel approaches to assess and recapitulate microenvironmental influences on hepatocyte function. Although these procedures, such as the micropatterning, bioreactor, and 3-D fabrication platforms, were developed and characterized specifically for the design of hepatic systems, the basic principles outlined in this chapter are intrinsic to the fabrication of tissue-engineered constructs for numerous organ systems.

2. PREPARATION OF REAGENTS

2.1. Krebs-Ringer Buffer (KRB)

For 500 ml: add 3.57 g of NaCl, 0.21 g of KCl, 0.50 g of D-glucose, 1.05 g of $NaHCO_3$, and 2.38 g of HEPES to 500 ml of ultrapure water (UPW). Adjust pH to 7.4 with 10 N NaOH, bubble with 5% CO_2-95% N_2 for 10 min, and sterilize by filtration.

2.2. KRB with EDTA

Add 0.19 g of ethylenediaminetetracetic acid disodium salt, dihydrate (EDTA) to 500 ml of KRB (above), to give 1 mM EDTA. Stir on low heat until dissolved and sterilize by filtration.

2.3. Collagenase, 0.055% (w/v)

Make 30–90 min before surgical procedure. Add 105 mg of type IV collagenase powder to 190 ml of KRB, add 9 ml of 0.11 M $CaCl_2$, and stir to ensure that collagenase is completely dissolved (~20 min). Sterilize by filtration. To maintain reliable collagenase activity, thaw for 15 min, measure, and then immediately return to $-20\,^{\circ}C$ storage.

2.4. Coating Buffer

Dissolve 1.61 g of Na_2CO_3 and 2.93 g $NaHCO_3$ in 975 ml of ddH_2O. Adjust pH to 9.6, bring volume to 1 L, and filter.

2.5. OPD Substrate Buffer

Dissolve 5.10 g of citric acid monohydrate and 13.78 g of sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$) in 975 ml of ddH_2O. Adjust pH to 5.0, bring volume to 1 L, and filter.

2.6. Complete Hepatocyte Culture Medium (CHCM)

Dulbecco's modified minimal essential medium (DMEM) with Phenol Red and 21.43 mM sodium bicarbonate, supplemented with 10% fetal bovine serum (FBS), 0.5 U/ml insulin (Lilly Islets II pork regular insulin), 7.5 μg/ml hydrocortisone sodium succinate, 14.28 ng/ml glucagon, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.7. Blocking Buffer

PBSA with 1% bovine serum albumin (BSA), 50 mM glycine, and 20% normal goat serum (same species as secondary antibody in staining procedure).

2.8. Washing Buffer

PBSA with 0.05% Tween 20 (polyoxyethylene-sorbitan monolaurate) and 1% normal goat serum.

2.9. Piranha Cleaning Solution

3:1 Mixture of H_2SO_4:30% H_2O_2, prepared at time of experiment. Extreme caution should be observed because of the corrosive nature of these chemicals.

2.10. Hepatocyte Culture Medium, Serum Free (HCM/SF)

Same formulation as for CHCM (See Section 2.6), but minus FBS and plus 0.26 mM (30 μg/ml) proline.

2.11. Fibroblast Culture Medium (FCM)

DMEM with 23.81 mM sodium bicarbonate, supplemented with 10% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.12. Hormonally Defined Hepatocyte Medium (HDHM)

DMEM-Ham's F-12, 1:1, supplemented with 5 μg/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM HEPES, 10^{-8} M dexamethasone, 5 μg/ml linoleic acid, 10^{-10} M $ZnSO_4$, 10^{-7} M $CuSO_4$, and 3×10^{-10} M H_2SeO_3.

2.13. SDS Lysis Buffer

Tris-HCl, 10 mM, pH 7.4 with 0.1% sodium dodecyl sulfate (SDS).

2.14. HepG2 Culture Medium

Eagle's minimal essential medium (MEM) supplemented with 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

3. ISOLATION OF PRIMARY RAT HEPATOCYTES

The following protocols for the isolation and purification of primary rat hepatocytes are an adaptation of procedures previously described [Seglen, 1976]. Rat model systems have been widely exploited for many years to study liver development, injury, and regenerative processes [Bankston and Pino, 1980; Godlewski et al., 1997;

Michalopoulos and DeFrances, 1997; Palmes and Spiegel, 2004], and, similarly, the culture of rat hepatocytes has been extensively characterized [Mitaka, 1998].

Protocol 15.1. Surgical Procedure for Rat Hepatocyte Isolation

Reagents and Materials

Sterile

- ❑ Krebs-Ringer Buffer (KRB) (See Section 2.1)
- ❑ KRB with 1 mM EDTA (See Section 2.2)
- ❑ Collagenase, 0.055% (w/v) (See Section 2.3)
- ❑ Autoclaved surgical instruments: partial curve microdissecting forceps, rat tooth tweezers, scalpel, fine sharp-tip scissors, and Mayo blunt scissors
- ❑ Cotton-tipped applicators
- ❑ Lengths of 3-0 silk suture, 6 in., 2
- ❑ Petri dishes

Nonsterile

- ❑ Rat, Female Lewis, 5–8 weeks old, 1
- ❑ Perfusion system: Water bath to warm perfusate to 39 °C outflow temperature and 95% O_2-5% CO_2 cylinder to equilibrate perfusate through semipermeable tubing. The end point is attached to a sterile 18-gauge Angiocath™ catheter
- ❑ Veterinary-grade isoflurane anesthesia device (Model 100F)
- ❑ Betadine
- ❑ Ethanol, 70%
- ❑ Beaker, 1 L with lid
- ❑ Hair clippers
- ❑ Vacuum
- ❑ Surgical tray (perforated) with collection tub

Protocol

(a) Flush perfusion reservoir and tubing with 70% ethanol, then 500 ml UPW, and then 100 ml KRB-EDTA. Add 400 ml KRB-EDTA, clear bubbles from line, and close the perfusion circuit. Set flow rate to 20 ml/min. Allow 15 min for warming and equilibration of KRB-EDTA.

(b) Anesthetize rat with vaporizer: Adjust flow rate on the anesthesia device to 1.0 L/min 95% O_2-5% CO_2, and set the isoflurane level to 5% v/v. Put exit tubing from the device with nose-cone end in a large beaker and cover, allowing a few minutes for the concentration of isoflurane to build. Transfer rat into the beaker, cover, and monitor until fully sedated.

(c) After initial sedation, remove rat from beaker and position on paper towels with nose-cone covering its mouth. Maintain portable anesthesia, with isoflurane set to 2.5% v/v. Shave abdomen fur and vacuum loose fur.

(d) Transfer rat to surgical tray with collection tub underneath, and tape limbs to restrain. Swab abdominal region generously with ethanol, then Betadine.

(e) Perform midline incision through skin from tip of xiphoid cartilage to groin, and then dissect percutaneous tissue free of underlying musculature laterally near incision. Cut through peritoneum from the groin to xiphoid, and perform lateral cuts perpendicular to the midline through skin and abdominal fascia in order to further open the abdominal cavity.

(f) Use cotton-tipped applicators moistened with KRB-EDTA to push aside the intestines and stomach. Move the liver gently, using a rolling motion with the applicators, until the portal vein is exposed.

(g) Loosely loop a suture around the portal vein near the hilus proximal to vessel branching and another one distally between the mesenteric veins.

(h) Insert the catheter into the portal vein, and position just beyond the proximal suture. Attach the perfusate line to the catheter, and immediately cut the inferior vena cava with a scalpel. It is imperative to establish flow quickly, to prevent clotting within the liver. Tie the distal suture, and then tie the proximal suture.

(i) Cut through the left-side rib cage to access the heart, and sever the left ventricle so that the heart stops. Cut through the sternum along the midline to the clavicles, and cut along the diaphragm and ribs on both sides to create room for the liver to expand. Turn off isoflurane.

(j) Trim connective tissue between liver and surrounding organs. Prop up liver with cotton-tipped applicators so that the liver is not stretched, to permit profuse flow of collagenase. When the reservoir is almost emptied, add in the collagenase. Allow the collagenase to perfuse the liver at 18 ml/min.

(k) Turn off the pump after deep fissures appear in the liver lobes, but before disintegration of the liver capsule and before the collagenase empties (approximately 11 min). Cut the catheter. Pick up the liver with forceps and sever connective tissue to completely free liver from the abdominal cavity. Place liver in Petri dish with KRB and take to laminar flow hood.

Protocol 15.2. Purification of Primary Rat Hepatocytes

Reagents and Materials

Sterile

- ❑ Collagenase perfused rat liver (See Protocol 15.1)
- ❑ Conical centrifuge tubes, 50 ml
- ❑ KRB (See Section 2.1)
- ❑ Large bowl covered with 30 × 30-cm, 62-μm nylon mesh
- ❑ 100 × 50-mm Pyrex dish covered with 15 × 15-cm, 250-μm nylon mesh
- ❑ 10× Hanks' balanced salt solution (HBSS), without calcium or magnesium
- ❑ Percoll
- ❑ Petri dish, 10 cm

Protocol

(a) In laminar flow hood, gently agitate the liver in the Petri dish containing KRB by holding the vascular tree and shaking until most of the cells have been dispersed.

(b) Place dish with 250-μm mesh filter on ice. Prewet filter with approximately 10 ml KRB. Transfer the cellular suspension from the two Petri dishes over the 250-μm filter. Use an additional 24 ml KRB to wash cells through the filter.

(c) Place bowl with 62-μm mesh filter on ice. Prewet filter with 10 ml KRB in the horizontal direction and 10 ml vertically in order to maximize coverage. Transfer cells to 62-μm filter covering Pyrex dish. Wash filter with approximately 36 ml KRB to ensure that the majority of the cells pass through.

(d) Aliquot cell suspension equally to two 50-ml centrifuge tubes and centrifuge at 50 *g* for 3 min at 4 °C. Aspirate supernate, leaving approximately 0.5 ml to avoid aspirating hepatocytes. Bring each volume of cell suspension to 12.5 ml with KRB, and resuspend cell pellet with slow rocking.

(e) Mix 10.8 ml Percoll and 1.2 ml 10× HBSS on ice. Add 12.5 ml Percoll-HBSS mixture to each tube of cellular suspension and mix by gently inverting. Centrifuge at 50 *g* for 5 min at 4 °C.

(f) Aspirate supernate, including cells suspended within Percoll layer. Resuspend pellet in KRB with gentle rocking and pool cells into one 50-ml conical tube. Bring volume to approximately 40 ml with KRB. Centrifuge at 50 *g* for 3 min at 4 °C.

(g) Aspirate supernatant, and resuspend to a final volume of 12.5 ml KRB. Count hepatocytes with a hemocytometer. With the procedure described here, an average yield of 1.9×10^8 viable hepatocytes per rat, with 90% overall viability, is obtained.

4. ASSAYS OF HEPATOCYTE FUNCTION

Several parameters are utilized when assessing primary hepatocyte stabilization in vitro. For example, the presence in culture of the classic hepatocyte morphology including a cuboidal shape and well-defined cell borders with intact bile canaliculi is considered suggestive of a mature stable phenotype. Furthermore, there are four categories of liver function: metabolism, synthesis, bile excretion, and detoxification (phase I and phase II). Specific markers are frequently analyzed as surrogate measures of these processes in hepatocytes within various in vitro contexts. These include albumin secretion, urea synthesis, and cytochrome P-450 (phase I) activity [Bhatia et al., 1999]. In addition to being surrogate markers, each of these functions represents an important hepatocyte process, and, accordingly, each is a critical component of a potentially effective bioartificial liver device. Although not measured routinely in our laboratory, assays for bile duct excretion and phase II activity have also been performed in our laboratory as well as others to assess the degree of hepatocyte stabilization. Also, when comprehensive analysis is required, microarray analysis of the expression of a broad range of functionally important genes can be utilized. In addition to the evaluation of isolated hepatocyte populations, the measurement of hepatocyte-specific functions is important in the assessment of hepatocyte differentiation from stem cell sources such as embryonic

stem cells or various adult stem cell populations [Dahlke et al., 2004; Kuai et al., 2003; Levenberg et al., 2003; Ruhnke et al., 2003; Schwartz et al., 2002]. Included in this section are protocols for determining the degree of albumin secretion, the identification of intracellular albumin, and the quantification of urea synthesis and cytochrome P-450 activity for rat hepatocytes.

4.1. Detection of Intracellular Albumin and Albumin Secretion

Protocol 15.3. Enzyme-Linked Immunosorbent Assay (ELISA) for Quantification of Albumin Secretion

Reagents and Materials

Nonsterile

- ❑ Purified rat albumin stock, 3.125 mg/ml
- ❑ Coating Buffer (See Section 2.4)
- ❑ Multiwell plates, flat bottom, 96-well
- ❑ Adhesive plate sealer
- ❑ Rabbit anti-rat albumin antibody, horseradish peroxidase (HRP) conjugated
- ❑ *o*-Phenylenediamine (OPD) tablets
- ❑ OPD Substrate Buffer (See Section 2.5)
- ❑ Tween, 0.05% in PBSA
- ❑ CHCM (See Section 2.6)
- ❑ H_2O_2
- ❑ H_2SO_4, 8 N
- ❑ Spectrophotometer

Protocol

(a) Remove supernatant from hepatocyte cultures at the time point determined by the particular experiment of interest and store at 4 °C. Calculate number of wells required for the assay based on 24 wells per plate for albumin concentration standards, and triplicates of experimental samples.

(b) For each plate in the assay, make 10 ml albumin solution (0.05 mg/ml) by adding 160 μl rat albumin stock to 10 ml coating buffer.

(c) Add 100 μl albumin solution to each well of 96-well plate, and cover with adhesive plate sealer. Incubate at 4 °C overnight.

(d) Make series of albumin standards, using serial dilution of albumin stock in same medium as experimental samples (normally CHCM). The normal standard range required is 2-fold dilutions from 100 μg/ml to 1.5625 μg/ml, as well as a 0 μg/ml control.

(e) Shake liquid out of plate, and tap plate on paper towels to thoroughly expel liquid. Wash 4× with Tween-PBSA, shaking liquid out of the plate between each wash.

(f) Load 50 μl of standards in triplicate to the appropriate wells of the 96-well plate.

(g) Load 50 μl of experimental samples also in triplicate to the 96-well plate.

(h) Prepare 1/10,000 dilution of HRP-conjugated anti-rat albumin antibody in Tween-PBSA.
(i) Add 50 μl antibody solution to each well containing either standards or experimental samples. Cover with adhesive plate sealer, and incubate at 4 °C overnight.
(j) Dissolve one OPD tablet per 25 ml OPD substrate buffer. Add 10 μl H_2O_2 per 25 ml OPD substrate buffer.
(k) Wash plate 4× with Tween-PBSA.
(l) Add 100 μl OPD solution with H_2O_2 to each well, and incubate for 5–10 min, for complete color change.
(m) Stop reaction by adding 50 μl of 8N H_2SO_4 to each well.
(n) Read absorbance at 490 nm with spectrophotometer, and, utilizing standard curve, calculate concentration of albumin within experimental samples. For many cases the OPD substrate is sufficient. However, for the detection of low concentrations of albumin (0–10 μg/ml), an alternative substrate, Ultra-TMB (3,3′,5,5′-tetramethylbenzidine), can be used.

Protocol 15.4. Immunofluorescence Assay for Detection of Intracellular Albumin

Reagents and Materials

Nonsterile

- ❑ PBSA, pH 7.4
- ❑ Paraformaldehyde, 4% in PBSA
- ❑ Triton X-100, 0.1% in PBSA
- ❑ Blocking Buffer (See Section 2.7)
- ❑ Washing Buffer (See Section 2.8)
- ❑ Rabbit anti-rat albumin antibody
- ❑ Goat anti-rabbit IgG antibody, fluorescein (FITC) conjugated
- ❑ Glass slide, 75 × 38 mm
- ❑ Vectashield
- ❑ Clear nail polish
- ❑ Fluorescence microscope

Protocol

(a) This protocol is based on hepatocyte cultures on 34-mm coverglasses in 6-well plates. Volumes given are on a per well basis, and can be easily scaled for the use with cultures of different sizes. In general, this procedure can be performed in a nonsterile manner. However, if samples will be stored at any stage before mounting, aseptic techniques should be utilized to prevent potential contamination.
(b) Aspirate culture supernatant and fix cells in 1 ml of 4% paraformaldehyde for 10 min at room temperature.

(c) Remove fixative, add 2 ml PBSA, and incubate for 5 min. Aspirate PBSA and repeat PBSA wash an additional 2× for 5 min each. Either store at 4 °C in PBSA or proceed with staining procedure.
(d) Add 1 ml 0.1% Triton X-100 for 10 min at room temperature to permeabilize cells.
(e) As previously, wash 3× with 2 ml PBSA for 5 min each.
(f) Incubate in 1 ml Blocking Buffer for 15 min at room temperature.
(g) Prepare a 1/133 dilution of rabbit anti-rat albumin antibody. For each ml, add 7.5 μl antibody to 100 μl Blocking Buffer and 892.5 μl PBSA.
(h) Aspirate Blocking Buffer, add 0.5 ml diluted anti-albumin antibody, and incubate for 2 h at 37 °C.
(i) Wash 4× with 2 ml Washing Buffer for 5 min each on orbital rocker.
(j) Prepare a 1/100 dilution of goat anti-rabbit IgG-FITC. For each ml, add 10 μl antibody to 100 μl Blocking Buffer and 890 μl PBSA.
(k) Aspirate final wash with Washing Buffer, add 0.5 ml of diluted anti-rabbit IgG antibody, and incubate in the dark for 1 h at 37 °C.
(l) Wash 3× with 2 ml Washing Buffer for 5 min each on rocker, with a final immersion in PBSA or other standard mounting buffer.
(m) Remove coverglass from buffer and mount. Hold coverglass with tweezers, touch edge to towel, and aspirate at edge to remove excess liquid. Turn coverslip upside down and add one small drop Vectashield. Slowly sandwich together coverslip and clean glass slide, and seal coverslip edges with clear nail polish.
(n) Store in the dark at 4 °C. Prepared specimens should retain fluorescent staining for several weeks when stored properly.
(o) Examine fluorescence, indicating presence of intracellular albumin, using standard fluorescence microscopy techniques with FITC filters.

Protocol 15.5. Immunohistochemistry Assay for Detection of Intracellular Albumin

Immunohistochemistry can be utilized as an alternative for immunofluorescence for the detection of intracellular albumin within hepatocytes. The following method utilizes the DAKO LSAB® horseradish peroxidase (HRP) system, and elements of this protocol are adapted from the manufacturer's procedure.

Reagents and Materials

Nonsterile

- ❑ PBSA, pH 7.4
- ❑ Rabbit anti-rat albumin antibody
- ❑ Bovine serum albumin (BSA)
- ❑ Paraformaldehyde, 4% in PBSA
- ❑ Triton X-100, 0.1% in PBSA

- ❑ Parafilm
- ❑ Biotin Blocking System
- ❑ Peroxidase Blocking Reagent
- ❑ Blocking Buffer (See Section 2.7)
- ❑ Washing Buffer (See Section 2.8)
- ❑ DAKO LSAB® System containing: biotinylated anti-rabbit Ig antibody, HRP-conjugated streptavidin, and chromagen substrate.

Protocol

(a) Perform Steps (a) through (e) from Protocol 15.4.
(b) Transfer coverslips to Parafilm to minimize the amount of liquid required to maintain coverage on slips.
(c) Incubate with 10 drops Avidin Blocking Agent, from Biotin Blocking System, for 20 min to suppress endogenous avidin. Wash 2×, each with 2 ml PBSA.
(d) Similarly, incubate with 10 drops Biotin Blocking Agent, from Biotin Blocking System, for 20 min to suppress endogenous biotin. Wash 2×, each with 2 ml PBSA.
(e) Incubate with 12 drops Peroxidase Blocking Reagent for 8 min to quench endogenous peroxidases. Wash 2×, each with 2 ml PBSA.
(f) Add 10 drops of Blocking Buffer and incubate for 5 min at room temperature. Use paper tissue to remove excess Blocking Buffer; do not rinse.
(g) Prepare 1/100 dilution of rabbit anti-rat albumin antibody in PBSA with 1% BSA. Add 600 μl diluted antibody solution to each coverslip. Incubate at 37 °C for 90 min, gently rocking every 30 min to ensure even coverage on substrate.
(h) Wash 3× with 2 ml Washing Buffer, for 5–10 min each on rocker.
(i) Incubate with 10 drops Biotinylated anti-rabbit Ig antibody solution for 20 min at room temperature. Wash 2× with 2 ml Washing Buffer, for 5–10 min each on rocker.
(j) Incubate with 10 drops HRP-conjugated streptavidin solution (including, according to manufacturer's instructions, 4 ml UPW, 4 drops buffer concentrate, and 1 drop concentrated HRP-streptavidin) for 20 min at room temperature. Wash 2×, each with 2 ml PBSA.
(k) Prepare substrate solution according to manufacturer's instructions: 2 ml substrate buffer, 1 drop H_2O_2, and 1 drop 3-amino-9-ethylcarbazole chromagen substrate in dimethyl formamide. Incubate for 10 min at 37 °C. Watch closely after 10 min for color change; do not overdevelop. Wash 2×, each with 2 ml PBSA.
(l) Remove coverglass from PBSA and mount. Hold coverglass with tweezers, touch edge to towel, and aspirate at edge to remove excess liquid. Turn coverslip upside-down and add one small drop Vectashield. Slowly sandwich together coverslip and clean glass slide, and seal coverslip edges with clear nail polish.
(m) Record images by photomicroscopy.

4.2. Quantitative Measure of Urea Synthesis

Protocol 15.6. Assay of Urea Synthesis in Cultured Hepatocytes

The following procedure has been adapted from Stanbio Urea Nitrogen Procedure No. 0580.

Reagents and Materials

Nonsterile

- ❑ Urea/nitrogen stock samples; 75, 50, and 25 mg/ml
- ❑ Blood Urea Nitrogen (BUN) Color Reagent
- ❑ BUN Acid Reagent
- ❑ 96-well plates, flat bottom
- ❑ Adhesive plate sealer
- ❑ Oven, 60 °C (or water bath alternatively)
- ❑ Spectrophotometer

Protocol

(a) Remove supernatant from hepatocyte cultures as indicated by the particular experiment, and store at 4 °C. Calculate number of wells required for assay based on 24 wells per plate for concentration standards, and triplicates of experimental samples.

(b) Prepare urea standards by serially diluting stock samples in same medium as experimental samples (normally CHCM). The normal range used is the same as for the albumin ELISA above (100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0 μg/ml). Alternatively, if hepatocytes are suspected to be highly functional and/or at high density prepare the following range of standards: 200, 100, 50, 25, 12.5, 6.25, 3.125, 0 μg/ml.

(c) Transfer 10 μl standards and experimental supernatants to individual wells of 96-well plate.

(d) Mix BUN Color and Acid reagents at the following ratio: 1/3 BUN Color Reagent plus 2/3 BUN Acid Reagent. Transfer 150 μl of this mixture to each well containing either standards or experimental samples. Seal plate tightly with adhesive plate sealer and incubate at 60 °C for 90 min.

(e) After color has developed, put plates on ice for 5–15 min, but no longer than 20 min.

(f) Read absorbance at 540 nm with spectrophotometer and, utilizing standard curve, calculate concentration of urea within experimental samples.

4.3. Measurement of Cytochrome P-450 Enzyme Activity

Protocol 15.7 has been adapted in our laboratory from several previous publications [Behnia et al., 2000; Burke and Mayer, 1974; Burke et al., 1985; Kelly and Sussman, 2000]. The formation of resorufin from ethoxyresorufin (EROD) reflects the cytochrome P-450 (CYP) activity of rat hepatocytes, in particular,

predominantly CYP1A1 activity. The procedure for examining EROD conversion is included in this chapter as a model assay investigating hepatocyte CYP activity. Although not explicitly shown below, a similar procedure can be utilized to assess the activity of other P-450 enzymes in rat hepatocytes, for example, conversion of pentoxyresorufin (PROD) as a measure of predominantly CYP2B1 activity, benzyloxyresorufin (BROD) for CYP2B2, or methoxyresorufin (MROD) for CYP1A2.

Protocol 15.7. Assay of Cytochrome P-450 1A1 Activity in Cultured Hepatocytes

Reagents and Materials

Sterile

- ❑ Ethoxyresorufin, 1 mM stock in dimethyl sulfoxide (DMSO)
- ❑ Dicumarol, 2 mM stock in 75 mM NaOH
- ❑ 3-Methylcholanthrene (3MC), 10 mM stock in DMSO
- ❑ CHCM (See Section 2.6)
- ❑ CHCM (See Section 2.6), without phenol red

Nonsterile

- ❑ 96-Well plate
- ❑ 24-Well plate
- ❑ 0.1 M NaOH
- ❑ β-Glucuronidase, 1600 U/ml in 0.1 M sodium acetate buffer
- ❑ Resorufin, 1 mM stock in DMSO
- ❑ Spectrofluorometer

Protocol

(a) This protocol is based on hepatocyte cultures in 6-well plates. Volumes given are on a per well basis, and can be easily scaled for the use with cultures of various dimensions.

(b) At the time point indicated by the particular experiment, remove culture medium from hepatocyte cultures and add CHCM containing 2 μM 3MC. Incubate cultures in 3MC-containing medium for 48–72 h, adding fresh medium with 3MC every 24 h, to induce sufficiently the expression of CYP1A. Alternatively, a 5 μM concentration of β-napthoflavone can be used in place of 3MC to induce CYP1A expression by rat hepatocytes. For experiments requiring the induction of CYP2B expression, 1 mM phenobarbital can be used.

(c) Aspirate medium and carefully wash wells with CHCM without phenol red. Add 750 μl solution of 5 μM EROD and 10 μM dicumarol in CHCM without Phenol Red. Incubate for 20 min at 37 °C. Dicumarol is a competitive inhibitor of cytosolic oxidoreductases and prevents diaphorase-mediated metabolism of resorufin.

(d) Transfer 750 μl aliquot to 24-well plate. Add 150 μl 1600 U/ml β-glucuronidase solution and incubate at 37 °C for a minimum of 2 h (maximum overnight).

(e) Terminate β-glucuronidase reaction by adding 200 μl 0.1 M NaOH. Mix thoroughly, and transfer 200 μl from each well to the appropriate wells of a 96-well plate in triplicate.

(f) Prepare resorufin standards by diluting 1 mM resorufin stock to the following concentration range in CHCM without phenol red: 1000, 500, 250, 125, 62.5, 31.25, 15.625, and 0 nM. Add standards in triplicate to the appropriate wells of the 96-well plate containing experimental samples.

(g) Use spectrofluorometer to quantify fluorescence at 590 nm emission, with 530 nm excitation. Generate standard curve, and utilize curve to calculate resorufin concentration in the culture supernatants. The presence of resorufin in the hepatocyte culture medium, after incubation with EROD, is a reproducible indicator of CYP activity, predominantly CYP1A1, in rat hepatocytes.

5. MICROPATTERNED CELL CULTURES

Cell-cell and cell-extracellular matrix interactions are critical for proper cellular processes in numerous organ systems. Specifically, such interactions have been demonstrated to be key determinants of hepatocyte function. For example, it has been well documented that isolated hepatocytes cultured in monolayer culture display a rapid loss of phenotype [Bissell et al., 1973; Dunn et al., 1989; Leffert and Paul, 1972; Reid et al., 1980; Selden et al., 1999], preventing the effectiveness of these cells in long-term applications. Cell-cell interactions, both homotypic (hepatocyte-hepatocyte) and heterotypic (hepatocyte-nonparenchymal), have been demonstrated to exhibit positive effects on hepatocyte function. In particular, hepatocyte viability and a variety of liver functions have been shown to be stabilized for weeks in vitro on cocultivation with liver-derived cell types as well as some non-liver-derived populations [Bhatia et al., 1999]. However, despite the significant amount of data existing on potential mediators of cell communication in cocultures, the mechanisms by which coculture of hepatocytes with other cells induces and stabilizes liver-specific function and viability remain primarily undefined. Current experiments in our laboratory are examining these processes.

To study the role of cell-cell interactions in hepatocellular function, we previously developed and characterized a flexible method of micropatterning cocultures of hepatocytes and an embryonic fibroblast cell line (3T3-J2) on solid substrates (U.S. Patent No. 6,133,030) [Bhatia et al., 1997]. In this approach, photolithographic techniques were used to pattern collagen I on glass substrates, which served as an adhesive substrate for primary rat hepatocytes. After hepatocyte attachment and spreading, 3T3-J2 fibroblasts were plated, by serum-mediated adhesion, in the remaining intermixed regions. The versatility of this technique allowed the design of culture configurations that included variations in homotypic hepatocyte interactions, fibroblast cell number, as well as heterotypic interactions. Cocultures were conducted in which the heterotypic interface could be varied over three orders of magnitude, with the ratio of cell populations remaining constant (See Fig. 15.3) [Bhatia et al., 1999]. Total cell numbers were held constant by holding

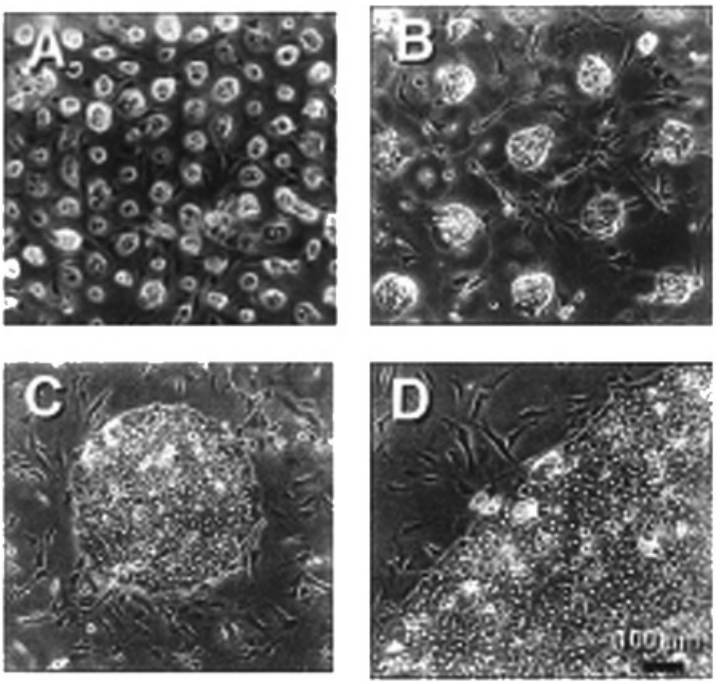

Figure 15.3. Micropatterned cocultures with constant ratio of cell populations. Phase-contrast micrographs of micropatterned cocultures indicate a broad range of heterotypic interface achieved despite similar cellular constituents. Four of five patterns used in study are shown. Diameters of hepatocyte islands were 36 μm (A), 100 μm (B), 490 μm (C), and 6800 μm (D). From Bhatia et al. [1999].

surface area of each domain (collagen I/bare glass) equal for each condition. In these experiments, liver functions, including albumin secretion and urea synthesis, were increased in cocultured configurations compared to hepatocytes alone (Fig. 15.4) (See Section 4 for the procedures for assaying albumin secretion and urea synthesis) [Bhatia et al., 1999]. The degree of upregulation varied with culture configuration, whereby cocultures with a larger initial heterotypic interface (i.e., single-cell islands) exhibited increased levels of liver-specific function.

In addition to bulk measures of hepatocyte function, in situ markers are equally important, particularly for the analysis of the role of the microenvironment on individual hepatocyte function. Immunohistochemical staining (See Protocol 15.5) indicated that hepatocytes near the heterotypic interface had a relative increase in liver-specific function (Fig. 15.5, See Color Plate 10B) [Bhatia et al., 1998b, 1999], which correlated with our data on bulk tissue function (increased heterotypic interactions equaled increased function). Notably, utilization of micropatterning techniques enabled a 12-fold reduction in fibroblast numbers with only a 50% reduction in albumin secretion, due to the ability to control the extent of heterotypic interactions [Bhatia et al., 1998a]. Reduction in the required numbers of nonparenchymal cells, which would occupy precious substrate surface area within a potential bioreactor, would essentially increase hepatocyte functionality per unit

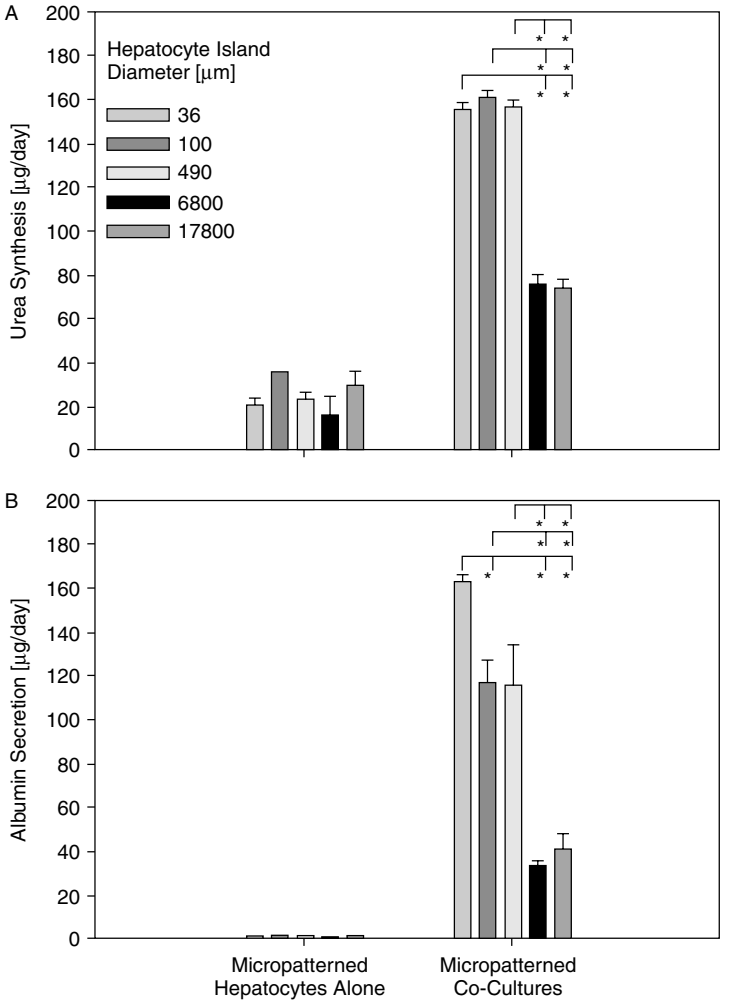

Figure 15.4. Liver-specific function of micropatterned cocultures with constant ratio of cell populations. Urea synthesis (A) and albumin secretion (B) on day 11 of culture were detected in micropatterned cocultures with varying heterotypic interactions despite similar cell numbers. Micropatterned hepatocyte-only cultures were utilized as a control. Statistical significance (*) was determined by one-way ANOVA with Tukey HSD post hoc analysis with $P < 0.05$. From Bhatia et al. [1999].

area. Thus micropatterning approaches would allow for significant improvements in the design of a projected coculture-based bioreactor.

The protocols outlined in this section describe this photopatterning procedure, which represents a multifaceted approach to investigation of complex mechanisms of cell-cell interaction, and specifically for our purposes, a means to identify the critical factors that mediate stabilization of the hepatocyte phenotype. Elucidation of these elements would have important implications in fundamental studies of cell communication as well as the development of highly functional tissue-engineered therapies for the liver.

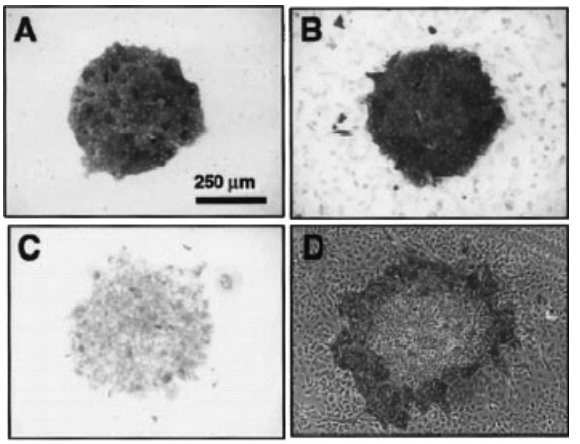

Figure 15.5. Intracellular albumin in micropatterned hepatocytes. Immunohistochemical staining of intracellular albumin in micropatterned hepatocytes in a representative (490 μm) pattern. Bright-field microscopy of hepatocytes (alone) on days 1 and 6 (A, C) and cocultures on days 1 and 6 (B, D). In coculture, albumin expression was highest near the heterotypic interface. From Bhatia et al. [1999]. (See Color Plate 10B.)

5.1. Microfabrication of Substrates

This section outlines a process for the localized modification of experimental substrates utilizing standard microfabrication techniques. In general, most integrated circuit manufacturing facilities can be used to perform these procedures.

Protocol 15.8. Photolithographic Patterning of Surface Modifications

Reagents and Materials

Nonsterile

- ❑ Circular borosilicate glass wafers, 5 cm (2 in.)
- ❑ Mask with desired pattern: Chrome masks are fabricated by a high-precision photolithographic process from Corel Draw. Alternatively, emulsion masks are commercially printed with a Linotronic-Hercules 3300 dpi high-resolution line printer.
- ❑ Piranha Cleaning Solution (See Section 2.9). Extreme caution must be observed during handling because of the corrosive nature of these chemicals. Pyrex containers must be utilized, and chemical removal should be performed via aspiration and dilution of acid mixture.
- ❑ Photoresist: Photoresist is a light-sensitive polymeric material. Any photoresist can be utilized that will adhere sufficiently to clean borosilicate, remain intact during subsequent processing, and yet be removed with relative ease after surface modification. We use one of the following positive photoresists: OCG 825–835 St, Shipley 1813, or Shipley 1818. UV exposure causes a positive photoresist to become more sensitive to developer, thereby resulting in a photoresist pattern after development that is identical to the mask pattern.

- Developer: Use developer appropriate for selected photoresist. We use OCG 934, MF-319, or Shipley 354.

Protocol

(a) Place borosilicate wafers in wafer carrier. Place carrier in Pyrex vat, pour Piranha Solution over wafers, and wait 10 min. Rinse wafers three times in a "dump-to-resistivity tank," a washing station that rinses wafers to an acceptable resistivity level ($>$10 MΩ-cm). Next, transfer wafers to a "spin-dryer." which uses air and spin cycles to dry the wafers. If a spin-dryer is not available, manual drying is done with a N_2 gas stream.

(b) Dehydrate wafers to promote adhesion of photoresist by baking for 60 min at 200 °C.

(c) Mount wafers on vacuum of a spin-coater chuck and coat with positive photoresist to a uniform layer of approximately 1 μm, by spinning at 5000 rpm for 30 s.

(d) Soft-bake for 30 min at 90 °C to drive out excess solvent and anneal any stress in the film.

(e) Expose coated substrates to 365-nm UV light in a Bottom Side Mask Aligner (Karl Suss) through patterned mask under vacuum-enhanced contact for 30–70 s at a dose of 10 mW/cm^2. Intensity and exposure time are dependent on type of photoresist and mask design.

(f) Immerse exposed photoresist in appropriate developer. Complete removal of photoresist in exposed areas is critical to subsequent surface modification. Presence of residual photoresist can be assessed by inspection under light interference or fluorescent microscopy. We develop by immersion and agitation in a bath of developer for 70 s. Surfaces should then be rinsed three times under running deionized water and cascade rinsed (if possible) for 2 min.

(g) Postbake patterned wafers for 30 min at 120 °C to drive off residual solvent and promote film adhesion.

(h) Wafers can be stored in closed containers (preferably within a nitrogen box) at room temperature for at least 1 month.

(i) Expose substrates 24 h to oxygen plasma before patterning of adhesive proteins and cell culture (See Protocol 15.8), to dry-etch (remove) a small layer of photoresist. This ensures complete removal of photoresist from exposed borosilicate; however, if substrates are well developed and pattern dimensions are larger than 10 μm, this step may be omitted. We use a parallel-plate Plasma Day Etcher at a base vacuum of 50 mtorr in an O_2 atmosphere and pressure of 100 mtorr at a power of 100 W for 2–4 min, which corresponds to an etch rate of approximately 0.1 μm/min.

The method described here was intended to be robust. In many instances, users may be able to eliminate certain elements of this process. Exposure to plasma oxygen may be unnecessary if patterns are well developed and not contaminated during

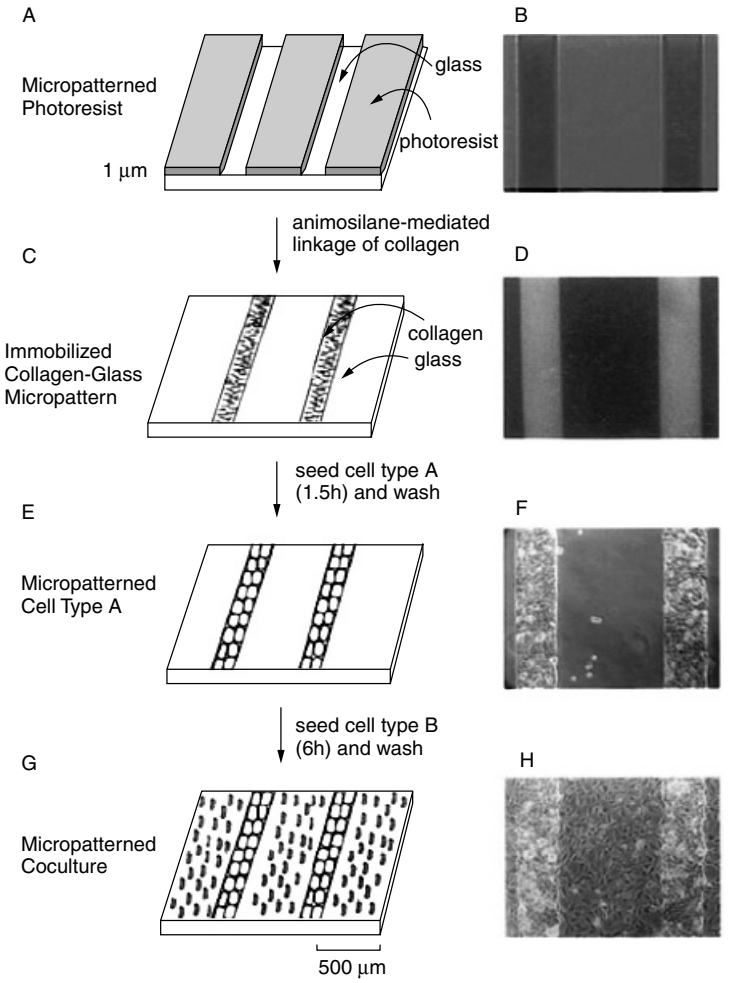

Figure 15.6. Schematic of method for generating micropatterned cocultures. Borosilicate substrates were coated with photoresist (a UV-sensitive polymer) and exposed to light through a mask, creating a photoresist pattern (A). Photoresist was visualized with epifluorescence microscopy (B) (excitation: 550 nm, emission: 575 nm). Collagen I was immobilized, followed by removal of photoresist, yielding a collagen-glass pattern (C). Indirect immunofluorescence allowed verification of collagen immobilization in appropriate locations (D). Patterned substrates were exposed to hepatocytes in serum-free media and rinsed, resulting in micropatterned hepatocytes (E). (F) Phase-contrast micrograph of 200 mm lanes of hepatocytes with 500 mm lane spacing. Addition of 3T3-J2 fibroblasts in medium supplemented with serum resulted in generation of micropatterned cocultures (G). Phase-contrast microscopy allowed morphological identification of 2 distinct cell types in "micropatterned coculture" (H). From Bhatia et al. [1999].

storage. Similarly, baking of wafers after AS modification may be unnecessary in some protocols.

Potential problems include peeling of photoresist during processing and difficulty with lift-off of photoresist. The integrity of the photoresist coating varies with solvents. In some instances, photoresist will peel away from the wafer surface prematurely. This indicates either insufficient adhesion of the photoresist to glass

(often because of an insufficient dehydration bake before photoresist coating) or insufficient baking after development to harden the photoresist. Difficulty with photoresist removal resulting in extended sonication in acetone often indicates exposure of photoresist to elevated temperatures.

5.2. Cellular Micropatterning on Modified Surfaces

The localized modifications of surface chemistry described above can be utilized to pattern cells by patterning adhesive molecules. There are a number of techniques for immobilizing adhesive proteins on solid substrates [Drumheller and Hubbell, 1995]. In Protocol 15.9, we describe techniques for covalent coupling of proteins to the surface by a modified technique of Lom et al. [1993], and Britland et al. [1992], as well as direct adsorption of proteins from solution. Subsequently, these modified substrates can be utilized for the micropatterning of primary hepatocytes and an embryonic fibroblast cell line. In this approach, hepatocytes attach specifically to the collagen I patterned regions, and fibroblasts undergo nonspecific, serum-mediated attachment to the remaining unmodified areas (Fig. 15.6).

Protocol 15.9. Micropatterning of Hepatocyte/Fibroblast Cocultures

Reagents and Materials

Sterile

- ❑ Freshly isolated primary rat hepatocytes (See Section 3)
- ❑ 3T3-J2 mouse fibroblast cell line
- ❑ CHCM (See Section 2.6)
- ❑ HCM/SF (See Section 2.10)
- ❑ FCM (See Section 2.11)
- ❑ Bovine serum albumin (BSA), 0.05% (w/w) in water. Sterilize by filtration through a 0.45-μm filter and store at 4 °C.
- ❑ Deionized water (DI). Autoclave to sterilize.
- ❑ Petri dishes, 6 cm
- ❑ Mitomycin C, 10 μg/ml in fibroblast medium
- ❑ Trypsin, 0.25%, EDTA, 5 mM (0.2g/L) in CMF-HBSS

Nonsterile

- ❑ Microfabricated glass wafers (See Protocol 15.8)
- ❑ 3-[(2-Aminoethyl)amino]propyltrimethoxysilane (AS), 2% in water
- ❑ Glutaraldehyde, 2.5% (v/v) in PBSA, pH 7.4
- ❑ PBSA
- ❑ Collagen type I (rat tail), final concentration approximately 500 μg/ml
- ❑ Acetone
- ❑ 70% Ethanol
- ❑ Glass Petri dish, 10 cm
- ❑ Wafer tweezers
- ❑ Petri dish, 6 cm

Protocol

(a) Rinse microfabricated wafers (See Protocol 15.8) by immersion in distilled DI in a glass 10-cm Petri dish. Repeat. Handle substrates with wafer tweezers, and pay special attention to the orientation of the patterned surface of the wafer-transparent substrates, as they can be easily inverted without any obvious differences in appearance.

(b) To pattern a protein such as collagen by adsorption, immerse sample in 4 ml collagen solution in a 6-cm Petri dish for 1 h at 37 °C and skip to step (h).

(c) To covalently link a protein to the patterned regions, first immobilize AS by immersion of samples in AS solution for 30 s at room temperature, followed by two rinses in DI water.

(d) Dry wafers with a stream of N_2 gas to avoid drying artifacts.

(e) Bake wafers in a closed container for 10 min at 120 °C. ***Note:*** Temperatures greater than 150 °C will cause hardening of many photoresists, and removal will be difficult.

(f) Soak disks in a covered container of 2.5% glutaraldehyde in PBSA for 1 h at 25 °C, followed by two rinses in fresh PBSA. Visually inspect wafers every 15 min to evaluate the integrity of the photoresist—if the glutaraldehyde solution causes peeling of the photoresist, the process will need to be abbreviated.

(g) To immobilize collagen, immerse wafers in 4 ml collagen solution in a 6-cm Petri dish for 30 min at 37 °C.

(h) To remove photoresist and expose underlying unmodified glass, float each wafer in acetone in a glass container and sonicate the container in a bath sonicator for 1–15 min. The duration of sonication is empirically determined by observation of the first wafer in each batch—examine wafers for complete removal of photoresist (previously "pink" wafers will appear clear). Treat all wafers in an experimental batch identically to ensure comparability of immobilized protein layers on all substrates. We use 10 ml acetone in a 10-cm glass Petri dish.

(i) Rinse wafers twice by immersion in DI. As previously shown, modified areas should display differential wetting on removal of substrate from water, thereby indicating successful patterned surface modification.

(j) Wafers can be stored dry in a covered container at 4 °C for at least 2 weeks. We store wafers on a piece of filter paper (to absorb residual water and prevent sticking) in 6-cm Petri dishes. Storage of wafers in solution (i.e., PBSA or ethanol) results in transfer of patterned protein to unmodified areas, presumably via desorption from modified areas and adsorption to unmodified areas; therefore, if the immobilized protein will tolerate dehydrated storage conditions, wafers should be stored dry. If immobilized protein requires hydration to maintain its bioactivity, storage time must be empirically determined—in our case, less than 48 h.

(k) Soak premodified wafer in 70% ethanol for sterilization. Use wafer tweezers to place wafer in 6-cm dish with 5 ml ethanol solution for at least 1 h, but not more than 24 h, at room temperature in a sterile laminar flow hood.

(l) Pour autoclaved water into sterilized beaker. Flame-sterilize wafer tweezers, allow to cool, and remove wafer from 70% ethanol under sterile conditions. Immerse wafer in water and agitate gently for approximately 10 s, being sure to preserve orientation of the wafer.

(m) If using wafers modified with adhesive species, coat wafers with bovine serum albumin (BSA) to deter nonspecific cell adhesion on unmodified regions. For rat hepatocytes, BSA coating reduces nonspecific cell attachment to glass from 30% to negligible levels. Place sterilized wafers in sterile 6-cm dishes, add 4 ml BSA solution (0.1%–1.0% wt/v, in PBSA) to each dish, and place in incubator for 45 min at 37 °C.

(n) To remove residual BSA solution, use sterile tweezers to remove wafers from dishes under sterile conditions, and immerse in autoclaved water in sterile beaker, gently agitating for 10 s.

(o) Dilute stock suspension of freshly isolated rat hepatocytes in HCM/SF to a final concentration of $1–2 \times 10^6$ hepatocytes/ml.

(p) Seed hepatocytes by placing 2 ml hepatocyte solution on each wafer. Agitate solution to disperse cell suspension and place in incubator for 1–1.5 h. Wafers should be periodically agitated (i.e., every 15 min) horizontally to promote maximal cell attachment.

(q) At this point, selective cell adhesion on collagen regions should render pattern features visible. Typically, to ensure 100% confluence on adhesive areas, hepatocyte seeding is repeated two to three times. Surfaces should be rinsed twice by pipetting and then aspirating 4 ml media, reseeded with hepatocytes for 1.5 h, and rinsed again. Repeat as necessary.

(r) After seeding, aspirate HCM/SF and replace with hepatocyte culture medium containing serum (10%). Hepatocytes are then allowed to spread over the remaining modified sites. Rat hepatocytes take more than 10 h to spread, so incubate patterned hepatocytes overnight. These micropatterned cell cultures can be used in experimental studies; otherwise proceed with addition of fibroblasts.

(s) To generate hepatocyte-fibroblast coculture, fibroblasts are trypsinized, resuspended in fibroblast media, and plated in 3 ml fibroblast media per micropatterned hepatocyte culture. Typically 750,000 fibroblasts per dish are sufficient; however, in some cases it is necessary to plate growth-arrested fibroblasts in greater numbers.

(t) Growth-arrested fibroblasts are generated by incubating each 150-cm^2 fibroblast flask with 15 ml mitomycin C solution for 2 h.

(u) Incubate cocultures in fibroblast media for 24 h.

(v) Change medium to Complete Hepatocyte Media (with serum) and continue culture in this medium for duration of the experimental investigation. Preservation of pattern integrity is dependent on both cell types (competence, migration rates, etc.) and pattern dimensions—for example; our patterns are stable for several weeks for collagen-modified areas larger than a few hundred microns.

The above process is adaptable for use with many different cell types. However, the procedure will vary with the characteristics of the desired protein. In our case, collagen type I retained its bioactivity for hepatocyte attachment and spreading despite treatment with acetone, ethanol, and dehydration. Other proteins may require modifications of this protocol to retain their bioactivity. Additionally, in our experience, covalent binding of collagen is not required for hepatocyte patterning and simple adsorption to the glass substrate is adequate. Yet it should be noted that reliance on adsorption alone is likely to result in different immobilized protein conformations than covalently bound proteins, and these potential differences should be kept in mind if modifying this procedure for other adhesive molecules.

Furthermore, selection of medium for two cell types must be considered. Medium must be selected that provides adequate nutrients and buffering for each population. Also, because of the rapid mitotic rate of fibroblasts, our medium contains hydrocortisone as a growth inhibitor. Alternatively, as suggested above, under some circumstances fibroblasts can be chemically growth arrested with mitomycin C.

Potential problems include minimal cell adhesion to the substrate and, conversely, uniform cell adhesion to the substrate. Lack of cell adhesion to the substrate can indicate many problems. The most common, however, is underdevelopment of the exposed photoresist (See Protocol 15.8, Step (e)), resulting in a lack of exposed borosilicate for protein immobilization. This can be alleviated by increasing development time, or increasing exposure to oxygen plasma before surface modification. This effect is often exacerbated with small (<5 μm) pattern dimensions. Alternatively, contaminants that coat the glass and prevent protein immobilization, or commonly utilized undercoatings for promoting photoresist adhesion, could produce this effect. On the other hand, a lack of discernible pattern because of exuberant cell adhesion is frequently caused by a defect in the original photoresist coating. Photoresist can degrade and crack over time, allowing exposure of all areas of the substrate to surface modification. Also, excessive exposure to oxygen plasma will strip the photoresist from the surface of the wafer completely, allowing for homogeneous protein adsorption.

In summary, the above protocols describe a method for micropatterning hepatocyte-fibroblast cocultures based on standard microfabrication techniques. Utilization of this system enables the controlled investigation of the complex cell-cell interactions important in the stabilization of the hepatocyte phenotype.

6. BIOREACTOR SYSTEM FOR THE REGULATION OF HEPATOCYTE ZONAL HETEROGENEITY

Numerous hepatocyte functions are known to vary along the length of the liver sinusoids from the portal triad (i.e., portal vein and hepatic artery) to the central vein, a feature termed "liver zonation" (Fig. 15.7) [Kietzmann and Jungermann, 1997]. For example, urea synthesis and gluconeogenesis are localized near the portal triad (periportal region), whereas conversely, the processes of cytochrome P450 activation and detoxification, as well as glycolysis, are enriched near the

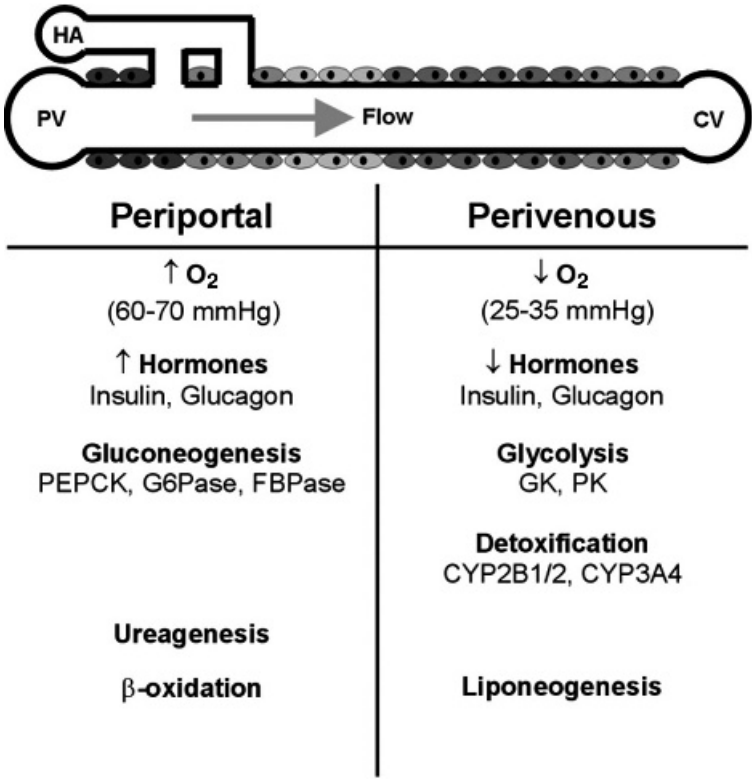

Figure 15.7. Zonated features along the sinusoid. The metabolic activity of hepatocytes along the liver sinusoid creates oxygen and hormone gradients from the periportal region to the perivenous. As a result, metabolic and detoxification functions are regionally dominant to one zone or the other, as indicated. Some specific zonated markers are listed under each process. Abbreviations: HA, hepatic artery; PV, portal vein; CV, central vein; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase; FBPase, fructose-1,6-bisphosphatase; GK, glucokinase; PK, pyruvate kinase; CYP, cytochrome P-450. From Allen and Bhatia [2003], adapted from Kietzmann and Jungermann [1997].

central vein (perivenous region). Notably, the zonation of enzymes involved in carbohydrate metabolism enables a mechanism for the liver to maintain blood glucose levels relatively constant during either fasting or feeding states [Jungermann, 1992]. In addition, certain agents, such as carbon tetrachloride and acetaminophen, exhibit zonal toxicity as a result of the localized P450 induction [Lindros, 1997]. In vivo, hepatocytes experience simultaneous gradients of O_2, hormones, and extracellular matrix, which are each thought to participate to some degree in the zonation process [Jungermann and Kietzmann, 2000; Kietzmann and Jungermann, 1997]. The development of in vitro models of zonation would enable the study of these important components, thereby providing a means to predict in vivo responses such as metabolic disturbances as well as zonal hepatotoxicity.

In particular, our laboratory and others have demonstrated the critical role of O_2 concentration in lipid metabolism, urea synthesis, gluconeogenesis, and xenobiotic metabolism of isolated hepatocytes [Bhatia et al., 1996; Holzer and Maier, 1987;

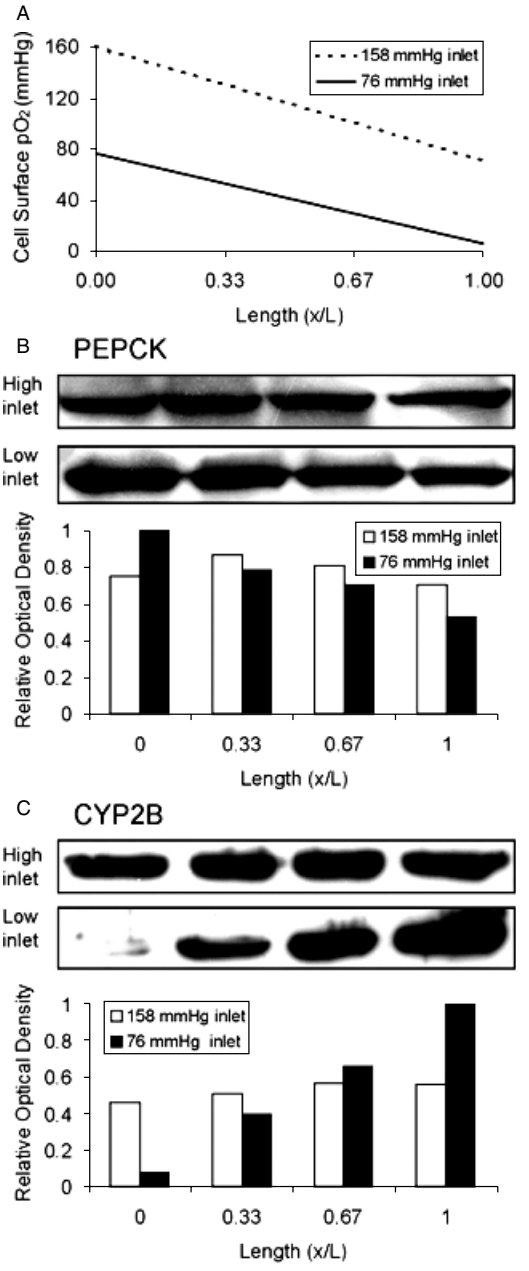

Figure 15.8. Heterogeneous induction of PEPCK and CYP2B by oxygen gradients. Bioreactors were operated with an inlet pO_2 of 76 and 158 mmHg and flow rate of 0.5 ml/min. The resulting cell surface oxygen gradients are shown schematically as calculated from the numerical model (A). Western blots of PEPCK (B) and CYP2B (C) protein levels from four regions along the chamber were analyzed to determine relative optical density. In both cases, when the bioreactor was operated with physiologic gradient (low inlet), a heterogeneous induction was observed, whereas imposing a supraphysiologic gradient (control, high inlet) resulted in a more uniform protein distribution. Blots were processed in separate experiments, enabling only qualitative comparison between conditions. Normalization of band densities to the maximal density from both experiments is meant to facilitate comparison. From Allen and Bhatia [2003].

Nauck et al., 1981; Suleiman and Stevens, 1987]. To examine the dynamic effects of O_2 gradients on hepatocyte function, we developed an in vitro perfusion system that allows exposure of cultured cells to a continuous range of O_2 tensions [Allen and Bhatia, 2003]. These controlled O_2 gradients contributed to the heterogeneous distribution of phosphoenolpyruvate carboxykinase (PEPCK), elevated upstream, and cytochrome P450 2B (CYP2B), elevated downstream (Fig. 15.8), which

correlates with the distribution of these enzymes in vivo. Thus the integration of specified O_2 gradients within this bioreactor system facilitated the establishment of hepatocyte zonal variations, thereby highlighting the importance of O_2 gradients in this process. Furthermore, the incorporation of O_2 gradients represents a means for enhancing the functionality of in vitro liver models as well as bioartificial liver devices. Although the oxygen-sensing mechanism is yet to be determined, several candidates are suggested by previous studies, including various transition metals and H_2O_2 in collaboration with the transcription factor HIF-1α [Huang et al., 1996; Jungermann and Kietzmann, 2000; Kietzmann et al., 1996; Maxwell and Ratcliffe, 2002]. Ongoing experiments in our laboratory are examining the molecular mechanisms responsible for the induction of zonated gene expression and, specifically, the role of HIF-1α in the regulation of gene expression in hepatocytes in response to hypoxic conditions.

6.1. Formation of Steady-State Oxygen Gradients

The perfusion bioreactor system described below exhibits parallel-plate geometry with a uniform flow field. A schematic of this system is displayed in Fig. 15.9. Consequently, the O_2 concentration profile can be modeled as a combination of

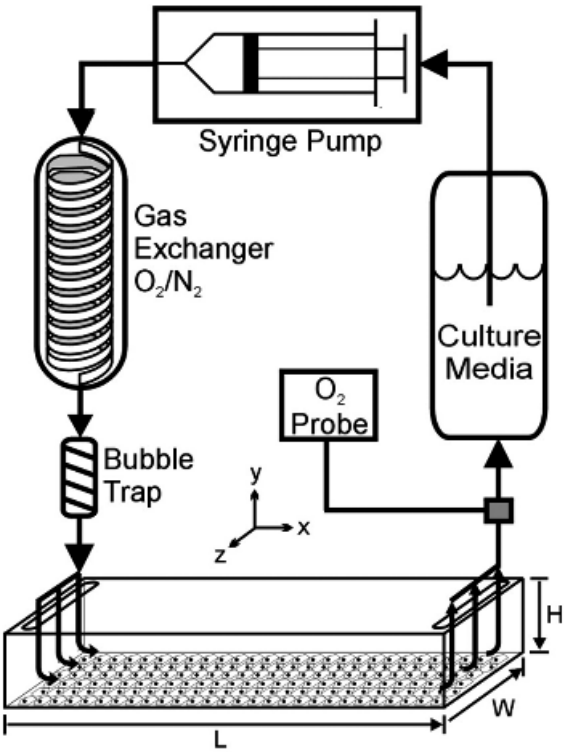

Figure 15.9. Schematic of bioreactor circuit. This parallel-plate bioreactor system containing cultured hepatocytes results in the formation of steady-state O_2 gradients, thereby generating zonated hepatocyte phenotypes.

O_2 diffusion to the cell, uptake by the cell, and convection along the length of the flow region. Both analytical and numerical models were shown to correlate closely with measured oxygen outputs. A detailed description of the mathematical modeling for this system has been reported previously [Allen and Bhatia, 2003]. Given an inlet O_2 concentration representative of physiologic periportal levels, 76 mmHg (10% O_2), a flow rate of 0.5 ml/min was determined to result in the exposure of cells in the final 50% of the chamber length to perivenous O_2 levels (<35 mmHg). Subsequently, this O_2 distribution resulted in zonated hepatocyte phenotypes within the bioreactor.

Protocol 15.10. Assembly of Bioreactor and Flow Circuit for Hepatocyte Micropatterning

Reagents and Materials

Sterile

- ❑ Freshly isolated primary rat hepatocytes (See Section 3)
- ❑ CHCM (See Section 2.6)
- ❑ HDHM (See Section 2.12)
- ❑ 70% Ethanol
- ❑ Collagen type I (rat tail), final concentration approximately 500 μg/ml

Nonsterile

- ❑ Perfusion circuit (Fig. 15.9) including: medium reservoir, gas exchanger with gas permeable Silastic tubing, O_2 probe (Clark-type electrode), syringe pump, bubble trap
- ❑ Flow deck: polycarbonate block containing inlet and outlet ports and 100-μm recess over which slide can be placed
- ❑ Stainless steel bracket, screws
- ❑ Inert silicone lubricant
- ❑ 38 mm × 75 mm glass microscope slides
- ❑ Incubator, PID controlled

Protocol

(a) Sterilize 38 mm × 75 mm microscope slide with 70% ethanol and treat with type I collagen as described in Protocol 15.9.

(b) Culture freshly isolated hepatocytes on slide to confluence with 2 seedings of 3×10^6 cells in Complete Hepatocyte Medium, followed by gentle horizontal shaking every 15 min for 1 h.

(c) After additional 2-h incubation, change medium to HDHM. Proceed with the following steps on day 1 or 2 after isolation. The hormonally defined preparation has been utilized in this procedure in order to help stabilize hepatocytes in monolayer culture, as previously described [Enat et al., 1984]. In addition, for the development of potentially clinical extracorporeal devices, it is important to optimize bioreactor parameters without the presence of nonhuman serum, which is incompatible with clinical approaches.

(d) Assemble flow chamber WITHOUT cells by adding blank microscope slide to polycarbonate block and closing with stainless steel bracket. Connect blank flow chamber to flow circuit and start flow (0.5 ml/min), in order to preequilibrate O_2 within lines and calibrate O_2 probe. Maintain all components of the flow circuit except the syringe pump within an incubator set at 37 °C. Allow an equilibration period of at least 15 min before continuing with the following steps.
(e) On a separate polycarbonate block, apply inert silicone lubricant to the outer edge of recessed section, outside the flow region.
(f) Invert slide with hepatocytes and lower onto this second flow deck. Overlay the stainless steel bracket and tighten with screws. The resultant flow field dimensions are 28 mm (width) $\times$ 55 mm (length) $\times$ 100 μm (height).
(g) Stop flow and remove flow chamber without cells. Quickly insert chamber with cultured hepatocytes into flow circuit as illustrated in Fig. 15.9, and restart flow (0.5 ml/min).

This bioreactor system represents a well-characterized platform in which O_2 gradients can be regulated by altering inlet concentration and flow rate. As expected at higher flow rates ($\geq$1.0 ml/min), given an inlet concentration of 76 mmHg, O_2 tension does not fall to perivenous levels in the length of the flow chamber. Increasing inlet O_2 can also prevent the decrease to the perivenous range. Notably, previous strategies for bioartificial liver devices have utilized preoxygenation of inlet streams to supraphysiologic levels in order to prevent inadequate O_2 delivery in parts of the bioreactor [Custer and Mullon, 1998]. However, as we have illustrated, O_2 can modulate hepatocyte function and therefore constitutes an important design criterion for an effective bioreactor system.

Another important design issue for bioreactors is the shear stress experienced by the cultured cells [Ledezma et al., 1999]. In the system described above, a flow rate of 0.5 ml/min corresponds to 1.25 dyn/cm^2, which is well below the shear stress of 5 dyn/cm^2 that was previously demonstrated to decrease rat hepatocyte function [Tilles et al., 2001]. Consequently, in our system, a flow rate of 0.5 ml/min and inlet of 76 mmHg represent the ideal parameters for preventing cellular damage due to shear stress or hypoxia, and concurrently generating adequate O_2 concentration gradients for the establishment of hepatocyte zonation.

6.2. Assessment of Zonated Features

Protocol 15.11. Examination of Zonated CYP2B Expression Resulting from Bioreactor Culture

Reagents and Materials

Sterile

- ❑ HDHM (See Section 2.12)
- ❑ Epidermal growth factor (EGF), 16 nM

- ❑ Phenobarbital (PB), 75 mM
- ❑ HDHM supplemented with 0.75 mM PB and 0.16 nM EGF [Kietzmann et al., 1999], made up just before use

Nonsterile

- ❑ Functioning perfusion bioreactor system (described above, Protocol 15.10)
- ❑ SDS lysis buffer (See Section 2.13)
- ❑ Protease inhibitor cocktail
- ❑ Microcentrifuge tubes (2 ml)
- ❑ Pestle

Protocol

(a) As described above, assemble bioreactor and flow circuit, and allow to reach steady state (approximately 15 min).
(b) Switch perfusion medium to supplemented HDHM and perfuse for 36 h.
(c) Stop flow, and remove flow chamber from flow circuit.
(d) Carefully disassemble flow chamber, and remove slide with hepatocytes.
(e) Scrape and lyse distinct sections of the chamber slide in SDS lysis buffer.
(f) Add samples to microcentrifuge tubes with protease inhibitor cocktail, homogenize with pestle, and centrifuge at 16,200 *g* for 5 min.
(g) Examine CYP2B protein expression with standard gel electrophoresis and Western blotting techniques.

Experiments examining CYP2B expression in four distinct regions of the described bioreactor have been performed (See Fig. 15.8) [Allen and Bhatia, 2003]. A similar procedure can be utilized to examine the zonated expression of other factors. For example, in place of infusion of PB and EGF, infusion of 10 nM glucagon for 8 h allows for the sufficient induction of PEPCK [Hellkamp et al., 1991], with an elevated level in the upstream section of this bioreactor system (See Fig. 15.8) [Allen and Bhatia, 2003].

Although this bioreactor system provides numerous advantages, the system is limited to short-term experimentation (3–4 days) because of the loss of differentiated functions by primary hepatocytes in monolayer culture even in the presence of the HDHM. Recent work in our laboratory has adapted hepatocyte and fibroblast coculture (described in Section 5.2), to this bioreactor system, to generate long-term stable hepatocyte cultures with defined O_2 gradients [Allen et al., 2005]. For longer-term studies, more rigorous sterilization procedures must be utilized, including the use of a polysulfone, which can be autoclaved, instead of polycarbonate for the flow deck.

In summary, the above protocols describe the assembly and utility of a perfusion bioreactor that exhibits steady-state O_2 gradients in an in vitro hepatocyte culture model. This system facilitates the investigation of the processes of liver zonation and provides a foundation for the development of bioartificial liver devices

exhibiting these zonated features. In addition, future studies utilizing microscale fabrication techniques are aimed at miniaturizing this system toward the development of a parallel array of bioreactors for the high-throughput examination of hepatotoxicity.

7. PHOTOPATTERNED THREE-DIMENSIONAL HYDROGELS CONTAINING LIVING CELLS

To develop implantable tissue constructs that can be successfully incorporated into host tissue and recapitulate the desired functions, it is important to simulate sufficiently normal tissue structure, including 3-D organization. Specifically, for the design of implantable liver schemes, it would be particularly advantageous to disseminate cells three-dimensionally within a thick construct, without requiring subsequent population or recruitment of cells. Hydrogels, which are cross-linked polymer networks based on chemistry originally derived by Hubbell and colleagues [Pathak et al., 1992], can be polymerized in the presence of cells, thereby generating gels with a homogeneous cellular distribution. As a result, hydrogels represent an ideal platform for the development of these types of implantable systems. In addition, hydrogels are biocompatible and exhibit high water content, which provides them with mechanical properties similar to tissues. Furthermore, several reports indicate that cellular phenotypes are different in 2-D versus 3-D cell culture [Katz et al., 2000; Poznansky et al., 2000; Wang et al., 1998], suggesting that the development of in vitro. 3-D culture models will be required to mimic more closely in vivo environments, an application for which hydrogels are also ideally suited.

Poly(ethylene glycol) (PEG)-based hydrogels are widely used in tissue engineering applications because of their biocompatibility and hydrophilicity resulting in resistance to nonspecific protein adsorption and the ability to alter the properties of the hydrogel by modifying the PEG chain length [Peppas et al., 2000]. Specifically, encapsulation within PEG-based hydrogels has been utilized for numerous cell types, including vascular smooth muscle cells [Mann et al. 2001a,b], chondrocytes [Bryant and Anseth, 2002; Elisseeff et al., 2000], fibroblasts [Gobin and West, 2002; Hern and Hubbell, 1998], and mesenchymal stem cells [Nuttelman et al., 2004] (See also Chapter 9). In addition to cellular encapsulation, the development of tissue-engineered constructs for spatially complex organs, such as the liver, will also require the ability to form specified 3-D features and control cellular orientations.

We have recently developed a photopatterning technique to generate hydrogels containing living cells with a defined architecture [Liu and Bhatia, 2002]. Explicitly, the combined methods of hydrogel polymerization and photolithography, in which UV cross-linking is performed through an emulsion mask, enabled the localized photopolymerization of hydrogel structures containing cells. Utilizing this method, we formed single-layer hydrogels of specified dimensions containing living cells as well as composite single-layer structures containing distinct cellular domains (Fig. 15.10, See Color Plate 10C) [Liu and Bhatia, 2002]. Furthermore, we demonstrated the formation of multiple-layer hydrogel structures

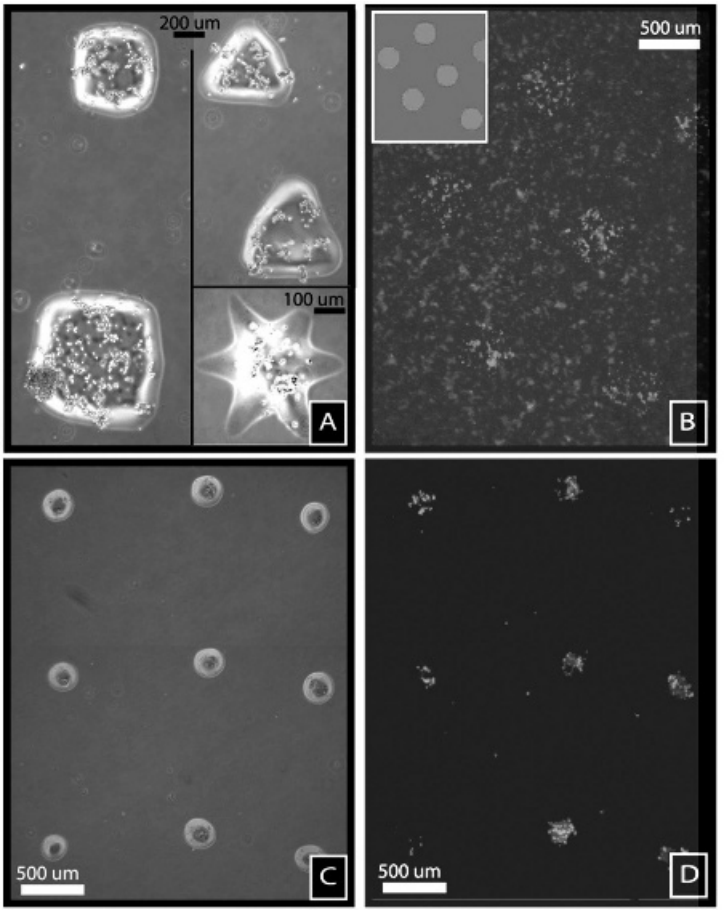

Figure 15.10. Hydrogel microstructures containing living cells. A) Cells entrapped in PEGDA hydrogels patterned in various shapes. B) Red and green labeled cells patterned within distinct domains of a single hydrogel layer. C) Phase microscopy of cellular array covalently linked to glass substrate. D) Fluorescent image of the cellular array, showing two different cell types (green, red). From Liu and Bhatia [2002]. (See Color Plate 10C.)

containing patterned cellular regions (Fig. 15.11) [Liu and Bhatia, 2002]. Protocol 15.12 describes this photopatterning procedure with HepG2 cells, a human hepatoma cell line, although this method is amenable to various cells types. Optimization parameters critical for adapting this system to other cell types are highlighted in this section.

Protocol 15.12. Photopatterning of Hydrogel with Living Cells

Reagents and Materials

Sterile

- ❑ HepG2 cells, human hepatoma cell line
- ❑ HepG2 Culture Medium (See Section 2.14)

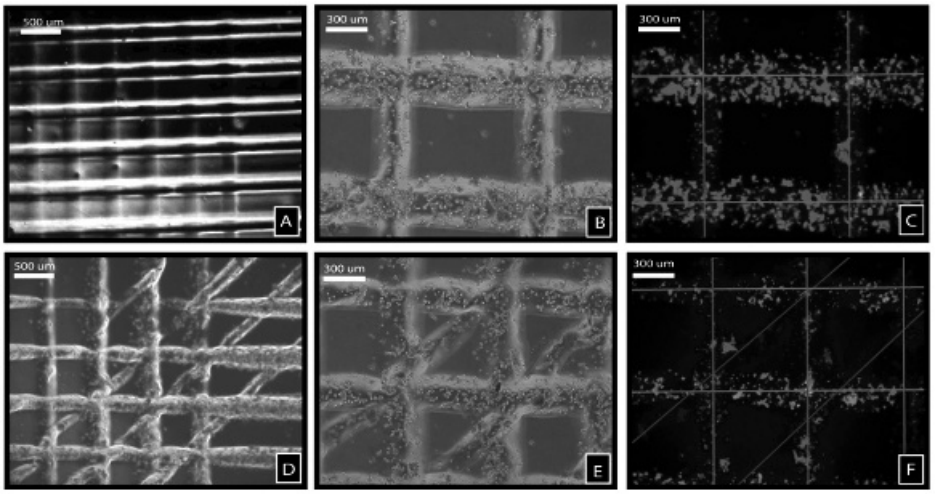

Figure 15.11. Multilayer hydrogel microstructures containing living cells. A) Two layers of patterned PEGDA lines. B) Two layers of patterned PEGDA lines containing cells. C) Fluorescent image of patterned hydrogel lines containing red and green tracked cells, demonstrating different cells in each layer. D) Three layers of patterned PEGDA lines containing cells at low magnification. E) Three layers of patterned PEGDA lines containing cells at higher magnification. F) Fluorescent image of three layers, with one layer containing red labeled cells, one layer containing green labeled cells, and all layers counterstained blue. From Liu and Bhatia [2002]. (See Color Plate 10D.)

- ❑ Microcentrifuge tubes (1.5 ml)
- ❑ Syringe filters (0.45 μm, 0.2 μm)
- ❑ Syringes (1 ml)
- ❑ Phosphate buffered saline (PBSA)

Nonsterile

- ❑ Poly(ethylene glycol) diacrylate (PEGDA), 3.4 kDa
- ❑ Photoinitiator: Irgacure 2959; 4-(2-hydroxyethoxy)phenyl-(2-hydroxy-2-propyl) ketone. Dissolved in 1-vinyl-2-pyrrolidinone to form 10% (w/v) stock solution.
- ❑ Circular borosilicate glass wafer, 2 in.
- ❑ 95% Ethanol (pH 5 with acetic acid)
- ❑ 100% Ethanol
- ❑ 70% Ethanol
- ❑ 2% (v/v) solution of 3-(trimethoxysilyl)propylmethacrylate in 95% ethanol (pH 5 with acetic acid)
- ❑ UV light source: Exfo Lite UV spot cure system with 5-mm LG Adapter, collimating single lens, and 365-nm filter
- ❑ Emulsion mask with desired pattern
- ❑ Polymerization apparatus (Fig. 15.12) including: foundation with stainless steel bracket and caliper screws, Teflon base with inlet and outlet channels, glass slide, and silicone spacers (100- to 500-μm thickness)

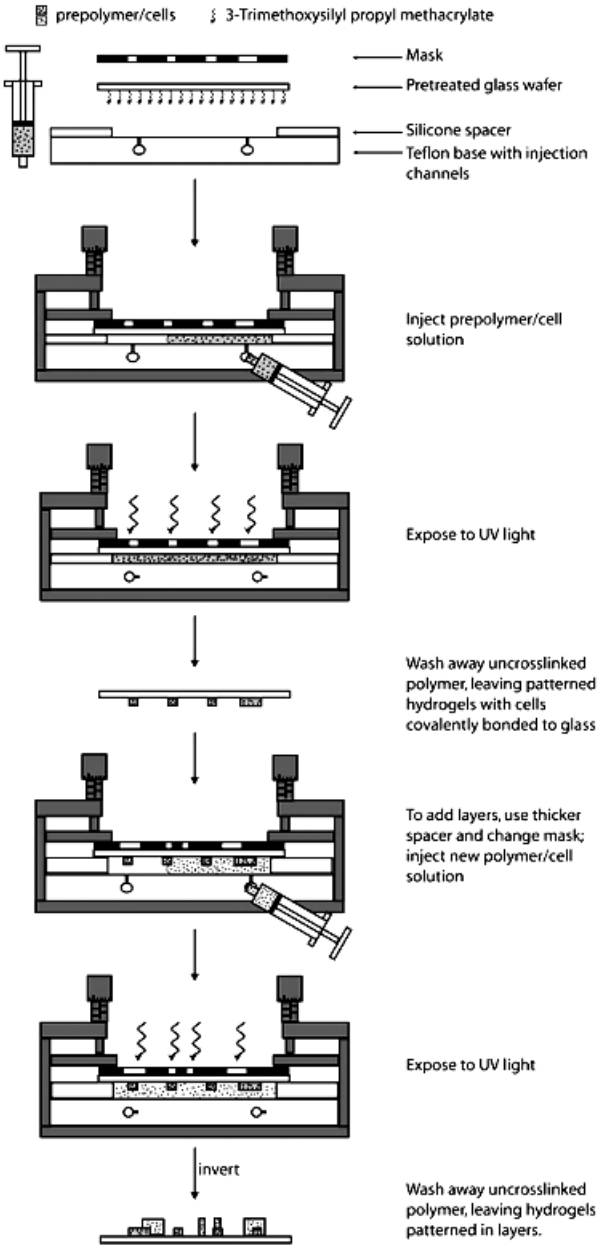

Figure 15.12. Process for formation of hydrogel microstructures containing living cells. The apparatus is assembled, including a pretreated glass wafer with reactive methacrylate groups on its surface, and a Teflon base with an inlet and outlet. Once the cells and prepolymer solution are injected, the inlet and outlet are closed, and the unit is exposed to UV light. The resulting patterned hydrogels containing cells are covalently bound to the glass wafer. At this time, a thicker spacer can be used in conjunction with a new mask to add another layer of cells. This process can be repeated several times. From Liu and Bhatia [2002].

Protocol

(a) Design emulsion mask(s) with drawing software, such as Corel Draw 11.0, and print with commercial Linotronic-Hercules 3300 dpi high-resolution line printer. For the formation of multiple layers, masks should contain reference points, such as cross hairs, for alignment purposes.

(b) Treat clean 2-in. borosilicate glass wafer with 2% 3-(trimethoxysilyl)propyl-methacrylate for 2 min. Rinse with 100% ethanol, and bake at 100 °C for 5–10 min. This process generates free methacrylate groups on the glass, which will react with the PEGDA during UV exposure and will prevent detachment of the hydrogel structures from the glass support. Alternatively, we have utilized 34-mm circular coverglasses, as opposed to 2-in. wafers, in some experiments because of their decreased thickness, for example, for situations in which confocal microscopy is required of the resultant structures.

(c) Etch marking on glass wafer outside the intended polymerization area, for alignment with cross hairs on masks.

(d) Sterilize masks, Teflon base, and silicone spacers by ultrasonic agitation (Sonicator) in 70% ethanol for 10–15 min. Let components dry in laminar flow hood.

(e) Resuspend HepG2 cells in HepG2 culture medium to 2× desired final concentration within the hydrogel. The final concentration is determined based on the particular experiment of interest.

(f) Dissolve PEGDA in PBSA to form 40% (w/v) solution.

(g) Add to the PEGDA solution the appropriate amount of photoinitiator stock (10% w/v) to achieve a photoinitiator concentration of 0.2% (w/v).

(h) Sterile filter PEGDA-photoinitiator solution, using sequentially a 0.45-μm, then a 0.2-μm syringe filter, and protect from light exposure.

(i) Assemble in laminar flow hood the polymerization apparatus illustrated in Fig. 15.12. Add sequentially the Teflon base, silicone spacer, methacrylate-treated glass wafer, mask, glass slide, and stainless steel bracket.

(j) Hold components in place by carefully tightening caliper screws on the bracket.

(k) Add cell suspension to PEGDA-photoinitiator prepolymer solution in 1:1 ratio, and mix gently.

(l) Use syringe to inject prepolymer solution with cells through the inlet port into the chamber formed by the glass wafer and silicone spacer. Alternatively, if the volume of the prepolymer solution with cells is limited, this solution can be added directly on to the Teflon base (without using inlet port), before adding the glass wafer, mask, slide, and bracket.

(m) Expose to UV light at 100 mW/cm^2 for 1.5 min. The exposed regions defined by the mask will be cross-linked to form the hydrogel. The exposure time and intensity may vary depending on the mask features.

(n) Wash away non-cross-linked polymer with PBSA, resulting in a patterned hydrogel containing cells.

To form a composite single-layer structure or cellular domains in an array format, after rinsing away the non-cross-linked polymer, a second prepolymer solution (with or without cells) can be added in the same manner and cross-linked by uniform exposure or exposure through a second mask. To form multilayer hydrogel structures, the thickness of the spacer is increased for the ensuing cross-linking steps. Alignment of the subsequent masks is achieved using the cross hairs, and the resultant structure is a complex 3-D construct containing living cells.

The above polymerization conditions have been optimized for experiments in our laboratory and can serve as a guideline, although the optimal conditions may vary depending on the cell type and desired hydrogel pattern. There are several design criteria to consider in adapting this procedure to a specific application. First, the cross-linking reaction proceeds because of the formation of free radicals from the photoinitiator, and these radicals can induce cell death. Consequently, photoinitiator concentration, as well as the intensity and duration of UV exposure, must be optimized for the particular cell type of interest. For multiple-layer structures, if possible, masks should be designed to prevent repeated UV exposure to the bottom layer. In addition, UV light is absorbed in each layer, including the glass slide on top of the apparatus and the mask. As a result, this can decrease the actual UV intensity reaching the hydrogel solutions, which is an important aspect to keep in mind when optimizing the intensity.

To increase pattern resolution, we have previously illustrated that UV exposure should be limited [Liu and Bhatia, 2002]. In addition, utilizing a collimated light source can improve pattern fidelity. Interestingly, we have found that photoinitiator concentration does not affect pattern resolution, so the lowest possible photoinitiator concentration can be used to minimize potential toxicity. Utilizing the parameters in the protocol described above, we have achieved 100-μm features with an accuracy of ± 5 μm. In general, increasing exposure intensity and decreasing the PEG chain length may enhance resolution. However, these alterations, if too extreme, can potentially decrease cell viability depending on the cell type utilized.

In summary, the protocol outlined in this section describes a method for constructing patterned 3-D hydrogel structures containing living mammalian cells. This technique can be broadly utilized for the design of implantable tissue constructs, development of defined 3-D in vitro culture models, and formation of immobilized cellular arrays as cell-based assays for pharmaceutical drug development. In particular, the formation of specified 3-D cellular structures represents an important step toward the development of tissue engineering therapies for spatially complex organs, such as the liver. Furthermore, several laboratories have demonstrated the incorporation of adhesive ligands [Hern and Hubbell, 1998; Hersel et al., 2003; Shin et al., 2003], degradable motifs [Bryant and Anseth, 2003; Lutolf et al., 2003; Metters et al., 2000; Sawhney et al., 1993; Seliktar et al., 2004], or growth factors [Gobin and West, 2003; Mann et al., 2001b; Seliktar et al., 2004; Zisch et al., 2003] into PEG-based hydrogels, providing a means to regulate cell attachment and spreading, growth, and other biological functions such as matrix deposition.

The combination of these techniques with the patterning method described here will provide a means for recapitulating within a tissue-engineered liver construct the intricate cell-cell and cell-matrix interactions critical for liver function.

8. DISCUSSION

This chapter has described several methods important for the fundamental investigation of hepatocyte biology and the development of engineered liver tissue. These included the isolation procedure for primary rat hepatocytes and numerous assays of rat hepatocyte function. Also included were protocols describing the micropatterning of hepatocyte-fibroblast cocultures, a bioreactor system for the generation of steady-state oxygen gradients and establishment of zonated features, as well as the three-dimensional fabrication of cellular constructs through photopatterning of hydrogels. Together, these procedures, and, similarly, alternative approaches developed in other laboratories, demonstrate numerous possibilities for both in vitro and clinical applications. Specifically, our laboratory's overall goals and approach for the development of engineered liver tissue are summarized in Fig. 15.13.

8.1. In Vitro Applications

The closely interconnected structure-function relationship in the liver dictates that tissue-engineered liver constructs must recapitulate, at least in part, the normal liver environment. Accordingly, much research in the field of liver tissue

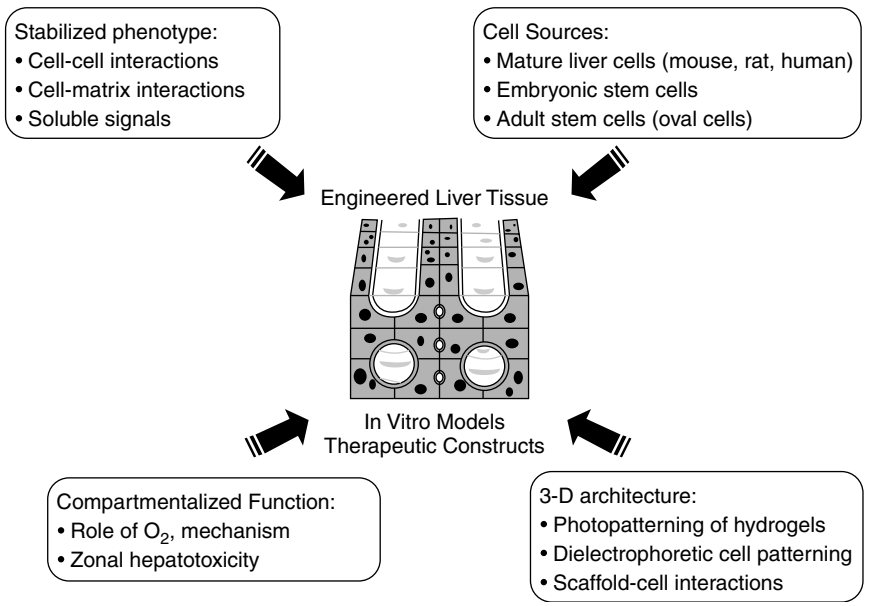

Figure 15.13. Overall approach for the design of engineered liver tissue. Each of these aspects will provide fundamental insight into liver biology and constitute important components contributing to the future development of highly functional tissue-engineered liver constructs.

engineering has initially focused on the development of in vitro culture models for liver cells, in order to characterize cellular responses in distinct environments and to systematically delineate the required extrinsic factors. For example, studies have assessed the in vitro morphogenesis of hepatocytes in pure cultures and cocultures [Berthiaume et al., 1996; Bhatia, 1997; Elcin et al., 1998; Haruyama et al., 2000; Powers and Griffith, 1998; Ranucci et al., 2000], as well as hepatocyte metabolic requirements [Balis et al., 1999; Kim et al., 2000; McClelland et al., 2003; Patzer, 2004; Rotem et al., 1994]. In addition, in our laboratory we are currently utilizing microfabrication approaches, such as the patterning procedure (See Section 5), to delineate the molecular mechanisms by which fibroblasts stabilize hepatocyte function, and the dynamics of this process. Identification of the important components involved, including soluble factors and key cell-cell and cell-matrix interactions, would enable the specified incorporation of these factors into a hepatocyte-only system, which would be more suitable for potential clinical applications.

Another important application for functional in vitro liver culture models is as a method for pharmaceutical drug development and the assessment of the risk of hepatotoxicity due to exposure to environmental toxicants. Liver toxicity is the major factor contributing to drug failures in clinical trials, and, furthermore, it has been reported that the most common cause of acute liver failure in the United States, accounting for up to one-half of cases, is drug-induced liver disease [Shakil et al., 2000; Kaplowitz and DeLeve, 2003]. Stabilized hepatocyte culture systems would represent a platform to thoroughly characterize drug metabolism, examine toxicity, and investigate drug-drug interactions. Notably, compartmentalized liver features such as zonation can regulate the degree of toxicity in response to various drugs [Kera et al., 1987; Lindros, 1997; Oinonen and Lindros, 1998; Pronko et al., 2002]. As a result, culture systems (such as the bioreactor platform described in this chapter) that not only stabilize hepatocyte function, but also reestablish certain heterogeneous elements observed in vivo, would be extremely useful in drug toxicity studies. For example, we have recently identified a fourfold decrease in the acetaminophen TD_{50} (toxic dose for 50% of cells) in the outlet region of this bioreactor [Allen et al., 2005]. Furthermore, microfabrication of functional hepatic elements would enable high-throughput analysis, in which multiple drug-drug combinations at varying doses could be analyzed in parallel.

8.2. Clinical Applications

The development of cell-based therapies for liver treatment aimed at the eventual replacement of damaged or diseased liver tissue represents a potential alternative to organ transplantation. One prospective cell-based approach is the transplantation of isolated mature hepatocytes. In experiments utilizing rodent models, transplanted hepatocytes were demonstrated to exhibit substantial proliferative capacity and the ability to replace diseased tissue, under some limited conditions [Overturf et al., 1997; Rhim et al., 1994; Sokhi et al., 2000]. However, the in vivo proliferation of transplanted hepatocytes is highly dependent on the presence of an adequate

"regenerative" environment, which would be difficult to control in a clinical setting. Furthermore, survival and engraftment of transplanted hepatocytes has been reported to be quite low, only 20–30% [Gupta et al., 1999b]. Several studies have demonstrated methods to improve engraftment and enhance the selective proliferation of transplanted hepatocytes [Guha et al., 1999; Joseph et al., 2002; Laconi et al., 1998; Malhi et al., 2002; Mignon et al., 1998; Slehria et al., 2002], although the clinical utility of these approaches remains to be determined.

As an alternative to mature hepatocytes, numerous stem cell populations are currently being investigated as potential cell sources, including pluripotent embryonic stem cells and various adult stem cell types. One adult stem cell of interest is the hepatic oval cell, which mediates liver regeneration in certain cases of severe and chronic liver injury [Oh et al., 2002; Sell, 2001]. Oval cells exhibit bipotential differentiation, defined by the ability to differentiate into both hepatocytes and bile duct epithelial cells. Oval cells can be isolated and significantly expanded in culture [Petersen et al., 1998; Yang et al., 2002], and, furthermore, recent experiments in mice illustrate the oval cell-mediated therapeutic repopulation of the liver [Wang et al., 2003]. Elucidation of the mechanisms regulating the proliferation and hepatic differentiation of oval cells, as well as other stem cell populations, could lay the groundwork for the development of robust cell-based liver transplantation therapies.

Another cell-based strategy for liver treatment is the implantation of hepatocellular constructs. Similar to various tissue engineering schemes in other organ systems, for this approach in the liver, hepatocytes are cultured within or immobilized on synthetic or biological scaffolds and surgically implanted [Allen and Bhatia, 2002]. Substantial current research is focused on the development of scaffolds that promote proper hepatic function. It is clear that several hepatocyte processes, such as survival and morphogenesis, can be influenced by the scaffold characteristics [Demetriou et al., 1986a; Dixit et al., 1992; Dvir-Ginzberg et al., 2003; Elcin et al., 1998; Glicklis et al., 2000; Hasirci et al., 2001; Kim et al., 1998; Li et al., 2003; Pollok et al., 1998; Powers and Griffith, 1998; Ranucci et al., 2000; Risbud et al., 2003]. Consequently, one significant challenge for the future is the design of platforms that enable the highly specified regulation of cellular orientations in three dimensions, as well as the controlled incorporation into the host environment. A recent study utilizing a focused laser to activate locally a photolabile hydrogel structure demonstrated the patterned immobilization of an adhesive peptide, GRGDS, which subsequently exhibited a guidance effect on neurite growth [Luo and Shoichet, 2004]. Several other techniques have also been used to pattern hydrogel networks, including microfluidics [Koh et al., 2003; Tan and Desai, 2003] and photolithography [Beebe et al., 2000; Koh et al., 2002; Ward et al., 2001; Yu and Ober, 2003]. Our laboratory has developed an adaptation of standard photolithography techniques resulting in the photopatterning of hydrogel structures containing living cells [Liu and Bhatia, 2002] (See Section 7). In addition, our laboratory has recently demonstrated the dielectrophoretic positioning of cells within a hydrogel in the 10-μm range [Albrecht et al., 2002] and the combination of this technique with the photopatterning procedure described here [Albrecht et al., 2005]. Notably,

scale-up of 3-D patterning of cellular constructs will likely occur via computer-aided design (CAD)-based rapid prototyping tools such as stereolithography, as part of the nascent field of "organ printing" [Jakab et al., 2004]. Taken together, the combination of these approaches allowing the spatial control of cells and bioactive factors represents an important step toward the formation of engineered liver systems more closely recapitulating normal liver structure and function.

An additional major challenge for the design of implantable liver systems is the need to overcome transport limitations within the grafted construct due to the lack of functional vasculature. Normally in vivo, hepatocytes are supplied by an extensive vasculature consisting of sinusoids with minimal extracellular matrix and a lining of fenestrated (sinusoidal) endothelial cells [Enzan et al., 1997]. Together these sinusoidal features allow for the efficient transport of nutrients to the hepatocytes, which are highly metabolic. Strategies to incorporate vasculature into engineered systems include the in vitro microfabrication of vascular units with accompanying surgical anastomosis during implantation [Griffith et al., 1997; Kaihara et al., 2000]. For example, polymer molding using microetched silicon has been shown to generate extensive channel networks with four dimensions in the order of capillaries [Borenstein et al., 2002]. An alternative approach is the incorporation of angiogenic factors within the implanted scaffolds. In particular, integration of cytokines important in angiogenesis, such as VEGF [Smith et al., 2004]. bFGF [Lee et al., 2002], and VEGF in combination with PDGF [Richardson et al., 2001], has been shown to promote the recruitment of host vasculature to implanted constructs.

An integral aspect in the development of clinically relevant cell-based liver therapies of all types is the utilization of adequate animal models in order to examine therapeutic effects as well as safety considerations. Numerous distinct large-animal models have been used to examine the efficacy of extracorporeal support systems in situations of liver failure [van de Kerkhove et al., 2004]. These systems fall into four major categories: partial hepatectomy, total hepatectomy, toxic, and ischemic models. Several extensive reviews have addressed the important criteria for developing animal models of fulminant hepatic failure [Newsome et al., 2000; Terblanche and Hickman, 1991; van de Kerkhove et al., 2004].

In addition to liver failure models, animal models have been used extensively to investigate liver regeneration. These can be broadly classified into two categories: surgically induced and chemically induced [Palmes and Spiegel, 2004]. Well-characterized models in the chemical category include exposure to toxic doses of carbon tetrachloride or acetaminophen, both situations that result in localized centrilobular necrosis [Anundi et al., 1993; Lindros et al., 1990]. Chemical-induced injury models are of particular interest for testing the utility of cell-based liver therapies, as these systems more closely mimic liver injuries commonly occurring in humans (i.e., drug toxicity). In the surgical category, the 2/3 partial hepatectomy model in rat has been widely utilized as a condition in which the injury stimulus is well-defined [Higgins and Anderson, 1931]. Interestingly, parabiotic systems have demonstrated that after partial hepatectomy, factors regulating liver

cell proliferation are present in the circulation [Fisher et al., 1971; Moolten and Bucher, 1967; Roesel et al., 1989]. Consequently, although the hepatectomy model is less relevant clinically, it may serve as a well-controlled system to investigate the role of regenerative cues in the engraftment and proliferation of hepatic constructs implanted in extrahepatic sites [Jirtle and Michalopoulos, 1982]. Overall, utilization of each of these types of systems (surgical and chemical) will likely be important in testing the utility of cell-based liver therapies. In addition, knowledge of the mechanisms of liver injury and regeneration gleaned from these animal models will provide an important blueprint for the design of clinically effective engineered liver tissue.

In summary, although many challenges remain for the improvement of tissue-engineered liver systems; substantial progress has been made toward a thorough understanding of the necessary components. Technologies such as hydrogel chemistries and microfabrication represent enabling tools for investigating the critical role of the microenvironment in liver function and, subsequently, the development of structurally complex and highly functional engineered liver constructs.

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SOURCES OF MATERIALS

Item	*Supplier*
1-Vinyl-2-pyrrolidinone	Sigma
250-μm nylon mesh (#CMN-250-D)	Small Parts
3-(Trimethoxysilyl) propyl methacrylate	Aldrich
3-0 Silk suture	Harvard Apparatus
3T3-J2 mouse fibroblast cell line	H. Green, Harvard Medical School
62-μm nylon mesh (#CMN-62-D)	Small Parts
96-Well plates, flat bottom	Nunc
Albumin, rat	ICN
Anesthesia device, veterinary grade (Model 100F)	SurgiVet
Angiocath™ catheter	Becton Dickinson
Anti-albumin antibody, rabbit anti-rat, HRP conjugated	ICN
Anti-albumin antibody, rabbit anti-rat, unconjugated	ICN
Anti-IgG antibody, goat anti-rabbit, FITC conjugated	Santa Cruz Biotechnology
Betadine	Owens and Minor, Inc.
Biotin Blocking System	DakoCytomation
Blood Urea Nitrogen (BUN) Acid Reagent	Stanbio Labs
Blood Urea Nitrogen (BUN) Color Reagent	Stanbio Labs
Borosilicate glass wafers, circular, 2 in.	Erie Scientific
Bottom Side Mask Aligner	Karl Suss
Bovine serum albumin	Sigma
Collagenase, type IV (C-5138)	Sigma
Coverglasses, circular, 34 mm	Fisher

Item	*Supplier*
DAKO LSAB® System-HRP	DakoCytomation
Developer appropriate for selected photoresist. We use OCG 934, MF-319, or Shipley 354	
Dulbecco's modified minimal essential medium	Invitrogen
Fetal bovine serum	Invitrogen
Glucagon	Bedford Laboratories
Goat serum	Sigma
HBSS, 10×, without calcium or magnesium	Invitrogen
HepG2 cells, human hepatoma cell line	ATCC
Hydrocortisone sodium succinate	Pharmacia
Insulin (Regular ILETIN® II)	Eli Lilly
Irgacure 2959	Ciba Specialty Chemicals
MF-319	Microchem Corp.
Minimal essential medium	Gibco
Mitomycin C	Boehringer
Nylon mesh	
O_2 probe	Microelectrodes
OCG 825–835 St	Olin-Ciba-Geigy
OCG 934	Olin-Ciba-Geigy
o-Phenylenediamine (OPD) tablets	Sigma
Paraformaldehyde	Electron Microscopy Sciences
Penicillin-streptomycin	GIBCO
Percoll	Amersham
Peroxidase Blocking Reagent	DakoCytomation
Positive photoresists, OCG 825–835 St, Shipley 1813, or Shipley 1818	
Poly(ethylene glycol) diacrylate (PEGDA), 3.4 kDa	Nektar Transforming Therapeutics
Protease inhibitor cocktail	Roche Diagnostics
Rats, female Lewis	Charles River
Shipley 1813	Microchem Corp.
Shipley 1818	Microchem Corp.
Shipley 354	Microchem Corp.
Silicone lubricant, inert	Dow Corning
Slides, glass (75 × 38 mm)	Fisher
Stanbio Urea Nitrogen Procedure No. 0580	Stanbio Labs
Syringe pump	Harvard Apparatus
Triton X-100	Fisher
Trypsin (0.25%)-EDTA (0.2 g/L)	Invitrogen
Ultra-TMB (3,3′,5,5′-tetramethylbenzidine	Research Diagnostics, Inc.
Urea/nitrogen stock samples	Stanbio Labs
UV light source: Exfo Lite	Exfo
Vectashield	Vector Laboratories
Wafer tweezers	Fluoroware

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