Effect of cell–cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells

S. N. BHATIA, U. J. BALIS, M. L. YARMUSH, AND M. TONER[1]

Center for Engineering in Medicine and Surgical Services, Massachusetts General Hospital, Harvard Medical School, and Shriners Hospital for Children, Boston, Massachusetts 02114, USA

ABSTRACT **Heterotypic cell interaction between parenchymal cells and nonparenchymal neighbors has been reported to modulate cell growth, migration, and/or differentiation. In both the developing and adult liver, cell–cell interactions are imperative for coordinated organ function. *In vitro*, cocultivation of hepatocytes and nonparenchymal cells has been used to preserve and modulate the hepatocyte phenotype. We summarize previous studies in this area as well as recent advances in microfabrication that have allowed for more precise control over cell–cell interactions through 'cellular patterning' or 'micropatterning'. Although the precise mechanisms by which nonparenchymal cells modulate the hepatocyte phenotype remain unelucidated, some new insights on the modes of cell signaling, the extent of cell–cell interaction, and the ratio of cell populations are noted. Proposed clinical applications of hepatocyte cocultures, typically extracorporeal bioartificial liver support systems, are reviewed in the context of these new findings. Continued advances in microfabrication and cell culture will allow further study of the role of cell communication in physiological and pathophysiological processes as well as in the development of functional tissue constructs for medical applications.—Bhatia, S. N., Balis, U. J., Yarmush, M. L., Toner, M. Effect of cell–cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells. *FASEB J.* 13, 1883–1900 (1999)**

Key Words: liver · coculture · bioartificial liver · hepatocyte morphology

Cell–cell interactions are central to the function of many organ systems. A common theme for heterotypic cell interactions is the interaction of parenchymal cells with nonparenchymal neighbors with resultant modulation of cell growth, migration, and/or differentiation. Specifically, these interactions are of fundamental importance in physiology (1, 2), pathophysiology (3, 4), cancer (5, 6), developmental biology (7, 8), wound healing (9, 10), and attempts to replace tissue function through 'tissue engineering' (11, 12). Further understanding of how cell–cell interactions modulate tissue function will allow us to gain fundamental biological insight as well as suggest approaches that will allow the manipulation of tissue function *in vitro* for therapeutic applications.

In particular, heterotypic interactions play a fundamental role in liver function. The formation of this vital organ from the endodermal foregut and mesenchymal vascular structures is thought to be mediated by heterotypic interactions (13, 14). Heterotypic interactions have also been implicated in adult liver physiology (i.e., localization of enzymes in zones of the liver) and pathophysiology (i.e., cirrhosis, and response to injury) (15–17). As we describe in this review, even *in vitro*, heterotypic interactions have proved useful in stabilizing liver-specific functions in isolated hepatocytes.

Despite extensive work in this area, the details by which cell–cell interactions modulate the hepatocyte phenotype *in vitro* remain unelucidated. Here, we summarize the existing works on cocultivation of hepatocytes with nonparenchymal cells: the experimental approaches, the outcome, and proposed mechanisms of interaction. In addition, recent advances in cell culture techniques (micropatterning) are discussed as they facilitate examination of these model systems. Finally, we present and discuss various approaches to the incorporation of hepatocyte cocultures into clinical liver support systems.

CELL–CELL INTERACTIONS IN THE LIVER *IN VIVO*

The liver arises as a bud from part of the foregut. The 'hepatic diverticulum' extends into the septum transversum, where it rapidly enlarges and divides into two parts: *1)* the primordium of the liver and the intrahe-

[1] Correspondence: Center for Engineering in Medicine, Massachusetts General Hospital, Bigelow 1401, 55 Fruit St., Boston, MA 02114, USA. E-mail: mtoner@sbi.org

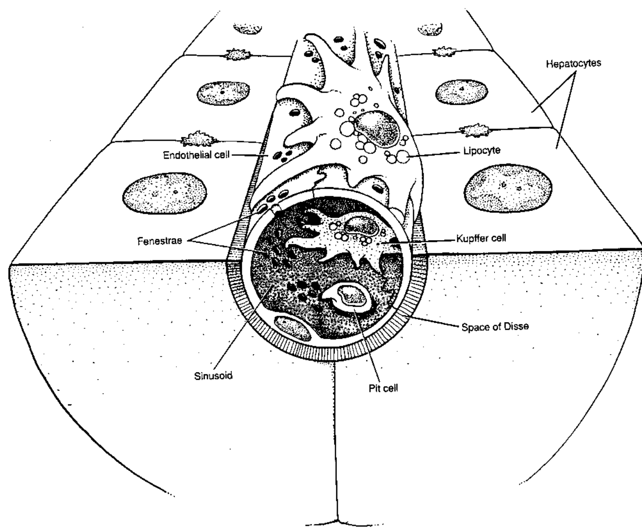

Figure 1. Schematic of the liver sinusoid (with permission from Kaplowitz (22). The adult consists of differentiated hepatocytes (H) separated from a fenestrated endothelium (E) by the Space of Disse. Lipocytes (stellate or Ito cells) are elaborate, extensive processes that encircle the sinusoid. Biliary ductal cells contact hepatocytes toward the end of the hepatic sinusoid (not depicted); Kupffer (the resident macrophage), and Pit cells (a type of natural killer cell) are free to roam through the blood and tissue compartment. Thus, the adult liver provides a scaffold for many complex cell–cell interactions that allow for effective, coordinated organ function.

patic portion of the biliary apparatus, and *2)* the gall bladder and cystic duct. The proliferating endodermal cells give rise to interlacing cords of liver cells and the epithelial lining of the intrahepatic biliary apparatus. As the liver cords penetrate the mesodermal septum transversum, they break up the mesodermal umbilical and vitelline veins, forming the hepatic sinusoids (18). The fibrous and hemopoietic tissue and Kupffer cells of the liver are also derived from the mesodermal septum transversum. It is thought that the mesenchyme induces the endoderm to proliferate, branch, and differentiate (19).

It has been shown experimentally in chimeric avian and mouse livers that differentiated hepatocytes arise from the endodermal compartment and mesenchyme gives rise to the endothelial lining of the adult sinusoids (13). In addition, when endoderm was cultivated alone, it failed to differentiate; however, tissue interactions between hepatic endoderm and mesenchyme induced hepatocyte differentiation *in vitro*. More recently, specific cytokines and transcription factors have been identified as important mediators of this process (20, 21).

In contrast, the adult form of the liver, a complex multicellular structure, is seen in **Fig. 1** (reprinted from Kaplowitz, ref 22). It consists of differentiated hepatocytes (H) separated from a fenestrated endothelium (E) by the Space of Disse. Lipocytes (stellate or Ito cells) are elaborate, extensive processes that encircle the sinusoid, well-positioned for both communication with hepatocytes and the potential to modify the extracellular space by secretion of extracellular matrix. Biliary ductal cells contact hepatocytes toward the end of the hepatic sinusoid (not depicted) and Kupffer cells (the resident macrophage), and Pit cells (a type of natural killer cell) are free to roam through the blood and tissue compartment. Thus, the adult liver provides a scaffold for many complex cell–cell interactions that allow for effective, coordinated organ function.

The information about cell–cell interactions in liver development and terminal differentiation implies an essential role for cell signaling between parenchymal and nonparenchymal tissue compartments. Cocultivation of hepatocytes with other cell types *in vitro* offers a unique model for in-depth study of these critical pathways.

COCULTIVATION OF HEPATOCYTES AND NONPARENCHYMAL CELLS

Hepatocyte viability and liver-specific function have been shown to be stabilized for several weeks *in vitro* upon cocultivation with a variety of other cell types. The resultant cocultures have been widely used in studies of various physiological and pathophysiologic processes including host response to sepsis (23, 24), mutagenesis (25–28), xenobiotic toxicity (29, 30), response to oxidative stress (31), lipid metabolism (32, 33), and induction of the acute-phase response (34–37). In addition, the ability to preserve key features of the hepatocyte phenotype *in vitro* may have important applications in hepatocyte-based therapies for liver disease.

First noted by Langenbach et al. in 1979 (38) through work with hepatocytes atop irradiated feeder layers of human fibroblasts and later elucidated by Guguen-Guillouzo et al. (39) by a mixed coculture of hepatocytes with live isolated rat liver epithelial cells, the effect of cell–cell interactions on the hepatocyte phenotype has become an active area of investigation. **Figure 2** shows the earliest images, to our knowledge, of retained hepatocyte morphology and function *in vitro* due to cocultivation with another live cell type (39). Note intracellular albumin staining throughout the hepatocyte island regardless of proximity to the heterotypic interface. In this review, the term heterotypic interface will be used to describe the spatial dimension over which fibroblasts and hepatocytes undergo coplanar cell–cell contact (i.e., in an island of hepatocytes surrounded by fibroblasts, this would correspond to the island perimeter). **Figure 3** depicts the functional outcome of this culture method and the clear demonstration of retention of a liver-specific function, albumin secretion, for many weeks (>5 wk) (37). In

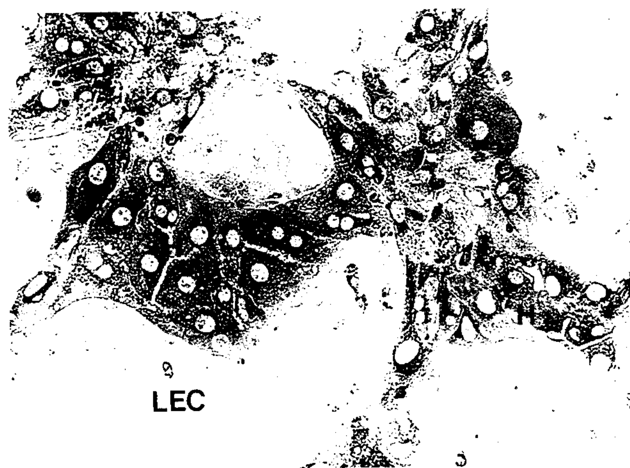

Figure 2. Randomly distributed coculture of hepatocytes and liver epithelial cells (with permission from Guguen-Guillouzo et al. (39). Hepatocytes exhibit stereotypical morphology—distinct nuclei and nucleoli as well as well-demarcated cell–cell borders. Localization by peroxidase-labeled antibodies of albumin 10 days after hepatocyte seeding. Almost all of the hepatocytes (H), but no liver epithelial cells (LEC), contain albumin. In addition, intracellular albumin staining is present throughout the hepatocyte island regardless of proximity to the heterotypic interface.

fact, relatively stable albumin production has been observed as long as 65 days (40).

This stabilization of liver-specific functions has been reported for cocultures with both liver-derived cell types as well as non-liver-derived endothelia and fibroblasts. **Table 1** summarizes studies of both liver and non-liver-derived cell types in hepatocyte cocultures. Liver-derived cell types include rat liver epithelial cells of presumed biliary origin (31, 37, 39–46), stellate (Ito, fat-storing) cells (47–49), sinusoidal endothelial cells (50, 51), Kupffer cells (24, 52–56), and the entire 'nonparenchymal' fraction of isolated liver cells (57–59). Although this effect on morphological and functional differentiation was originally thought to be species specific, many other cell types from other organ systems and species have since been shown to influence isolated rat hepatocytes. This effect has been demonstrated with rat hepatocytes, to varying degrees, using embryonic murine 3T3 and C3H 10T½ cells (29, 32, 45, 51, 60–64), rat dermal fibroblasts (51), Chinese hamster cells (25, 28), canine kidney epithelia (65), bovine aortic endothelia (51, 66), and human fibroblasts and lung epithelia (26, 27, 65, 67, 68). In addition, similar findings have been observed for adult human and fetal rat, chick, and porcine hepatocytes (28, 30, 68–71). Finally, the effects of cell–cell communication are also reciprocal; stabilization of function and responsiveness of nonparenchymal cells when cocultured with hepatocytes has also been reported (48, 59).

Typically, the time course of events in hepatocyte cocultures is similar, independent of the choice of secondary cell type, culture configuration, or cell concentration. Most cultures have preserved hepatospecific synthetic functions for prolonged periods (1 to 10 wk). The effects on hepatocyte function are inducible for 3–7 days, after which hepatocyte 'rescue' is unattainable (41, 69). This is noted graphically in Fig. 3, which demonstrates the comparable efficacy of initiating coculture on both day 1 and day 7 of hepatocyte culture. In addition, the time course over which albumin synthetic capability increases before stabilization appears to remain fairly constant, 6–10 days.

Culture configurations for many of these systems employed variations in the ratio of cell types and media composition. Typically, investigators have explored ratios of cell numbers of ~1:1 (nonparenchymal: hepatocyte); however, this has varied among studies from 10:1 to 1:10 (56, 58). In addition, many media formulations have included additions of insulin and glucocorticoids such as hydrocortisone or dexamethasone to inhibit fibroblastic overgrowth. Last, both serum-free and fetal bovine serum formulations have been used successfully. In addition to viable cells in the above culture configurations, experiments have been performed with feeder layers, including irradiated (38), desiccated and heated (58), glutaraldehyde-fixed (72), or mitomycin C-treated (61) nonparenchymal cells. One study compared the relative effect of viable cells vs. feeder layers and reported comparable effects on the exam-

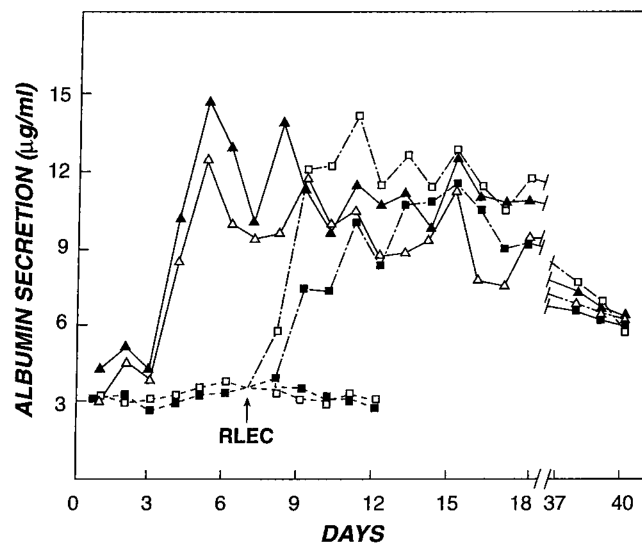

Figure 3. Albumin secretion by human hepatocytes cultured alone or in association with rat liver epithelial cells (with permission from Clement et al (69). The influence of late addition of the epithelial cells in depicted: these cells were added 3 h (solid line) or 7 days (dashed line) after hepatocyte plating in media supplemented (open) or not (filled) with serum. Pure cultures were maintained in an arginine-free medium before addition of epithelial cells on day 7. Late addition of epithelial cells allowed 'rescue' of pure hepatocyte cultures. Subsequent daily albumin secretion rates indicate retention of liver-specific function for many weeks.

TABLE 1. *Cell types used in cocultures for stabilization of rat hepatocyte phenotype*

Liver-derived	Non-liver-derived
Rat liver epithelial (presumed biliary origin) (31, 37, 39, 40–42, 44–46, 59)	Bovine aortic endothelia (51, 66)
Stellate (47–49)	Canine kidney epithelia (65)
Sinusoidal endothelial (50, 51)	Chinese hamster epithelia (25, 28)
Kupffer (24, 52–56)	Embryonic murine (3T3, C3H 10T$\frac{1}{2}$) (29, 32, 45, 51, 60, 63–65)
'Nonparenchymal' fraction of isolated population (57–59)	Human fibroblast (26, 27, 67, 68)
	Human lung epithelia (65)
	Human venous endothelia (42)
	Monkey kidney epithelia (65, 78)
	Rat dermal fibroblast (51)

ined markers, DNA synthesis (58). Similarly, glutaraldehyde-fixed endothelial cells elicited a comparable response to viable cells (72) when cocultured with hepatocytes.

In general, a variety of coculture models have met with significant success in maintenance of many hepatospecific functions. A summary of the existing data on the morphological, mitotic, and biochemical effects of coculture on hepatocytes is presented below.

EFFECT OF COCULTURE ON HEPATOCYTE MORPHOLOGY AND FUNCTION

In vivo, hepatocytes are large, compact polyhedral cells with round nuclei and prominent nucleoli (see Fig. 3); however, when isolated and cultured on tissue culture plastic, they adhere and spread, losing many of their characteristic features (73). Over time, the nuclei undergo karyolysis (loss of nucleus associated with cell death), the cell borders become indistinct, the cytoskeleton undergoes rearrangement with actin stress fiber formation and a 'fibroblastic' appearance, and cells ultimately detach and die. In contrast, hepatocyte cocultures exhibit stereotypical polygonal morphology with distinct nuclei and nucleoli, well-demarcated cell–cell borders, and a visible bile canalicular network for many weeks.

Cocultures have also been shown to express many liver-specific proteins such as albumin (**Table 2**). Murine 3T3's have been shown to induce the highest levels of albumin secretion by hepatocytes (4.2 to 15 $\mu g/10^6$ cells/h) (74, 75), followed by rat liver endothelial cells (3.1 $\mu g/10^6$ cells/h) (51), rat dermal fibroblasts (3.1 $\mu g/10^6$ cells/h) (51), rat liver epithelial cells (2.9 $\mu g/10^6$ cells/h) (39), and bovine aortic endothelial cells (1 $\mu g/10^6$ cells/h) (51).

TABLE 2. *Measured hepatocyte functions in cocultures*

Albumin secretion
Cytochrome P-450 activity (isoenzymes 1A1, 2B1, 3A1) and inducibility
Glutathione S-transferase
Tight junctions (detection of ZO-1)
Gap junctions (detection of connexin 32, microinjection)
Other: pyruvate kinase, transferrin, DNA synthesis, UDP-glucoronyl transferase

The level of regulation involved in induction of liver-specific protein production has also been investigated. The cause of the increases observed in protein synthesis and mRNA was studied using *in vitro* transcriptional assays from isolated nuclei as well as 'rescue' experiments wherein mRNA was allowed to decline and then observed to reappear. Albumin, pyruvate kinase, transferrin, and various subunits of glutathione S-transferase were found to be regulated primarily at the transcriptional level, with at least some component of post-transcriptional mRNA stabilization (41, 44).

Markers of detoxification capability, such as cytochrome P-450 enzyme activity, have also been observed to increase in amount and stability. For the most part, P-450 isoenzymes 1A1, 2B1 and 3A1 seem to be the best stabilized after 1 wk (45, 46, 65, 76–81). In comparison to conventional hepatocyte cultures such as Matrigel, total P-450 content was found to be elevated twofold in uninduced cocultures (62). In addition, hepatocytes retained inducibility of P-450 enzymes by prototypic inducers (61, 62, 77, 79–81). One study showed a 12- to 15-fold increase in mRNA levels for CYP2B1 after 7 days of induction of cocultures by phenobarbital as compared to hepatocytes cultured alone (81). Although each isoenzyme shows a different induction pattern, some have been reported to be inducible for up to 2 months (82). On the other hand, cocultivation does not preserve all isoenzyme activities; some, such as 2C11, 2C6, and 2E1, were reported to decline continuously (45, 46, 79, 80).

The influence of hepatocyte coculture on other markers of detoxification pathways such as the conjugating (phase II) systems have also been studied. The glutathione-S-transferase (GST) family, a family of dimeric enzymes that catalyze the conjugation of reduced glutathiones to electrophiles, is the most commonly studied of the phase II systems. Like the

cytochrome P-450 isoenzymes, GSTs are generally more stable in coculture than pure hepatocyte culture (31, 43–46, 83, 84). Stable expression of some subunits has been noted for 12 days in coculture, with significant quantitative differences between various nonparenchymal cell sources (44, 78). In addition, as seen in the P-450 family, quantitative differences for each subunit are noted, with subunits 3 and 4 being the most stable. The mechanism of stabilization of the GSTs in coculture is thought to be both at the transcriptional and mRNA stabilization level (44). GSTs may also be induced in cocultures by stereotypical inducers (i.e., phenobarbital), with each subunit responding differentially (43, 46). Finally, the GST family differs from the cytochrome P-450 family in one important arena: expression of a fetal GST (7) is induced in both pure hepatocyte and hepatocyte cocultures, though not typically seen in the adult liver (43, 44, 83, 84).

Functional contacts were also observed in hepatocyte cocultures. Tight junctions were detected by the presence of ZO-1 in cocultures (47). Gap junctions (connexin 32) were localized by indirect immunofluorescence and/or by microinjection of Lucifer yellow. In general, gap junctions were formed only in homotypic hepatocyte interactions (40). One notable exception is the formation of heterotypic functional gap junctions between hepatocytes and fat-storing cells (connexin 43) (49). In addition, the degree of induction of albumin synthesis correlated with increased levels of connexin 43 in various fat-storing cell clones. This observation is particularly significant because fat-storing cells maintain direct contact with hepatocytes *in vivo*. This model suggests that hepatocyte function may be influenced by the degree of cell–cell interaction (and thus the formation of heterotypic gap junctions). Therefore, though homotypic hepatocyte gap junctions are commonly noted in coculture, the presence and influence of heterotypic gap junctions are cell type dependent.

Spatial and temporal distribution of gap junctions between hepatocytes was also examined under various coculture conditions. Mesnil et al. (40) noted that the number of dye-coupled hepatocytes per injection gradually increased with coculture time from a single cell early in coculture to 9 cells by 25 days. Once hepatospecific functions stabilized, all hepatocytes in a given colony were found to express both functional gap junctions and albumin regardless of the proximity to the heterotypic nonparenchymal cell type (i.e., even hepatocytes that do not undergo direct contact with nonparenchymal cells retain function). This data provided indirect evidence that the signal for induction of liver-specific function is not confined to the heterotypic interface, i.e., signals may propagate through confluent hepatocyte populations. Thus, studies of gap junction expression in hepatocyte cocultures suggested that both temporal and spatial variations exist.

Even though these model systems offer the opportunity to study complex modes of cell–cell communication, there are significant confounding factors in such randomly distributed cocultures. Hepatocyte colony size varies with cell seeding density as well as hepatocyte adhesion, aggregation, and migration. The approximate size of hepatocyte colonies (estimated from published micrographs) in these studies was 100–200 μm in diameter containing 5–15 cells. These phenomena may ultimately be better examined by using a culture system that produces spatially uniform cell–cell interactions.

An additional notable feature of certain hepatocyte cocultures as compared to pure hepatocyte cultures is the ability to synthesize DNA *in vitro*. This effect has been noted in hepatocyte cocultures of both liver-derived and non-liver-derived cell types. An important distinction must be made between DNA synthesis and cell growth *per se*, especially in light of the known ability of hepatocytes to multinucleate both *in vivo* and *in vitro*. Given this caveat, two investigators have reported significant levels of DNA synthesis/division in cocultures. When rat liver cells were cocultured with the entire nonparenchymal liver fraction on felt templates, parenchymal cells of 15–30 μM diameter increased in number by 10-fold over 48 days as measured by enzymatic separation of cultures and counts of cell populations by size. In addition, thymidine incorporation was measured over 48 days and found to reach a maximum at 24 days of culture (82). In contrast, Shimaoka et al. (58) found an increase of labeling index from 13% of hepatocytes in pure cultures to 35% of hepatocytes in cocultures with nonparenchymal cells. This stimulatory effect of nonparenchymal cells on DNA synthesis by adult hepatocytes varied in a dose-dependent manner, where cultures with low hepatocyte densities demonstrated a twofold increase in labeling index over high hepatocyte densities. Furthermore, DNA synthesis reached a maximum at 3 days of culture.

DNA synthesis was also examined by coculture with non-liver-derived 3T3 clones, producing varied results. Some investigators have reported 20–30% labeling indices (61) whereas others have reported minimal thymidine uptake (58, 62). In other non-liver-derived cell types such as human embryonic lung, canine kidney, and monkey kidney epithelia, minimal thymidine uptake was reported (65). Thus, in general, it appears that very little hepatocyte growth occurs in coculture configurations with non-liver-derived nonparenchymal cells. This suggests that growth-arrest of the ‘alternative cell type’ in this type of hepatocyte cocultures may afford adequate

control over preservation of approximately constant cell numbers for precise study of both subpopulations.

In summary, cocultivation of hepatocytes with nonparenchymal cells has been shown to preserve stereotypical hepatocyte morphology and a variety of synthetic, metabolic, and detoxification functions of the liver. Although cell communication clearly plays a role in the regulation of these hepatospecific functions, the complex rules that govern the influence of homotypic cell interactions, heterotypic cell interactions, cell density, and ratio of cell populations remain undetermined. These issues may be elucidated by use of a model system that allows precise control over these interactions. One such model system, based on cellular micropatterning techniques, was recently developed and is discussed in detail elsewhere in this review (85).

MECHANISM OF INDUCTION OF LIVER-SPECIFIC FUNCTION IN HEPATOCYTES

The precise mechanisms that regulate increases in liver-specific function in hepatocyte cocultures have not yet been elucidated. The potential mediators of cell–cell communication include 'freely secreted' signals (i.e., cytokines) or 'cell-associated' signals (i.e., insoluble extracellular matrix or membrane-bound proteins).

Many studies attempting to discern the contribution of soluble factors in coculture systems have produced contradictory results. Morin et al. (72) reported that a transmembrane culture system using hepatocytes seeded on a 0.45 μM pore size filter and endothelial cells in an underlying well induced similar levels of albumin secretion as control cocultures with sinusoidal cells in contact with hepatocytes on similar filters. In contrast, Donato et al. (79) reported no significant improvement in P450 activity when hepatocytes were cultured on the bottom of a similar trans-well system with a 0.4 μM pore size and MS epithelial cells on top of the insert over pure hepatocyte cultures. The differences in these findings suggest that perhaps culture configuration (i.e., hepatocyte adhesion to a transwell filter as compared to tissue culture dishes) is important. In addition, use of media conditioned by the second cell type on pure hepatocyte cultures has been shown to be almost universally ineffective (58, 61, 62). At least one dissenting study showed a partial effect of rat liver epithelial cell conditioned media on hepatocyte cultures (half-maximal increases in levels of glutamine synthetase activity relative to control cocultures). Conditioned media obtained from cocultures showed no effect on glutamine synthetase activity (the only function tested). This implies that any potential soluble factor is not present in excess in cocultures due to its uptake/degradation by hepatocytes (42).

Comparatively, studies of extracellular matrix-mediated effects on liver-specific gene expression have been even less conclusive. Although many groups have reported matrix deposition patterns specific to cocultures, no causative effects of this matrix have been shown. In particular, reticulin fibers were observed in cocultures but absent in both types of pure culture (39, 51, 86). Other extracellular matrix components have been observed in cocultures with indirect immunofluorescent techniques including collagens I, IV, fibronectin, laminin, and entactin (37, 47, 51).

Mesenchymal cells typically are characterized by their ability to produce collagen I and fibronectin matrix molecules, whereas hepatocytes have been shown to primarily produce collagen IV and laminin. As a result, the cellular source of extracellular (ECM) deposition in cocultures is unclear. In addition, endothelial cells were found to produce perlecan *in vivo* (heparan sulfate-proteoglycan), a known mediator of some liver-specific functions (87), which may implicate proteoglycans in some component of the coculture effect. However, this ECM effect on liver cells is unlikely to be descriptive of the mechanism by which stellate cells induce hepatospecific function since they were consistently negative for perlecan. Finally, two groups have attempted to modulate the effect of potentially ECM-mediated events by (1) crudely assessing the distance over which the signal can travel from the heterotypic interface (42) and by (2) treating feeder layers with enzymes specific for ECM destruction (58). Shrode et al. (42) found up-regulation of glutamine synthetase production up to a few millimeters from the heterotypic interface; they suggest that large, insoluble ECM molecules are likely mediators since they would have limited diffusivity at critical concentrations. In contrast, the effects of direct cell contact communicated via gap junctions are discounted by the authors as they hypothesize that such a signal would travel over a limited distance. Finally, Shimaoka et al. (58) reported that the DNA synthesis they monitored in cocultures was acid-, trypsin-, and collagenase-sensitive, implicating some protein containing collagen. In addition, precultured feeder layers induced DNA synthesis earlier than fresh feeder layers, indicating that the presence of some material was rate-limiting. The authors suggest that the insoluble molecules (ECM or membrane receptors) in the feeder layers were responsible for the observed effects, although soluble factors entrapped in the feeder layers may also have played a role.

Until recently, the role of direct contact of cells, the other potential mechanism involved with induction of liver-specific function, has remained unclear. Mesnil et al. (40) showed that only hepatocytes in

close proximity to epithelial cells in sparse cultures remained viable and differentiated as compared to those that appeared to lack heterotypic contact. The authors suggest the importance of cell contact based on this indirect evidence; however, it seems clear that local deposition of ECM or local concentrations of critical soluble factors cannot be ruled out as causes for the preservation of viability and differentiation. More rigorous evidence supporting the role of membrane contact as a potential mechanism was reported in 1991 by Corlu et al. (88). These authors identified a cell surface protein (liver-regulating, or LRP) that seemed to be involved in the establishment and maintenance of hepatocyte differentiation in coculture with liver epithelial cells. They demonstrated the ability to modulate albumin secretion, cytoskeletal organization, and ECM deposition by addition of a monoclonal antibody against LRP. Furthermore, the authors discount extracellular matrix as potential ligand for LRP due to the inability of anti-LRP antibody to modulate cell adhesion to immobilized ECM. In addition, this inhibitory effect was produced only upon addition of the antibody early in culture. The authors suggest that this time dependence supports the role of cell–cell contact in the coculture effect due to the indirect evidence that establishment of cell–cell contacts occurs during the same time frame in culture. Finally, it seems that LRP is almost certainly not the whole story; although some cell types that induce liver-specific functions in hepatocytes stained positive for LRP (sinusoidal cells and Ito cells), other cell types did not (vascular endothelia, biliary ductal cells) (89). Therefore, although the presence of LRP may modulate hepatocyte function in epithelial coculture, the absence of LRP in coculture with other cell types does not seem to prevent induction of liver-specific functions.

Other modes of direct contact such as gap junctional communication may also play a role in cell signaling. In one study, levels of connexin 43 expressed by fat-storing cell subclones correlated with albumin mRNA levels in cocultured hepatocytes. Functional heterotypic gap junctions were observed as a result of connexin 43 protein synthesis (49). This mode of cell signaling may be particularly important in hepatocyte interaction with Ito cells compared to other cell types due to the potential relevance of this signaling mechanism *in vivo* I(90). In addition, communication between cells has also been implicated in transport of reactive intermediates (91).

Due to the relationships described between dedifferentiation in tumors and decrease in gap junctions, studies were also done to assess the necessity of homotypic gap junctional communication for the stabilization of differentiated functions. Traiser et al. (92) found that gap junction intercellular communication could be effectively blocked with minimal effects on the stabilization of xenobiotic metabolic enzyme activities (another liver-specific marker), suggesting they may be unimportant in preservation of the hepatocyte phenotype. However, the results of this study may not be conclusive due to the potential effects of the compounds used for interfering with gap junctional communication on induction of P-450 enzymes. The notion that hepatocyte gap junctions may be decoupled from liver-specific functions is also supported by the lack of observable gap junctions in well-established hepatic culture systems after 24 h (73, 93).

In summary, despite the substantial data existing on potential mediators of cell communication in cocultures (receptors, gap junctions, cytokines, ECM), the mechanisms by which coculture of hepatocytes with other cell types induce and stabilize liver-specific function and viability are undefined. Indeed, many distinct mechanisms may operate in concert, each modulating a subset of hepatospecific functions. For example, expression of glutamine synthetase, albumin, and connexin 32 may each be independently regulated both in the time course of expression and rates of secretion. The difficulty with which homotypic and heterotypic interactions can be experimentally uncoupled, however, has made their role in these processes difficult to assess. Here we review methods that have been used to study the role of cell–cell interactions, including a 'micropattern'-based technique that has recently facilitated some novel insights in this area.

METHODS TO EXAMINE INFLUENCE OF CELL–CELL INTERACTIONS ON LIVER-SPECIFIC FUNCTIONS

The coculture systems discussed above used a variety of techniques to examine the role of cell–cell interactions in induction of liver-specific functions in isolated hepatocytes as depicted in **Fig. 4**. Control of cell–cell interactions fall into two general categories: *1)* prevention of contact or *2)* modulation of the degree of contact. Prevention of contact has been achieved by cocultures with porous filter inserts, insertion of crude spacers, or conditioned media experimentation. As detailed above, these culture configurations have led to some novel insights but have also been limited by variations in media sampling, storage, filter material, and cell seeding densities. In addition, absolute lack of contact is difficult to ensure; for example, transfer of detached cells in conditioned media or protrusion of cell processes through the porous filter is difficult to completely rule out.

Another approach at prevention of contact was reported by Shrode et al. (42). Creation of a cell-free

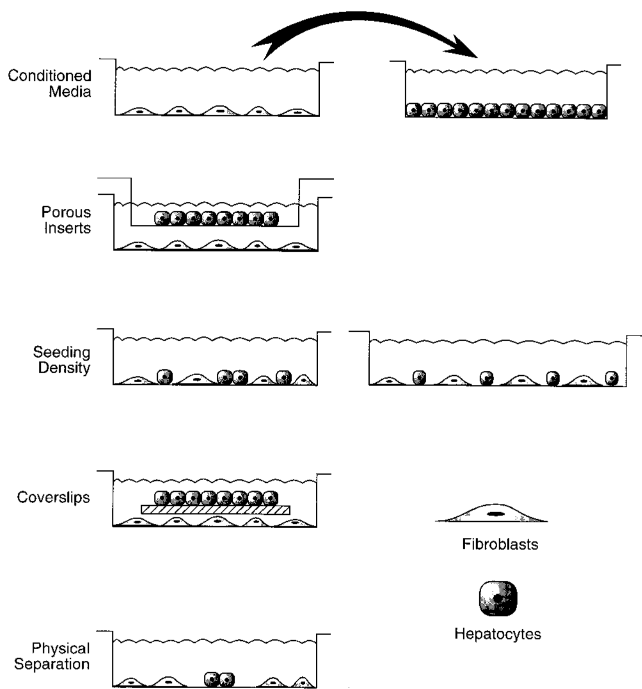

Figure 4. Schematic of previous attempts to control cell–cell interactions in hepatocyte cocultures. Media that has been conditioned by incubation with nonparenchymal cells is transferred to a pure hepatocyte population to examine the role of soluble factors. Porous membrane inserts are used in trans-well configurations. Mixed cocultures of various seeding densities have been performed to investigate the effect of local cell–cell interactions. Confluent coverslips have been incubated within confluent tissue culture dishes to examine the heterotypic interface. Finally, cell populations have been spatially separated by use of polymeric washers.

annulus was achieved through the addition of a polymer spacer to a culture dish by use of rubber cement adhesive. This spacer was then removed, resulting in a defined, relatively large (~ mm) cell-free annulus between the cell populations. Cell populations then grew together, allowing study of the role of local cell contact in zonation of the liver. Although this method did show that hepatocyte populations can undergo induction locally, the method is limited by the undefined underlying substrate (residual adhesive) and relegation to relatively large dimensions of annuli (spacers must be large enough to manipulate manually).

In addition to control of cell–cell interactions by prevention of contact, modulation of the degree of cell contact has also been attempted. Both conventional techniques (variations in seeding density) as well as more specialized systems (addition of confluent coverslips to confluent cultures) have been used. Variations in seeding density were used by Guguen-Guillouzo (94) to study the effects of cell contact on hepatocyte differentiation. They examined effects of lower seeding densities by seeding the same cell numbers in a two different size flasks. This method is simple and reproducible, but heterotypic cell contacts occur due to random events such as attachment during cell seeding. In addition, a confounding factor in these experiments may be the ability of the nonparenchymal cell type to divide: lower seeding densities may permit increases in the nonparenchymal population and the accompanying soluble factors synthesized by these cells.

Another study examined the role of cell contact by addition of confluent cultures of hepatocytes on a coverslip to the center of confluent cultures of either fibroblasts or fibroblast/hepatocyte cocultures (58). This technique also attempts to examine the role of local contact, and these studies succeeded in probing the role of soluble factors in a novel way; however, it is likely that the results were confounded by cell death underlying the coverslip and the significant topological variations in the culture (height of a coverslip is typically 100–300 μ). Another similar study using coverslip inserts examined the role of local stellate cell–hepatocyte interactions (48). This study demonstrated a localized 'paracrine' signal ~10 cell widths from the heterotypic interface, providing valuable insight into the potential mediators of this signaling process. However, this technique is also relegated to relatively large dimensions and significant potential for artifacts secondary to local cell damage.

Although some of these models have successfully examined the outcome associated with a complete lack of heterotypic cell contact, no existing experimental techniques have conferred the ability to systematically and uniformly vary the degree of local heterotypic cell interaction. Rather, cell–cell interaction has been typically dictated by poorly controlled parameters such as cell attachment, aggregation, and migration or by gross manipulations of culture configurations. Recently, a method was reported that significantly advance the current state-of-the-art reviewed here (85). These techniques allow control over the spatial distribution of two cell types in planar cultures and systematic investigation of the effects of cell–cell interactions on tissue function.

MICROFABRICATED COCULTURES: CONTROL OF HOMOTYPIC AND HETEROTYPIC CELL INTERACTIONS

Recently, 'cellular patterning' techniques were used to quantitatively control heterotypic interactions and to study how local tissue microenvironments modulate bulk tissue function (74, 75, 85, 95, 96). Indeed, cellular 'micropatterning' has already been useful in the study of many diverse biological phenomena. Spatial control over cell attachment and spreading has facilitated an un-

precedented level of sophistication in the investigation of mechanisms of nerve growth cone guidance, influence of cell shape on growth and apoptosis, and growth and orientation of fungal pathogens (97–102). Improvements on existing micropatterning technology recently allowed control over the degree of interaction between two different cell populations. These techniques were used to explore the effects of homotypic and heterotypic cell interaction on tissue function.

The photolithographic technique developed for the micropatterning of cells to allow spatial control over two distinct cell populations is depicted in **Fig. 5**. Borosilicate wafers were patterned with photoresist (a polymer that has variable solubility with exposure to ultraviolet light) by exposure to light through a prefabricated chrome mask (Fig. 5*A*). Patterned substrates were used to control subsequent immobilization of collagen I (103, 104) (Fig. 5*B*). The localization of adhesive extracellular matrix (here, collagen I) allowed for patterning of the first cell type, primary hepatocytes (Fig. 5*C*). Hepatocytes exhibited a well-spaced morphology with distinct nuclei and bright intercellular borders. Subsequent deposition of a nonparenchymal cell type (here, 3T3-J2 fibroblasts) allowed for spatial control over heterotypic cell interactions in the cellular microenvironment (Fig. 5*D*). This technique offers the ability to present different adhesive ligands to each population within a single culture (here, collagen I to hepatocytes, and serum-adsorbed proteins to fibroblasts), which cannot be achieved with many conventional methods. The versatility of this technique is derived from the ability to alter cell–cell interactions with ease via use of different chrome masks; therefore, the size of each cell subpopulation may be maintained while allowing variation in the extent of heterotypic interaction. Conversely, the level of heterotypic interaction may be held constant while allowing variation of the number of cells in each subpopulation.

Although the techniques described above enable the investigation of complex interactions between two cell types, the existing technology suffers from a number of limitations. First, although cells are patterned initially, tissue morphogenesis is not restricted in these cultures. Reorganization through cell motility was observed to be dependent both on hepatocyte island diameter as well as center-to-center spacing, with islands greater than or equal to 490 μM retaining an observable pattern for at least 2 wk whereas smaller patterns reorganized into cord-like structures on the order of days. Second, the success of this technique is dependent on the relative cell–cell and cell–substrate adhesiveness of each cell type, i.e., the relative preference of nonparenchymal adhesion to the substrate rather than the preseeded hepatocyte surface. This aspect of a micropatterned coculture could be studied using labeling with fluorescent vital dyes and/or confocal microscopy. Finally, the method depicted by Fig. 5 is limited to confluent cocultures on glass substrates; however, many applications may require separation of cell populations and/or the flexibility to use a variety of underlying substrates. Recent reports on use of polymeric microchannels to direct protein immobilization provide methods to pattern cells on a variety of surfaces from polystyrene to thin metal films (105–107), thus broadening the potential of the techniques described in this review.

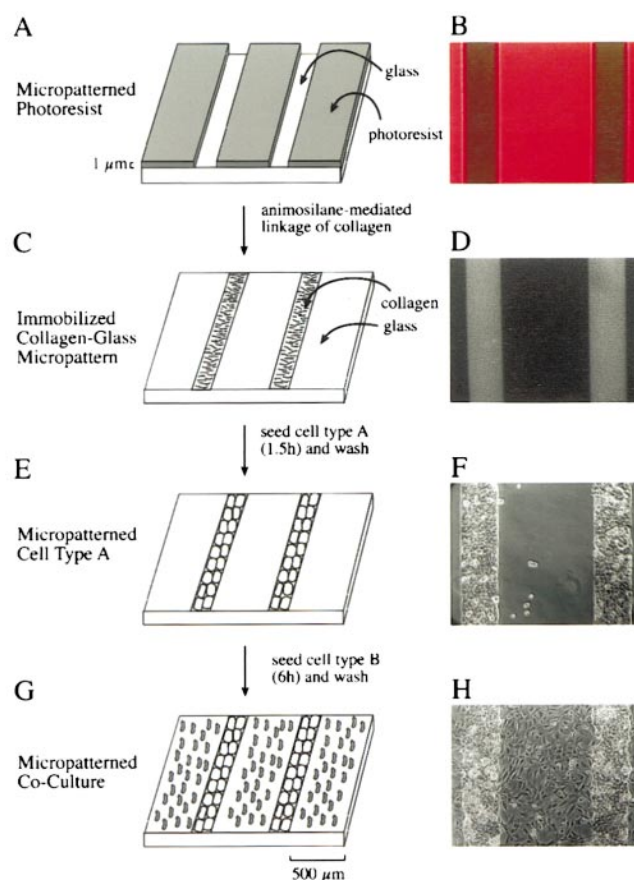

Figure 5. Schematic of novel method for generating micropatterned cocultures. Borosilicate substrates were coated with photoresist (a UV-sensitive polymer) and exposed to light through a mask, creating a photoresist pattern (*A*). Photoresist was visualized using epifluorescent microscopy (*B*) (ex: 550 nm, em: 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*). Phase contrast micrograph of 200 μM lanes of hepatocytes with 500 μM lane spacing (*F*). Addition of 3T3-J2 fibroblasts in media supplemented with serum resulted in generation of micropatterned cocultures (*G*). Phase contrast microscopy allowed morphological identification of 2 distinct cell types in a 'micropatterned coculture' (H).

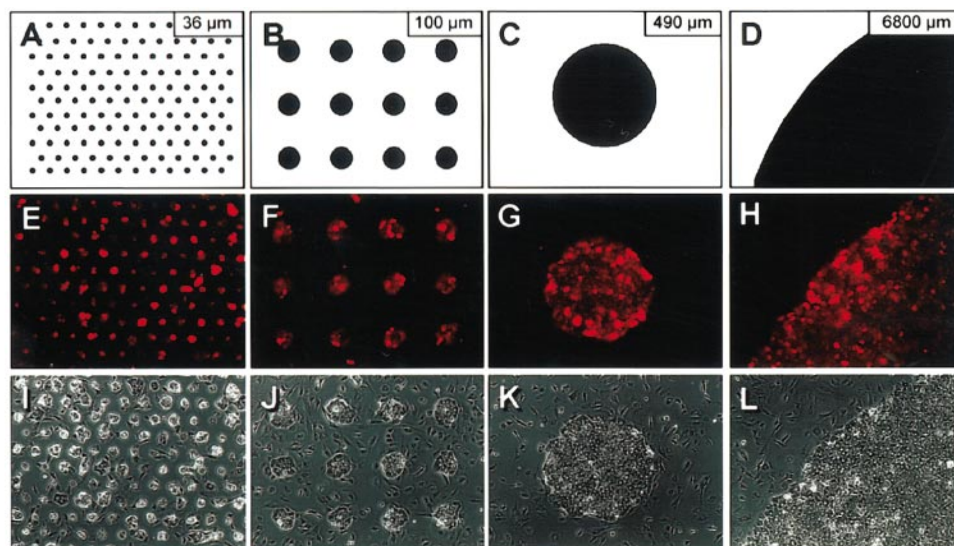

Figure 6. Micropatterned cocultures with constant ratio of cell populations. Schematic of 4 of 5 patterns used in this study (*A–D*). Black circles correspond to hepatocyte-adhesive areas. Total hepatocyte-adhesive area remained constant over a 2 inch diameter substrate. Inset indicates diameter of hepatocyte islands. The largest dimension consisted of a single island of 17.8 mm diameter (not pictured). Fluorescent visualization of hepatocytes in cocultures indicated fidelity of cell pattern to theoretical configuration (*E–H*). Phase contrast micrographs of micropatterned cocultures indicate broad range of heterotypic interface achieved despite similar cellular constituents (*I–L*).

Role of the Extent of Heterotypic Interactions in Induction of Liver-Specific Function

Micropatterned cocultures facilitate a number of different experimental approaches to the role of cell–cell interactions in modulating the hepatocyte phenotype. One study design allowed systematic variation of heterotypic cell interactions without modifications in individual cell populations (and thereby the hepatocyte:nonparenchymal cell ratio). Micropatterned cocultures were generated with variations in heterotypic interface, yet an identical surface area dedicated to both hepatocyte and fibroblast adhesion. Arrays were hexagonally packed, circular hepatocyte islands of varying dimensions and center-to-center spacing where total hepatocyte-adhesive surface area was held constant at 2.5 cm^2. Five different configurations for cocultures ranged from maximal heterotypic contact (smallest islands of 36 μM) to minimal heterotypic contact (single large island of 17.8 mm). Variations in spatial configurations were used to generate 'theoretical' differences in total perimeter of hepatocyte islands from 5.6 cm to 3224 cm, which, upon addition of fibroblasts, should correspond to variations in the total heterotypic interface with preservation of similar cell numbers in each subpopulation (**Fig. 6*A–D***). Micropatterned hepatocytes visualized with a fluorescent vital dye and were found to adhere predominantly to collagen-modified islands (Fig. 6*E–H*). Addition of 3T3-J2 fibroblasts to micropatterned hepatocytes resulted in micropatterned cocultures with marked alterations in initial heterotypic interface despite similar numbers of fibroblasts and hepatocytes across conditions, as depicted in Fig. 6 (*I–L*).

To determine the effect of modulation of heterotypic cell interactions on liver-specific function, cultures were characterized for expression of liver-specific biochemical (urea and albumin secretion) and immunohistochemical markers (intracellular albumin staining) and overall DNA content. We measured these markers in five different micropatterned cocultures with varying degrees of heterotypic interaction, each with a matched control of micropatterned hepatocytes in the identical configuration (i.e., no heterotypic interaction). In all micropatterned cocultures, urea synthesis was found to be significantly increased by 2.5- to 6-fold over micropatterned hepatocyte (only) controls on day 11, indicating that the induction of urea synthesis in hepatocytes was due to cocultivation with fibroblasts (**Fig. 7*A***). The degree of improved function over control micropatterned hepatocyte (only) cultures varied with the degree of heterotypic interaction. Two patterns of up-regulation of this liver-specific marker emerged: *1)* the three smallest island configurations (36,100, 490 μM, with relatively increased heterotypic interaction) showed up-regulation of urea synthesis to similar levels, whereas *2)* the two larger island configurations (6.8, 17.8 mm) showed relatively little up-regulation (~50% of cultures with greater heterotypic interaction). Therefore, a statistically significant increase in urea synthesis production was achieved in certain pattern configurations by modulation of the initial cellular microenvironment despite similar cellular constituents.

Similarly, all micropatterned cocultures had marked induction of albumin secretion when compared to micropatterned hepatocyte (alone) controls (Fig. 7*B*). By day 11, all micropatterned hepatocyte (alone) conditions had negligible levels of albumin secretion. In contrast, cocultivation with fibroblasts produced variations in the degree of up-regulation of this marker with the degree of heterotypic interaction. Again, two patterns emerged: *1)* dramatic up-regulation to similar levels of albumin secretion

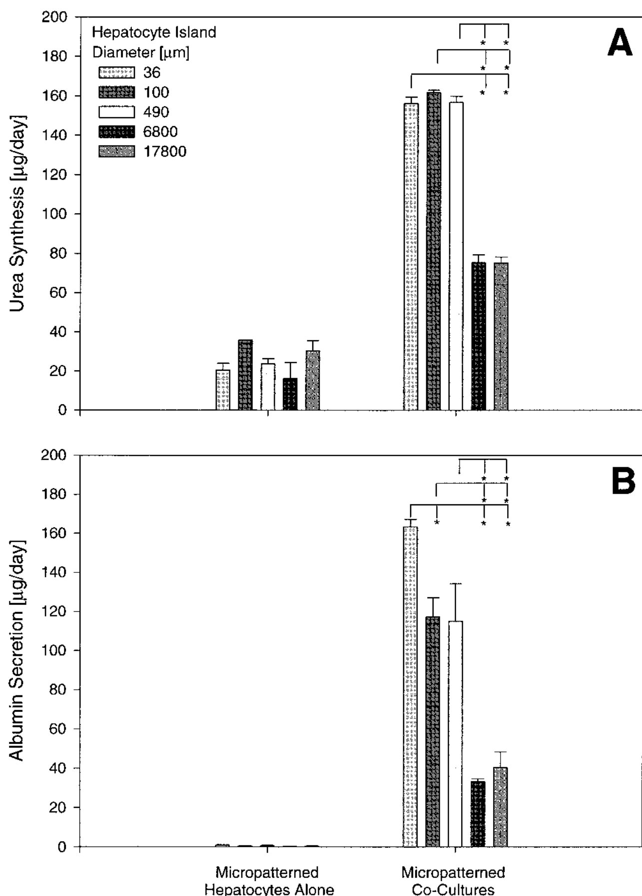

Figure 7. Liver-specific function of micropatterned cocultures with constant ratio of cell populations. Urea synthesis (*A*) and albumin secretion (*B*) on day 11 of culture were detected in micropatterned cocultures with varying heterotypic interactions despite similar cell numbers as well as micropatterned hepatocytes (only) controls. Statistical significance (*) was determined by one-way ANOVA with Tukey HSD post-hoc analysis with $P<0.05$. Note: To ensure that any observed functional differences were not due to variations in initial hepatocyte number, hepatocyte DNA was measured at 24 h (119). Most micropatterned configurations had statistically similar levels (8±1.8 μg DNA); however, the smallest islands had twofold elevated levels of DNA, due perhaps to the potential for more than one unspread hepatocyte (20 μM diameter) to adhere to 36 μM islands or the potential increased mitotic index of hepatocytes under sparse seeding densities (120). In either case, data from 36 μM islands may not be directly comparable to other configurations given the altered initial hepatocyte/fibroblast ratio.

in the three smallest island configurations (with relatively increased heterotypic interactions) and *2)* relatively modest up-regulation (~30% of cultures with greater heterotypic interaction) in the two larger island configurations. Therefore, a substantial increase in albumin production was achieved in certain pattern configurations by modulation of the initial heterotypic cellular microenvironment.

Thus, variation of initial heterotypic cell–cell interactions was found to modulate long-term bulk tissue function for at least two liver-specific functions. The kinetics of this response are described in detail elsewhere (75). Briefly, micropatterned cocultures demonstrated increased albumin synthesis rates until stabilization at day 9 for all configurations, whereas urea synthesis was either stabilized or increased to a plateau by day 3. Thus, despite the similarity in long-term effects of heterotypic interaction on two different markers of liver-specific function, the kinetic response of this induction varied. This finding is consistent with known differences in the patterns of recovery for various liver-specific functions in other hepatocyte culture systems (73). In addition, randomly distributed cocultures (i.e., not micropatterned) in the same model system had similar kinetics for induction of albumin secretion, but induction of urea synthesis was delayed until stabilization at day 7–10 (74). Differences in the kinetic response of randomly distributed cocultures from micropatterned cocultures may be due to reorganization of cell populations over time, artifactual due to differences in culture conditions (i.e., fibroblast adhesion to collagen I vs. serum-adsorbed proteins), or reflective of a time delay in signal propagation through randomly distributed cocultures.

As previously mentioned, extensive studies of the effect of initial cellular microenvironment on liver-specific function in cocultures are scant due to the limitations of existing experimental methods. One study attempted to examine the effect of local microenvironment by variation in size of culture plate (94). This study of human hepatocytes cocultured with rat liver epithelial cells (RLEC) used the same numbers of cells in 25 cm^2 and 75 cm^2 dishes. Heterotypic cell interactions were largely dictated by seeding density, plate size, and random cell aggregation. Their results suggest twofold higher albumin secretion in sparser cultures; however, this result may have been affected by RLEC number and associated cellular products (due to potential for increased RLEC growth on larger plate), differences in nutrient supply (oxygen, glucose, essential amino acids due to differences in amount of media), increased heterotypic interactions, or some combination thereof. In contrast, these novel microfabrication techniques allowed the demonstration of a threefold increase in albumin secretion due solely to variations in initial heterotypic cell–cell interaction. Thus, it seems that the local cellular microenvironment has been definitively isolated as an important modulator of liver-specific function.

Role of Heterotypic Interactions in Localized Induction of Liver-Specific Function

Although bulk markers of liver-specific function such as albumin and urea secretion are valuable in assessing the potential for heterotypic cell–cell interactions to modulate tissue-specific function, these

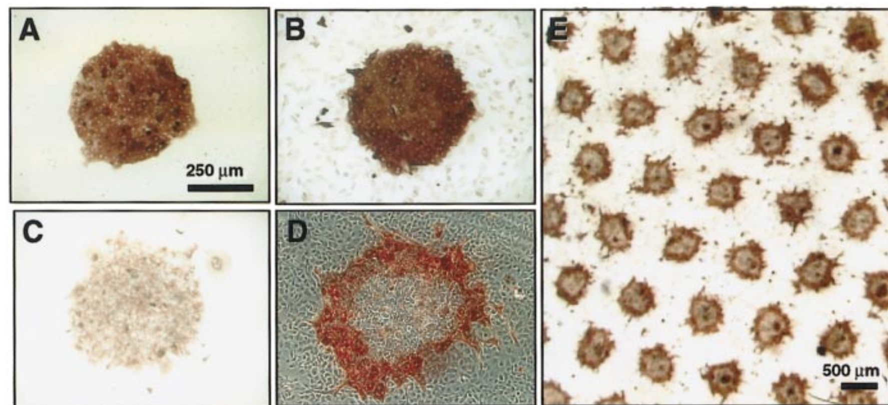

Figure 8. Immunohistochemical staining of intracellular albumin in micropatterned hepatocytes in a representative (490 μM) pattern. Bright-field microscopy of micropatterned hepatocytes (alone) on days 1 and 6 (*A, C*) Micropatterned cocultures on days 1 and 6 (*B, D*) did not display a uniform decline in albumin content. Presence of fibroblasts can be verified in periphery in phase contrast micrograph (*D*). Low power bright-field image (*E*) of 490 μM pattern stained for intracellular albumin indicates reproducibility of annular staining.

markers do not allow examination of individual cellular function in relation to the heterotypic interface. *In situ* markers of liver-specific function could elucidate the relationship between hepatocyte function and fibroblast interaction and perhaps implicate underlying mechanisms for this response. **Figure 8** depicts *in situ* immunostaining of intracellular albumin for various culture configurations. Micropatterned cocultures (Fig. 8*B, D*) were compared with matched micropatterned hepatocyte (only) controls (Fig. 8*A, C*) for 490 μM hepatocyte islands. These results show that micropatterned hepatocytes (only) stained uniformly for intracellular albumin at day 2 after isolation (Fig. 8*A*). As with secreted albumin, the level of detectable protein subsequently declined on the order of days in the absence of fibroblasts (Fig. 8*C*). These data are also consistent with previous studies showing detectable levels of albumin mRNA in freshly isolated hepatocytes with decline of mRNA over 1 wk of culture (i.e., no fibroblasts) (108). In comparison, micropatterned cocultures displayed a more complex behavior. They, too, displayed initial uniform staining for intracellular albumin at day 2 (Fig. 8*B*). Over 6 days, however, hepatocytes close to the heterotypic interface stained for high levels of intracellular albumin whereas protein levels in hepatocytes far from the heterotypic interface (> three or four cells) continued to decline as in the pure hepatocyte cultures. Thus, proximity to the heterotypic interface correlated with high levels of intracellular albumin. To ensure that the annulus of intense staining was due to variations in intracellular albumin content of hepatocytes as opposed to the detachment of hepatocytes or fibroblasts from the lightly stained areas, phase contrast microscopy of these cultures was performed. Figure 8*D* clearly depicts the presence of fibroblasts in the periphery of the hepatocyte islands and cellular structures in the center of the hepatocyte islands. Finally, Fig. 8*E* demonstrates the reproducibility of this peripheral annulus of intense staining observed across a 490 μM micropatterned coculture. Analysis of smaller islands (100 μM islands) showed staining throughout hepatocyte regions whereas cultures with decreased heterotypic interface (6800 μM islands) showed well-demarcated annular staining in the vicinity of the heterotypic interface, indicating that increased heterotypic interaction led to a larger population of highly functional hepatocytes (75).

These *in situ* immunostaining data contradict some existing reports (39, 69, 94). In 10-day-old cocultures of hepatocytes and rat liver epithelial cells, hepatocyte colonies were stained uniformly for albumin. Some investigators (40) have suggested the potential for hepatocytes to communicate with one another since hepatocytes away from the heterotypic interface stained for albumin; however, micropatterning cocultures allowed the creation of larger hepatocyte colonies than those that come about by random aggregation and ultimately indicated a finite penetration length of the differentiation signal to the interior of a large hepatocyte colony. This result demonstrated that, under some circumstances, hepatocytes may not communicate effectively throughout a hepatocyte colony. This type of evidence may lead to new mechanistic information with regard to intercellular signal transduction.

In summary, experiments on micropatterned cocultures indicate that tissue-specific function can be modulated by variation of initial heterotypic cell–cell interactions despite utilization of identical cellular constituents. Increasing heterotypic interaction correlated with a relative increase in all measured markers of liver-specific function including urea synthesis, albumin secretion, and intracellular albumin staining. Furthermore, these techniques allowed elucidation of potential mechanisms underlying this response. In this particular model system, it ap-

peared that the differences in bulk tissue properties as a function of cellular microenvironment were caused by induction of spatial heterogeneity in hepatocyte functions, i.e., hepatocytes close to the heterotypic interface were more functional and therefore patterns with greater interfacial regions displayed superior overall function.

Role of Nonparenchymal/Parenchymal Cell Ratio in Modulation of Liver-Specific Function

Another potential modulator of tissue function is the relative cell number in each constituent population independent of the heterotypic interface. *In vivo*, the liver is composed of ~33% nonparenchymal cells and 67% parenchymal cells (hepatocytes), a ratio of 0.5:1 (109). As noted earlier, studies have been performed with ratios of cell populations in coculture varying between 10:1 to 1:10. Using conventional methods, however, the ratio of cell populations is difficult to study in isolation. Typically, alteration of cell ratio (i.e., by variations in cell seeding densities) will vary local cell–cell interactions in the resulting coculture (see Fig. 4). Micropatterning techniques depicted in Fig. 5 combined with use of polymeric washers to limit the seeded surface area of glass substrates recently allowed independent study of the influence of nonparenchymal:parenchymal cell ratio, with preservation of heterotypic interface (94).

Micropatterned cocultures were generated with approximately equivalent total initial heterotypic interface and equivalent hepatocyte number, yet varying ratios of fibroblasts to hepatocytes of 6, 3, 1, and 0.5. Cocultures were probed for urea and albumin production as markers of liver-specific function. Although all culture configurations produced stabilization of urea synthesis due to coculture of hepatocytes with fibroblasts, levels of urea production declined with decreasing fibroblast number. The smallest ratio of fibroblasts: hepatocytes (0.5) produced ~15 $\mu g/10^6$ hepatocytes/h, close to *in vivo* levels of 5–8 $\mu g/10^6$ hepatocytes/h. Similarly, albumin production was up-regulated for all culture configurations; however, only physiological ratios of nonparenchymal to parenchymal cells (0.5) approached physiological levels of albumin (~4 as compared to *in vivo* value of 2–3 $\mu g/10^6$ hepatocytes/h) (110). Finally, the dependence of steady-state liver-specific functions on fibroblast number was remarkably similar in both cases (urea and albumin), suggesting the potential for an underlying common mechanism.

Thus, reduction of fibroblast number produced a decline in liver-specific function despite the preservation of the heterotypic interface, a parameter shown to be critical in earlier experiments. Bhatia et al. (95) speculate that modulation of liver-specific function based on fibroblast number may be due to either modification of homotypic fibroblast signaling producing variations in hepatocyte microenvironment or variations in bulk concentrations of heterotypic signaling cytokines. Cytokines, mesenchymal matrix products, and membrane-bound proteins known to have homotypic fibroblast signaling capability include interleukin-1, basic fibroblast growth factor, transforming growth factor-β, collagen I, fibronectin, and connexin 43 (111–115); therefore, changes in these signals could result in an altered composition of the heterotypic interface either by modification of fibroblast membrane-bound proteins, locally secreted extracellular matrix, or variation in the matrix or fibroblast-associated cytokines that are presented to the hepatocyte surface. Alternatively, concentrations of soluble heterotypic signaling cytokines will be diminished with decreasing fibroblast number; any contribution to induction of liver-specific function by such humoral factors may also be modulated by variation in fibroblast cell number.

CLINICAL APPLICATIONS OF HEPATOCYTE COCULTURES

New insights into the role of the heterotypic interface and ratio of cell populations allow re-evaluation of the role of cocultivation in existing model systems intended for use in clinical support of liver failure. Gerlach et al. (116) have developed a capillary-based reactor with an intraluminal endothelial cell compartment and extraluminal hepatocyte seeding (i.e., separated by a semipermeable membrane). The hydrophilic polypropylene membrane separates hepatocytes from nonparenchymal liver cells by ~300 μM. Review of the literature, along with recent data using microfabricated cocultures, suggests that induction of liver-specific function in hepatocytes due to the presence of nonparenchymal cells requires heterotypic cell contact (or at least close proximity). Thus, induction of liver-specific functions due solely to nonparenchymal cells (and not extracellular matrix or hormonally defined media) may be limited in this setting. This issue may be further evaluated as more data become available on the role of coculture in this particular device configuration.

Similarly, Koike et al. (117) have attempted cocultivation of hepatocytes with nonparenchymal cells in a perfused multiplate reactor that allows heterotypic contact. Although this reactor design does allow cell contact between hepatocytes and hepatic nonparenchymal cells, studies did not demonstrate a significant advantage of coculture over simple manipulations of extracellular matrix environment. In this case, perhaps the level of heterotypic interaction was suboptimal due to a relatively small number of

nonparenchymal cells (ratio of 1:1). Recent studies of hepatocytes cocultured with fibroblasts performed at this ratio of fibroblasts to hepatocytes resulted in decreased heterotypic interactions and a resultant decline in function (95). Novel culture methods that allow an increase in heterotypic interface without increasing fibroblast cell number, such as micropatterning, may improve performance of these bioreactors.

Another approach, presented by Griffith et al. (66) and Kim et al. (118), incorporates use of cocultures in a perfused array of 3-dimensional synthetic biodegradable polymer scaffolds. Endothelial cells (bovine) or rat nonparenchymal cells were used in conjunction with rat hepatocytes. In these studies, morphology and function of perfused systems were compared to those under static conditions; however, the role of nonparenchymal cells in induction of liver-specific function is not specifically addressed. The authors suggest that reorganization of cell populations within 3-dimensional channels may produce physiological 'bridging structures' akin to hepatic cords. Although the formation of these structures may increase available surface area for mass transfer, there is no clear evidence that increased hepatocellular function will result. *In vivo*, endothelial cells line hepatic sinusoids; however, efficient mass transfer between the blood and hepatocytes is maintained by fenestrations in the endothelium as well as a Space of Disse with minimal extracellular matrix to act as a diffusional barrier. In fact, even in the setting of nonparenchymal cell 'sorting' to the outside of 'bridging structures', the resulting structure may or may not recapitulate these critical features. Perhaps the endothelium offers the potential to incorporate additional functionality into the bioreactor (i.e., nonthrombogenic surface or responsiveness to biochemical stimuli); however, these aspects of endothelial function have not been explored in this setting. Thus, the benefit of nonparenchymal cells in this system has yet to be demonstrated. Indeed, the role of homotypic and heterotypic interactions in modulating hepatocellular function and reorganization in this model system will be important to systematically investigate for effective bioreactor design.

Finally, Bhatia et al. (74, 75, 85, 95) have recently conducted studies of the role homotypic and heterotypic cell interactions in hepatocyte cocultures toward design of a multiplate micropatterned bioreactor with rat hepatocytes and murine 3T3-J2 fibroblasts. Studies indicated that the murine embryonic fibroblast cell line, 3T3-J2, is superior to other nonparenchymal cells in its ability to induce liver-specific functions in isolated rat hepatocytes (3- to 15 fold increased albumin secretion per hepatocyte). Therefore, use of this cell line will decrease the required hepatocyte cell mass, a critical limitation found in the use of isolated cells in bioreactors, by ~10-fold. The use of nonparenchymal cells in a multiplate reactor configuration creates a paradox: on the one hand, nonparenchymal cells occupy substrate surface area that could be dedicated to hepatocyte adhesion, yet they also provide the necessary cues to induce hepatocellular function. The effectiveness of nonparenchymal cells was maximized at relatively large nonparenchymal to parenchymal ratios (i.e., 6:1); however, in randomly distributed cocultures, a 12-fold reduction of fibroblast number resulted in 13-fold reduction of hepatocellular pattern. Use of micropatterning techniques and polymeric elastomers to preserve heterotypic interactions generated micropatterned cocultures with only a 50% of maximal albumin secretion with a 12-fold reduction in fibroblast number (95). Thus, use of micropatterning techniques to optimize the cell ratio in a proposed bioreactor resulted in design criteria for maximal hepatocellular function per unit area at a ratio of 1:1. Finally, these studies showed that the induction of liver-specific functions correlated with the extent of initial heterotypic interface. Indeed, 500 μm hepatocyte islands showed signs of central diminished liver-specific function (Fig. 8), suggesting that smaller hepatocyte islands will ultimately prove most useful. Thus, in this setting, micropatterning techniques allowed significant improvements in the design of a proposed hepatocyte coculture bioreactor.

Given the importance of initial heterotypic cell interaction and the likelihood that heterotypic cell contact is necessary to induce stable liver-specific functions in cocultures, some preliminary design criteria for a BAL can be established. A comparison of various nonparenchymal cell types indicates the superiority of murine 3T3-J2 fibroblasts over other cell types. In addition, evidence that the induction signal is linked to the nonparenchymal surface and has a limited distance of propagation implies that coculture configurations in a BAL device should maximize heterotypic interactions and, if possible, allow a large proportion of hepatocytes to remain within three to four cell widths from the heterotypic interface. Conversely, bioreactor design may be optimized by balancing hepatocellular function and surface area available for cell seeding. Since nonparenchymal cells occupy precious surface area for mass transfer, yet provide important cues for induction of hepatocyte function, guidelines on effective ratios of cell populations should be included. These criteria and others that can be derived from similar studies will provide design guidelines that may be fulfilled in the future by incorporating both conventional as well as novel cell culture technologies. Ultimately, a better understanding of the role of

heterotypic and homotypic interactions in coculture based bioreactors will improve efficiency, require less hepatocellular mass, reduce cost, and increase the lifetime of these devices.

SUMMARY

In this review, we have presented a summary of various approaches to the cocultivation of primary hepatocytes and nonparenchymal cells. The model systems, influence on liver-specific functions, proposed mechanisms of cell–cell interaction, and clinical applications of hepatocyte cocultures were detailed. Recent advances in microfabrication techniques allowed novel studies of the role of the heterotypic interface and the ratio of cell populations as independent variables, resulting in some new insights on the complex modes of cell–cell communication in these cocultures. Future improvements in microfabrication technology will allow these approaches to be tailored to explore specific hypotheses on a broad range of materials, with a variety of biomolecules and an achievable spatial resolution in the submicron range. Furthermore, some preliminary design criteria were determined for use of cocultures in bioreactors for clinical support of liver failure. In addition to their utility in bioreactors for clinical support, hepatocyte cocultures will have applications both in fundamental studies of cell communication, organogenesis, and physiology as well as development of functional tissue constructs for medical applications. In the area of tissue engineering, the ability to modulate function of multicellular systems by manipulation of the spatial relationship between cell populations will facilitate more effective *in vitro* reconstruction of liver, skin, vascular grafts, muscle, and many other tissues. FJ

This study was supported in part by the National Institutes of Health (DK 3371 and RR 13322).

REFERENCES

1. van Breemen, C., Skarsgard, P., Laher, I., McManus, B., and Wang, X. (1997) Endothelium-smooth muscle interactions in blood vessels. *Clin. Exp. Pharmacol. Physiol.* **24,** 989–992
2. Clark, B. R., and Keating, A. (1995) Biology of bone marrow stroma. *Ann. N.Y. Acad. Sci.* **770,** 70–78
3. Davies, P. F. (1986) Biology of disease: vascular cell interactions with special reference to the pathogenesis of atherosclerosis. *Lab. Invest.* **55,** 5–24
4. Grinnell, A. D. (1995) Dynamics of nerve-muscle interaction in developing and mature neuromuscular junctions. *Physiol. Rev.* **75,** 789–834
5. Camps, J. L., Chang, S. M, Hsu, T. C., Freeman, M. R., Hong, S. J., Zhau, H. E, von Eschenbach, A. C., and Chung, L. W. (1990) Fibroblast-mediated acceleration of human epithelial tumor growth *in vivo. Proc. Natl. Acad. Sci. USA* **87,** 75–79
6. Hornby, A. E., and Cullen, K. J. (1995) In *Epithelial–Mesenchymal Interactions in Cancer* (Goldberg, I .D., and Rosen, E. M., eds) pp. 249–272, Birkhauser, Verlag
7. Aufderheide, E., Chiquet-Ehrismann, R., and Ekblom, P. (1987) Epithelial-mesenchymal interactions in the developing kidney lead to expression of terascin in the mesenchyme. *J. Cell Biol.* **105,** 599–608
8. Taderera, J. V. (1967) Control of lung differentiation *in vitro. Dev. Biol.* **16,** 489–512
9. Grinnell, F. (1992) Wound repair, keratinocyte activation and integrin modulation. *J. Cell Sci.* **101,** 1–5
10. Brown, L. F., Yeo, K. T., Berse, B., Yeo, T. K., Senger, D. R., Dvorak, H. F., and Van De Water, L. (1992) Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. *J. Exp. Med.* **176,** 1375–1379
11. Morgan, J. R., and Yarmush, J. R. (1997) Bioengineered skin substitutes. *Sci. Med.* **4,** 6–16
12. L'Heureux, N., Paquet, S., Labbe, R., Germain, L., and Auger, F.A. (1998) A completely biological tissue-engineered human blood vessel. *FASEB J.* **12,** 1331–1340
13. Houssaint, E. (1990) Differentiation of the mouse hepatic primordium. I. an analysis of tissue interactions in hepatocyte differentiation. *Cell Differ.* **9,** 269–279
14. Le Douarin, N. M. (1975) An experimental analysis of liver development. *Med. Biol.* **53,** 427–455
15. Gebhardt, R., and Mecke, D. (1983) Heterogeneous distribution of glutamine synthetase among rat liver parenchymal cell *in situ* in primary culture. *EMBO J.* **2,** 567–570
16. Olson, M. J., Mancini, M. A., Venkatachalam, M. A., and Roy, A. K. (1990) Hepatocyte cytodifferentiation and cell-to-cell communication. In *Cell Intercommunication* (De Mello, W. C., ed) pp. 71–92, CRC Press, Boca Raton, Florida
17. Michalopoulos, G. K., and DeFrances, M. C. (1997) Liver Regeneration. *Science* **276,** 60–66
18. Sadler, T. W. (1990) In *Langman's Medical Embryology* (Gardner, J. N., ed) pp.222–224, Williams & Wilkins, Baltimore, Maryland
19. Gilbert, S.F. (1991) Early vertebrate development: mesoderm and endoderm. In *Developmental Biology*, pp. 201–246, Sinauer Associates, Sunderland, Massachusetts
20. Bezerra, J. A. (1998) Liver development: a paradigm for hepatobiliary disease in later life. *Semin. Liver Dis.* **18,** 203–216
21. Cereghini, S. (1996) Liver-enriched transcription factors and hepatocyte differentiation. *FASEB J.* **10,** 267–282
22. Kaplowitz, N. (1992) Structure and function of the liver. In *Liver and Biliary Diseases* (Kaplowitz, N., ed) pp. 5, Williams & Wilkins, Baltimore, Maryland
23. Monden, K., Arii, S., Itai, S., Sasaoki, T., Adachi, Y., Funaki, N., and Tobe, T. (1991) Enhancement of hepatic macrophages in septic rats and their inhibitory effect on hepatocyte function. *J. Surg. Res.* **50,** 72–76
24. West, M. A., Manthei, R., and Bubrick, M. P. (1993) Autoregulation of hepatic macrophage activation in sepsis. *J. Trauma* **34,** 473–479
25. Langenbach, R., Freed, H. J., and Huberman, E. (1978) Liver cell-mediated mutagenesis of mammalian cells by liver carcinogens. *Proc. Natl. Acad. Sci. USA* **75,** 2864–2867
26. Michalopoulos, G., Strom, S. C., Kligerman, A. D., Irons, G. P., and Novicki, D. L. (1981) Mutagenesis induced by procarcinogens at the hypoxanthine-guanine phosphoribosyl transferase locus of human fibroblasts cocultured with rat hepatocytes. *Cancer Res.* **41,** 1873–1878
27. Strom, S., Kligerman, A. D., and Michalopoulos G. (1981) Comparisons of the effects of chemical carcinogens in mixed cultures of rat hepatocytes and human fibroblasts. *Carcinogenesis* **2,** 709–715
28. Jongen, W. M., Sijtsma, S. R., Zwijsen, R. M., and Temmink, J. H. (1987) A co-cultivation system consisting of primary chick embryo hepatocytes and V79 Chinese hamster cells as a model for metabolic cooperation studies. *Carcinogenesis* **8,** 767–772
29. Mendoza-Figueroa, T., Hernandez, A., and Lopez, M. L. (1989) Triglyceride accumulation in long-term cultures of adult rat hepatocytes by chronic exposure to Aroclor 1254. *J. Toxicol. Environ. Health* **26,** 293–308
30. Guillouzo, A., Morel, F., Fardel, O., and Meunier, B. (1993) Use of human hepatocyte cultures for drug metabolism studies. *Toxicology* **82,** 209–219

31. Mertens, K., Rogiers, V., and Vercruysse, A. (1993) Glutathione dependent detoxication in adult rat hepatocytes under various culture conditions. *Arch. Toxicol.* **67,** 680–685
32. De La Vega, F. M., and Mendoza-Figueroa, T. (1991) Dimethyl sulfoxide enhances lipid synthesis and secretion by long-term cultures of adult rat hepatocytes. *Biochimie (Paris)* **73,** 621–624
33. Rincon-Sanchez, A. R., Hernandez, A., Lourdes Lopez, M. de, and Mendoza-Figueroa, T. (1992) Synthesis and secretion of lipids by long-term cultures of female rat hepatocytes *Biol. Cell* **76,** 131–138
34. Guillouzo, A., Delers, F., Clement, B., Bernard, N., and Engler, R. (1984) Long term production of acute-phase proteins by adult rat hepatocytes co-cultured with another liver cell type in serum-free medium *Biochem. Biophys. Res. Commun.* **120,** 311–317
35. Lebreton, J. P., Daveau, M., Hiron, M., Fontaine, M., Biou, D., Gilbert, D., and Guguen-Guillouzo, C. (1986) Long-term biosynthesis of complement component C3 and alpha-1 acid glycoprotein by adult rat hepatocytes in a co-culture system with an epithelial liver cell-type. *Biochem. J.* **235,** 421–427
36. Conner, J., Vallet-Collom, I., Daveau, M., Delers, F., Hiron, M., Lebreton, J. P., and Guillouzo, A. (1990) Acute-phase-response induction in rat hepatocytes co-cultured with rat liver epithelial cells. *Biochem. J.* **266,** 683–688
37. Clement, B., Guguen-Guillouzo, C., Grimaud, J., Bissel, M., and Guillouzo, A. (1988) Effect of hydrocortisone on deposition of types I and IV collagen in primary culture of rat hepatocytes *Cell. Mol. Biol.* **34,** 449–460
38. Langenbach, R., Malick, L., Tompa, A., Kuszynski, C., Freed, H., and Huberman, E. (1979) Maintenance of adult rat hepatocytes on c3H/10T1/2 cells. *Cancer Res.* **39,** 3509–3514
39. Guguen-Guillouzo, C., Clement, B., Baffet, G., Beaumont, C., Morel-Chany, E., Glaise, D., and Guillouzo, A. (1983) Maintenance and reversibility of active albumin secretion by adult rat hepatocytes co-cultured with another liver epithelial cell type. *Exp. Cell Res.* **143,** 47–54
40. Mesnil, M., Fraslin, J., Piccoli, C., Yamasaki, H., and Guguen-Guillouzo, C. (1987) Cell contact but not junctional communication (dye coupling) with biliary epithelial cells is required for hepatocytes to maintain differentiated functions. *Exp. Cell Res.* **173,** 524–533
41. Fraslin, J. M., Kneip, B., Vaulont, S., d. Glaise, Muunich, A., and Guguen-Guillouzo, C. (1985) Dependence of hepatocyte-specific gene expression on cell–cell interactions in primary culture. *EMBO J.* **4,** 2487–2491
42. Schrode, W., Mecke, D., and Gebhardt, R. (1991) Induction of glutamine synthetase in periportal hepatocytes by cocultivation with a liver epithelial cell line. *Eur. J. Cell Biol.* **53,** 35–41
43. Vandenberghe, Y., Morel, F., Pemble, S., Taylor, J. B., Rogiers, V., Ratanasavanh, D, Vercruysse, A., Ketterer, B., and Guillouzo, A. (1990) Changes in expression of mRNA coding for glutathione S-transferase subunits 1–2 and 7 in cultured rat hepatocytes. *Mol. Pharmacol.* **37,** 372–376
44. Vandenberghe, Y., Tee, L., Rogiers, V., and Yeoh, G. (1992) Transcriptional- and post-transcriptional-dependent regulation of glutathione S-transferase expression in rat hepatocytes as a function of culture conditions. *FEBS Lett.* **313,** 155–159
45. Utesch, D., Molitor, E., Platt, K.-L., and Oesch, F. (1991) Differential stabilization of cytochrome p-450 isoenzymes in primary cultures of adult rat liver parenchymal cells. *In Vitro Cell Dev. Biol.* **27A**, 858–863
46. Lerche, C., Fautrel, A., Shaw, P. M., Glaise, D., Ballet, F., Guillouzo, A., and Corcos, L. (1997) Regulation of the major detoxication functions by phenobarbital and 3-methylcholanthrene in co-cultures of rat hepatocytes and liver epithelial cells. *Eur. J. Biochem.* **244,** 98–106
47. Loreal, O., Levavasseur, F., Fromaget, C., Gros, D., Guillouzo, A., and Clement, B. (1993) Cooperation of Ito cells and hepatocytes in the deposition of an extracellular matrix *in vitro*. *Am. J. Pathol.* **143,** 538–544
48. Bedossa, P., Houglum, K., Trautwein, C., Holstege, A., and Chojkier, M. (1994) Stimulation of collagen alpha 1(I) gene expression is associated with lipid peroxidation in hepatocellular injury: a link to tissue fibrosis? *Hepatology* **19,** 1262–1271
49. Rojkind, M., Novikoff, P. M., Greenwel, P., Rubin, J., Rojas-Valencia, L., Campos de Carvalho, A., Stocker, R., Spray, D., Hertzberg, E. L., and Wolkof, A. W. (1995) Characterization and functional studies of rat liver fat-storing cell line and freshly isolated hepatocyte coculture system. *Am. J. Pathol.* **146,** 1508–1520
50. Morin, O., and Normand, C. (1986) Long-term maintenance of hepatocyte functional activity in co-culture: requirements for sinusoidal endothelial cells and dexamethasone. *J. Cell. Physiol.* **129,** 103–110
51. Goulet, F., Normand, C., and Morin, O. (1988) Cellular interactions promote tissue-specific function, biomatrix deposition and junctional communication of primary cultured hepatocytes. *Hepatology* **8,** 1010–1018
52. Sibille, J. C., Kondo, H., and Aisen, P. (1988) Interactions between isolated hepatocytes and Kupffer cells in iron metabolism: a possible role for ferritin as an iron carrier protein. *Hepatology* **8,** 296–301
53. Billiar, T. R., Maddaus, M. A., West, M. A., Dunn, D. L., and Simmons, R. L. (1988) The role of intestinal flora on the interactions between nonparenchymal cells and hepatocytes in coculture. *J. Surg. Res.* **44,** 397–403
54. Billiar, T. R., Curran, R. D., West, M. A., Hofmann, K., and Simmons, R. L. (1989) Kupffer cell cytotoxicity to hepatocytes in coculture requires L-arginine. *Arch. Surg.* **124,** 1416–1420
55. Billiar, T. R., Curran, R. D., Stuehr, D. J., West, M. A., Bentz, B. G., and Simmons, R. L. (1989) An L-arginine-dependent mechanism mediates Kupffer cell inhibition of hepatocyte protein synthesis *in vitro*. *J. Exp Med* **1,** 169(4):1467–1472
56. Matsuo, R., Ukida, M., Nishikawa, Y., Omori, N., and Tsuji, T. (1992) The role of Kupffer cells in complement in D-galactosamine/lipopolysaccharide-induced hepatic injury of rats. *Acta Med Okayama* **46,** 345–354
57. Fry, J. R., and Bridges, J. W. (1977) A novel mixed hepatocyte-fibroblast culture system and its use as a test for metabolism-mediated cytotoxicity. *Biochem. Pharmacol.* **26,** 969–973
58. Shimaoka, S., Nakamura, T., and Ichihara, A. (1987) Stimulation of growth of primary cultured adult rat hepatocytes without growth factors by coculture with nonparenchymal liver cells. *Exp. Cell Res.* **172,** 228–242
59. Villafuerte, B. C., Koop, B. L., Pao, C.I., Gu, L., Birdsong, G. G., and Phillips, L. S. (1994) Coculture of primary rat hepatocytes and nonparenchymal cells permits expression of insulin-like growth factor binding protein-3 *in vitro*. *Endocrinology* **134,** 2044–2050
60. Mendoza-Figueroa, T., Hernandez, A., De Lourdes Lopez, M., and Kuri-Harcuch, W. (1988). Intracytoplasmic triglyceride accumulation produced by dexamethasone in adult rat hepatocytes cultivated on 3T3 cells. *Toxicology* **52,** 273–286
61. Kuri-Harcuch, W., and Mendoza-Figueroa, T. (1989) Cultivation of adult rat hepatocytes on 3T3 cells: expression of various liver differentiated functions. *Differentiation* **41,** 148–157
62. Donato, M. T., Gómez-Lechón, M. J., and Castell, J. V. (1990) Drug metabolizing enzymes in rat hepatocytes co-cultured with cell lines. *In Vitro Cell Dev. Biol.* **26,** 1057–1062
63. Hernandez, A., Lourdes Lopez, M., Chamorro, G., and Mendoza-Figueroa, T. (1993) Inhibition of lipid synthesis and secretion in long-term cultures of adult rat hepatocytes by alpha-asarone. *Planta Med.* **59,** 121–124
64. Lourdes Lopez, Ma., Hernandez, A., Chamorro, G., and Mendoza-Figueroa, T. (1993) Alpha-asorone toxicity in long-term cultures of adult rat hepatocytes. *Planta Med.* **59,** 115–120
65. Donato, M. T., Castell, J. V., and Gomez-Lechon, M. J. (1991) Co-cultures of hepatocytes with epithelial-like cell lines: expression of drug-biotransformation activities by *hepatocytes Cell Biol. Toxicol.* **7,** 1–14
66. Griffith, L. G., Wu, B., Cima, M. J., Powers, M. J., Chaignaud, B., and Vacanti, J. P. (1997) *In vitro* organogenesis of liver tissue. *Ann. N.Y. Acad. Sci.* **831,** 382–397
67. Michalopoulos, G., Russell, F., and Biles, C. (1979) Primary cultures of hepatocytes on human fibroblasts. *In Vitro* **15,** 796–806
68. Guguen-Guillouzo, C., Clement, B., Lescoat, G., Glaise, D., and Guillouzo, A. (1984) Modulation of human fetal hepatocyte survival and differentiation by interactions with a rat liver epithelial cell line. *Dev. Biol.* **105,** 211–220
69. Clement, B., Guguen-Guillouzo, C., Campon, J.-P, Glaise, D., Bourel, M., and Guillouzo, A. (1984) Long-term co-cultures of adult human hepatocytes with rat liver epithelial cells: modu-

lation of albumin secretion and accumulation of extracellular material. *Hepatology* **4,** 373–380

70. Lescoat, G., Theze, N., Clement, B., Guillouzo, A., and Guguen-Guillouzo, C. (1985) Modulation of fetal and neonatal rat hepatocyte functional activity by glucocorticoids in coculture. *Cell Differ.* **16,** 259–268
71. Talbot, N. C., Pursel, V. G., Rexroad, C. E., Jr., Caperna, T. J., Powell, A. M., and Stone, R. T. (1994) Colony isolation and secondary culture of fetal porcine hepatocytes on STO feeder cells. *In Vitro Cell Dev. Biol. Anim.* **30A,** 851–858
72. Morin, O, Goulet, F., and Normand, C. (1988) Liver sinusoidal endothelial cells: isolation, purification, characterization and interaction with hepatocytes. In *Cell Biology Reviews* (Morin, O, Goulet, F., and Normand, C. eds) Vol. 15, pp. 1–73, Springer International, New York
73. Berthiaume, F., Moghe, P. V., Toner, M., and Yarmush, M. L. (1996) Effect of extracellular matrix topology on cell structure, function, and physiological responsiveness: hepatocytes cultured in a sandwich configuration. *FASEB J.* **10,** 1471–1484
74. Bhatia S. N., Yarmush M. L., and Toner M. (1997) Engineered substrates for controlling cell–cell Interactions. *Adv. Heat Mass Transfer Biotech. ASME* HTD-355**,** BED-**37,** 99–103
75. Bhatia, S. N., Balis, U. J., Yarmush, M. L., and Toner, M. (1998) Probing heterotypic cell interactions: hepatocyte function in microfabricated co-cultures. *J. Biomater. Sci. Polymer Ed.* **9,** 1137–1160
76. Begue, J. M., Guguen-Guillouzo, C., Pasdeloup, N., and Guillouzo, A. (1984) Prolonged maintenance of active cytochrome P-450 in adult rat hepatocytes co-cultured with another liver cell type. *Hepatology* **4,** 839–842
77. Guillouzo, A. (1986) Use of isolated and cultured hepatocytes for xenobiotic metabolism and cytotoxicity studies. In *Isolated and Cultured Hepatocytes* (Guillouzo, A., and Guguen-Guillouzo, C. eds) pp. 313–332, John Libbery Eurotext, France
78. Donato, M. T., Gomez-Lechon, M. J., and Castell, J. V. (1991) Rat hepatocytes cultured on MS cells: expression of biotransformation and metabolic activity. *Toxicol. In Vitro* **5,** 435–438
79. Donato, M. T., Castell, J. V., and Gomez-Lechon, M. J. (1994) Cytochrome P450 activities in pure and co-cultured rat hepatocytes: effects of model inducers. *In Vitro Cell Dev. Biol.* **30A,** 825–832
80. Perrot, N., Chesne, C., De Waziers, I., Conner, J., Beaune, P. H., and Guillouzo, A. (1991) Effects of ethanol and clofibrate on expression of cytochrome P-450 enzymes and epoxide hydrolase in cultures and cocultures of rat hepatocytes. *Eur. J. Biochem.* **200,** 255–261
81. Akrawi, M., Rogiers, V., Vandenberghe, Y., Palmer, C. N., Vercruysse, A., Shephard, E. A., and Phillips, I. R. (1993) Maintenance and induction in co-cultured rat hepatocytes of components of the cytochrome P450-mediated mono-oxygenase. *Biochem. Pharmacol.* **45,** 1583–1591
82. Naughton, B. A., Roman, J. S., Sibanda, B., Weintraub, J. P., and Kamali, V. (1994) Stereotypic culture systems for liver and bone marrow: evidence for the development of functional tissue *in vitro* and following implantation *in vivo. Biotech. Bioengin.* **43,** 810–825
83. Vandenberghe, Y., Ratanasavanh, D., Glaise, D., and Guillouzo, A. (1988) Influence of medium composition and culture conditions on glutathione S-transferase activity in adult rat hepatocytes during culture. *In Vitro Cell Dev. Biol.* **24,** 281–288
84. Vandenberghe, Y., Tee, L., Morel, F., Rogiers, V., Guillouzo, A., and Yeoh, G. (1991) Modulation of GST gene expression by phenobarbital in cultured adult rat hepatocytes *Fed. Eur. Biochem. Soc.* **284,** 103–108
85. Bhatia, S. N., Yarmush, M. L., and Toner, M. (1997) Controlling homotypic vs heterotypic interactions by micropatterning: co-cultures of hepatocytes and 3T3 fibroblasts *J. Biomed. Materials Res.* **34,** 189–199
86. Baffet, G., Loyer, P., Glaise, D., Corlu, A., Etienne, P., and Guguen-Guillouzo, C. (1991) Distinct effects of cell–cell communication and corticosteroids on the synthesis and distribution of cytokeratins in cultured rat hepatocytes. *J. Cell Sci.* **99,** 609–615
87. Fujita, M., Spray, D. C., Choi, H., Saez, J. C., Watanabe, T., Rosenberg, L. C., Hertzberg, E. L., and Reid, L. M. (1987) Glycosaminoglycans and proteoglycans induce gap junction expression and restore transcription of tissue-specific mRNAs in primary liver cultures. *Hepatology* **7** (Suppl. 1), 1S–9S
88. Corlu, A., Kneip, B., Lhadi, C., Leray, G., Glaise, D., Baffet, G., Bourel, D., and Guguen-Guillouzo, C. (1991) A plasma membrane protein is involved in cell contact-mediated regulation of tissue-specific genes in adult hepatocytes. *J. Cell Biol.* **115,** 505–515
89. Corlu, A., Ilyin, G. P., Gerard, N., Kneip, B., Rissel, M., Jegou, B., and Guguen-Guillouzo C. (1994) Tissue distribution of liver regulating protein. Evidence for a cell recognition signal common to liver, pancreas, gonads, and hemopoietic tissues. *Am. J. Pathol.* **145,** 715–727
90. Clemens, M. G., Bauer, M., Gingalewski, C., Miescher, E., and Zhang, J. (1994) Hepatic intercellular communication in shock and inflammation. *Shock* **2,** 1–9
91. Jongen, W. M., van der Leede, B. J., Chang, C. C., and Trosko, J. E. (1987) The transport of reactive intermediates in a co-cultivation system: the role of intercellular communication. *Carcinogenesis* **8,** 1239–1243
92. Traiser, M., Diener, B., Utesch, D., and Oesch, F. (1995) The gap junctional intercellular communication is no prerequisite for the stabilization of xenobiotic metabolizing enzyme activities in primary rat liver parenchymal cells *in vitro. In Vitro Toxicol.* **31,** 266–273
93. Reid, L. M., and Jefferson, D. M. (1984) Culturing hepatocytes and other differentiated cells. *Hepatology* **4,** 548–559
94. Guguen-Guillouzo, C. (1986) Role of homotypic and heterotypic cell interactions in expression of specific functions by cultured hepatocytes. In *Isolated and Cultured Hepatocytes* (Guillouzo, A., and Guguen-Guillouzo, C., eds) pp. 259–284, John Libbery Eurotext, France
95. Bhatia, S. N., Balis, U., Yarmush, M. L., and Toner, M. (1998) Microfabrication of hepatocyte/fibroblast co-cultures: role of homotypic cell interactions. *Biotech. Prog.* **14,** 378–387
96. Bhatia, S. N., and Toner, M. (1998) Micropatterning cells in tissue engineering. In *Methods in Molecular Medicine, Vol 18: Tissue Engineering Methods and Protocols* (Morgan, J., and Yarmush, M. L., eds) pp. 349–363, Humana Press, Clifton, N.J.
97. Hammarback, J. A., McCarthy, J. B., Palm, S. L., Furcht, L. T., and Letourneau, P. C. (1988) Growth cone guidance by substrate-bound laminin pathways is correlated with neuron-to-pathway adhesivity. *Dev. Biol.* **126,** 29–39
98. Hammarback, J. A., and Letourneau, P. C. (1986) Neurite extension across regions of low cell-substratum adhesivity: implications for the guidepost hypothesis of axonal pathfinding. *Dev. Biol.* **117,** 655–671
99. Clark, P., Britland, S., and Connolly, P. (1993) Growth cone guidance and neuron morphology on micropatterned laminin. *J. Cell Sci.* **105,** 203–212
100. Chen, C. S., Mrksich, M., Huang, S., Whitesides, G. M., and Ingber, D. E. (1997) Geometric control of cell life and death. *Science* **276,** 1425–1428
101. Singhvi, R., Kumar, A., Lopez, G. P., Stephanopoulos, G. N., Wang, D. I. C, Whitesides, G. M., and Ingber, D. E. (1994) Engineering cell shape and function. *Science* **264,** 696–698
102. Hoch, H. C., Staples, R. C., Whitehead, B., Comeau, J., and Wolf, E. D. (1987) Signaling for growth orientation and cell differentiation by surface topography in *Uromyces. Science* **235,** 1659–1662
103. Lom, B., Healy, K. E., and Hockberger, P. E. (1993) A versatile technique for patterning biomolecules onto glass coverslips. *J. Neurosci. Methods* **50,** 385–397
104. Britland, S., Perez-Arnaud, E., Clark, P., McGinn, B., Connolly, P., and Moores, G. (1992) Micropatterning proteins and synthetic peptides on solid supports: a novel application for microelectronics fabrication technology. *Biotech. Prog.* **8,** 155–160
105. Delamarche, E., Bernard, A., Schmid, H., Michel, B., and Biebuyck, H. (1998) Patterned delivery of immunoglobulins to surfaces using microfluidic networks. *Science* **276,** 779–781
106. Delamarche, E., Bernard, A., Schmid, H., Bietsch, A., Michel, B., and Biebuyck, H. (1998) Microfluidic networks for chemical patterning of substrates: design and application to bioassays. *J. Am. Chem. Soc.* **120,** 500–508
107. Folch, A., and Toner, M. (1998) Cellular micropatterns on biocompatible materials *Biotechnol. Prog.* **14,** 388–392

108. Dunn, J. C. Y., Tompkins, R. G., and Yarmush, M. L. (1992) Hepatocytes in collagen sandwich: evidence for transcriptional and translational protein. *J. Cell Biol.* **116,** 1043–1053
109. Naughton, B. A. (1995) The importance of stromal cells. In *Biomedical Engineering Handbook* (Bronzino, J. D., ed) pp. 1710–1722, CRC Press, Boca Raton, Florida
110. Rodwell, V. W. (1993) Catabolism of Protein and amino acid metabolism. In *Harpers Biochemistry* (Murray, R. K., Granner, D. K., Mayer, P. A., and Rodwell, V. W., eds) pp. 293–302, Appleton and Lange, Norwalk, Connecticut
111. Baird, A., and Klagsbrun, M. (1991) The fibroblast growth factor family. *Cancer Cells* **3,** 239–243
112. Bergsteindottir, K., Kingston, A., Mirsky, R., and Jessen, K. R. (1991) Rat Schwann cells produce interleukin-1. *J. Neuroimmunol.* **34,** 15–23
113. Roberts, A. B., and Sporn, M. B. (1993) Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta). *Growth Factors* **8,** 1–9
114. Fini, M. E., Strissel, K. J., Girard, M. T., Mays, J. W., and Rinehart, W. B. (1994) Interleukin 1 alpha mediates collagenase synthesis. *J. Biol. Chem.* **269,** 11291–11298
115. Ruch, R., Guan, X., and Sigler, K. (1995) Inhibition of gap junctional intercellular communication and enhancement of growth in BALB/c 3T3 cells treated with connexin43 antisense oligonucleotides. *Mol. Carcinog.* **14,** 269–274
116. Gerlach, J. C., Schnoy, N., Encke, J., Smith, M. D., Muller, C., and Neuhaus, P. (1995) Improved hepatocyte *in vitro* maintenance in a culture model with woven multicompartment capillary systems: electron microscopy studies. *Hepatology* **22,** 546–552
117. Koike, M., Matsushita, M., Taguchi, K., and Uchino, J. (1996) Function of culturing monolayer hepatocytes by collagen gel coating and coculture with nonparenchymal cells. *Artificial Organs* **20,** 186–192
118. Kim, S. S., Utsonomiya, A., Koski, J. A., Wu, B. M., Cima, M. J., Sohn, J., Mukai, K., Griffith, L. G., and Vacanti, J. P. (1998) Survival and function of hepatocytes on a novel 3-dimensional synthetic biodegradable polymer scaffold with an intrinsic network of channels. *Ann. Surg.* **228,** 8–13
119. MacDonald, C., and Pitt, A. M. (1991) Rapid DNA quantitation using Hoechst 33258 dye in a multiwell fluorescent plate reader. *J. Cell Biol.* **115,** 79a–80a
120. Nakamura, T., Tomitaka, Y., and Ichihara, A. (1983) Density dependent growth control of adult rat hepatocytes in coculture. *J. Biochem.* **94,** 1029–1035