

Chapter 2

In Vitro Systems for Hepatotoxicity Testing

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Abstract

Hepatotoxicity is the most frequent reason of drug withdrawal from the market. Therefore, hepatocyte in vitro systems that predict human hepatotoxicity are of high importance. Although some progress has been achieved in predicting toxicity formation of major metabolites and enzyme induction (Hewitt et al., *Drug Metab Rev* 39:159–234, 2007; Hengstler et al., *Chem Biol Interact* 125:51–73, 2000) it is still difficult to reliably predict idiosyncratic drug-induced liver injury (iDILI), a particularly worrying form of hepatotoxicity that can arise from many commonly prescribed drugs (Godoy et al., *Arch Toxicol* 87:1315–1530, 2013; Amacher, *Expert Opin Drug Metab Toxicol* 8:335–347, 2012). This chapter describes currently available hepatocyte in vitro systems and their possibilities as well as limitations in studying hepatotoxicity and ADME.

Key words Hepatotoxicity, Hepatocytes, Kupffer cells, Stellate cells

1 Organization and Cellular Composition of the Liver

1.1 Lobular Architecture

To understand the possibilities and limitations of liver in vitro systems it is crucial to be aware of the organization principles of this organ. The smallest functional unit of the liver is the lobule (Fig. 1a). The human liver is composed of approximately one million lobules. Each lobule is supplied by branches of the portal vein which carries blood from the intestine (about 80 % of the liver's blood). Moreover, arterial blood is supplied by branches of the liver artery (about 20 %). The blood enters the lobules in the periphery, passes through microvessels where it is in close contact with hepatocytes, is finally drained off into the central veins, and leaves the liver by the hepatic vein. The oxygen concentration is about 13 % v/v (60–65 mmHg) in the periportal zone and drops to about 4 % v/v (30–35 mmHg) in the central vein [3].

1.2 Non-parenchymal Cells

Hepatocytes contribute about 60 % of the cells of the liver; 40 % are non-parenchymal cells, which closely cooperate with the hepatocytes (Fig. 1b): (1) Sinusoidal endothelial cells (SECs) line the

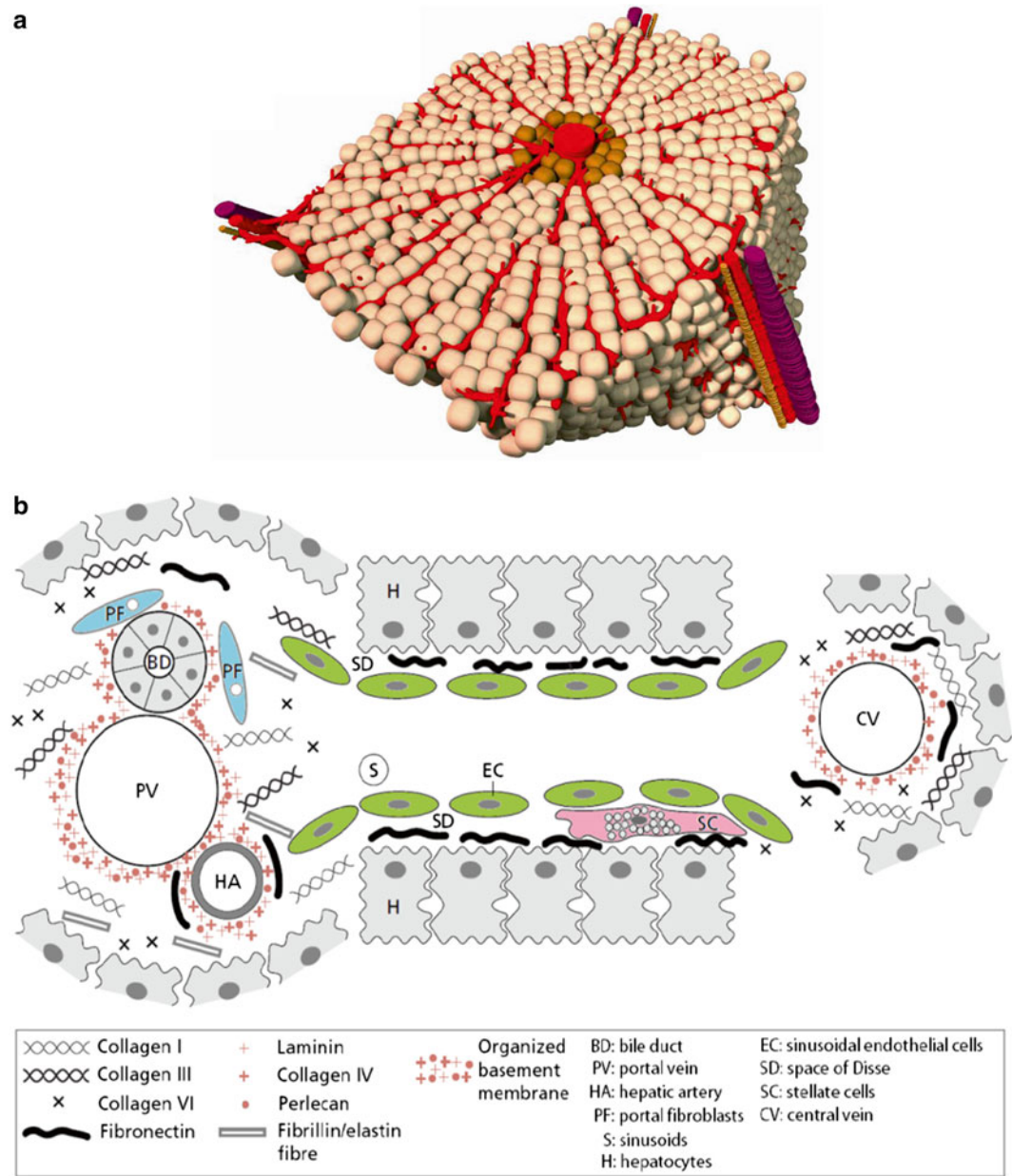


Fig. 1 Organization and cellular composition of the liver (from Godoy et al. [3]). **(a)** Overview over a liver lobule, the smallest functional unit to the liver. Only the sinusoidal but not the bile canalicular network is shown. **(b)** Non-parenchymal cell types and their position in the lobule. **(c)** Sinusoidal (red) and bile canalicular (green) vessel networks of the liver. The structures have been reconstructed from confocal laser scans of a mouse liver, Hoehme et al. [5]. **(d)** Zonation of liver functions along the lobule

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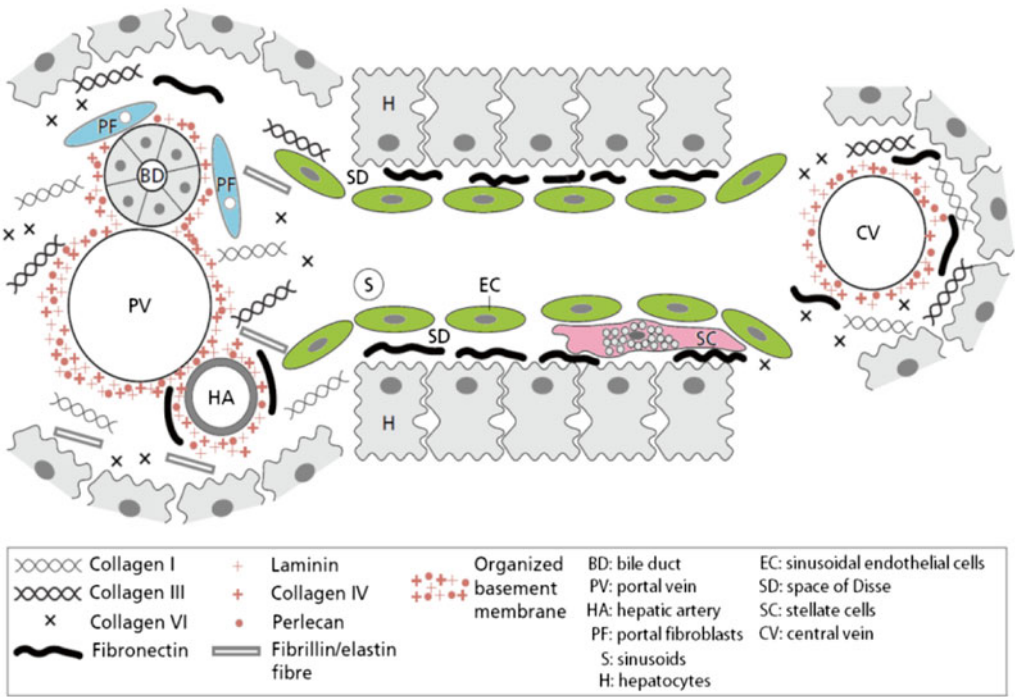
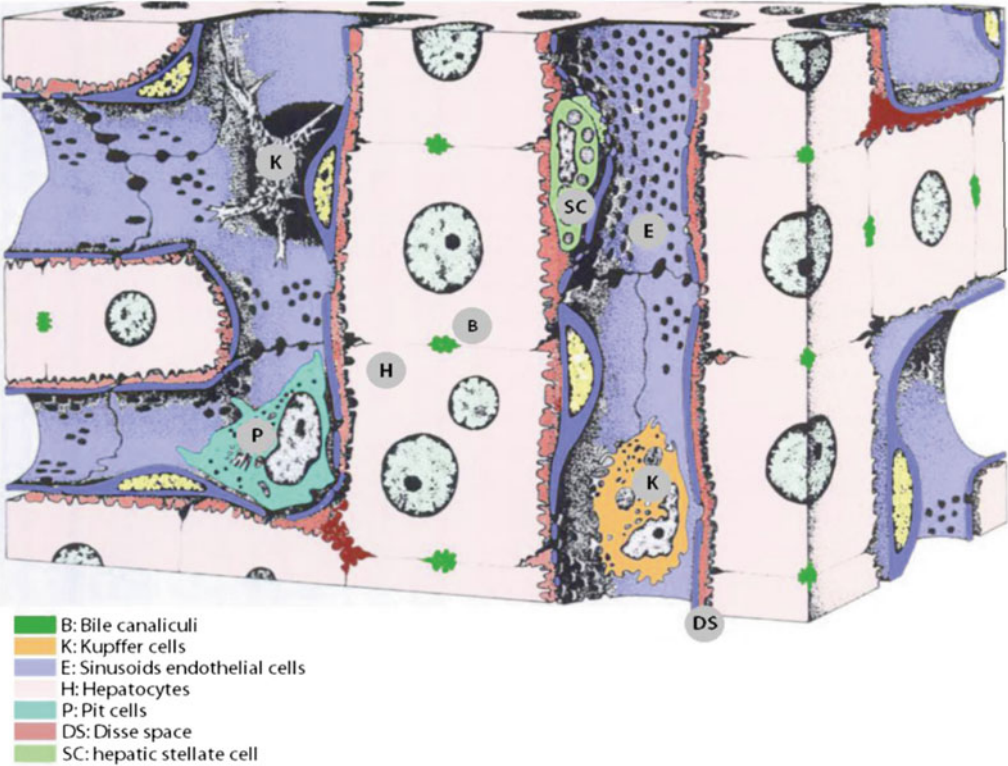


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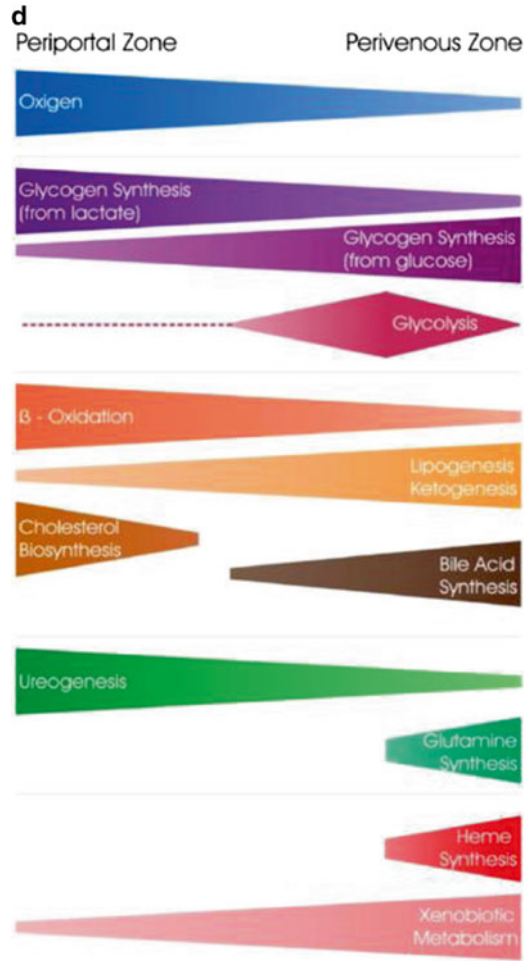


Fig. 1 (continued)

microvessels (sinusoids) of the liver lobule. Besides their function as fenestrated endothelial cells they also play a critical role in hepatotoxicity and regeneration [5, 6]. By secreting cytokines they influence the architecture of hepatocyte sheets which align along the sinusoids [5]. Moreover, SECs sense hepatocyte damage which in turn leads to secretion of cytokines (including HGF and Wnt factors) finally stimulating hepatocytes to proliferate. (2) Kupffer cells are resting macrophages which upon liver damage secrete pro- and anti-inflammatory cytokines. Although the role of Kupffer cells in hepatotoxicity is still discussed controversially it seems that they predominantly mediate hepatoprotective effects by induction of export pumps and by anti-inflammatory cytokines (review: Godoy et al. [3]). (3) Stellate cells (HSCs) reside in the space of Disse between SECs and hepatocytes. In normal liver they represent a storage site for vitamin A. After induction of liver injury they acquire a myofibroblast-like cell type which is involved in repair of damaged tissue. Upon repeated induction of liver damage

activated HSCs contribute to fibrosis and cirrhosis by excessive formation of scar tissue. (4) Infiltrating macrophages and their contribution to hepatotoxicity remain controversial. As reported for Kupffer cells they can produce pro-inflammatory cytokines, such as $\text{TNF}\alpha$ and $\text{IL-1}\beta$ [7]. However, they can also secrete the anti-inflammatory IL-10 , $\text{TGF}\beta$, and IL-18 -binding proteins [8]. In recent years it has become clear that “pro-” and “anti-inflammatory” macrophages exist, playing different roles after induction of liver damage. (5) Neutrophils are rapidly recruited to sites of inflammation [9]. They can contribute to tissue damage in cholestasis, ischemia/reperfusion, and endotoxic shock. However, their role in chemically induced liver damage is far from being understood (review: Godoy et al. [3]). (6) Natural killer cells (NK cells) are large granular lymphocytes that represent key components of the innate immune system. NK cells contribute to inflammation by releasing cytokines such as IFN gamma [10]. Hepatocytes seem to be particularly susceptible to the damaging effects of NK cells. They express relatively low levels of MHC class I molecules that inhibit NK cells [11]. Moreover, the liver contains a population of Ly-49 -deficient cells, an inhibitory receptor that recognizes MHC class I [11]. (7) Biliary epithelial cells (synonym: cholangiocytes) line the bile ducts. These cells are often targets in cholestatic liver diseases and hepatotoxicity.

1.3 Blood and Bile Flow

A key feature of the liver is the antidromic blood and bile flow system (Fig. 1c). Blood enters the lobules in the periphery, passes the sinusoids, and is drained off into the central vein. Bile is secreted by the hepatocytes into the apical bile canaliculi, which form a network organized parallel as well as perpendicular to the sinusoidal vessels. Bile flows to the bile ducts in the periportal field and is finally drained off into the gall bladder and the small intestine. The bile canalicular network is not composed of conventional vessels with endothelial cells. It is formed by the apical membrane of hepatocytes and thereby is a consequence of the polar structure of these cells which have an apical pole (facing the bile canaliculus) and a basolateral side (facing the sinusoid). The direct contact to bile leads to a high vulnerability of hepatocytes. Destruction of the apical hepatocyte membrane may lead to bile acids entering the cell, hepatocyte killing, and inflammation.

1.4 Zonation

Hepatocytes show a remarkable heterogeneity and functional specialization which systematically differs depending on the cell's position in the lobule (simplified overview: Fig. 1d). For hepatotoxicity it is particularly relevant that many cytochrome P450 enzymes (e.g., CYP2E1 and CYP3A4) are preferentially expressed in the center of the lobule. Since many hepatotoxic compounds such as paracetamol or CCl_4 are metabolically activated by these enzymes they lead to a specific pericentral pattern of hepatotoxicity.

1.5 Inevitable Limitations of Current Liver In Vitro Systems

This brief overview about liver physiology and hepatotoxicity remains incomplete and has been comprehensively summarized elsewhere [3]. However, this background is essential to understand the following inevitable limitations of hepatocyte in vitro systems:

- Despite some progress in this field, current liver in vitro systems do not yet sufficiently include communication of hepatocytes and non-parenchymal cells.
- With the exception of the isolated perfused liver current in vitro systems do not contain the antidromic blood and bile circulation systems. This may lead to cholestasis and inflammation in vitro.
- Current hepatocyte in vitro system, including 3D cultures and microtissues, do not yet form a lobular zonation.
- The highly specific liver tissue microenvironment tightly controls numerous factors ranging from local cytokine concentrations, cell contacts with NPCs, pH value, and oxygen as well as carbogen tension within a narrow range. When primary cells are taken out of their natural microenvironment they undergo manifold alterations, reflected, e.g., by up and downregulation of numerous genes. Currently, it is not yet possible to sufficiently simulate the complex in vivo microenvironment in cell culture.
- Although co-culture systems with hepatocytes and macrophages have been successfully established it is not yet possible to simulate in vitro hepatocyte-immune cell interactions.

1.6 Hepatocyte Isolation

Since several decades a principally identical hepatocyte isolation technique is recommended (published standard operation procedures: Godoy et al. [3]): This procedure is based on antegrade (via the portal vein) or retrograde (via the vena cava) perfusion, removing the blood in a first step followed by EGTA collagenase perfusion, release of enzymatically dissociated cells from the perfused liver tissue, washing of the cells, and enrichment of hepatocytes by centrifugation at low speed. In principle, similar techniques are used to isolate hepatocytes from rodents, dogs, pigs, and humans (Fig. 2). The major technical difference is that rodents (and rabbits) are perfused in situ (with the liver remaining in the animal), while for larger animals, including humans, resected pieces of the liver are perfused by cannulation of vessels at the cut surface. Currently, there is no alternative to the well-established “two-step EGTA collagenase” procedure to isolate primary hepatocytes for all types of in vitro systems. However, often experimentalists are not aware of one major problem associated with this procedure, the so-called burning hepatocyte phenomenon. Due to the perfusion/digestion procedure cytokines in the extracellular matrix become activated and numerous signaling pathways in hepatocytes are massively stimulated in freshly isolated hepatocytes. This is easily

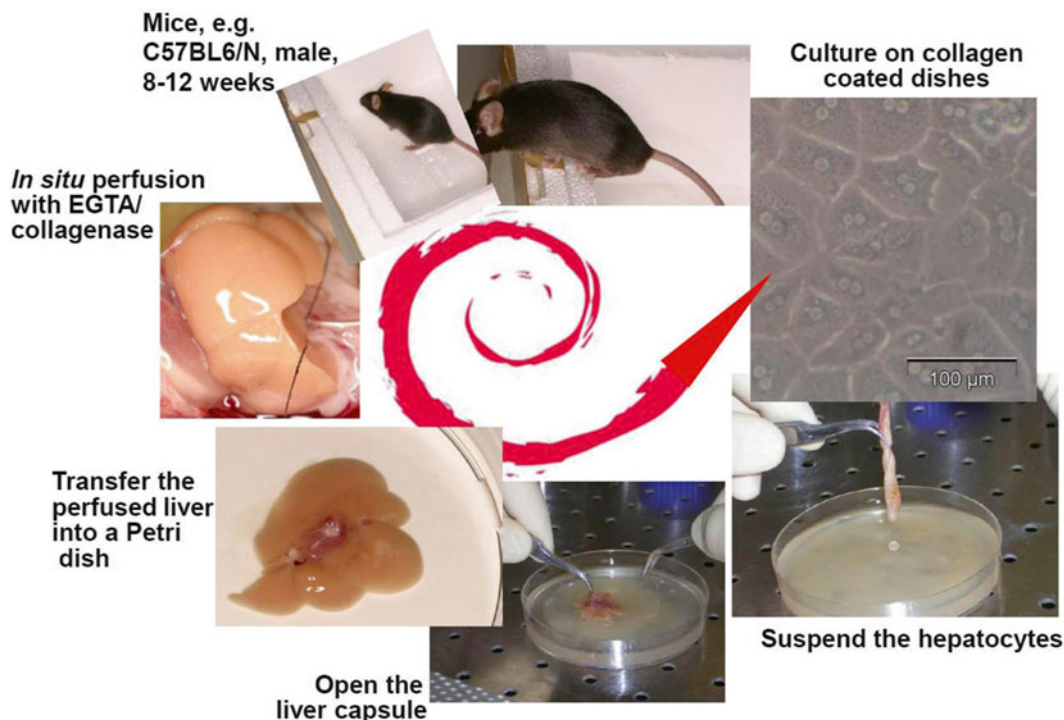


Fig. 2 Isolation of hepatocytes by the “two-step EGTA collagenase” procedure

analyzed by a simple Western blot of phosphorylated ERK 1/2 where freshly isolated hepatocytes show dramatically increased levels compared to liver tissue. However, the transcriptional (i.e., transcriptome) and metabolic features of these cells are quite similar to those of normal liver tissue [12]. Yet, it is likely that this “burning hepatocyte” phenomenon contributes to later alterations of the phenotype in culture. Therefore, a possible future development may be the addition of small-molecule inhibitor cocktails to the perfusion media. However, this is not yet part of the routinely used isolation protocol. In the following paragraphs we discuss how the isolated hepatocytes can be used for different forms of cell culture.

2 Hepatocyte Culture Systems

2.1 Hepatocytes Cultivated on Collagen-Coated Dishes

The traditional way to cultivate primary hepatocytes is on culture dishes coated with stiff and dry collagen that has been isolated from rat tails. This technique is also named 2D culture. 2D cultures are known to represent the in vitro system which most rapidly dedifferentiates. However, it should be considered that dedifferentiation shows major interspecies differences. While hepatocytes of rats and mice rapidly dedifferentiate showing features of epithelial

to mesenchymal transition (EMT) [13] this process is much slower for human hepatocytes. Despite their limitations 2D cultures are nevertheless successfully used for short-term studies (1–3 days) of enzyme induction and drug metabolism [14–18]. Moreover, subconfluent rodent hepatocytes in 2D cultures can easily be stimulated to proliferate using HGF and EGF and are therefore used to study proliferation-associated signaling [19]. 2D cultures of rodent hepatocytes have been used to understand the mechanisms why cultivated hepatocytes dedifferentiate. These studies have shown that 2D cultures induce the activation of two major signaling pathways, namely ERK 1/2 and Akt [13]. These studies demonstrated that dedifferentiation (i.e., EMT) is an active process driven by Ras/Mek/ERK signaling. Suppression of ERK 1/2 phosphorylation by inhibitors delays hepatocyte dedifferentiation [13]. Conversely, the increased Akt signaling promotes an apoptosis-resistant phenotype [13]. Noteworthy, the activation of these cultivation-dependent pathways can be largely prevented by using a soft, 3D extracellular matrix, such as the collagen sandwich technique, which is explained in detail in the following section.

2.2 Sandwich Cultures

Hepatocytes cultivated between two layers of soft gel collagen represent the most frequently used hepatocyte in vitro system. They establish an apical pole between the cells which contains bile canaliculi (Fig. 3). The hepatocyte membrane facing the collagen gel corresponds to the basolateral side. Therefore, hepatocyte sandwich cultures represent the easiest to handle 3D culture system, although only one sheet of hepatocytes is represented. The hepatocyte phenotype in sandwich culture is characterized by (1) maintenance of susceptibility to apoptosis, (2) a delayed decrease of drug-metabolizing activities compared to monolayer cultures, (3) establishment and maintenance of bile canaliculi, and (4) a resting cell state where stimulation by HGF and EGF induces almost no proliferation events. As previously mentioned, this cultivation system effectively prevents the spontaneous activation of ERK and Akt which occurs in 2D systems [13]. Consistent with the effects of small chemical inhibitors in 2D cultures, expression of a constitutively active form of Ras in sandwich-cultured hepatocytes induces features of EMT and stress fibers. In contrast to Ras, expression of constitutive active Akt in hepatocytes induces an antiapoptotic phenotype and does not cause EMT [13].

Considering that sandwich cultures are relatively cheap, easy to handle, and applicable to high throughput it is not surprising that most studies in drug metabolism, enzyme induction, and hepatotoxicity have relied on this culture system. Despite its advantage it should be considered that also sandwich cultures do not guarantee an in vivo-like state. Gene array studies show that culture between soft gel collagen only ameliorates and delays the gene expression alterations that occur in conventional 2D monolayer culture.

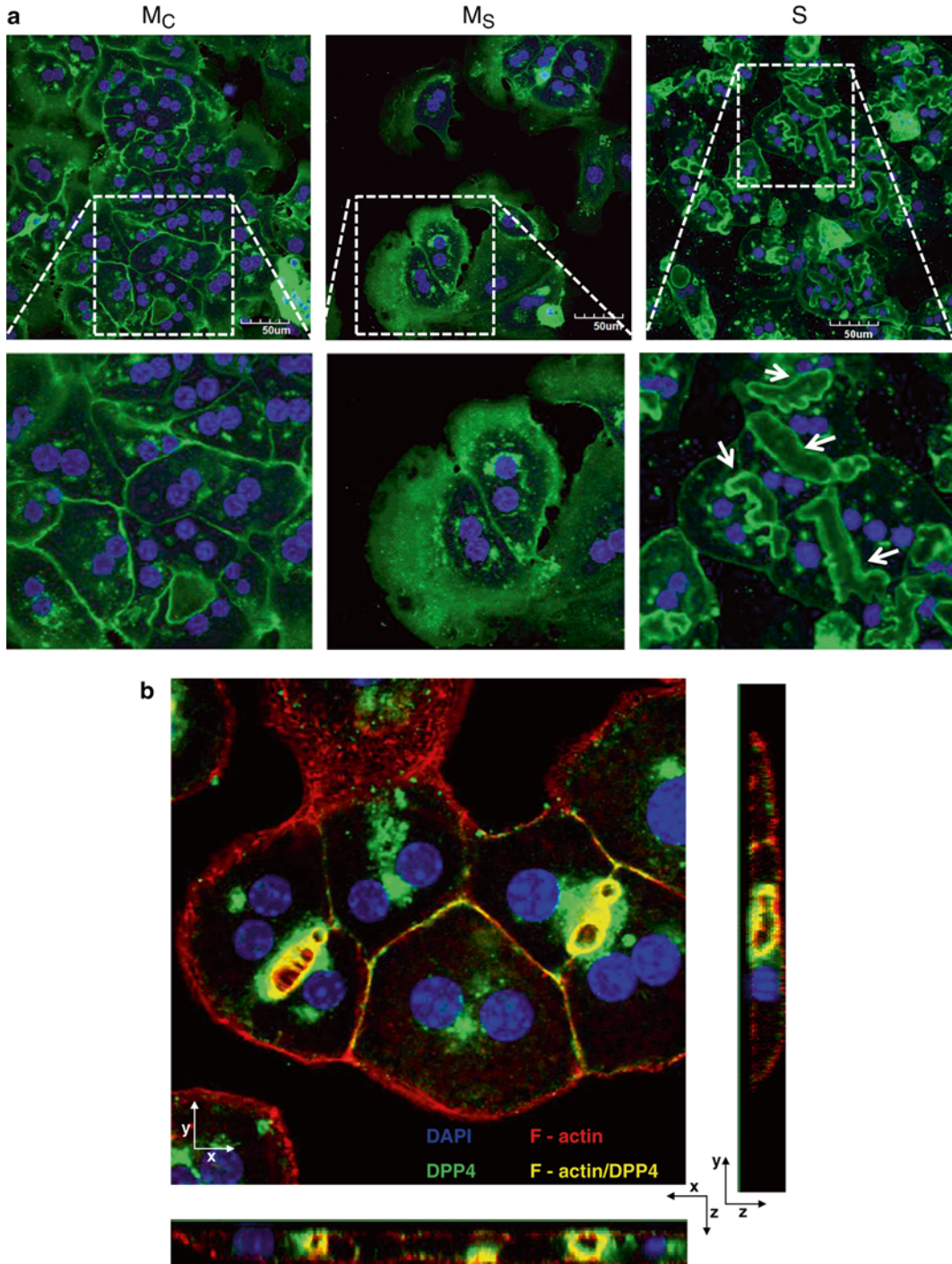


Fig. 3 Hepatocyte polarity in different culture conditions. (a) Confocal microscopy reveals formation of bile canaliculi (*white arrows*) in primary mouse hepatocytes. These structures are formed within 24 h when hepatocytes are cultivated between two layers of soft gel collagen (i.e., sandwich culture S) but not in monolayer confluent (M_C) or monolayer subconfluent (M_S) cultures. *Green fluorescence* corresponds to DPP4V staining (a marker for bile canaliculi). Nuclei appear *blue* (DAPI staining). (b) Bile canaliculi lumen is further revealed in z-stack confocal imaging in sandwich-cultured hepatocytes. *Red* corresponds to F-actin and *green* to DPP4V. Co-localization of the two markers is seen in *yellow* and corresponds to bile canaliculi. Nuclei appear *blue* (DAPI staining)

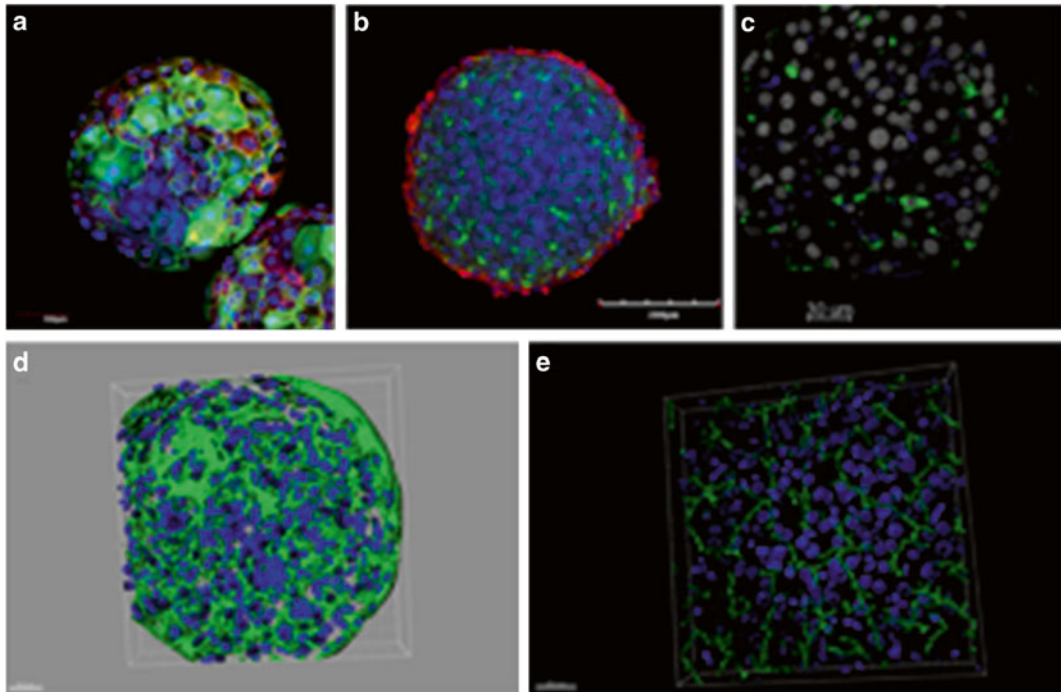


Fig. 4 Confocal laser scans of human hepatocyte spheroids. **(a)** Human hepatocyte spheroid. Nuclei appear *blue* (DAPI staining). Bile canaliculi are visualized by green fluorescence (staining of the bile canalicular marker DPPIV). **(b)** Similar spheroid as shown in **(a)**, with inclusion of sinusoidal endothelial cells (*red*) into the hanging drop culture. The endothelial cells do not form vessel-like structures as *in vivo* but build an epithelium at the surface of the spheroid. **(c)** Kupffer cells integrated into a spheroid of hepatocytes. **(d)** Reconstructed bile canalicular network of a spheroid of human hepatocytes. **(e)** Bile canalicular network of liver tissue

Again, the interspecies difference should be taken into account. Because rodent hepatocytes tend to dedifferentiate more rapidly they “profit” much more from sandwich culture conditions than human hepatocytes which have a lower propensity to show EMT-like features.

2.3 Spheroids and Microtissues

Hepatocyte spheroids represent an *in vitro* system that has been used since decades [20–22]. The concept is that suspended hepatocytes are capable of forming 3D tissue or “spheroids,” if adhesion of the hepatocytes to a substrate is prevented. In recent years a specific technique based on the hanging drop principle has become particularly popular, because it can also be applied in 96-well dishes [23]. Hepatocytes in spheroids spontaneously secrete extracellular matrix (ECM) components that closely resemble the normal ECM of the liver [24]. It has been reported that the 3D configuration in hepatospheres offers some advantages with respect to activities of drug-metabolizing enzymes and transporters (review: Godoy et al. [3]). Indeed, hepatospheres establish a bile canalicular network which in several aspects resembles the *in vivo* situation (Fig. 4a). However, the bile flow dynamics have not

yet been studied. Moreover, spheroids have not yet been systematically compared to sandwich cultures using genome-wide techniques, such as gene arrays. Hepatospheres can also be used to integrate non-parenchymal cells, such as Kupffer cells, into the “microtissue” (Fig. 4b). However, it has not been possible to establish sinusoidal microvessels in liver “microtissues.” When SECs are added together with hepatocytes to the hanging drop culture they form an epithelium covering the core of hepatocytes (Fig. 4a). Some researchers report about necrotic regions in “hepatospheres.” However, in our hands this occurred only when the initial viability of the primary hepatocytes was not adequate (trypan blue exclusion should be at least 90 %) or if too many hepatocytes were added to the hanging drops (2,000 hepatocytes per hanging drop seems to be adequate). In conclusion, “hepatospheres” established by hanging drop cultures represent an easy-to-handle, robust system whose potential also in comparison to other culture forms still has to be evaluated.

2.4 Hydrogels and Scaffolds

Hepatocytes are attachment-dependent cells [3, 25]. Without adequate extracellular matrix they lose their liver-specific functions. Therefore, it has been studied intensively which types of matrix, hydrogels, and scaffolds support differentiated hepatocyte functions and which are the key properties. One of the first commercially available hydrogels was Matrigel, an extract from mouse sarcoma cells [26]. Later Extragel, another collagen-based hydrogel [27]; Alimatrix, a porous 3D scaffold [28]; and PuraMatrix [29], a peptide hydrogel, became available. Moreover, 3D HGF/heparin-immobilized collagen matrices have been recommended [30]. Some hepatocyte functions have been preserved by embedding hepatocytes into alginate [31]. Further improvements have been reported by alginate including additionally galactosylated chitosan and heparin [32]. The multitude of techniques that successfully preserved hepatocyte functions makes it difficult to recommend a specific method or hydrogel type. Possibly, several techniques by which hepatocytes are embedded into hydrogels may be similarly useful. A limitation of many previously published studies is that specific hepatocyte functions, such as albumin secretion or cytochrome P450 activities of hepatocytes in hydrogels, were compared only to hepatocytes in 2D culture. In this constellation it will not be difficult to obtain a favorable comparison, since 2D cultures actively promote hepatocyte dedifferentiation (as reported above). More helpful would be comparisons to the *in vivo* situation (liver tissue) or to freshly isolated hepatocytes which at the transcriptional and metabolic level resemble very closely the state of intact liver tissue, in spite of the strong activation of signal transduction pathways. Also systematic unbiased comparisons of whole-genome gene expression sets of hepatocytes would help to identify advantages and limitations of specific hydrogel types.

Numerous studies have reported that hepatocytes cultivated on 3D scaffolds proliferate, migrate, and express differentiated functions [33, 34]. The conventional way for producing 3D scaffolds includes fiber bonding, phase separation, particulate leaching, melt molding, gas foaming, and freeze-drying (review: Godoy et al. [3]). An advantage of the freeze-drying technique is that the pore size of scaffolds can be controlled by the cooling rate of the hydrated gel and thereby be optimized for the specific needs of hepatocytes [35]. Even more controlled properties may be achieved by computational scaffold design [36]. A feature of scaffolds is that galactose can be presented on their surface. This can be advantageous because of the specific affinity of hepatocytes to galactose residues [37]. Recently, much excitement has surrounded the establishment of decellularized liver-derived extracellular matrix (e.g., Uygun et al. [38]; Bao et al. 2011). The 3D scaffold obtained from a decellularized rat liver lobe was repopulated with liver cells which exerted liver-specific functions and could be transplanted into rats after partial hepatectomy. However, gene expression patterns of hepatocytes in matrix of decellularized livers showed alterations similar to those described in other culture forms. For example, mRNA expression levels of phase 1 and phase 2 metabolism-associated genes were downregulated to similar levels in both systems compared to freshly isolated hepatocytes or liver tissue [38]. Furthermore, a proper vascularization of the recellularized liver was not achieved, since only hepatocytes were transplanted and not SECs. This may also account for a lack of proper zonation, since zonation depends on Wnt factors secreted from NPCs [39]. In conclusion, although hydrogels and 3D scaffolds maintain some liver functions it has not yet been analyzed on a genome-wide level to which degree they avoid gene expression alterations compared to the *in vivo* situation. Based on our previous experience it appears questionable whether an optimized matrix alone will sufficiently mimic the *in vivo* situation.

3 Liver Slices

Liver slices have been used since the 1920s [3, 40, 41]. However, reproducibility was initially limited. With the development of precision tissue slicers the technique became more popular since the 1980s [42]. Today precision-cut liver slices are mostly used for toxicity and drug metabolism studies [43–45]. The obvious advantage of liver slices is the maintenance of liver microarchitecture with all liver cell types; the major difficulty is the loss of blood perfusion leading to physiological changes. As a consequence, expression of xenobiotic metabolizing enzymes tends to decrease and cells in the tissue slice become necrotic. Two technical factors are particularly critical for the successful maintenance of liver slices. First, an optimized slicing technique must be used which allows reproducible

slicing without bruising of the tissue. The optimal thickness has been reported to range between 100 and 250 μm [44, 46]. Moreover, it is critical to use a carbogenated (95 % oxygen and 5 % carbon dioxide) slicing buffer (e.g., Krebs–Henseleit, pH 7.4, 4 °C) with high glucose (25 mM). A second critical factor is the incubation system. It seems to be critical to use a gas phase with relatively high oxygen tension (e.g., up to 95 % oxygen and 5 % CO_2) and to shake during the culture [47–49]. Maximum incubation periods of 96 h have been reported [50, 51]. However, a limitation remains that depending on the effort invested in optimization of the technique, several functions tend to decrease already during the first 24 h. In conclusion, liver slice cultures represent an attractive in vitro system, because all liver cell types are included. However, careful controls are required to guarantee that the function of interest was stable during the investigated time period.

4 The Isolated Perfused Liver

The isolated perfused liver differs from the decellularized liver model in that the NPCs, including the vascular endothelial cells, are preserved. Hence, it represents the only in vitro system that includes perfusion of the sinusoids in a physiological state. It has been introduced by Miller et al. [52] and has been further improved in the following decades (e.g., Sies [53], Häussinger [54], vom Dahl and Häussinger [55]). For this system usually rat livers are used, but the technique has also been adapted to the smaller size of mouse livers. The perfusion fluid is usually delivered from a reservoir by a roller pump and enters the liver by the cannulated portal vein [3]. It is drained into a cannula in the superior vena cava. Moreover, the bile duct can be cannulated for sampling. The most frequent application of the isolated perfused liver is determination of metabolic flux rates. When the substrate concentration of a non-recirculating inflow is constant, the concentration of metabolites in the effluent can be analyzed. This can be used to study metabolites of xenobiotics. It has also been successfully used to determine the detoxification of ammonia to urea and glutamine (review: Godoy et al. [3]). A particularly valuable technical possibility of the isolated perfused liver is to switch the direction of the flow of the perfusion buffer. Antegrade perfusion means a flow of the buffer in the “normal” direction from the portal vein to the vena cava, whereas for retrograde perfusion the buffer is pumped into the vena cava and leaves the liver by the portal vein. This switch between ante- and retrograde perfusion is a key method for studying the functional heterogeneity of the liver lobule. It has, for example, been used to prove that ammonia is detoxified to urea in a periportal compartment of the liver lobule, whereas a ring of pericentral hepatocytes metabolizes ammonia to glutamine. The principle of the antegrade–retrograde perfusion change is that one

of the two metabolic pathways will be favored depending on the perfusion direction. A precondition is that different compartments of the lobule compete for the same substrate. Moreover, it is critical to use rate-limiting substrate concentrations for this type of experiment (review: Godoy et al. [3]). In conclusion, the isolated perfused liver represents the most complex model and is the only system that allows functional analysis of metabolic zonation of the liver. Nevertheless, this system is not suitable for screenings or for long-term toxicity testing.

5 Alternative Cells to Primary Human Hepatocytes

Alternative cell systems to primary hepatocytes have recently been reviewed comprehensively (e.g., Godoy et al. [3]). Here, we only summarize implications for studies of in vitro toxicity and metabolism with human cells. Several human hepatoma cell lines are of practical relevance (e.g., HepG2, Hep3B, Huh7, Fa2N4, or HepaRG). Under specific cultivation conditions, such as high cell densities, 3D culture systems, or high DMSO concentrations, they can express relatively high levels of phase I and phase II metabolizing enzymes and may therefore be useful in studies of drug metabolism and metabolism-associated toxicity (review: Godoy et al. [3]). By transfection of drug-metabolizing genes the metabolizing capacity could be further enhanced. Numerous studies have been performed to immortalize human hepatocytes (e.g., Tsuruga et al. [56]; Wege et al. [57]; Kobayashi et al. [58]). A general difficulty of these approaches is the propensity of immortalized hepatocytes to lose differentiated functions. Human embryonic stem cells or induced pluripotency cells have been extensively used to generate differentiation protocols for hepatocytes. In these cell types it has been possible to induce expression of genes typically observed in hepatocytes. However, unbiased analysis, e.g., by gene arrays demonstrates that “hepatocyte-like cells” derived from stem cells still show huge differences compared to freshly isolated primary hepatocytes or to liver tissue [59]. Therefore, it is still not recommended to replace primary hepatocytes by stem cell-derived cell types. However, these limitations are not surprising. When primary hepatocytes are isolated from their physiological environment hundreds of genes are massively up- and downregulated during the cultivation period. Culture conditions avoiding these alterations of primary cells are not yet available. It would therefore be surprising if stem cells could be differentiated into a state similar to real hepatocytes when we have not yet found in vitro conditions to maintain the phenotype of the primary cells. Although a lot has already been invested into research on hepatocytes from alternative sources, the hunt for the “hepatocyte-like cell” that deserves this name has only just begun.

References

- Hewitt NJ, Lechon MJ, Houston JB, Hallifax D, Brown HS, Maurel P, Kenna JG, Gustavsson L, Lohmann C, Skonberg C, Guillouzo A, Tuschl G, Li AP, LeCluyse E, Groothuis GM, Hengstler JG (2007) Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev* 39(1):159–234
- Hengstler JG, Ringel M, Biefang K, Hammel S, Milbert U, Gerl M, Klebach M, Diener B, Platt KL, Bottger T, Steinberg P, Oesch F (2000) Cultures with cryopreserved hepatocytes: applicability for studies of enzyme induction. *Chem Biol Interact* 125(1):51–73
- Godoy P, Hewitt NJ, Albrecht U, Andersen ME, Ansari N, Bhattacharya S, Bode JG, Bolleyn J, Borner C, Bottger J, Braeuning A, Budinsky RA, Burkhardt B, Cameron NR, Camussi G, Cho CS, Choi YJ, Craig Rowlands J, Dahmen U, Damm G, Dirsch O, Donato MT, Dong J, Dooley S, Drasdo D, Eakins R, Ferreira KS, Fonsato V, Fraczek J, Gebhardt R, Gibson A, Glanemann M, Goldring CE, Gomez-Lechon MJ, Groothuis GM, Gustavsson L, Guyot C, Hallifax D, Hammad S, Hayward A, Haussinger D, Hellerbrand C, Hewitt P, Hoehme S, Holzthutter HG, Houston JB, Hrach J, Ito K, Jaeschke H, Keitel V, Kelm JM, Kevin Park B, Kordes C, Kullak-Ublick GA, Lecluyse EL, Lu P, Luebke-Wheeler J, Lutz A, Maltman DJ, Matz-Soja M, McMullen P, Merfort I, Messner S, Meyer C, Mwinyi J, Naisbitt DJ, Nussler AK, Olinga P, Pampaloni F, Pi J, Pluta L, Przyborski SA, Ramachandran A, Rogiers V, Rowe C, Schelcher C, Schmich K, Schwarz M, Singh B, Stelzer EH, Stieger B, Stober R, Sugiyama Y, Tetta C, Thasler WE, Vanhaecke T, Vinken M, Weiss TS, Widera A, Woods CG, Xu JJ, Yarborough KM, Hengstler JG (2013) Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Arch Toxicol* 87(8):1315–1530
- Amacher DE (2012) The primary role of hepatic metabolism in idiosyncratic drug-induced liver injury. *Expert Opin Drug Metab Toxicol* 8(3):335–347
- Hoehme S, Brulport M, Bauer A, Bedawy E, Schormann W, Hermes M, Puppe V, Gebhardt R, Zellmer S, Schwarz M, Bockamp E, Timmel T, Hengstler JG, Drasdo D (2010) Prediction and validation of cell alignment along microvessels as order principle to restore tissue architecture in liver regeneration. *Proc Natl Acad Sci U S A* 107(23):10371–10376
- Ding BS, Nolan DJ, Butler JM, James D, Babazadeh AO, Rosenwaks Z, Mittal V, Kobayashi H, Shido K, Lyden D, Sato TN, Rabbany SY, Rafii S (2010) Inductive angiocrine signals from sinusoidal endothelium are required for liver regeneration. *Nature* 468(7321):310–315
- Goldin RD, Ratnayaka ID, Breach CS, Brown IN, Wickramasinghe SN (1996) Role of macrophages in acetaminophen (paracetamol)-induced hepatotoxicity. *J Pathol* 179(4):432–435
- Gordon S (2003) Alternative activation of macrophages. *Nat Rev Immunol* 3(1):23–35
- Mantovani A, Cassatella MA, Costantini C, Jaillon S (2011) Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* 11(8):519–531
- Notas G, Kisseleva T, Brenner D (2009) NK and NKT cells in liver injury and fibrosis. *Clin Immunol* 130(1):16–26
- Ochi M, Ohdan H, Mitsuta H, Onoe T, Tokita D, Hara H, Ishiyama K, Zhou W, Tanaka Y, Asahara T (2004) Liver NK cells expressing TRAIL are toxic against self hepatocytes in mice. *Hepatology* 39(5):1321–1331
- Tuschl G, Hrach J, Walter Y, Hewitt PG, Mueller SO (2009) Serum-free collagen sandwich cultures of adult rat hepatocytes maintain liver-like properties long term: a valuable model for in vitro toxicity and drug-drug interaction studies. *Chem Biol Interact* 181(1):124–137
- Godoy P, Hengstler JG, Ilkavets I, Meyer C, Bachmann A, Muller A, Tuschl G, Mueller SO, Dooley S (2009) Extracellular matrix modulates sensitivity of hepatocytes to fibroblastoid dedifferentiation and transforming growth factor beta-induced apoptosis. *Hepatology* 49(6):2031–2043
- Reder-Hilz B, Ullrich M, Ringel M, Hewitt N, Utesch D, Oesch F, Hengstler JG (2004) Metabolism of propafenone and verapamil by cryopreserved human, rat, mouse and dog hepatocytes: comparison with metabolism in vivo. *Naunyn Schmiedeberg's Arch Pharmacol* 369(4):408–417
- Carmo H, Hengstler JG, de Boer D, Ringel M, Remiao F, Carvalho F, Fernandes E, dos Reis LA, Oesch F, de Lourdes Bastos M (2005)

- Metabolic pathways of 4-bromo-2,5-dimethoxyphenethylamine (2C-B): analysis of phase I metabolism with hepatocytes of six species including human. *Toxicology* 206(1): 75–89
16. Ullrich A, Berg C, Hengstler JG, Runge D (2007) Use of a standardised and validated long-term human hepatocyte culture system for repetitive analyses of drugs: repeated administrations of acetaminophen reduces albumin and urea secretion. *ALTEX* 24(1): 35–40
 17. Ullrich A, Stolz DB, Ellis EC, Strom SC, Michalopoulos GK, Hengstler JG, Runge D (2009) Long term cultures of primary human hepatocytes as an alternative to drug testing in animals. *ALTEX* 26(4):295–302
 18. Knobloch D, Ehnert S, Schyschka L, Buchler P, Schoenberg M, Kleff J, Thasler WE, Nussler NC, Godoy P, Hengstler J, Nussler AK (2012) Human hepatocytes: isolation, culture, and quality procedures. *Methods Mol Biol* 806:99–120
 19. Klingmuller U, Bauer A, Bohl S, Nickel PJ, Breitkopf K, Dooley S, Zellmer S, Kern C, Merfort I, Sparna T, Donauer J, Walz G, Geyer M, Kreutz C, Hermes M, Gotschel F, Hecht A, Walter D, Egger L, Neubert K, Borner C, Brulport M, Schormann W, Sauer C, Baumann F, Preiss R, MacNelly S, Godoy P, Wiercinska E, Ciucan L, Edelmann J, Zeilinger K, Heinrich M, Zanger UM, Gebhardt R, Maiwald T, Heinrich R, Timmer J, von Weizsacker F, Hengstler JG (2006) Primary mouse hepatocytes for systems biology approaches: a standardized in vitro system for modelling of signal transduction pathways. *Syst Biol (Stevenage)* 153(6):433–447
 20. Peshwa MV, Wu FJ, Sharp HL, Cerra FB, Hu WS (1996) Mechanistics of formation and ultrastructural evaluation of hepatocyte spheroids. *In Vitro Cell Dev Biol Anim* 32(4): 197–203
 21. Tzanakakis ES, Hansen LK, Hu WS (2001) The role of actin filaments and microtubules in hepatocyte spheroid self-assembly. *Cell Motil Cytoskeleton* 48(3):175–189
 22. Chang TT, Hughes-Fulford M (2009) Monolayer and spheroid culture of human liver hepatocellular carcinoma cell line cells demonstrate distinct global gene expression patterns and functional phenotypes. *Tissue Eng Part A* 15(3):559–567
 23. Kelm JM, Timmins NE, Brown CJ, Fussenegger M, Nielsen LK (2003) Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. *Biotechnol Bioeng* 83(2):173–180
 24. Landry J, Bernier D, Ouellet C, Goyette R, Marceau N (1985) Spheroidal aggregate culture of rat liver cells: histotypic reorganization, biomatrix deposition, and maintenance of functional activities. *J Cell Biol* 101(3): 914–923
 25. Kim BS, Park IK, Hoshiba T, Jiang HL, Choi YJ, Akaike T, Cho CS (2011) Design of artificial extracellular matrices for tissue engineering. *Prog Polym Sci* 36(2):239–268
 26. Kleinman HK, McGarvey ML, Hassell JR, Star VL, Cannon FB, Laurie GW, Martin GR (1986) Basement membrane complexes with biological activity. *Biochemistry* 25(2): 312–318
 27. Ranucci CS, Kumar A, Batra SP, Moghe PV (2000) Control of hepatocyte function on collagen foams: sizing matrix pores toward selective induction of 2-D and 3-D cellular morphogenesis. *Biomaterials* 21(8):783–793
 28. Rowley JA, Madlambayan G, Mooney DJ (1999) Alginate hydrogels as synthetic extracellular matrix materials. *Biomaterials* 20(1): 45–53
 29. Semino CE, Merok JR, Crane GG, Panagiotakos G, Zhang S (2003) Functional differentiation of hepatocyte-like spheroid structures from putative liver progenitor cells in three-dimensional peptide scaffolds. *Differentiation* 71(4–5):262–270
 30. Hou YT, Ijima H, Matsumoto S, Kubo T, Takei T, Sakai S, Kawakami K (2010) Effect of a hepatocyte growth factor/heparin-immobilized collagen system on albumin synthesis and spheroid formation by hepatocytes. *J Biosci Bioeng* 110(2):208–216
 31. Ringel M, von Mach MA, Santos R, Feilen PJ, Brulport M, Hermes M, Bauer AW, Schormann W, Tanner B, Schon MR, Oesch F, Hengstler JG (2005) Hepatocytes cultured in alginate microspheres: an optimized technique to study enzyme induction. *Toxicology* 206(1): 153–167
 32. Seo SJ, Choi YJ, Akaike T, Higuchi A, Cho CS (2006) Alginate/galactosylated chitosan/heparin scaffold as a new synthetic extracellular matrix for hepatocytes. *Tissue Eng* 12(1): 33–44
 33. Sachlos E, Czernuszka JT (2003) Making tissue engineering scaffolds work. Review: the application of solid freeform fabrication technology to the production of tissue engineering scaffolds. *Eur Cell Mater* 5:29–39, discussion 39–40
 34. Putnam AJ, Mooney DJ (1996) Tissue engineering using synthetic extracellular matrices. *Nat Med* 2(7):824–826

35. Chung TW, Yang J, Akaike T, Cho KY, Nah JW, Kim SI, Cho CS (2002) Preparation of alginate/galactosylated chitosan scaffold for hepatocyte attachment. *Biomaterials* 23(14): 2827–2834
36. Hollister SJ (2005) Porous scaffold design for tissue engineering. *Nat Mater* 4(7):518–524
37. Cho CS, Goto M, Kobayashi A, Kobayashi K, Akaike T (1996) Effect of ligand orientation on hepatocyte attachment onto the poly(N-p-vinylbenzyl-o-beta-D-galactopyranosyl-D-gluconamide) as a model ligand of asialoglycoprotein. *J Biomater Sci Polym Ed* 7(12):1097–1104
38. Uygun BE, Soto-Gutierrez A, Yagi H, Izamis ML, Guzzardi MA, Shulman C, Milwid J, Kobayashi N, Tilles A, Berthiaume F, Hertl M, Nahmias Y, Yarmush ML, Uygun K (2010) Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. *Nat Med* 16(7): 814–820
39. Zeng G, Awan F, Otruba W, Muller P, Apte U, Tan X, Gandhi C, Demetris AJ, Monga SP (2007) Wnt'er in liver: expression of Wnt and frizzled genes in mouse. *Hepatology* 45(1): 195–204
40. Krebs HA (1933) Untersuchungen über den Stoffwechsel der Aminosäuren im Tierkörper. *Hoppe-Seyl Z* 217:190
41. Warburg O (1923) Versuche an u"berlebendem Karzinomgewebe. *Biochem Z* 142:317
42. Krumdieck CL, dos Santos JE, Ho KJ (1980) A new instrument for the rapid preparation of tissue slices. *Anal Biochem* 104(1):118–123
43. Worboys PD, Bradbury A, Houston JB (1997) Kinetics of drug metabolism in rat liver slices. III. Relationship between metabolic clearance and slice uptake rate. *Drug Metab Dispos* 25(4):460–467
44. Olinga P, Merema MT, Meijer DKF, Slooff MJH, Groothuis GMM (1993) Human liver slices express the same lidocaine biotransformation rate as isolated human hepatocytes. *ATLA* 21:466–468
45. Martin H, Sarsat JP, Lerche-Langrand C, Housset C, Balladur P, Toutain H, Albaladejo V (2002) Morphological and biochemical integrity of human liver slices in long-term culture: effects of oxygen tension. *Cell Biol Toxicol* 18(2):73–85
46. de Graaf IA, de Kanter R, de Jager MH, Camacho R, Langenkamp E, van de Kerkhof EG, Groothuis GM (2006) Empirical validation of a rat in vitro organ slice model as a tool for in vivo clearance prediction. *Drug Metab Dispos* 34(4):591–599
47. Olinga P, Groen K, Hof IH, De Kanter R, Koster HJ, Leeman WR, Rutten AA, Van Twillert K, Groothuis GM (1997) Comparison of five incubation systems for rat liver slices using functional and viability parameters. *J Pharmacol Toxicol Methods* 38(2):59–69
48. Olinga P, Elferink MG, Draaisma AL, Merema MT, Castell JV, Perez G, Groothuis GM (2008) Coordinated induction of drug transporters and phase I and II metabolism in human liver slices. *Eur J Pharm Sci* 33(4–5): 380–389
49. de Graaf IA, Olinga P, de Jager MH, Merema MT, de Kanter R, van de Kerkhof EG, Groothuis GM (2010) Preparation and incubation of precision-cut liver and intestinal slices for application in drug metabolism and toxicity studies. *Nat Protoc* 5(9):1540–1551
50. Klassen LW, Thiele GM, Duryee MJ, Schaffert CS, DeVeney AL, Hunter CD, Olinga P, Tuma DJ (2008) An in vitro method of alcoholic liver injury using precision-cut liver slices from rats. *Biochem Pharmacol* 76(3):426–436
51. Vickers AE, Saulnier M, Cruz E, Merema MT, Rose K, Bentley P, Olinga P (2004) Organ slice viability extended for pathway characterization: an in vitro model to investigate fibrosis. *Toxicol Sci* 82(2):534–544
52. Miller LL, Bly CG, Watson ML, Bale WF (1951) The dominant role of the liver in plasma protein synthesis; a direct study of the isolated perfused rat liver with the aid of lysine-epsilon-C14. *J Exp Med* 94(5): 431–453
53. Sies H (1978) The use of perfusion of liver and other organs for the study of microsomal electron-transport and cytochrome P-450 systems. *Methods Enzymol* 52:48–59
54. Haussinger D (1987) Isolated perfused rat liver: an experimental model for studies on ammonium and amino acid metabolism. *Infusionsther Klin Ernahr* 14(4):174–178
55. vom Dahl S, Haussinger D (1997) Experimental methods in hepatology. Guidelines of the German Association for the Study of the Liver (GASL). Liver perfusion—technique and applications. *Z Gastroenterol* 35(3):221–226
56. Tsuruga Y, Kiyono T, Matsushita M, Takahashi T, Kasai H, Matsumoto S, Todo S (2008) Establishment of immortalized human hepatocytes by introduction of HPV16 E6/E7 and hTERT as cell sources for liver cell-based therapy. *Cell Transplant* 17(9): 1083–1094
57. Wege H, Le HT, Chui MS, Liu L, Wu J, Giri R, Malhi H, Sappal BS, Kumaran V, Gupta S,

- Zern MA (2003) Telomerase reconstitution immortalizes human fetal hepatocytes without disrupting their differentiation potential. *Gastroenterology* 124(2):432–444
58. Kobayashi N, Noguchi H, Westerman KA, Watanabe T, Matsumura T, Totsugawa T, Fujiwara T, Leboulch P, Tanaka N (2001) Cre/loxP-based reversible immortalization of human hepatocytes. *Cell Transplant* 10(4–5):383–386
59. Hart SN, Li Y, Nakamoto K, Subileau EA, Steen D, Zhong XB (2010) A comparison of whole genome gene expression profiles of HepaRG cells and HepG2 cells to primary human hepatocytes and human liver tissues. *Drug Metab Dispos* 38(6):988–994

In Vitro Toxicology Systems

Bal-Price, A.; Jennings, P. (Eds.)

2014, XXI, 583 p. 64 illus., 54 illus. in color., Hardcover

ISBN: 978-1-4939-0520-1

A product of Humana Press