Published in final edited form as:
Toxicol Res (Camb). 2013 January 1; 2(1): 23–39. doi:10.1039/C2TX20051A.

In vitro models for liver toxicity testing

Valerie Y. Soldatow[1], **Edward L. LeCluyse**[2], **Linda G. Griffith**[3], and **Ivan Rusyn**[1]

[1]Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill, NC 27599, USA

[2]The Hamner Institutes for Health Sciences, 6 Davis Drive, Research Triangle Park, NC 27709, USA

[3]Department of Biological and Mechanical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA

Abstract

Over the years, various liver-derived *in vitro* model systems have been developed to enable investigation of the potential adverse effects of chemicals and drugs. Liver tissue slices, isolated microsomes, perfused liver, immortalized cell lines, and primary hepatocytes have been used extensively. Immortalized cell lines and primary isolated liver cells are currently most widely used *in vitro* models for liver toxicity testing. Limited throughput, loss of viability, and decreases in liver-specific functionality and gene expression are common shortcomings of these models. Recent developments in the field of *in vitro* hepatotoxicity include three-dimensional tissue constructs and bioartificial livers, co-cultures of various cell types with hepatocytes, and differentiation of stem cells into hepatic lineage-like cells. In an attempt to provide a more physiological environment for cultured liver cells, some of the novel cell culture systems incorporate fluid flow, micro-circulation, and other forms of organotypic microenvironments. Co-cultures aim to preserve liver-specific morphology and functionality beyond those provided by cultures of pure parenchymal cells. Stem cells, both embryonic- and adult tissue-derived, may provide a limitless supply of hepatocytes from multiple individuals to improve reproducibility and enable testing of the individual-specific toxicity. This review describes various traditional and novel *in vitro* liver models and provides a perspective on the challenges and opportunities afforded by each individual test system.

1. Chemical Toxicity Testing

The gold standard toxicological approach for evaluating chemical toxicity involves complex *in vivo* studies which are both time consuming and costly. Due to concerns about animal welfare, time and cost constraints, and the ever increasing number of chemicals that need testing, establishing workable *in vitro* culture systems has become a priority for the toxicology community. In addition, the predictive accuracy of rodent *in vivo* testing for human adverse health effects has become a matter of dispute in recent years, in part due to poor concordance of animal study results to disease phenotypes observed in heterogeneous human populations.[1, 2] The use of *in vitro* model systems in toxicity testing has many advantages including the decrease in animal numbers, the reduced cost of animal maintenance and care, small quantity of a chemical needed for testing, shortening of the time needed, and increase in throughput for evaluating multiple chemicals and their metabolites.[3, 4] *In vitro* systems also allow to study chemical metabolism, evaluate the

Corresponding author: Ivan Rusyn, MD, PhD Department of Environmental Sciences and Engineering University of North Carolina Chapel Hill, NC 27599, USA T/F: +1-919-843-2596 iir@unc.edu.

mechanisms of toxicity, measure enzyme kinetics, and examine dose-response relationships.[4]

The Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) at the U.S. National Toxicology Program (NTP) works in conjunction with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) to establish and validate alternative methods to *in vivo* toxicological testing. The three main tenants for animal toxicity studies of these agencies are known as the three R's: refinement, reduction, and replacement. The ultimate goal is “the validation and regulatory acceptance of test methods that are more predictive of adverse human and ecological effects than currently available methods, supporting improved protection of human health and the environment.”[5]

The landmark report released by the National Research Council (NRC), “Toxicity Testing in the 21st Century: A Vision and a Strategy”,[2] identified the challenges of modern toxicology and provided strategies for developing alternatives to *in vivo* research. The report supports the movement towards the use of *in vitro* systems instead of *in vivo* toxicological studies and, as requested by the U.S. Environmental Protection Agency (EPA), has developed a long-term objective of decreasing the use of *in vivo* studies for toxicity testing and proposed an initial strategy towards achieving that goal. In addition to the limitations mentioned above, the report acknowledges that *in vivo* study results cannot evaluate the much lower concentrations and mixtures of chemicals that humans are exposed to, lack information regarding modes and mechanisms of actions, and cannot account for human variability in responses and susceptibility.[2] The report asserts that *in vitro* work can elucidate cellular-response networks and toxicity pathways, modes and mechanisms of action, allow for high-throughput studies, enhance dose-response relationships, evaluate many more concentrations than *in vivo* work, use concentrations relative to human exposure, provide information for generation of pharmaco-kinetic and -dynamic models, and lead to genome based investigations into perturbations of toxicity pathways.

There are three important factors which hinder the ability to use animal models to predict human adverse effects and the National Research Council report posits that the use of *in vitro* models should aid in overcoming this challenge. First, *in vivo* studies typically use high doses of compounds which are orders of magnitude greater than those humans are exposed to. Dose-response relationships are complex so extrapolation from these high doses to lower, human, exposure levels is difficult and results in many inaccuracies. Second, *in vivo* studies examine the response of a standard laboratory animal to a toxicant. This response may or may not occur in humans. Even more important is that the human population is very heterogeneous and a single strain of animal cannot accurately predict the variability in responses seen in the human population. Finally, *in vivo* exposures in toxicity testing are usually composed of a single compound. While this allows for close examination of the results of that single compound, humans are constantly exposed to mixtures of compounds daily and the effects of these co-exposures need to be examined. Conducting *in vivo* studies using mixtures of chemicals would be a monumental task due to the number of different chemicals and combinations of chemicals as well as the time it takes to complete each *in vivo* study.

To overcome these limitations to *in vivo* studies, the National Research Council developed four criteria important to designing a new toxicity-testing paradigm: 1) broaden the studies to include a greater number of chemicals and chemical mixtures as well as more end points and life-stages; 2) decrease the time it takes to obtain results for risk assessment by reducing costs and length of tests and increasing efficiency and flexibility of said tests; 3) reduce the number and suffering of animals involved in testing; and 4) more closely examine

mechanistic and dosimetry information to provide risk assessment organizations with a broader range of information. The major components of the proposed strategy to establish this paradigm are chemical characterization, toxicity testing, and dose-response and extrapolation modeling. The National Research Council has recognized the fact that this new toxicity-testing paradigm will require years of research and assay validation and may never fully eliminate the need for *in vivo* work; however the benefits of reducing the number of animal studies are well worth the effort.

One of the current major benefits of *in vitro* systems is their utility for screening chemicals for prioritization purposes. The EPA ToxCast™ project was established mainly for the purpose of prioritization of chemicals for further toxicological study by using high-throughput *in vitro* assays to establish "toxicity signatures" of chemicals, identify toxicity pathways, and predict potential toxicity.[6] To do this, ToxCast™ uses a large number of cell-free or cell-based *in vitro* high-throughput assays. The different assays measure perturbations in pathways that could lead to cellular toxicity and the results of ToxCast™ assays will be used to link particular genes to toxicity endpoints. Researchers hope to use data produced by the high-throughput assays of ToxCast™ to supplement data from genomics, proteomics, and metabolomic studies to further elucidate toxicity pathways.[7] Unfortunately, the high-throughput screening results do not correlate well with *in vivo* toxicity and cannot account for biological processes related to toxicity such as exposure, biotransformation, toxicokinetics, and individual genetic diversity.[8] Thus, other *in vitro* methods must be examined to supplement these high-throughput assays.

The liver is the major source of metabolism and drug biotransformation, thus liver cells are a logical choice for toxicological and pharmacological testing. Indeed, *in vivo* liver toxicity testing comprises a large portion of *in vitro* toxicology. High throughput *in vitro* liver models would be a great resource and enable the shift to a greater use of the alternative toxicity testing methods. In this review we discuss the benefits and pitfalls of traditional liver-derived *in vitro* systems and examine novel *in vitro* liver models and their usefulness for toxicity testing.

2. Traditional Liver-derived In Vitro Systems

Many different *in vitro* liver models have been employed over the years with the hopes that the effects after chemical treatment will be predictive of *in vivo* responses. Liver slices, cell lines, and primary hepatocytes have consistently remained the leading models in *in vitro* liver toxicity testing. Advantages and disadvantages of these systems vary greatly.

Liver Slices

Liver tissue slices can be a beneficial model as they retain liver structure, contain all the cell types found *in vivo*, have good *in vitro*/*in vivo* correlation of xenobiotic metabolism, and maintain zone-specific cytochrome activity (allowing for cellular and zonal toxicity) and mechanisms of toxicity.[9] Phase II enzymes, albumin production, and gluconeogenesis were shown to decrease slightly but remain fairly stable for up to 20-96 hours when slices were cultured.[10-13] Toxicogenomics studies found that rat liver slices correlated more closely to *in vivo* rat livers over 24 hours when compared to two cell lines and primary hepatocytes in both conventional and sandwich culture[14] and human liver slices showed low levels of CYP down-regulation over the same time period.[15]

Studies using tissue slices typically range in culture length from 30 minutes to 5 days[10, 16-18] and culture conditions such as oxygen tension, media and supplements, and culture system (i.e. shaken flasks, multiwall plates, stirred wells, etc.) have been modified to increase cell viability and reduce degenerative changes in the tissue over the culture period.[9]

Unfortunately, even with these modifications, necrosis still occurs after 48-72 hours in culture,[10, 18, 19] and metabolic enzyme levels are greatly reduced after 6-72 hours.[17, 20, 21] In addition, rates of drug metabolism and intrinsic clearance were found to be lower in liver slices than in isolated hepatocytes,[22, 23] K_m values are usually higher in slices than in isolated hepatocytes,[22] and investigators surmise that a gradient in chemical exposure exists within the tissue slice resulting in not all hepatocytes participating in the metabolism of compounds.[9]

Immortalized Cell Lines

Most of the available liver-derived immortalized cell lines do not possess phenotypic characteristics of the liver tissue.[24-26] Common immortalized liver-derived cell lines in use are Fa2N-4, HepG2, Hep3B, PLc/PRFs Huh7, HBG, and HepaRG.[27, 28] The HepG2 line was generated in the 1970s and expresses many liver-specific genes;[29] however, the expression profile of genes involved in phase I and phase II metabolism has been shown to vary between passages and as a result data can be difficult to interpret across laboratories and passages.[28] The more recently developed human hepatoma cell line, HepaRG, retains the expression of many liver-specific functions as well as many cytochrome P450s, nuclear receptors, membrane transporters, and phase II enzymes.[30] HepaRG cells have a stable karyotype, can differentiate into either hepatocyte or biliary lineages, have a high proliferative capacity, and have shown to produce data that is both reproducible and consistent among experiments.[28, 31, 32] Still, the expression of liver-specific functions in HepaRG cells is still much lower on average than that of primary hepatocytes[31] and they represent a phenotype from a single donor, thereby reducing their predictive value for the human population.

Primary Hepatocyte Suspensions

Most hepatocyte isolation protocols use collagenase digestion to disrupt the bonds between cells and allow for single-celled suspensions. Suspensions of hepatocytes are an easy to use method for moderately high-throughput toxicity studies. Griffin and Houston [33] demonstrated that suspensions of hepatocytes provided a more accurate estimate of internal clearance rate when compared to conventional monolayer cultures. Suspensions also retain high levels of functionality allowing for a more accurate correlation to *in vivo* toxicity than cultured cells.[34-37] However, it is well known that most isolation protocols result in damage to cell surface receptors and antigens, cell junctions, cell membranes, and cytosolic contents.[9, 38] Collagenase digestion also induces oxidative stress in the hepatocytes leading to a loss of cytochrome enzyme activity with greatest loss observed 4-8 hours after isolation.[39-41] The loss of contact with extra cellular matrix and other cell types results in loss of cell polarity, integrity, and differentiation[42, 43] which can only be re-established after restoring normal intercellular contacts. Finally, as Hewitt *et al.* [35] and Burke *et al.* [44] describe, often hepatotoxicity is a process that occurs over several hours (such as with acetaminophen toxicity) and thus, hepatocytes in suspension cannot maintain viability for the time necessary for the development of toxicity.

Primary Hepatocyte Cultures

Cultures of primary hepatocytes have been the gold standard for *in vitro* testing as they can maintain functional activities for 24-72 hours, can be used for enzyme induction and inhibition studies, allow for medium-throughput screening of compounds, and are ideal for examining interspecies and inter-individual differences in metabolism.[34, 45] Primary hepatocytes are often employed with the expectation that chemicals will affect or be affected by an isolated cell in the same manner that would occur in the whole organ and will in this way be a predictive model.[4] Unfortunately, primary hepatocyte cultures are limited as *in vitro* systems as well.

Traditional primary hepatocyte cell culture involves the method of plating cells on a rigid substratum in which they proceed to form a monolayer across the bottom of the culture plate well. Under these conditions, primary hepatocytes undergo changes in cell morphology, structure, polarity, gene expression, and liver-specific functions,[3, 46, 47] a process referred to as de-differentiation. Hepatocyte de-differentiation is an important limitation to this *in vitro* system. As the isolated cells have lost their normal microenvironment structure, cell to cell interactions, and cell membrane structures, response to chemical exposures can be different than those occurring *in vivo*. Finally, one of the most problematic disadvantages of the use of primary hepatocytes for toxicological research is the drastic decrease in cell functionality over time. Liver specific functions such as albumin production and cytochrome P450 expression decline quickly over the first 24-48 hours of culture as the cells begin to lose their differentiated status.[3, 34, 46, 48] Extending culture longevity, both in terms of liver specific functions as well as basic cellular functionality, has been a great obstacle the field has not yet been able to overcome.

In an effort to maintain liver-specific functionality over longer culture periods, a simple sandwich configuration was developed. In this system, hepatocytes are placed between two layers of matrix (traditionally collagen or Matrigel®.[47] Maintaining hepatocytes in a sandwich culture configuration increases and maintains albumin secretion, restores polygonal morphology similar to that of *in vivo* cells, prevents loss of cell viability, increases basal and induced enzyme activities, and mimics *in vivo* biliary excretion rates.[47-51] Some of these functions are retained for relatively long periods of 24 days to 7 weeks in rat hepatocytes. Investigators attribute these positive effects to cell polarity which was induced by the ECM.[47] Research has shown that sandwich culture prevents the formation of stress fibers in addition to increasing transferrin, fibrinogen, and bile salt secretion, and stabilizing urea secretion in rat hepatocytes over 42 days.[52]

Despite the positive effects attributed to the sandwich culture technique, expression of genes responsible for many liver-specific functions decreases over time (90 hours). Even though phase I enzyme gene expression decreases over time and phase II enzymes remain expressed at relatively high levels, data indicate that P450 gene expression in sandwich cultured mouse hepatocytes is still more stable than in monolayers of primary hepatocytes.[53] It has been suggested that the sandwich culture model is most useful for the mechanistic studies of hepatobiliary toxicity.[54]

Approaches to surmount the challenges of the *in vitro* liver test systems have been proposed, including adjusting components of the culture medium, altering the extracellular matrix, changing the cell culture format (such as monolayer, spheroid, or 3D cultures), adding flow to the culture system, and culturing hepatocytes with other cell types, all of which are summarized in multiple reviews.[3, 34, 54-57] Although a number of investigators have found certain approaches to benefit hepatocyte culture in their own laboratories, difficulties in comparison of experimental results occur and continue to hinder the identification and adoption of optimal culture conditions. In addition, choice of species, strain, and sex of animal vary greatly across experiments. Conditions of cell isolation are also variable as are the methods for cell purification, conditions of cell culture, and endpoint assay methods. Yields and viabilities of final cell preparations can differ depending on types of collagenase and perfusion methods used. Species differences in optimal culture conditions abound as well. These factors must be taken into consideration when employing primary hepatocyte cultures for toxicity testing and developing the proper procedures in one's own laboratory.

Primary Hepatocyte Cultures Combined with Inflammatory Mediators

Traditional 2D hepatocyte cultures are not particularly well suited for high-throughput screening applications. Still, the availability of the robotics-equipped facilities and a

relatively low per sample cost of data generation in these systems has prompted exploration of novel culture formats. For example, a well-established role for inflammation in toxicity – both developmental and postnatal – has been demonstrated in many organ systems and disease states, from endometriosis[58] to idiosyncratic drug toxicity.[59]

An *in vitro* drug/inflammatory cytokine/inflammatory mediator co-treatment approach was used to reproduce clinical drug hepatotoxicity signatures, particularly for idiosyncratic drugs, in cultured primary human and rat hepatocytes, as well as HepG2 cells.[60] Using this approach, drug-cytokine hepatotoxicity synergies were shown to be detectable for multiple idiosyncratic hepatotoxicants, but not for non-toxic. The authors ascertained that TNF, IL-1 alpha, and LPS were especially informative within the context of multi-cytokine mixes. Further studies using this method (i.e., combinations of hepatotoxic drugs and cytokines) combined with multiplex phosphoprotein signaling and cytotoxicity measurements in cultured hepatocytes from multiple human donors produced multi-dimensional data amenable to modeling the signal-response data.[61] The authors showed that drug/cytokine liver toxicity is integratively controlled by four key signaling pathways: Akt, p70 S6 kinase, MEK-ERK, and p38-HSP27. Furthermore, this modeling predicted, and experimental studies confirmed, that the MEK-ERK and p38-HSP27 pathways contribute pro-death signaling influences in drug-cytokine synergy. These studies may be especially useful for illuminating consensus mechanisms of toxicity in human cells as they are amenable to high throughput, multiplexed intracellular signaling measurements, and reveal signaling networks that are difficult to ascertain from either *in vivo* or traditional *in vitro* culture systems.

3. Novel Liver-derived Cell Culture Systems

Primary hepatocytes and hepatocyte-like cells have limited utility under standard culture conditions for certain types of toxicity testing. A continuing challenge in the culture of both primary hepatocytes and hepatocyte-like cells is the long-term maintenance of their functionality.[3, 34, 46, 48, 62, 63] In addition, lack of proper absorption, distribution, metabolism, and excretion (ADME) properties as a result of cellular disconnect from the circulatory and other organ systems also make assessment of chemical exposure results difficult with primary hepatocyte cultures.[4, 34] At this time, novel culture strategies are being devised to counteract the de-differentiation process, establish a more heterotypical microenvironment, and create more predictive *in vitro* liver toxicity models. In this section, four of those strategies will be reviewed in more detail: three-dimensional culture systems, bioartificial livers, co-cultures, and stem cell models.

Three-dimensional Culture Systems

Three-dimensional culture of hepatocytes is a rapidly expanding field of study as engineers, biomaterial scientists and biologists attempt to recreate the complex cellular microenvironment of the liver in hopes of extending primary hepatocyte culture longevity and functionality. Three-dimensional cultures range in complexity from monolayer sandwich culture and spheroids to more advanced systems involving porous materials, packed-bed reactors, hollow fibers, and perfusion flow.

The microenvironment of the hepatocyte *in vivo* is very important to the maintenance of normal function, including its response to endogenous and exogenous substrates, and can be complex to mimic. Limitations of traditional two-dimensional culture, namely reduction of gene activity and expression of genes involved in drug metabolism, mainly occur within the first 24-48 hours and these cultures have been shown to have a low sensitivity of drug hepatotoxicity detection.[54] Reestablishment of cellular polarity has been suggested by many investigators as being crucial to maintaining gene activity and expression as well as proper function of hepatocytes while in culture.[52] Hepatocytes possess not only a single apical and

basal surface, but have multiple apical surfaces (bile canalicular surfaces) and two basolateral surfaces.[52] For this reason, re-establishing cell polarity in vitro is more challenging than with other types of epithelial cells. Different extracellular matrices (ECMs) have been extensively evaluated in 2D and 3D hepatocyte culture in an attempt to re-establish and subsequently maintain hepatocyte cell polarity. Many configurations of matrices have been evaluated to determine optimal conditions for restoration of hepatocyte polarity. The use of 3D liver cultures can overcome deficits of the 2D culture system by providing models that reestablish cellular polarity and create more complex local environments.

Three-dimensional culture systems have evolved greatly within the last decade in an effort to extend culture period and retain hepatocyte function. Initially, hepatocyte spheroids were constructed, with the assumption that cellular aggregates better mimic liver tissue characteristics. Working with self-forming hepatocyte spheroids was initially a complex undertaking involving a prolonged culture period for spheroid formation prior to the hepatocytes being available for any toxicological or pharmacological experimentation. In 1996, Wu and colleagues[64] optimized a protocol for quick production of rat hepatocyte spheroids through use of spinner flasks. They observed rat hepatocyte spheroid physiology and found that oxygen supply was critical in the proper formation of spheroids and that these cells maintained a more differentiated state as compared to the monolayer culture. *Cyp1a2* and *Cyp1a1* expression dropped rapidly in a traditional 2D monolayer culture of mouse hepatocytes, while in 3D spheroids the levels remained high over 5 days,[65, 66] which indicates that spheroid culture is preferred over monolayers for studies evaluating levels of gene expression. Spheroid culture of mouse hepatocytes can also maintain expression of *Cyp2b9* and *Cyp2b10* for several days at levels equal to *in vivo.*[67]

Du *et al.* [68] were able to develop a unique spheroid-based monolayer culture configuration by conjugating cell adhesion peptides Arg-Gly-Asp (RGD) with galactose ligand and polyethylene terephthalate. They determined that the rat hepatocyte 3D spheroid configuration was ideal; by using this new culture system cells would anchor well to the substrata and have a limited ability to spread while still keeping the functional characteristics of spheroids. Liver-specific functions of cells on the new substratum, such as albumin secretion and urea synthesis, were similar to that of traditional spheroids. These cells also exhibited greater sensitivity to acetaminophen-induced hepatotoxicity than those cultured on traditional 2D collagen. However, due to oxygen and nutrient diffusion difficulties, spheroid culture is limited in its ability to be used for bioartificial liver models or long-term culture; thus, additional strategies for 3D models are being explored.

Gelled materials have been devised to combine the complexity of a Matrigel®-type extracellular matrix with spheroid formation. The idea of culturing hepatocytes encapsulated within a gel was originally developed for use in a bioartificial liver where semi-permeable hollow fiber cartridges were used as a scaffold for hepatocyte attachment.[54] Miccheli et al.[69] entrapped rat hepatocytes in alginate beads and placed them in a bioreactor system with a continuous flow of medium. In this system, energy metabolism, viability, and redox state was stable within 3 hours and the proper shape, microvilli, tight junctions, and bile canaliculi were reformed within 8 hours. The authors confirmed previous observations that adequate supply of oxygen and substrates is critical for proper cellular reorganization in these systems. They observed increased glycerophosphorylethanolamine levels in the static cultures indicating problems with membrane phospholipid metabolism regulation contrasting low levels in the bioreactor system. Gels of agarose have been employed to sustain viability of mouse hepatocyte cultures for 21 days. Within these gels, hepatocytes typically form aggregates and excrete significantly higher amounts of albumin than their 2D counterparts.[70] Shen *et al.* [71] demonstrated that gel-entrapped hepatocytes expressed higher

levels of liver-specific functions and phase I metabolism, maintained higher intracellular ATP levels and mitochondrial membrane potential, and accumulated less lipids within the cytoplasm than those cultured in traditional 2D monolayer. In addition, measurements of phase I metabolic enzymes were similar to levels found *in vivo*. Furthermore, gel entrapped cultures were able to accurately reflect hepatotoxicity in over twenty reference compounds.[72] A general limitation of the gel culture systems is impairment of oxygen and nutrient transfer to cells. In addition, these systems are labor-intensive, low throughput and require large amounts of reagents.

An extension of this line of research was the development of models that use scaffolding to provide structural support. A semi-permeable hollow fiber-based system serves as an example. Some of the fiber systems incorporated gel entrapment while others did not. Rat hepatocyte cultures with increased functionality have been obtained through use of scaffolding composed of polyvinyl formal resin and nanofibers.[73, 74] The scaffolding increased cell adhesion, even at higher seeding densities, exhibited lower LDH release, and maintained biochemical functions such as albumin secretion, urea synthesis, and glycogen synthesis. Stabilization of albumin and urea levels after an initial decline was noted in a functional 20-day rat culture.[73] Shen and colleagues[75] examined a simple micro-hollow fiber reactor system for rat hepatocytes and determined that functionality (in terms of urea synthesis and albumin secretion) was increased in this system when compared to simple monolayer culture. Such a system also improves the metabolic capability of cells, as indicated by greater sensitivity to chemical insult than seen in cells in monolayer culture. Still, these models are difficult to produce in large quantities and also lack flow to facilitate oxygen and nutrient delivery to the cultured cells.

Incorporating fluid flow into three-dimensional culture systems was an important step in tissue engineering. Packed-bead, hollow fiber, or other type of “reactors” integrate micro-circulation of medium to recreate the fluid flow within the liver. Addition of flow to 3D cultures is important for combating the issues of poor oxygen and nutrient diffusion through spheroids and aggregates of cells and extracellular matrices. Systems incorporating shear flow have shown to promote round cell aggregates that are more similar to *in vivo* morphology and increase and maintain liver-specific functions.[76] Incorporating flow into the culture system has been shown to increase and maintain a higher level of urea synthesis[77] and to increase albumin, lactate, and glucose secretion.[78] Physiologic oxygen gradients can be established in these microfluidic systems which can assist in elucidating the physiological mechanisms involved in hepatotoxicity.[79] Furthermore, the organotypic physiological geometries and flow properties in these systems allow for the study of drug-drug and cell-cell interactions.[80] Although these methods of three-dimensional hepatocyte culture show improvement over traditional two-dimensional cultures they are still insufficient for long-term culture or use in clinical therapy (such as bioartificial livers) due to the continued loss of metabolic gene expression after cell isolation.

There continues to be a need for more complex 3D systems. There are a number of efforts to engineer more advanced 3-D culture systems by applying the existing knowledge regarding important cellular environmental dynamics between extracellular matrix, micro-circulation, and cell type and density. The overall goal of many of these efforts is to form a fully functional liver culture model that can be used for toxicological and pharmacological research or that can be modified into a bio-artificial liver for clinical use. A few systems will be briefly discussed here.

HμREL® Biochip—Chao *et al.* [80] published an evaluation of a novel microfluidic device, the HμREL® biochip (an earlier model of this system was previously described by Sin *et al.* [81]). Four biochips are enclosed in a polycarbonate housing connected, by tubing lines, to

a fluid reservoir and peristaltic pump. Each biochip has one or more separate compartments in which different cells can be housed. The compartments are microfluidically connected, in a linear path, to allow for interaction between the cell types. A new design allows for separate microfluidic experiments to be run in parallel on a larger set of housing plates. Evaluation of the system using primary human hepatocytes indicated the system preserved cell viability and metabolic competency at least as high as, and sometimes higher than, the traditional static culture conditions.[80, 82] The utility of this model for toxicity testing was explored through prediction of *in vivo* clearance rates. It was demonstrated that data from this model system was more correlative with in vivo data than that derived from the static hepatocyte cultures and this correlation further improved when co-cultures were used.[82] In addition, *in vivo*-like absorption, distribution, metabolism, bioaccumulation, and toxicity of naphthalene was demonstrated when lung, adipose, and liver cells were fluidically connected.[83] The microscale design allows for microscopic imaging, oxygen sensing, physiologically relevant ratios of chamber sizes and liquid residence times in each compartment, physiological hydrodynamic shear stress, physiological liquid to cell ratios, and requires less media and cells. The model is severely limited, however, by the fact that sample removal is difficult without disturbing the system dynamics. Furthermore, the recirculation of the medium involves a complex set of tubing lines and reservoirs, and cells on chips form monolayers and not physiological tissue constructs.

Hollow-Fiber Reactor—A hollow-fiber based bioreactor was described by Schmelzer *et al.*[84] for the regeneration and culture of human hepatocytes. Cells are seeded into the extracapillary space and are surrounded by three independent capillary membrane systems. The capillary systems are composed of porous polyethersulphone and hydrophobic multilaminate hollow fiber membranes which allow for gas exchange. The capillary layers are interwoven around the extracapillary space and two of the capillary systems are perfused in a counter-current flow with either culture medium or plasma while the third allows for decentralized oxygenation and supply of nutrients. The bioreactor functions through use of a perfusion device that uses pressure-regulated pumps to control the medium flow. A gas mixing unit is also used to provide the system with, and control the rates of, air, O_2, and CO_2. Utilizing this system, human hepatocyte functionality was stable for up to four weeks in this bioreactor system. Although this bioreactor system allowed for a microenvironment more similar to *in vivo* conditions, the system was large and required high numbers of cells and large amounts of reagents. A microscale prototype version of this bioreactor was then developed.[85] In this smaller model, the bioreactor is comprised of four cell chambers each of which contains four compartments. The first compartment houses the cells, two contain culture medium, and the last provides the oxygen supply. All compartments are connected to provide the cells with a physiologically-based environment. This micro-bioreactor has thus far been used for human fetal liver cell culture and has sustained cell viability and differentiation for ten days. The prototype allows for small numbers of cells and limited reagent use, microscopic evaluation of the cells, and monitoring of oxygen concentrations. In addition, the counter-directional perfusion method is unique in providing a more physiologically similar flow. Subsequently, a similar system was used for studies of pharmacokinetics and drug toxicity. Albumin synthesis and CYP activity was maintained for 2-3 weeks in co-cultures of parenchymal and non-parenchymal liver cells.[86] Limitations of this system include a lack of physiologic gradients normally seen in liver tissue, the complication of numerous tubing lines, and limited throughput as only a small number of different culture conditions can be evaluated on a single system.

Single- and Multi-Well Perfused Bioreactor—Sivaraman *et al.* [45] used a 3D bioreactor system to evaluate the functionality of rat hepatocyte spheroids. They had hypothesized that a system that included heterotypic cell interactions, fluid flow stresses,

and microarchitecture similar to those *in vivo* would provide the appropriate microenvironment for cells to function as they do *in vivo*. This system housed spheroids of rat hepatocytes attached within the many channels of a silicon scaffold. Perfusion of the medium was directed both over the top of the channels as well as through the tissue in the channels. A high-resistance filter controlled the perfusion through the channels so flow was uniform through all channels of the scaffold. Flow was initially in a downward direction to trap the cells in the channels and later, after the cells had adhered to the channel walls, the flow was reversed. Spheroids seeded in the bioreactor system had an increased and sustained functionality when compared to spheroids in both static culture and single cells seeded into the bioreactor system. Both urea synthesis and albumin excretion were increased and more stable with the spheroid bioreactors. This bioreactor system can be scaled up easily to incorporate more channels (and thus, more cells) and up to 12 different reactors controlled by the same unit while still maintaining cell viability and albumin production for 7 days.[87] The throughput and ease of handling of this model system is greater as compared to other 3D systems. Toxicity testing-relevant uses of this system include studies showing that clearance rates of compounds with known liver metabolism are comparable to those obtained in vivo, and promising results with known inflammatory-mediated idiosyncratic toxicants.[88] The scaffolds in these perfused bioreactors promote tissue morphogenesis and allow for microscopic examination via light or two-photon microscopes; however this cell culture system still lacks high-throughput capabilities, does not accommodate large numbers of cells, and needs to be examined with hepatocytes from other species.

Although much progress has been made over the traditional two-dimensional static culture system, there still is a need for a model in which all levels of liver-specific function can be maintained for long periods of time. Many of the available models have yet to establish whether the systems can incorporate and sustain hepatocytes from different species (e.g., rodents and humans). Furthermore, studies of applications of these methods in toxicity testing have thus far been limited and further research is needed to establish whether they may provide increased benefit over traditional culture systems.

Bioartificial Livers

Bioartificial livers (BALs) are a promising avenue for *ex vivo* therapies for patients with liver disease or failure. If properly designed and manufactured, the devices could be used for toxicological studies as well. The most common designs for BALs are hollow-fiber systems and flat-plate systems, using aggregates or single layers of hepatocytes respectively.[89] In these systems plasma can either be in direct contact to, or separated from, the hepatocytes.

Three primary criteria must be fulfilled in the design of a fully functional bioartificial liver: the device must be able to (1) use and maintain a large number of differentiated hepatocytes, (2) decrease transport limitations, and (3) prevent the need for large amounts of plasma/medium.[90]

A number of bioartificial liver models are under development and many use selective membranes to prevent direct contact between the blood and hepatocytes.[89] The ability to evaluate hepatotoxicity in the presence of human blood allows for a more predictive model by providing non-hepatic soluble factor influences. Zhang *et al.* [91] developed a multilayer flat-plate BAL that caused a reduced immune response in the recipient. These researchers co-cultured fresh porcine hepatocytes with bone marrow mesenchymal stem cells and used the BAL device to treat beagles in acute liver failure. Low levels of antibodies and complement indicate that this BAL has a high level of immunosafety. Davidson and colleagues[92] determined that zonal distribution of oxygen is one of the most important factors in BAL design. Their results showed that flow rate of plasma/medium was key to creating metabolic zones in such devices. A liver organoid system was developed by Lu and

associates[93] which utilized hollow fibers to assemble collagen-hepatocyte mixtures into three-dimensional constructs. In this system, glucose-related metabolic activity and hormonal responses were maintained at high levels over the culture period. Another device used microcarriers and alginate to allow for a large number of cells, minimal migration, and sufficient oxygen diffusion.[94]

The spatial heterogeneity that a bioartificial liver provides would be a great advantage for toxicological studies. For example, urea cycle enzymes are expressed in high levels in the periportal and centrilobular regions, however not in the perivenous hepatocytes.[90] Hepatotoxins such as acetaminophen can, over a period of repeat exposure, modulate expression of cytochrome P450 enzymes across the acinus, leading to new expression patterns.[89] Bioartificial liver devices can create a more similar environment to that of *in vivo* liver than 2D and 3D culture and can allow for maintenance of functional heterogeneity.

Although advances in BALs are promising, Bikhchandani and colleagues[95] indicate that the clinical relevance of BALs is still questionable and no statistically significant benefit in regards to patient survival has been shown by any single device. However, the lack of survival benefits for patients is not a disadvantage to their use in toxicity studies. Yet there are other attributes of BALs that are detrimental to toxicity studies, including their need for large numbers of highly functional cells (not widely available), lack of proper microenvironment for hepatocytes (including structure and interactions with other important non-parenchymal cell types), short life-span of hepatocytes in culture, and lack of proper complex membranes (to allow for oxygen, nutrient, and small molecule exchange).[96]

It has been suggested that any bio-artificial device composed of primary cells will require non-parenchymal cells in order to maintain tissue-specific functions.[97] Bioartificial liver devices are being developed to not only assist in the treatment of liver failure but also for use in drug metabolism and toxicological studies.[98] Whether or not a bioartificial liver or other type of three-dimensional model would best predict *in vivo* responses to chemical exposure remains to be seen; however the incorporation of key micro-environmental requirements, such as the presence of non-parenchymal cells, is critical to the development and application of these systems.

Co-culture Systems

In the body, cells function by way of complex interactions and signals from other cells. For this reason, it is safe to assume that cultures of hepatocytes alone may not properly represent *in vivo* functionality especially when examining drug related hepatotoxicity. Hepatocyte co-cultures are usually comprised of hepatocytes with one other cell type, often other liver cells,[99-102] non-liver epi- or endo-thelial cells,[103, 104] fibroblasts or cell lines.[105-107] Co-cultures have also been prepared using hepatocytes and more than one additional cell type, such as hepatocyte-non-parenchymal cell (NPC) cultures. In these cultures the hepatocytes are cultured with other liver cells: Kupffer cells (resident macrophages), sinusoidal endothelial cells, and stellate cells (stromal cells). Co-culturing hepatocytes with other cell types have been shown to be one of the most successful techniques for maintaining hepatocyte function under *in vitro* conditions.[28, 108-110]

Griffith and colleagues[111] demonstrated that hepatocytes seeded with bovine aortal endothelial cells could form three dimensional structures in a polymer scaffolding system with flow. Within these structures hepatocytes bound to the substrata and the endothelial cells flattened and “covered” the hepatocytes. These studies were later expanded to demonstrate that hepatocyte co-cultures with liver-derived endothelial cells in a microfabricated perfusion reactor resulted in formation of endothelial network structures and greater retention of hepatocellular function that those without co-cultured cells.[11287, 88] Of

note, co-cultures including liver-derived immune cells, along with other liver non-parenchymal cells, were responsive to inflammatory cues, rendering cultures susceptible to idiosyncratic toxicity of certain drugs that display toxicity when their metabolism synergizes with inflammation.[88] Other investigators have found increases in albumin secretion and urea synthesis in rat hepatocytes when cultured with mouse embryonic fibroblasts, 3T3-J2, or NIG-3T3 cells. Other benefits included retention of hepatic polygonal morphology, visible bile canaliculi, and distinct nuclei.[113] Mouse hepatocytes demonstrated better maintenance of albumin secretion and urea synthesis over a 7 day culture period when allowed to form 3D aggregates with mouse liver fibroblasts. The importance of the physical interactions between the two cell types was evident. Increased liver-specific functionality developed when the cells were in contact in the same culture rather than on different substrata.[114]

Results from a study culturing micropatterned hepatocytes (produced by seeding on a stencil and then removing the stencil) on a 3T3-J2 fibroblast feeder layer indicate that this type of co-culture can lead to increased and maintained albumin and urea production as well as retained hepatocyte morphology for up to 42 days.[115] This collagen micropatterned substrate system is now used by Hepregen Corporation in their HepatoPac™ Bioengineered Microliver Platform. Hepatocytes selectively adhere to ECM-coated domains while stromal cells attach to remaining areas surrounding the parenchymal cells.[115] When compared to human liver microsomes, S-9 fractions, and primary hepatocyte suspensions, the Hepregen system allowed for longer incubations with 27 known liver-metabolized compounds and was able to generate a greater proportion of the major human metabolites normally found in vivo.[116] A similar system is produced by Transparent, Inc. in which mouse fibroblasts are used as feeder cells. In this system, the feeder cells migrate from underneath and surround the 3D hepatocyte structure and form a Disse-like space between the two cell types. Results from this system indicate that hepatocyte CYP basal expression and induction and transporter activity can be maintained, increased, or measured in levels higher than traditional monolayer culture for 7, 14, or even 54 days.[117] The importance of the heterotypic interface between the hepatocytes and fibroblasts further exemplifies the importance of cell-cell contacts in hepatocyte culture.[118]

Not only can cell-cell contacts with fibroblasts, cell lines, and epi- and endothelial cells affect hepatocytes in culture, but the soluble factors excreted by other cell types have been shown to be extremely important as well. Cultures of rat hepatocytes in medium conditioned by cardiac endothelial cells functioned similarly to hepatocytes cultured under a layer of endothelial cells and gel.[119] Hepatocytes were shown to modulate endothelial activation states as well.[120] Morin *et al.* [121] observed similar increases in albumin secretion in culture systems in which the endothelial cells and hepatocytes were not in contact as cultures in which the cells were in direct contact. These non-contact co-cultures have had varied success and limited reproducibility between laboratories, however, thus cell-cell contacts continue to be one of the most important features of co-cultures.[57, 122]

The addition of liver non-parenchymal cells to cultures of hepatocytes is required to reconstruct an organotypic environment that can approximate *in vivo* conditions. Many publications report on the benefit of non-parenchymal cells on hepatocyte function. Ries *et al.* [99] described a hepatocyte-NPC co-culture model in which hepatocytes were seeded on top of the NPC layer (containing Kupffer, stellate, and sinusoidal endothelial cells). In their cultures, sinusoidal endothelial cells survived and maintained their fenestrae for 6 days. Other cell types remained viable for up to 14 days. They were able to demonstrate that not only did the presence of the NPCs maintain hepatocyte differentiation, but the hepatocytes contributed to survival of the NP cells. Human hepatocytes co-cultured with non-parenchymal cells, in a system with flow, were shown to have greater drug metabolism capabilities and better maintenance of metabolic capacity than cultures composed solely of

hepatocytes.[82] Other cultures of hepatocytes with non-parenchymal cells found that stimulation of hepatocyte DNA replication after treatment with known hepatotoxicant WY-14,643 was enhanced compared to cultures of hepatocytes alone, indicating that the co-cultures result in more *in vivo*-like responses to treatment.[123] Tukov *et al.* [100] examined the effects of Kupffer cells cultured in contact with rat hepatocytes. They found that by adding Kupffer cells to the cultures they could mimic *in vivo* drug-induced inflammatory responses thus providing a model which could be useful in predicting such interactions prior to clinical trials. Co-cultures of rat primary hepatocytes and stellate cells on a poly (DL-lactic acid) surface quickly formed spheroidal aggregates and maintained liver-specific functions for over 2 months. Cytochrome P450 enzyme induction was maintained for almost the entire culture period.[124] Stellate cells have also been shown to promote cell proliferation when in contact with hepatocytes and maintain hepatocyte differentiation when cultured without direct contact.[102]

Finally, RegeneMed Inc. has demonstrated the beneficial effects of seeding primary hepatocytes on nylon screen mesh constructs containing established cultures of non-parenchymal cells. These co-cultures formed 3D-like tissue constructs and maintained albumin synthesis and cytochrome induction for up to 48 days in culture.[125] More recently, the company has established that cells within the 3D co-culture system can respond to chemical exposures (such as Wy-14643, Phenobarbital, TCDD, and IL-6) and induce gene expression in multiple pathways in a manner similar to that found in *in vivo* tissue.[126]

Stem Cells

Current procedures used to isolate primary hepatocytes tend to be labor intensive and results vary greatly between laboratories and protocols. Use of cryopreserved hepatocytes is becoming more popular; however cell quality upon thawing is not always consistent. A more robust and reproducible source of hepatocytes would greatly benefit the field of toxicity testing and assist in standardizing research. Theoretically, stem cells would represent a renewable source of cells and would potentially provide large numbers of functionally equivalent cells that could be stored for later use. A toxicity model which incorporates a relatively unlimited supply of human hepatocytes with defined phenotypes would allow for better predictivity in responses to drug treatment and could account for the genetic diversity within the human population as well. Two sources of stem cells will be discussed here: embryonic and induced pluripotent cells.

Embryonic Stem Cells—Many laboratories have, with some success, developed protocols to isolate embryonic stem cells (ESCs) and induce them to form hepatocyte-like cells. Hamazaki *et al.* [127] evaluated the capability of murine embryonic stem cells to differentiate into mature adult hepatocytes *in vitro*. The researchers found that although ESCs could not differentiate past the initial stages of mid-endodermal differentiation (expression of TTR, AFP, AAT, ALB) with simply basal media alone; the addition of growth factors allowed the cells to differentiate into further stages of hepatic lineage (expression of mature hepatic genes). Other investigators have produced metabolically active hepatocyte-like cells from human ESCs using supplemented media[128-130] and recently researchers used HepG2 pre-conditioned medium to induce similar formation.[131] The researchers treated the hepatocyte-like cells with ethanol and observed similar effects (down-regulation of genes and proteins as well as cell mortality) that are typical of this treatment *in vivo*.

Unfortunately, all differentiation protocols result in highly variable functionality within the cell population and cells begin to lose hepatic characteristics after a few days much like standard culture conditions.[62] Zamule *et al.* [132] employed WEM with normal hepatocyte

supplements (HEPES, glutamine, dexamethasone, etc.) and type I collagen extracellular matrix to culture human ESCs for 10-20 days. Over this period of time, and under these conditions, the cells became committed to hepatic lineage differentiation. Although the differentiated cells expressed many liver specific functions at high levels, the levels were still not comparable to primary human hepatocytes indicating that the cells were not fully mature. Other researchers have found many other substances that induce differentiation of human ESCs along the hepatic lineage: FGF,[128, 133-136] BMP,[129, 134, 135] HGF,[128, 136] dexamethasone,[135, 137, 138] insulin,[134, 138, 139] and transferrin.[134] Mouse and rat models have shown to support the proliferation and function of transplanted adult hepatocytes in cases where the liver has been severely injured.[140-142] Unfortunately, hepatocytes transplanted into normal functioning liver tissue do not respond in the same proliferative manner.[143]

Researchers are developing methods to overcome these disadvantages of functional variability, low expression levels, and loss of functionality over time, however embryonic stem cells are a controversial source of cells for scientific research and this is not likely to change in the near future. A different source of stem cells could eliminate this ethical controversy.

Induced Pluripotent Stem Cells—Induced pluripotent stem cells (iPSCs) transform into hepatocyte-like cells by following the same differentiation processes as stem cells; however they originate from somatic cell types which must first be “reprogrammed”. IPSCs retain great capacity for cell division and differentiation.[144] Since these cells do not come from an embryo, the use of these cells is not as ethically controversial.

In 2010, a method that produced hepatocyte-like cells from murine fibroblasts was published.[63] This study was important as it produced cells that had mRNA expression similar to that of hepatocyte-like cells derived from embryonic stem cells indicating iPSCs could be as good a model as ESCs. Liu *et al.* [145] noted that generating human iPSCs from hepatocytes was a faster process than generation from other somatic cell types, however functionality was not determined beyond minimal evaluation (albumin, CYP3a4, and CYP1a2). Si-Tayeb and colleagues[63] postulated that if enzyme expression was to remain at high levels proper culture methods would have to be established (similar to 2D culture). These scientists also used a method that did not use any undefined factors such as serum or primary feeder cells which increased reproducibility and allowed for a large proportion of cells to exhibit primary hepatocyte characteristics.

Regrettably, expression levels of xenobiotic metabolism genes in iPSCs are still not equal to those found in whole liver or freshly isolated primary hepatocytes, enzyme levels decrease quickly over the culture period (again, similar to 2D hepatocyte culture), and other minor obstacles continue to impede the progress towards using these cells for *in vitro* toxicology (variability in lines, incomplete programming within cell populations, uncharacteristic response to prototype hepatotoxicants, etc.).[146] Unexplained differences in protein-coding and miRNA gene expression between ES and iPS cells were reported.[147] It was also found that the origin of the iPSCs could influence their differentiation, tumorigenic properties, and gene expression and epigenetic features.[148, 149] Finally, Liu and colleagues[145] were not able to determine if the final hepatocyte-like cells derived from their iPSCs were mature hepatocytes containing proper gene expression profiles at levels comparable to whole liver.

Fully functional hepatocytes derived from induced pluripotent stem cells would be a valuable tool for drug development or evaluation of the contribution of genetic variation to variable responses. However, due to current disadvantages and technical limitations, neither

induced pluripotent nor embryonic stem cells are yet a widely accepted option for toxicological and pharmacological studies.

4. Conclusions

Much progress has been made towards improving traditional *in vitro* models that sustain liver-specific functions and can accurately predict *in vivo* responses to toxicants. These models, however, still lack the ability to provide long-term culture without cell necrosis or de-differentiation. Although each traditional *in vitro* system has advantages and disadvantages, the use of three-dimensional systems with dynamic flow for primary cell and/or stem cell cultures appears to be the most promising for toxicological studies. One can imagine the implications of a physiologically accurate 3D system or BAL and the availability of a large number of highly functional stem cell populations. In addition, the ability to provide large biomasses of human-disease relevant cells could significantly reduce the need for animal use in toxicological studies. The use of co-cultures could further enhance the *in vivo*–like characteristics of a culture device and would provide more predictive results. As the needs for toxicity testing of ever greater number of chemical substances are not likely to diminish in the next decade, the development of the models for reliable, quick, and economical toxicity testing is of major importance. The need for quantities of hepatocytes capable of *in vivo*-like functions over long-term culture is urgent and with these new developments it appears that this goal may soon be within reach.

Acknowledgments

Supported, in part, by grants from the National Institutes of Health: P42 ES005948, and R01 ES015241.

References

1. Holmes AM, Creton S, Chapman K. Working in partnership to advance the 3Rs in toxicity testing. Toxicology. 2010; 267:14–19. [PubMed: 19903508]
2. Council, NR. Toxicity testing in the 21st century: A vision and a strategy. National Academy Press; Washington, DC: 2007.
3. LeCluyse EL, Bullock PL, Parkinson A. Strategies for restoration and maintenance of normal hepatic structure and function in long-term cultures of rat hepatocytes. Adv Drug Deliver Rev. 1996; 22:133–186.
4. DelRaso NJ. In vitro methodologies for enhanced toxicity testing. Toxicol Lett. 1993; 68:91–99. [PubMed: 8516778]
5. NIEHS. [08/17/11, 2011] About the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). http://iccvam.niehs.nih.gov/about/about_NICEATM.htm
6. Judson R, Richard A, Dix DJ, Houck K, Martin M, Kavlock R, Dellarco V, Henry T, Holderman T, Sayre P, Tan S, Carpenter T, Smith E. The toxicity data landscape for environmental chemicals. Environ Health Perspect. 2009; 117:685–695. [PubMed: 19479008]
7. Judson RS, Houck KA, Kavlock RJ, Knudsen TB, Martin MT, Mortensen HM, Reif DM, Rotroff DM, Shah I, Richard AM, Dix DJ. In vitro screening of environmental chemicals for targeted testing prioritization: the ToxCast project. Environ Health Perspect. 2010; 118:485–492. [PubMed: 20368123]
8. Knudsen TB, Houck KA, Sipes NS, Singh AV, Judson RS, Martin MT, Weissman A, Kleinstreuer NC, Mortensen HM, Reif DM, Rabinowitz JR, Setzer RW, Richard AM, Dix DJ, Kavlock RJ. Activity profiles of 309 ToxCast chemicals evaluated across 292 biochemical targets. Toxicology. 2011; 282:1–15. [PubMed: 21251949]
9. Lerche-Langrand C, Toutain HJ. Precision-cut liver slices: characteristics and use for in vitro pharmaco-toxicology. Toxicology. 2000; 153:221–253. [PubMed: 11090959]

10. Toutain HJ, Moronvalle-Halley V, Sarsat JP, Chelin C, Hoet D, Leroy D. Morphological and functional integrity of precision-cut rat liver slices in rotating organ culture and multiwell plate culture: effects of oxygen tension. Cell Biol Toxicol. 1998; 14:175–190. [PubMed: 9689491]
11. Hashemi E, Till C, Ioannides C. Stability of Phase II Conjugation Systems in Cultured Precision-cut Rat Hepatic Slices. Toxicol In Vitro. 1999; 13:459–466. [PubMed: 20654503]
12. Ghantous HN, Fernando J, Gandolfi AJ, Brendel K. Sevoflurane is biotransformed by guinea pig liver slices but causes minimal cytotoxicity. Anesthesia and analgesia. 1992; 75:436–440. [PubMed: 1510266]
13. B. T. Gokhale MS, Zurlo J, Yager JD. Cytochrome P-450 1A1/1A2 induction, albumin secretion, and histological changes in cultured rat liver slices. In Vitro Toxicology. 1997; 8:357–368.
14. Boess F, Kamber M, Romer S, Gasser R, Muller D, Albertini S, Suter L. Gene expression in two hepatic cell lines, cultured primary hepatocytes, and liver slices compared to the in vivo liver gene expression in rats: possible implications for toxicogenomics use of in vitro systems. Toxicol Sci. 2003; 73:386–402. [PubMed: 12657743]
15. Elferink MG, Olinga P, van Leeuwen EM, Bauerschmidt S, Polman J, Schoonen WG, Heisterkamp SH, Groothuis GM. Gene expression analysis of precision-cut human liver slices indicates stable expression of ADME-Tox related genes. Toxicol Appl Pharmacol. 2011; 253:57–69. [PubMed: 21420995]
16. Olinga P, Groen K, Hof IH, De Kanter R, Koster HJ, Leeman WR, Rutten AA, Van Twillert K, Groothuis GM. Comparison of five incubation systems for rat liver slices using functional and viability parameters. Journal of pharmacological and toxicological methods. 1997; 38:59–69. [PubMed: 9403776]
17. Price RJ, Ball SE, Renwick AB, Barton PT, Beamand JA, Lake BG. Use of precision-cut rat liver slices for studies of xenobiotic metabolism and toxicity: comparison of the Krumdieck and Brendel tissue slicers. Xenobiotica. 1998; 28:361–371. [PubMed: 9604300]
18. S. R. Fisher RL, Jenkins PM, Austin ML, Roth GL, Gandolfi AJ, Brendel K. Dynamic organ culture is superior to multiwell plate culture for maintaining precision-cut tissue slices: optimization of tissue slice culture, part I. Toxicology Methods. 1995; 5:99–113.
19. Leeman WR, van de Gevel IA, Rutten AA. Cytotoxicity of retinoic acid, menadione and aflatoxin B(1) in rat liver slices using Netwell inserts as a new culture system. Toxicol In Vitro. 1995; 9:291–298. [PubMed: 20650090]
20. Wright MC, Paine AJ. Evidence that the loss of rat liver cytochrome P450 in vitro is not solely associated with the use of collagenase, the loss of cell-cell contacts and/or the absence of an extracellular matrix. Biochem Pharmacol. 1992; 43:237–243. [PubMed: 1310850]
21. Lake BG, Beamand JA, Wield PT, Price RJ. Use of precision-cut liver slices to evaluate species differences in 2-acetylaminofluorene-induced unscheduled DNA synthesis. Toxicol Appl Pharmacol. 1996; 138:231–241. [PubMed: 8658524]
22. Worboys PD, Bradbury A, Houston JB. Kinetics of drug metabolism in rat liver slices. II. Comparison of clearance by liver slices and freshly isolated hepatocytes. Drug Metab Dispos. 1996; 24:676–681. [PubMed: 8781785]
23. Ekins S, Murray GI, Burke MD, Williams JA, Marchant NC, Hawksworth GM. Quantitative differences in phase I and II metabolism between rat precision-cut liver slices and isolated hepatocytes. Drug Metab Dispos. 1995; 23:1274–1279. [PubMed: 8591730]
24. Yu LJ, Matias J, Scudiero DA, Hite KM, Monks A, Sausville EA, Waxman DJ. P450 enzyme expression patterns in the NCI human tumor cell line panel. Drug Metab Dispos. 2001; 29:304–312. [PubMed: 11181500]
25. Wooster R, Ebner T, Sutherland L, Clarke D, Burchell B. Drug and xenobiotic glucuronidation catalysed by cloned human liver UDP-Glucuronosyltransferases stably expressed in tissue culture cell lines. Toxicology. 1993; 82:119–129. [PubMed: 8236271]
26. MacDonald C, Vass M, Willett B, Scott A, Grant H. Expression of liver functions in immortalised rat hepatocyte cell lines. Hum Exp Toxicol. 1994; 13:439–444. [PubMed: 8086228]
27. Guguen-Guillouzo C, Corlu A, Guillouzo A. Stem cell-derived hepatocytes and their use in toxicology. Toxicology. 2010; 270:3–9. [PubMed: 19815049]

28. Guguen-Guillouzo C, Guillouzo A. General review on in vitro hepatocyte models and their applications. Methods Mol Biol. 2010; 640:1–40. [PubMed: 20645044]

29. Braiterman, LT.; Hubbard, AL. The Liver. John Wiley & Sons, Ltd; 2009. p. 73-105.

30. Aninat C, Piton A, Glaise D, Le Charpentier T, Langouet S, Morel F, Guguen-Guillouzo C, Guillouzo A. Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. Drug Metab Dispos. 2006; 34:75–83. [PubMed: 16204462]

31. Marion MJ, Hantz O, Durantel D. The HepaRG cell line: biological properties and relevance as a tool for cell biology, drug metabolism, and virology studies. Methods Mol Biol. 2010; 640:261–272. [PubMed: 20645056]

32. Lubberstedt M, Muller-Vieira U, Mayer M, Biemel KM, Knospel F, Knobeloch D, Nussler AK, Gerlach JC, Zeilinger K. HepaRG human hepatic cell line utility as a surrogate for primary human hepatocytes in drug metabolism assessment in vitro. Journal of pharmacological and toxicological methods. 2011; 63:59–68. [PubMed: 20460162]

33. Griffin SJ, Houston JB. Prediction of in vitro intrinsic clearance from hepatocytes: comparison of suspensions and monolayer cultures. Drug Metab Dispos. 2005; 33:115–120. [PubMed: 15608354]

34. LeCluyse EL. Human hepatocyte culture systems for the in vitro evaluation of cytochrome P450 expression and regulation. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences. 2001; 13:343–368. [PubMed: 11408150]

35. 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. 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. 2007; 39:159–234. [PubMed: 17364884]

36. O'Brien PJ, Siraki AG. Accelerated cytotoxicity mechanism screening using drug metabolising enzyme modulators. Current drug metabolism. 2005; 6:101–109. [PubMed: 15853762]

37. Guillouzo A. Liver cell models in in vitro toxicology. Environ Health Perspect. 1998; 106(Suppl 2):511–532. [PubMed: 9599700]

38. Jauregui HO, McMillan PN, Hevey K, Naik S. A quantitative analysis of lectin binding to adult rat hepatocyte cell surfaces. In vitro cellular & developmental biology : journal of the Tissue Culture Association. 1988; 24:401–412.

39. Duval DL, Sieg DJ, Billings RE. Regulation of hepatic nitric oxide synthase by reactive oxygen intermediates and glutathione. Archives of biochemistry and biophysics. 1995; 316:699–706. [PubMed: 7532384]

40. Wang H, Gao X, Fukumoto S, Tademoto S, Sato K, Hirai K. Post-isolation inducible nitric oxide synthase gene expression due to collagenase buffer perfusion and characterization of the gene regulation in primary cultured murine hepatocytes. J Biochem. 1998; 124:892–899. [PubMed: 9792910]

41. Tirmenstein MA, Nicholls-Grzemski FA, Schmittgen TD, Zakrajsek BA, Fariss MW. Characterization of nitric oxide production following isolation of rat hepatocytes. Toxicol Sci. 2000; 53:56–62. [PubMed: 10653521]

42. Dalet C, Fehlmann M, Debey P. Use of Percoll density gradient centrifugation for preparing isolated rat hepatocytes having long-term viability. Anal Biochem. 1982; 122:119–123. [PubMed: 6285762]

43. Maurice M, Rogier E, Cassio D, Feldmann G. Formation of plasma membrane domains in rat hepatocytes and hepatoma cell lines in culture. J Cell Sci. 1988; 90(Pt 1):79–92. [PubMed: 3058731]

44. Burke AS, Macmillan-Crow LA, Hinson JA. The Hepatocyte Suspension Assay Is Superior to the Cultured Hepatocyte Assay for Determining Mechanisms of Acetaminophen Hepatotoxicity Relevant to in Vivo Toxicity. Chem Res Toxicol. 2010

45. Sivaraman A, Leach JK, Townsend S, Iida T, Hogan BJ, Stolz DB, Fry R, Samson LD, Tannenbaum SR, Griffith LG. A microscale in vitro physiological model of the liver: predictive

screens for drug metabolism and enzyme induction. Current drug metabolism. 2005; 6:569–591. [PubMed: 16379670]

46. Nelson KF, Acosta D, Bruckner JV. Long-term maintenance and induction of cytochrome P-450 in primary cultures of rat hepatocytes. Biochem Pharmacol. 1982; 31:2211–2214. [PubMed: 7115442]
47. Dunn JC, Yarmush ML, Koebe HG, Tompkins RG. Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration. FASEB J. 1989; 3:174–177. [PubMed: 2914628]
48. Bader A, Borel Rinkes IH, Closs EI, Ryan CM, Toner M, Cunningham JM, Tompkins RG, Yarmush ML. A stable long-term hepatocyte culture system for studies of physiologic processes: cytokine stimulation of the acute phase response in rat and human hepatocytes. Biotechnology progress. 1992; 8:219–225. [PubMed: 1368259]
49. LeCluyse EL, Ahlgren-Beckendorf JA, Carroll K, Parkinson A, Johnson J. Regulation of glutathione S-transferase enzymes in primary cultures of rat hepatocytes maintained under various matrix configurations. Toxicol In Vitro. 2000; 14:101–115. [PubMed: 10793289]
50. Liu X, Chism JP, LeCluyse EL, Brouwer KR, Brouwer KL. Correlation of biliary excretion in sandwich-cultured rat hepatocytes and in vivo in rats. Drug Metab Dispos. 1999; 27:637–644. [PubMed: 10348791]
51. Mingoia RT, Nabb DL, Yang CH, Han X. Primary culture of rat hepatocytes in 96-well plates: effects of extracellular matrix configuration on cytochrome P450 enzyme activity and inducibility, and its application in in vitro cytotoxicity screening. Toxicol In Vitro. 2007; 21:165–173. [PubMed: 17141466]
52. Dunn JC, Tompkins RG, Yarmush ML. Long-term in vitro function of adult hepatocytes in a collagen sandwich configuration. Biotechnology progress. 1991; 7:237–245. [PubMed: 1367596]
53. Mathijs K, Kienhuis AS, Brauers KJ, Jennen DG, Lahoz A, Kleinjans JC, van Delft JH. Assessing the metabolic competence of sandwich-cultured mouse primary hepatocytes. Drug Metab Dispos. 2009; 37:1305–1311. [PubMed: 19251822]
54. Meng Q. Three-dimensional culture of hepatocytes for prediction of drug-induced hepatotoxicity. Expert opinion on drug metabolism & toxicology. 2010; 6:733–746. [PubMed: 20380484]
55. Mitaka T. The current status of primary hepatocyte culture. Int J Exp Pathol. 1998; 79:393–409. [PubMed: 10319020]
56. Griffith LG, Swartz MA. Capturing complex 3D tissue physiology in vitro. Nat Rev Mol Cell Biol. 2006; 7:211–224. [PubMed: 16496023]
57. Coecke S, Rogiers V, Bayliss M, Castell J, Doehmer J, Fabre G, Fry J, Kern A, Westmoreland C. The use of long-term hepatocyte cultures for detecting induction of drug metabolising enzymes: the current status. ATLA-NOTTINGHAM-. 1999; 27:579–638.
58. Herington JL, Bruner-Tran KL, Lucas JA, Osteen KG. Immune interactions in endometriosis. Expert review of clinical immunology. 2011; 7:611–626. [PubMed: 21895474]
59. Shaw PJ, Ganey PE, Roth RA. Idiosyncratic drug-induced liver injury and the role of inflammatory stress with an emphasis on an animal model of trovafloxacin hepatotoxicity. Toxicol Sci. 2010; 118:7–18. [PubMed: 20538741]
60. Cosgrove BD, King BM, Hasan MA, Alexopoulos LG, Farazi PA, Hendriks BS, Griffith LG, Sorger PK, Tidor B, Xu JJ, Lauffenburger DA. Synergistic drug-cytokine induction of hepatocellular death as an in vitro approach for the study of inflammation-associated idiosyncratic drug hepatotoxicity. Toxicol Appl Pharmacol. 2009; 237:317–330. [PubMed: 19362101]
61. Cosgrove BD, Alexopoulos LG, Hang TC, Hendriks BS, Sorger PK, Griffith LG, Lauffenburger DA. Cytokine-associated drug toxicity in human hepatocytes is associated with signaling network dysregulation. Molecular bioSystems. 2010; 6:1195–1206. [PubMed: 20361094]
62. Guillouzo A, Guguen-Guillouzo C. Evolving concepts in liver tissue modeling and implications for in vitro toxicology. Expert opinion on drug metabolism & toxicology. 2008; 4:1279–1294. [PubMed: 18798698]
63. Si-Tayeb K, Noto FK, Nagaoka M, Li J, Battle MA, Duris C, North PE, Dalton S, Duncan SA. Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology. 2010; 51:297–305. [PubMed: 19998274]

64. Wu FJ, Friend JR, Hsiao CC, Zilliox MJ, Ko WJ, Cerra FB, Hu WS. Efficient assembly of rat hepatocyte spheroids for tissue engineering applications. Biotechnology and bioengineering. 1996; 50:404–415. [PubMed: 18626989]
65. Nemoto N, Sakurai J. Activation of Cyp1a1 and Cyp1a2 genes in adult mouse hepatocytes in primary culture. Japanese journal of cancer research : Gann. 1993; 84:272–278. [PubMed: 8486528]
66. Nemoto N, Sakurai J. Differences in regulation of gene expression between Cyp1a-1 and Cyp1a-2 in adult mouse hepatocytes in primary culture. Carcinogenesis. 1992; 13:2249–2254. [PubMed: 1335375]
67. Nemoto N, Sakurai J, Funae Y. Maintenance of phenobarbital-inducible Cyp2b gene expression in C57BL/6 mouse hepatocytes in primary culture as spheroids. Archives of biochemistry and biophysics. 1995; 316:362–369. [PubMed: 7840637]
68. Du Y, Chia SM, Han R, Chang S, Tang H, Yu H. 3D hepatocyte monolayer on hybrid RGD/galactose substratum. Biomaterials. 2006; 27:5669–5680. [PubMed: 16904177]
69. Miccheli A, Tomassini A, Capuani G, Di Cocco ME, Sartori E, Falasca L, Conti Devirgiliis L, Manetti C, Conti F. Energy metabolism and re-establishment of intercellularadhesion complexes of gel entrapped hepatocytes. Cytotechnology. 2000; 32:219–228. [PubMed: 19002983]
70. Ise H, Takashima S, Nagaoka M, Ferdous A, Akaike T. Analysis of cell viability and differential activity of mouse hepatocytes under 3D and 2D culture in agarose gel. Biotechnology letters. 1999; 21:209–213.
71. Shen C, Zhang G, Qiu H, Meng Q. Acetaminophen-induced hepatotoxicity of gel entrapped rat hepatocytes in hollow fibers. Chem Biol Interact. 2006; 162:53–61. [PubMed: 16797510]
72. Lu Y, Meng Q, Zhang G, Bei X. Clozapine-induced hepatotoxicity in rat hepatocytes by gel entrapment and monolayer culture. Toxicol In Vitro. 2008; 22:1754–1760. [PubMed: 18761400]
73. Miyoshi H, Ookawa K, Ohshima N. Hepatocyte culture utilizing porous polyvinyl formal resin maintains long-term stable albumin secretion activity. Journal of biomaterials science. Polymer edition. 1998; 9:227–237. [PubMed: 9556759]
74. Chu XH, Shi XL, Feng ZQ, Gu ZZ, Ding YT. Chitosan nanofiber scaffold enhances hepatocyte adhesion and function. Biotechnology letters. 2009; 31:347–352. [PubMed: 19037598]
75. Shen C, Zhang G, Meng Q. An in vitro model for long-term hepatotoxicity testing utilizing rat hepatocytes entrapped in micro-hollow fiber reactor. Biochemical Engineering Journal. 2007; 34:267–272.
76. Kang IK, Kim GJ, Kwon OH, Ito Y. Co-culture of hepatocytes and fibroblasts by micropatterned immobilization of [beta]-galactose derivatives. Biomaterials. 2004; 25:4225–4232. [PubMed: 15046912]
77. Taguchi K, Matsushita M, Takahashi M, Uchino J. Development of a bioartificial liver with sandwiched-cultured hepatocytes between two collagen gel layers. Artificial organs. 1996; 20:178–185. [PubMed: 8712966]
78. Kim SS, Utsunomiya H, Koski JA, Wu BM, Cima MJ, Sohn J, Mukai K, Griffith LG, Vacanti JP. Survival and function of hepatocytes on a novel three-dimensional synthetic biodegradable polymer scaffold with an intrinsic network of channels. Annals of surgery. 1998; 228:8–13. [PubMed: 9671060]
79. Allen JW, Khetani SR, Bhatia SN. In vitro zonation and toxicity in a hepatocyte bioreactor. Toxicol Sci. 2005; 84:110–119. [PubMed: 15590888]
80. Chao P, Maguire T, Novik E, Cheng KC, Yarmush ML. Evaluation of a microfluidic based cell culture platform with primary human hepatocytes for the prediction of hepatic clearance in human. Biochem Pharmacol. 2009; 78:625–632. [PubMed: 19463793]
81. Sin A, Chin KC, Jamil MF, Kostov Y, Rao G, Shuler ML. The design and fabrication of three-chamber microscale cell culture analog devices with integrated dissolved oxygen sensors. Biotechnology progress. 2004; 20:338–345. [PubMed: 14763861]
82. Novik E, Maguire TJ, Chao P, Cheng KC, Yarmush ML. A microfluidic hepatic coculture platform for cell-based drug metabolism studies. Biochem Pharmacol. 2010; 79:1036–1044. [PubMed: 19925779]

83. Baxter GT. Hurel -- an in vivo-surrogate assay platform for cell-based studies. Alternatives to laboratory animals : ATLA. 2009; 37(Suppl 1):11–18. [PubMed: 19807199]
84. Schmelzer E, Mutig K, Schrade P, Bachmann S, Gerlach JC, Zeilinger K. Effect of human patient plasma ex vivo treatment on gene expression and progenitor cell activation of primary human liver cells in multi-compartment 3D perfusion bioreactors for extra-corporeal liver support. Biotechnology and bioengineering. 2009; 103:817–827. [PubMed: 19274748]
85. Schmelzer E, Triolo F, Turner ME, Thompson RL, Zeilinger K, Reid LM, Gridelli B, Gerlach JC. Three-dimensional perfusion bioreactor culture supports differentiation of human fetal liver cells. Tissue engineering. Part A. 2010; 16:2007–2016. [PubMed: 20088704]
86. Zeilinger K, Schreiter T, Darnell M, Soderdahl T, Lubberstedt M, Dillner B, Knobeloch D, Nussler AK, Gerlach JC, Andersson TB. Scaling down of a clinical three-dimensional perfusion multicompartment hollow fiber liver bioreactor developed for extracorporeal liver support to an analytical scale device useful for hepatic pharmacological in vitro studies. Tissue engineering. Part C, Methods. 2011; 17:549–556. [PubMed: 21210724]
87. Domansky K, Inman W, Serdy J, Dash A, Lim MH, Griffith LG. Perfused multiwell plate for 3D liver tissue engineering. Lab on a chip. 2010; 10:51–58. [PubMed: 20024050]
88. Dash A, Inman W, Hoffmaster K, Sevidal S, Kelly J, Obach RS, Griffith LG, Tannenbaum SR. Liver tissue engineering in the evaluation of drug safety. Expert opinion on drug metabolism & toxicology. 2009; 5:1159–1174. [PubMed: 19637986]
89. Chan C, Berthiaume F, Nath BD, Tilles AW, Toner M, Yarmush ML. Hepatic tissue engineering for adjunct and temporary liver support: critical technologies. Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society. 2004; 10:1331–1342. [PubMed: 15497161]
90. Tilles AW, Berthiaume F, Yarmush ML, Tompkins RG, Toner M. Bioengineering of liver assist devices. Journal of hepato-biliary-pancreatic surgery. 2002; 9:686–696. [PubMed: 12658402]
91. Zhang Y, Shi XL, Han B, Gu JY, Chu XH, Xiao JQ, Ren HZ, Tan JJ, Ding YT. Immunosafety Evaluation of a Multilayer Flat-Plate Bioartificial Liver. The American journal of the medical sciences. 2011
92. Davidson AJ, Ellis MJ, Chaudhuri JB. A theoretical approach to zonation in a bioartificial liver. Biotechnology and bioengineering. 2012; 109:234–243. [PubMed: 21809328]
93. Lu Y, Zhang G, Shen C, Uygun K, Yarmush ML, Meng Q. A novel 3D liver organoid system for elucidation of hepatic glucose metabolism. Biotechnology and bioengineering. 2012; 109:595–604. [PubMed: 22006574]
94. Jeffries RE, Macdonald JM. New advances in MR-compatible bioartificial liver. NMR in biomedicine. 2012; 25:427–442. [PubMed: 22351642]
95. Bikhchandani J, Metcalfe M, Illouz S, Puls F, Dennison A. Extracorporeal liver perfusion system for artificial liver support across a membrane. The Journal of surgical research. 2011; 171:e139–147. [PubMed: 21920550]
96. Sanal MG. Future of liver transplantation: non-human primates for patient-specific organs from induced pluripotent stem cells. World J Gastroenterol. 2011; 17:3684–3690. [PubMed: 21990949]
97. Soto-Gutierrez A, Navarro-Alvarez N, Yagi H, Nahmias Y, Yarmush ML, Kobayashi N. Engineering of an hepatic organoid to develop liver assist devices. Cell transplantation. 2010; 19:815–822. [PubMed: 20573303]
98. Sharma NS, Nagrath D, Yarmush ML. Metabolic profiling based quantitative evaluation of hepatocellular metabolism in presence of adipocyte derived extracellular matrix. PloS one. 2011; 6:e20137. [PubMed: 21603575]
99. Ries K, Krause P, Solsbacher M, Schwartz P, Unthan-Fechner K, Christ B, Markus PM, Probst I. Elevated expression of hormone-regulated rat hepatocyte functions in a new serum-free hepatocyte-stromal cell coculture model. In vitro cellular & developmental biology. Animal. 2000; 36:502–512. [PubMed: 11149749]
100. Tukov FF, Maddox JF, Amacher DE, Bobrowski WF, Roth RA, Ganey PE. Modeling inflammation-drug interactions in vitro: a rat Kupffer cell-hepatocyte coculture system. Toxicol In Vitro. 2006; 20:1488–1499. [PubMed: 16782301]

101. Krause P, Saghatolislam F, Koenig S, Unthan-Fechner K, Probst I. Maintaining hepatocyte differentiation in vitro through co-culture with hepatic stellate cells. In vitro cellular & developmental biology. Animal. 2009; 45:205–212. [PubMed: 19184253]
102. Higashiyama S, Noda M, Muraoka S, Uyama N, Kawada N, Ide T, Kawase M, Yagi K. Maintenance of hepatocyte functions in coculture with hepatic stellate cells. Biochemical Engineering Journal. 2004; 20:113–118.
103. Talamini MA, McCluskey MP, Buchman TG, De Maio A. Expression of alpha2-macroglobulin by the interaction between hepatocytes and endothelial cells in coculture. Am J Physiol. 1998; 275:R203–211. [PubMed: 9688980]
104. Gebhardt R, Schrode W, Eisenmann-Tappe I. Cellular characteristics of epithelial cell lines from juvenile rat liver: selective induction of glutamine synthetase by dexamethasone. Cell Biol Toxicol. 1998; 14:55–67. [PubMed: 9538945]
105. Kang IK, Kim GJ, Kwon OH, Ito Y. Co-culture of hepatocytes and fibroblasts by micropatterned immobilization of beta-galactose derivatives. Biomaterials. 2004; 25:4225–4232. [PubMed: 15046912]
106. Evenou F, Hamon M, Fujii T, Takeuchi S, Sakai Y. Gas-permeable membranes and co-culture with fibroblasts enable high-density hepatocyte culture as multilayered liver tissues. Biotechnology progress. 2011
107. Bhatia SN, Balis UJ, Yarmush ML, Toner M. Microfabrication of hepatocyte/fibroblast co-cultures: role of homotypic cell interactions. Biotechnology progress. 1998; 14:378–387. [PubMed: 9622518]
108. Guguen-Guillouzo C, Guillouzo A. Modulation of functional activities in cultured rat hepatocytes. Mol Cell Biochem. 1983; 53-54:35–56. [PubMed: 6621517]
109. Begue JM, Guguen-Guillouzo C, Pasdeloup N, Guillouzo A. Prolonged maintenance of active cytochrome P-450 in adult rat hepatocytes co-cultured with another liver cell type. Hepatology. 1984; 4:839–842. [PubMed: 6434390]
110. Kaihara S, Kim S, Kim BS, Mooney DJ, Tanaka K, Vacanti JP. Survival and function of rat hepatocytes cocultured with nonparenchymal cells or sinusoidal endothelial cells on biodegradable polymers under flow conditions. J Pediatr Surg. 2000; 35:1287–1290. [PubMed: 10999680]
111. Griffith LG, Wu B, Cima MJ, Powers MJ, Chaignaud B, Vacanti JP. In vitro organogenesis of liver tissue. Annals of the New York Academy of Sciences. 1997; 831:382–397. [PubMed: 9616729]
112. Hwa AJ, Fry RC, Sivaraman A, So PT, Samson LD, Stolz DB, Griffith LG. Rat liver sinusoidal endothelial cells survive without exogenous VEGF in 3D perfused co-cultures with hepatocytes. FASEB J. 2007 In press.
113. Khetani SR, Szulgit G, Del Rio JA, Barlow C, Bhatia SN. Exploring interactions between rat hepatocytes and nonparenchymal cells using gene expression profiling. Hepatology. 2004; 40:545–554. [PubMed: 15349892]
114. He J, Feng M. Primary 3-dimensional culture of mouse hepatocytes. Chinese Science Bulletin. 2000; 45:1878–1883.
115. Khetani SR, Bhatia SN. Microscale culture of human liver cells for drug development. Nat Biotechnol. 2008; 26:120–126. [PubMed: 18026090]
116. Wang WW, Khetani SR, Krzyzewski S, Duignan DB, Obach RS. Assessment of a micropatterned hepatocyte coculture system to generate major human excretory and circulating drug metabolites. Drug Metab Dispos. 2010; 38:1900–1905. [PubMed: 20595376]
117. Enosawa, YTS.; Jomura, T.; Ozeki, E.; Ikeya, T. JSSX Annual Meeting; Hiroshima, Japan. 2011;
118. Cho CH, Park J, Tilles AW, Berthiaume F, Toner M, Yarmush ML. Layered patterning of hepatocytes in co-culture systems using microfabricated stencils. Biotechniques. 2010; 48:47–52. [PubMed: 20078427]
119. Jindal R, Nahmias Y, Tilles AW, Berthiaume F, Yarmush ML. Amino acid-mediated heterotypic interaction governs performance of a hepatic tissue model. FASEB J. 2009; 23:2288–2298. [PubMed: 19246486]

120. Jindal R, Patel SJ, Yarmush ML. Tissue-Engineered Model for Real-Time Monitoring of Liver Inflammation. Tissue Engineering Part C: Methods. 2010; 17:113–122.

121. Morin O, Goulet F, Normand C. Liver sinusoidal endothelial cells: isolation, purification, characterization and interaction with hepatocytes. Revisiones sobre biologia celular : RBC. 1988; 15:1–85. [PubMed: 3057541]

122. Bhatia SN, Balis UJ, Yarmush ML, Toner M. Effect of cell-cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells. FASEB J. 1999; 13:1883–1900. [PubMed: 10544172]

123. Karam WG, Ghanayem BI. Induction of replicative DNA synthesis and PPAR alpha-dependent gene transcription by Wy-14 643 in primary rat hepatocyte and nonparenchymal cell co-cultures. Carcinogenesis. 1997; 18:2077–2083. [PubMed: 9395205]

124. Riccalton-Banks L, Liew C, Bhandari R, Fry J, Shakesheff K. Long-term culture of functional liver tissue: three-dimensional coculture of primary hepatocytes and stellate cells. Tissue Eng. 2003; 9:401–410. [PubMed: 12857408]

125. Naughton BA, Sibanda B, Weintraub JP, San Roman J, Kamali V. A stereotypic, transplantable liver tissue-culture system. Applied biochemistry and biotechnology. 1995; 54:65–91. [PubMed: 7486986]

126. Pearson, CI.; Applegate, DR.; New, L.; Thomas, RS.; Brennan, R.; Roter, AH.; Su, SV.; Naughton, BA. Society of Toxicology; Salt Lake City, UT: 2010.

127. Hamazaki T, Iiboshi Y, Oka M, Papst PJ, Meacham AM, Zon LI, Terada N. Hepatic maturation in differentiating embryonic stem cells in vitro. FEBS Letters. 2001; 497:15–19. [PubMed: 11376655]

128. Schwartz RE, Linehan JL, Painschab MS, Hu WS, Verfaillie CM, Kaufman DS. Defined conditions for development of functional hepatic cells from human embryonic stem cells. Stem cells and development. 2005; 14:643–655. [PubMed: 16433619]

129. Cai J, Zhao Y, Liu Y, Ye F, Song Z, Qin H, Meng S, Chen Y, Zhou R, Song X. Directed differentiation of human embryonic stem cells into functional hepatic cells. Hepatology. 2007; 45:1229–1239. [PubMed: 17464996]

130. Rambhatla L, Chiu CP, Kundu P, Peng Y, Carpenter MK. Generation of hepatocyte-like cells from human embryonic stem cells. Cell transplantation. 2003; 12:1–11. [PubMed: 12693659]

131. Pal R, Mamidi MK, Das AK, Gupta PK, Bhonde R. A Simple and economical route to generate functional hepatocyte-like cells from hESCs and their application in evaluating alcohol induced liver damage. J Cell Biochem. 2012; 113:19–30. [PubMed: 21956183]

132. Zamule SM, Coslo DM, Chen F, Omiecinski CJ. Differentiation of human embryonic stem cells along a hepatic lineage. Chemico-Biological Interactions. 2011

133. Lavon N, Yanuka O, Benvenisty N. Differentiation and isolation of hepatic-like cells from human embryonic stem cells. Differentiation. 2004; 72:230–238. [PubMed: 15270779]

134. Brolen G, Sivertsson L, Bjorquist P, Eriksson G, Ek M, Semb H, Johansson I, Andersson TB, Ingelman-Sundberg M, Heins N. Hepatocyte-like cells derived from human embryonic stem cells specifically via definitive endoderm and a progenitor stage. Journal of biotechnology. 2010; 145:284–294. [PubMed: 19932139]

135. Duan Y, Ma X, Zou W, Wang C, Bahbahan IS, Ahuja TP, Tolstikov V, Zern MA. Differentiation and characterization of metabolically functioning hepatocytes from human embryonic stem cells. Stem Cells. 2010; 28:674–686. [PubMed: 20135682]

136. Touboul T, Hannan NR, Corbineau S, Martinez A, Martinet C, Branchereau S, Mainot S, Strick-Marchand H, Pedersen R, Di Santo J, Weber A, Vallier L. Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. Hepatology. 2010; 51:1754–1765. [PubMed: 20301097]

137. Agarwal S, Holton KL, Lanza R. Efficient differentiation of functional hepatocytes from human embryonic stem cells. Stem Cells. 2008; 26:1117–1127. [PubMed: 18292207]

138. Shirahashi H, Wu J, Yamamoto N, Catana A, Wege H, Wager B, Okita K, Zern MA. Differentiation of human and mouse embryonic stem cells along a hepatocyte lineage. Cell transplantation. 2004; 13:197–211. [PubMed: 15191158]

139. Baharvand H, Hashemi SM, Kazemi Ashtiani S, Farrokhi A. Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems in vitro. The International journal of developmental biology. 2006; 50:645–652. [PubMed: 16892178]

140. Rhim JA, Sandgren EP, Degen JL, Palmiter RD, Brinster RL. Replacement of diseased mouse liver by hepatic cell transplantation. Science. 1994; 263:1149–1152. [PubMed: 8108734]

141. Laconi E, Oren R, Mukhopadhyay DK, Hurston E, Laconi S, Pani P, Dabeva MD, Shafritz DA. Long-term, near-total liver replacement by transplantation of isolated hepatocytes in rats treated with retrorsine. Am J Pathol. 1998; 153:319–329. [PubMed: 9665494]

142. Guha C, Sharma A, Gupta S, Alfieri A, Gorla GR, Gagandeep S, Sokhi R, Roy-Chowdhury N, Tanaka KE, Vikram B, Roy-Chowdhury J. Amelioration of radiation-induced liver damage in partially hepatectomized rats by hepatocyte transplantation. Cancer Res. 1999; 59:5871–5874. [PubMed: 10606225]

143. Grossman M, Rader DJ, Muller DW, Kolansky DM, Kozarsky K, Clark BJ 3rd, Stein EA, Lupien PJ, Brewer HB Jr. Raper SE, et al. A pilot study of ex vivo gene therapy for homozygous familial hypercholesterolaemia. Nat Med. 1995; 1:1148–1154. [PubMed: 7584986]

144. Shafritz, DA.; Oertel, M.; Dabeva, MD.; Grompe, M. The Liver: Biology and Pathobiology. 5 edn.. Arias, HJAIM.; Boyer, JL.; Cohen, DE.; Fausto, N.; Shafritz, DA.; Wolkoff, AW., editors. John Wiley & Sons; 2009. p. 577-595.

145. Liu H, Ye Z, Kim Y, Sharkis S, Jang YY. Generation of endoderm-derived human induced pluripotent stem cells from primary hepatocytes. Hepatology. 2010; 51:1810–1819. [PubMed: 20432258]

146. Si-Tayeb K, Noto FK, Nagaoka M, Li J, Battle MA, Duris C, North PE, Dalton S, Duncan SA. Highly efficient generation of human hepatocyte–like cells from induced pluripotent stem cells. Hepatology. 2010; 51:297–305. [PubMed: 19998274]

147. Chin MH, Mason MJ, Xie W, Volinia S, Singer M, Peterson C, Ambartsumyan G, Aimiuwu O, Richter L, Zhang J, Khvorostov I, Ott V, Grunstein M, Lavon N, Benvenisty N, Croce CM, Clark AT, Baxter T, Pyle AD, Teitell MA, Pelegrini M, Plath K, Lowry WE. Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. Cell stem cell. 2009; 5:111–123. [PubMed: 19570518]

148. Marchetto MC, Yeo GW, Kainohana O, Marsala M, Gage FH, Muotri AR. Transcriptional signature and memory retention of human-induced pluripotent stem cells. PloS one. 2009; 4:e7076. [PubMed: 19763270]

149. Miura K, Okada Y, Aoi T, Okada A, Takahashi K, Okita K, Nakagawa M, Koyanagi M, Tanabe K, Ohnuki M, Ogawa D, Ikeda E, Okano H, Yamanaka S. Variation in the safety of induced pluripotent stem cell lines. Nat Biotechnol. 2009; 27:743–745. [PubMed: 19590502]

Table 1

Summary of commonly used *in vitro* hepatotoxicity model systems.

Model System	Advantages	Disadvantages
Liver Slices [9-13, 17-23]	•Fairly high throughput • Retain liver structure; contain all cell types • Functional bile canaliculi • Good in vitro/in vivo correlation of xenobiotic metabolism • Maintain zone-specific CYP activity; maintain toxicity mechanisms • Stability of phase II enzymes, albumin production, gluconeogenesis for 20-96 hrs	• Cellular necrosis after 48-72 hrs • CYP levels quickly decrease (6-72 hrs) • Poor concordance with liver for intrinsic clearance rates and Km values • Diffusion-limited gradient of the exposure to a compound across the slice
Immortalized Hepatic Cell Lines [25, 26, 30, 31, 62]	•Throughput depends on application • Unlimited amount of cells available • Some cell lines retain expression of many liver-specific functions	• Lacking most phenotypic and functional characteristics of the liver tissue
Primary Hepatocyte Suspensions [33-37, 39-43]	• Fairly high throughput • Better estimate of internal clearance than monolayer cultures • Retain high level of enzyme functionality (close to *in vivo*)	•Loss of cell-cell interactions • Loss of cell-matrix interactions • Limited viability allows short-term use only (<4 hrs) • Loss of cellular polarity • No bile canaliculi
Primary Hepatocyte Cultures [3, 34, 46-48]	• Throughput depends on the application • Cells can re-establish cell-cell interactions and polarity • Cells retain some morphology and liver-specific functionality in short-term cultures (2-4 days) • Induction/inhibition of the metabolizing enzymes can be studied	•Inability to maintain *in vivo* liver-specific functionality for long-term culture • Quick reduction in functionality and phenotype (24-48 hrs) • May not develop functional bile canaliculi
Primary Hepatocyte Cultures – Sandwich [47, 48, 50-53]	• Throughput depends on the application • Restores *in vivo* polygonal morphology • Better maintains liver-specific functionality • Prevents loss of viability • Functional bile canaliculi	•Loss of liver-specific functionality, morphology and phenotype in long-term cultures • Decline in metabolic enzyme activity in long-term culture

Table 2

Summary of novel *in vitro* hepatotoxicity model systems.

Model System	Advantages	Disadvantages
Hurel® Biochip [80-83]	•Moderate throughput* •Allows for multiple cell types and interaction between cell types • Preservation of cell viability and metabolic competency • Microscopic imaging and oxygen sensing • Physiologically relevant ratios of liquid:cells and shear stress • Requires less media and cells than traditional culture • Good correlation with *in vivo* clearance rates	•A complex system to establish and maintain • Sample removal difficult • No 3D tissue constructs
Hollow-Fiber Reactor [84-86]	•Moderate throughput* • Counter-directional flow • Small cell numbers and media volumes • Microscopic evaluation is easy	•A complex system • Lack of physiological gradients
Multi-well Perfused Bioreactor [45, 87, 88]	•High throughput* • Cells form 3D tissue constructs • Sustained liver-like cell functionality • Physiological shear stress • Good correlation with *in vivo* clearance rates • Ability for microscopic examination	•Uses greater cell numbers and larger media volumes • Has been validated with rat and human hepatocytes
Bioartificial Livers [89, 90]	• Low throughput* • Microenvironment most similar to *in vivo* tissue • Allows for studies of functional heterogeneity • Ability to evaluate hepatotoxicity using human blood	•Requires large number of cells • Currently not in use with other cell types • Complex membranes needed for proper use • Does not maintain viability or functionality of hepatocytes longer than other methods
Primary Cell Co-cultures [28, 99-103]	•Throughput* depends on the application • Improved hepatocyte function • Allows for studies of immune-mediated toxicity • Retention of morphology, bile canaliculi, and cell-cell contacts • Improved longevity/functionality of all cell types • Good correlation with *in vivo* toxicity	• Best results shown with non-hepatic (e.g., fibroblasts) cells • No standard as to which other cell type to use • Methods vary greatly among laboratories
Embryonic Stem Cells [63, 131-136]	•Throughput* depends on the application • Relatively unlimited supply • Defined phenotype	•Ethical concerns • Highly variable functionality within cell populations • May be useful for short-term culture only (2-4 days) • Requires special media • Low expression levels of liver-specific metabolism genes
Induced Pluripotent Stem Cells [63, 145-149]	•Throughput* depends on the application • Not as controversial source of cells • Relatively unlimited supply • Defined phenotype • Allows studies of inter-individual variability	• Complex “reprogramming” steps • Low expression levels of liver-specific metabolism genes • May be useful for short-term culture only (2-4 days) • Variability among preparations • Few studies in toxicology yet

* Relative throughput comparison is referring to other model systems in this table.