Three-Dimensional Liver Culture Systems

Subjects: Toxicology

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Drug-induced liver injury (DILI) is the major reason for failures in drug development and withdrawal of approved drugs from the market. Two-dimensional cultures of hepatocytes often fail to reliably predict DILI: hepatoma cell lines such as HepG2 do not reflect important primary-like hepatic properties and primary human hepatocytes (pHHs) dedifferentiate quickly in vitro and are, therefore, not suitable for long-term toxicity studies. More predictive liver in vitro models are urgently required in drug development and compound safety evaluation. This review discusses available human hepatic cell types for in vitro toxicology analysis and their usage in established and emerging three-dimensional (3D) culture systems. Generally, 3D cultures maintain or improve primary hepatic functions (including expression of drug metabolizing enzymes) of different liver cells for several weeks of culture, thus allowing long-term and repeated-dose toxicity studies. Spheroid cultures of pHHs have been comprehensively tested, but also other cell types such as HepaRG benefit from 3D culture systems. Emerging 3D culture techniques include usage of induced pluripotent stem-cell-derived hepatocytes and primary-like upcyte cells, as well as advanced culture techniques such as microfluidic liver-on-a-chip models. In-depth characterization of existing and emerging 3D hepatocyte technologies is indispensable for successful implementation of such systems in toxicological analysis.

Keywords: 3D culture systems ; liver ; hepatocytes ; HepG2 ; HepaRG ; upcyte hepatocytes ; primary hepatic function ; toxicology ; spheroids ; perfused bioreactors ; liver-on-a-chip

1. Three-Dimensional Culture Models for Human Hepatocytes

Several cell types have been used in different types of 3D culture. 3D cultivation more closely mimics the human liver and, indeed, many hepatic functions and properties could be restored or improved upon 3D culture. The reason for this seems to be improved cell-cell- as well as cell-matrix-communication. Cell junctions between hepatocytes improve their functionality and it has been shown, for example, that albumin secretion and importantly also CYP expression rely on adherence junctions. Further, hepatic homeostasis is also maintained by cell-extracellular matrix interactions via integrin signaling pathways (reviewed in [1]). The following sections will discuss well-established and emerging 3D culture systems for human hepatocytes. Efforts in the field are being made to generate stable and reliable test systems for toxicology analyses of liver cells with the aim of creating an environment for the cells that allows researchers to mimic the human liver in vitro.

2. Liver Spheroid and Organoid Culture

Spheroids are formed by self-aggregation of hepatocytes when cultured in suspension without any substrates that promote cell attachment, such as collagen I. This can be achieved by the hanging drops method or by using ultra-low attachment (ULA) multiwell plates. As the starting cell number can be defined, those spheroids usually have equal sizes, which is a feature of importance for standardized measurements. Further, they can be kept in culture for several days to weeks. Spheroids can also be formed in stirred tank bioreactors. This method allows us to generate spheroids on a large scale, but sizes may vary greatly, which makes it difficult to compare and to reproduce data. Further, single spheroids must be isolated from the stirred tank bioreactor to perform individual experiments as spheroids are generated in a single reaction tank. Due to these reasons, the hanging drops or ULA method are the preferred ones for toxicological analyses and, if not otherwise stated, results described below refer to spheroids generated by those two methods.

Recent advances were also made for liver organoid cultures. Such organoids derive from adult liver cells or iPSCs, are more difficult to create and need a scaffold of extracellular matrix (often supplemented with growth factors and cytokines) for aggregation. In contrast to conventional spheroids, mature organoids consist of different cell types including also non-parenchymal cells (NPCs) or biliary epithelial cells.

2.1. Spheroids from Primary Human Hepatocytes

Many attempts were made to use pHHs in 3D culture with the aim to maintain and to prolong their viability and to prevent dedifferentiation. PHH spheroids were viable for at least 2 and up to 7 weeks with stable albumin production [2][3][4][5][6], urea synthesis [3][4] and glycogen storage [6]. Additionally, cellular polarization was clearly present in pHH spheroids as shown by MRP2 [3][5], P-glycoprotein (Pgp), [6], and BSEP expression [5][6]. Drug-metabolizing enzyme expression and activity of CYP1A2 [5][7][8][9], CYP2B6 [7], CYP2C9 [7], CYP2C19 [10], CYP2D6 [5][7][9], CYP3A4 [2][5][7][8][9][10][11], and UGTs [8][11] was stable over several weeks of pHH spheroid culture. CYP2C9 activity even increased with culture time [5][7][8][10], while CYP2C8 [5][8], and in some cases also CYP1A2, CYP2B6, CYP2D6 and CYP3A4 [7][10] activities decreased with time. In general, maintenance of their initial hepatic transcriptomic and metabolomic profiles was possible for at least 2–5 weeks [3][5][11][12], which allowed long-term and repeated-dose toxicity testing. EC50 values for acetaminophen, bosentan, diclofenac, troglitazone, and other hepatotoxic compounds significantly decreased upon long-term treatment [5][12] and were closer to the corresponding in vivo C_{max} than acute toxicity EC50 values [5][11]. Importantly, liver spheroids cultured in chemically-defined medium reached 100% specificity and 69% sensitivity when 123 hepatotoxic compounds were tested. The model system could reliably distinguish between hepatotoxic substances and their nontoxic structural analogues [13]. The authors used a repeated-dose approach for 14 days and an easy-to-handle ATP assay as readout. Such comprehensive studies corroborate the utility of pHH spheroids in drug development, toxicity testing and DILI prediction. Further, those studies used panels of drugs with different toxicity mechanisms, including mitochondrial dysfunction, steatosis, cholestasis, and fibrosis. This implies that spheroid cultures of pHHs can be used to study different mechanisms of drug induced hepatotoxicity [5][11][12][13][14][15].

Spheroids can also be formed by using pHHs together with non-parenchymal cells (NPCs). This approach intends to further improve hepatic characteristics. Those spheroids, often called microtissues, displayed pronounced glycogen storage and albumin synthesis capabilities as well as expression of CYP1A2, CYP2C9 and CYP3A4. Maintenance of cell polarity could be shown by MRP2 and BSEP expression [16][17][18]. Viability of the spheroids could be maintained for up to 7 weeks in one of the studies, but albumin secretion declined from day 28 on. Interestingly, *CYP1A1/2*, *CYP2C9* and *CYP3A4/5* transcript levels were higher in those microtissues than in clinical liver specimens or pHHs upon long-term culture [17][18]. However, on protein level, only CYP2B6 and UGT1A1 levels remained stable for 7 weeks of culture. Other CYP and phase II enzyme protein levels dropped already at day 7 of culture. Additionally, a decline of CYP1A2, CYP2B6, CYP2C9 and CYP2C19 activities could be observed after 4 weeks in culture, which is in accordance with the reduction in albumin production at this time-point [18]. Others directly compared hepatic spheroids in monoculture and in co-culture with NPCs over a culture time of 14 days. Spheroid functionality was increased in the co-culture setup as shown by expression of albumin (*ALB*) and *CYP3A4*, as well as by albumin synthesis [19]. A contradictory study, however, showed that *ALB* and *CYP1A2* and *CYP3A4* expression was higher in hepatocyte-only spheroids than in NPC-containing spheroids [20]. Thus, it remains to be shown whether co-culture with NPCs really adds a benefit to pHH spheroid culture.

Other studies used such 3D human liver microtissues for comprehensive toxicology analyses and tested a panel of 100 and 110 hepatotoxic compounds, respectively. Results were directly compared with 2D pHH cultures. It must be noted that spheroids were treated for 7 and 14 days, while monolayer cultures were treated for 24 and 72 h, respectively. Overall, specificity of spheroids for detection of hepatotoxic substances was high (79–85%), and sensitivity was moderate (19–61%) indicating that also NPC containing spheroid cultures of pHHs are suitable for hepatotoxicity risk assessment in drug development processes and for the study of toxicity mechanisms [21][22].

Further improvement of spheroid co-cultures might be achieved by using bioprinting techniques. Here, spheroids are formed by bioprinting pHHs together with non-parenchymal cells such as endothelial and stellate cells on transwell culture inserts of multi-well plates [23]. Upon this culture system, albumin synthesis and glycogen storage capacity could be sustained, and albumin secretion even increased over the culture period of 4 weeks. Expression levels of *CYP1A2*, *CYP2B6*, *CYP2C9*, *CYP2D6* and *CYP3A4* also increased significantly over time, with a peak at day 14 followed by a slight decrease at day 28. In addition, EC50 values for trovafloxacin were lower in those so-called 3D liver tissues than in corresponding 2D pHH cultures [23]. Bioprinting methods are emerging technologies, are often expensive and difficult in handling. Further studies are required to provide easy-to-use and reproducible protocols and to prove the utility of the method for toxicology analyses.

Primary liver characteristics could also be maintained when spheroids were generated in a stirred tank bioreactor. The authors described stable urea and albumin synthesis as well as stable expression of phase I enzymes (*CYP1A2*, *CYP2C9*, *CYP3A4*), the phase II enzymes *GSTA1* and *UGT2B7* and the polarization marker F-actin [24]. Gene expression of *CYP1A2*, *CYP2C9* and *CYP3A4* could be further improved in stirred tank bioreactors by adding an outer layer of bone marrow-derived mesenchymal stem cells to the liver spheroids, while albumin and urea production remained unaffected

with this strategy [25]. The greatest disadvantage of spheroids in stirred tank bioreactors is the single reaction vessel. Spheroids generated by this method a) vary greatly in size and b) need to be separated and transferred to other culture vessels for toxicology testing. Therefore, this method is not suitable for high-throughput analyses and spheroids generated by the hanging drops or ULA method should be preferred.

2.2. Spheroids from HepG2 Cells

Many groups use hepatoma cell lines as HepG2 for spheroid culture due to their ease of use and low cost. It could be shown that culture in this 3D format indeed improves hepatic properties at several levels.

Albumin synthesis [26][27][28][29][30] and urea production [28] were elevated when HepG2 cells were cultured as spheroids and compared to their 2D cultured counterparts. However, one study showed a contradictory result: albumin secretion levels were significantly higher in 2D than in 3D spheroid cultures [31]. Gene expression of drug-metabolizing enzymes such as *CYP1A1/2* [26][27][30][32], *CYP3A4* [26][27][32] and *UGT1A1* [26][27] could be increased upon 3D spheroid culture. Importantly, in addition, cellular polarization could be restored. This was shown by positive MRP2 and Pgp staining as well as by functional transporter assays indicating presence of canalicular-like structures [28].

Co-culture of HepG2 cells with endothelial cells and application of a cell coating technique resulted in elevated albumin synthesis, MRP2 expression and CYP enzyme activities [33]. Similarly, HepG2 cells cultured together with endothelial and mesenchymal cells as tubular 3D liver microtissues had higher expression levels of drug-metabolizing enzymes than 2D cultured counterparts [34].

Additionally, bioprinted 3D HepG2 spheroids showed stable albumin and urea synthesis for at least 7 days [35][36]. Such bioprinted HepG2 spheroids displayed higher *CYP1A2* gene expression and higher sensitivity towards acetaminophen than 2D cultured HepG2 cells [36]. Bioprinting HepG2 cells together with fibroblasts resulted in elevated albumin production [37].

Cell lines as HepG2 are by nature very well suited for high-throughput screens, which is desirable for any toxicological test system. Indeed, HepG2 cells cultured in 3D spheroids were more susceptible to hepatotoxic compounds and showed considerably lower EC50 values for many different drugs than 2D cultured HepG2 cells [28]. However, other studies have shown that HepG2 spheroids were more resistant to hepatotoxins than their 2D counterparts, both in acute toxicity and repeated-dose toxicity experiments [38]. Furthermore, they were still considerably less sensitive to hepatotoxic compounds than 3D cultured pHHs. This might be explained by the low expression of CYP and other drug-metabolizing enzymes. As their basal expression is very low to absent in HepG2 cells, also an increase in expression levels upon spheroid culture might not lead to sufficiently elevated enzyme activities. Therefore, the value of such cells for drug development and DILI prediction is questionable.

2.3 Spheroids from HepaRG Cells

HepaRG cells also benefit from spheroid culture conditions. They displayed improved albumin synthesis capacity upon culture as spheroids in hanging droplets and when compared to HepaRG monolayer culture. Those HepaRG spheroids needed several days for maturation. Albumin synthesis was elevated at day 6 of spheroid culture, and mRNA levels of *CYP1A2*, *CYP2B6* and *CYP3A4* first dropped, then slightly increased at day 4 and were elevated 1.2–3 -fold at day 7 when compared to day 0 [29]. Additionally, others have shown that HepaRG spheroids (self-aggregated or bioprinted) could be cultured over several weeks and that those cultures maintained several hepatic properties: HepaRG spheroids were capable of (a) albumin [39][40][41][42][43] and urea [39][42] production, (b) displayed expression and activity of CYP1A2 [40][41], CYP2B6 [41] and CYP3A4 [38][40][41][42][44], (c) showed phase II enzyme activity [41][43], and (d) displayed cellular polarization shown by MRP2 [39][40][42] and Pgp [43] expression, and by F-actin bands, indicative of bile canalicular structures [41][43][44]. Regarding expression and activity of phase I enzymes, however, several studies have shown that basal levels in spheroids were unaffected, in part even slightly lower than in monolayer cultures. This might be explained by the relatively high basal expression of several CYP enzymes in HepaRG cells. However, the authors of those studies have deemed it necessary to elevate expression and activity of CYPs by induction using β-naphthoflavone (for CYP1A2), phenobarbital (for CYP2B6) or rifampicin (for CYP2C9 and CYP3A4) to reach more relevant levels [39][41][43][45].

Importantly, and in accordance with the presence of hepatic characteristics, HepaRG spheroids were also more sensitive to hepatotoxic compounds than monolayer cultures. This was shown by smaller EC50 values in a range of substances including acetaminophen, tamoxifen, and aflatoxin B1 [38][40][42][43][46]. Interestingly, a recent study showed in a high-throughput 384-well format that HepG2 spheroids were more sensitive to hepatotoxic compounds than HepaRG spheroids. When 150 compounds were tested, HepG2 spheroids displayed a sensitivity of 58% and a specificity of 83%,

while HepaRG spheroids showed sensitivity of 47% and specificity of 86% [47]. Static HepaRG spheroid cultures could also be combined with lactate and oxygen microsensors. This allowed live, long-term, and fast measurement of the cellular metabolic activity. This test system was validated using antimycin A and bosentan as test substances, and lactate production increased or decreased accordingly [48]. Further, it was shown that HepaRG spheroids were able to predict steatosis and mitochondrial dysfunction, while cholestasis could only be predicted in 2D culture models in this work [40]. Another study presented spheroid models to study cholestatic liability of compounds and showed that HepaRG spheroids as well as pHH spheroids were both able to reliably predict cholestasis [14]. A model to detect liver fibrosis could be generated by co-culturing HepaRG cells and hepatic stellate cells in spheroid cultures [49]. Those studies imply that HepaRG cells are well suited to study different types of hepatotoxicity.

Culture of HepaRG as 3D spheroids indeed seems a promising system to maintain primary hepatic properties. However, some studies found that expression of drug-metabolizing enzymes needed to be elevated by prototypic CYP inducers. This, together with the required time-consuming differentiation procedure, renders the system somewhat complicated for toxicity testing. In summary, however, HepaRG cells constitute a more reliable test system than HepG2 cells, especially regarding their drug-metabolizing enzyme expression, and more closely resemble pHHs than other cell lines of cancerous origin.

2.4. Spheroids from iPSC-Derived Hepatocytes

Studies on spheroid cultures of iPSC-heps for toxicity testing are still rare. It was shown that hepatic properties improved upon 3D culture of those liver cells, but they still maintained a more fetal-like phenotype, and adult marker genes (including *CYP1A2*, *CYP2C9*, *CYP3A4* and *ALB*) were only expressed at low levels [50][51][52]. For example, albumin production was not significantly increased when iPSC-heps were cultured as spheroids instead of 2D monolayers. However, an increase in urea production could be observed at least at day 12 of spheroid culture [51]. In contrast, others have shown that spheroids of iPSC-heps were capable of albumin production, urea synthesis, glycogen storage and gene expression of drug-metabolizing enzymes (*CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, UGT2B7*). However, again, levels remained far below those of pHHs [53]. A very recent study compared 2D and 3D cultured iPSC-heps with pHHs cultured in 2D and 3D spheroids. Basal activities of CYP1A2, CYP2B6 and CYP3A4, as well as canaliculi formation, were similar in all culture systems, but CYPs could be induced successfully only in pHH cultures, which is an important feature of functional human hepatocytes [54].

Bioprinted 3D spheroids of iPSC-heps displayed higher albumin and urea synthesis as well as *CYP1A2* and *CYP3A4* expression than their 2D cultured counterparts [55]. Co-culturing of iPSC-hep spheroids with endothelial cells seems to improve maturity of the cells as shown by albumin and urea secretion and CYP enzyme activities [56].

Interestingly, iPSC-hep spheroids were more sensitive to a set of 24 hepatotoxic compounds than HepG2 spheroids in one study [52], while another study showed opposite results for 10 out of 23 tested compounds [57]. When compared to pHH spheroids, IC50 values of iPSC-hep spheroids were similar in 12/15 tested hepatotoxic compounds [53]. In another study, the authors tested a set of seven hepatotoxic compounds and found that pHHs generally displayed higher sensitivity [54].

Hepatic differentiation of iPSCs is a promising approach for modelling the human liver in vivo. To date, however, those hepatocytes have a more fetal-like than mature phenotype and efforts must be made to optimize the differentiation procedure towards adult characteristics. Three-dimensional cultures seem to add only little towards this goal, and contradictory data on utility for toxicological analyses render this system, still, an immature technology for such applications.

2.5. Spheroids from Upcyte and Primary-Like Hepatocytes

Data for spheroid culture of upcyte or other primary-like hepatocytes are even scarcer than those for iPSC-heps. Despite their characteristics seem to make them a promising tool for in vitro toxicology testing, they might be still rather unknown in the community. One study compared gene expression of drug-metabolizing enzymes in 2D and 3D spheroid cultures of upcyte hepatocytes. The results indicate that expression levels of *CYP2C19, CYP3A4, MRP2* and *OATP-C* in spheroids were slightly higher than in 2D cultures, while *CYP2C8* and *BSEP* expression was reduced in 3D cultures [58]. A second study used spheroids of primary-like hepatocytes generated from proliferation-competent liver progenitor cells. Those spheroids showed elevated *ALB* and *CYP1A2, CYP2D6, CYP3A4, UGT1A1* and *MRP2* expression, albumin and urea synthesis as well as glycogen storage. However, these levels did not reach those of pHHs. Gene-expression profiling showed that spheroids of primary-like hepatocytes clustered with pHHs and differentiated HepaRG cells, but not with HepG2 cells, indeed indicating a mature phenotype [59]. And a third study showed that spheroids of so called ProliHHs

were comparable to pHHs regarding albumin expression and secretion, but expression and activity levels of CYP1A2, CYP2B6 and CYP3A4, as well as *BSEP* and *MRP2* expression levels were considerably lower than in pHHs [60].

References

1. Vinken, M.; Papeleu, P.; Snykers, S.; De Rop, E.; Henkens, T.; Chipman, J.K.; Rogiers, V.; Vanhaecke, T. Involvement of cell junctions in hepatocyte culture functionality. Crit. Rev. Toxicol. 2006, 36, 299–318.
2. Riede, J.; Wollmann, B.M.; Molden, E.; Ingelman-Sundberg, M. Primary human hepatocyte spheroids as an in vitro tool for investigating drug compounds with low clearance. Drug Metab. Dispos. 2021.
3. Rose, S.; Ezan, F.; Cuvellier, M.; Bruyère, A.; Legagneux, V.; Langouët, S.; Baffet, G. Generation of proliferating human adult hepatocytes using optimized 3D culture conditions. Sci. Rep. 2021, 11, 515.
4. Mizoi, K.; Hosono, M.; Kojima, H.; Ogihara, T. Establishment of a primary human hepatocyte spheroid system for evaluating metabolic toxicity using dacarbazine under conditions of CYP1A2 induction. Drug Metab. Pharmacokinet. 2020, 35, 201–206.
5. Bell, C.C.; Hendriks, D.F.G.; Moro, S.M.L.; Ellis, E.; Walsh, J.; Renblom, A.; Fredriksson Puigvert, L.; Dankers, A.C.A.; Jacobs, F.; Snoeys, J.; et al. Characterization of primary human hepatocyte spheroids as a model system for drug-induced liver injury, liver function and disease. Sci. Rep. 2016, 6, 25187.
6. Messner, S.; Agarkova, I.; Moritz, W.; Kelm, J.M. Multi-cell type human liver microtissues for hepatotoxicity testing. Arch. Toxicol. 2013, 87, 209–213.
7. Kanebratt, K.P.; Janefeldt, A.; Vilén, L.; Vildhede, A.; Samuelsson, K.; Milton, L.; Björkbom, A.; Persson, M.; Leanderson, C.; Andersson, T.B.; et al. Primary Human Hepatocyte Spheroid Model as a 3D In Vitro Platform for Metabolism Studies. J. Pharm. Sci. 2021, 110, 422–431.
8. Vorrink, S.U.; Ullah, S.; Schmidt, S.; Nandania, J.; Velagapudi, V.; Beck, O.; Ingelman-Sundberg, M.; Lauschke, V.M. Endogenous and xenobiotic metabolic stability of primary human hepatocytes in long-term 3D spheroid cultures revealed by a combination of targeted and untargeted metabolomics. FASEB J. 2017, 31, 2696–2708.
9. Berger, B.; Donzelli, M.; Maseneni, S.; Boess, F.; Roth, A.; Krähenbühl, S.; Haschke, M. Comparison Of Liver Cell Models Using The Basel Phenotyping Cocktail. Front. Pharmacol. 2016, 7, 443.
10. Foster, A.J.; Chouhan, B.; Regan, S.L.; Rollison, H.; Amberntsson, S.; Andersson, L.C.; Srivastava, A.; Darnell, M.; Cairns, J.; Lazic, S.E.; et al. Integrated in vitro models for hepatic safety and metabolism: Evaluation of a human Liver-Chip and liver spheroid. Arch. Toxicol. 2019, 93, 1021–1037.
11. Bell, C.C.; Lauschke, V.M.; Vorrink, S.U.; Palmgren, H.; Duffin, R.; Andersson, T.B.; Ingelman-Sundberg, M. Transcriptional, Functional, and Mechanistic Comparisons of Stem Cell–Derived Hepatocytes, HepaRG Cells, and Three-Dimensional Human Hepatocyte Spheroids as Predictive In Vitro Systems for Drug-Induced Liver Injury. Drug Metab. Dispos. 2017, 45, 419–429.
12. Bell, C.C.; Dankers, A.C.A.; Lauschke, V.M.; Sison-Young, R.; Jenkins, R.; Rowe, C.; Goldring, C.E.; Park, K.; Regan, S.L.; Walker, T.; et al. Comparison of Hepatic 2D Sandwich Cultures and 3D Spheroids for Long-term Toxicity Applications: A Multicenter Study. Toxicol. Sci. 2018, 162, 655–666.
13. Vorrink, S.U.; Zhou, Y.; Ingelman-Sundberg, M.; Lauschke, V.M. Prediction of Drug-Induced Hepatotoxicity Using Long-Term Stable Primary Hepatic 3D Spheroid Cultures in Chemically Defined Conditions. Toxicol. Sci. Off. J. Soc. Toxicol. 2018, 163, 655–665.
14. Hendriks, D.F.; Fredriksson Puigvert, L.; Messner, S.; Mortiz, W.; Ingelman-Sundberg, M. Hepatic 3D spheroid models for the detection and study of compounds with cholestatic liability. Sci. Rep. 2016, 6, 35434.
15. Kozyra, M.; Johansson, I.; Nordling, Å.; Ullah, S.; Lauschke, V.M.; Ingelman-Sundberg, M. Human hepatic 3D spheroids as a model for steatosis and insulin resistance. Sci. Rep. 2018, 8, 14297.
16. Rubiano, A.; Indapurkar, A.; Yokosawa, R.; Miedzik, A.; Rosenzweig, B.; Arefin, A.; Moulin, C.M.; Dame, K.; Hartman, N.; Volpe, D.A.; et al. Characterizing the reproducibility in using a liver microphysiological system for assaying drug toxicity, metabolism, and accumulation. Clin. Transl. Sci. 2021, 14, 1049–1061.
17. Kukla, D.A.; Crampton, A.L.; Wood, D.K.; Khetani, S.R. Microscale Collagen and Fibroblast Interactions Enhance Primary Human Hepatocyte Functions in Three-Dimensional Models. Gene Expr. 2020, 20, 1–18.
18. Messner, S.; Fredriksson, L.; Lauschke, V.M.; Roessger, K.; Escher, C.; Bober, M.; Kelm, J.M.; Ingelman-Sundberg, M.; Moritz, W. Transcriptomic, Proteomic, and Functional Long-Term Characterization of Multicellular Three-Dimensional Human Liver Microtissues. Appl. In Vitro Toxicol. 2018, 4, 1–12.

19. Baze, A.; Parmentier, C.; Hendriks, D.F.; Hurrell, T.; Heyd, B.; Bachellier, P.; Schuster, C.; Ingelman-Sundberg, M.; Richert, L. Three-dimensional spheroid primary human hepatocytes in monoculture and coculture with nonparenchymal cells. Tissue Eng. Part C Methods 2018, 24, 534–545.

20. Bell, C.C.; Chouhan, B.; Andersson, L.C.; Andersson, H.; Dear, J.W.; Williams, D.P.; Söderberg, M. Functionality of primary hepatic non-parenchymal cells in a 3D spheroid model and contribution to acetaminophen hepatotoxicity. Arch. Toxicol. 2020, 94, 1251–1263.

21. Li, F.; Cao, L.; Parikh, S.; Zuo, R. Three-dimensional spheroids with primary human liver cells and differential roles of kupffer cells in drug-induced liver injury. J. Pharm. Sci. 2020, 109, 1912–1923.

22. Proctor, W.R.; Foster, A.J.; Vogt, J.; Summers, C.; Middleton, B.; Pilling, M.A.; Shienson, D.; Kijanska, M.; Ströbel, S.; Kelm, J.M.; et al. Utility of spherical human liver microtissues for prediction of clinical drug-induced liver injury. Arch. Toxicol. 2017, 91, 2849–2863.

23. Nguyen, D.G.; Funk, J.; Robbins, J.B.; Crogan-Grundy, C.; Presnell, S.C.; Singer, T.; Roth, A.B. Bioprinted 3D primary liver tissues allow assessment of organ-level response to clinical drug induced toxicity in vitro. PLoS ONE 2016, 11, e0158674.

24. Tostões, R.M.; Leite, S.B.; Serra, M.; Jensen, J.; Björquist, P.; Carrondo, M.J.T.; Brito, C.; Alves, P.M. Human liver cell spheroids in extended perfusion bioreactor culture for repeated-dose drug testing. Hepatology 2012, 55, 1227–1236.

25. Rebelo, S.P.; Costa, R.; Silva, M.M.; Marcelino, P.; Brito, C.; Alves, P.M. Three-dimensional co-culture of human hepatocytes and mesenchymal stem cells: Improved functionality in long-term bioreactor cultures. J. Tissue Eng. Regen. Med. 2017, 11, 2034–2045.

26. Štampar, M.; Breznik, B.; Filipič, M.; Žegura, B. Characterization of In Vitro 3D Cell Model Developed from Human Hepatocellular Carcinoma (HepG2) Cell Line. Cells 2020, 9, 2557.

27. Štampar, M.; Tomc, J.; Filipič, M.; Žegura, B. Development of in vitro 3D cell model from hepatocellular carcinoma (HepG2) cell line and its application for genotoxicity testing. Arch. Toxicol. 2019, 93, 3321–3333.

28. Gaskell, H.; Sharma, P.; Colley, H.E.; Murdoch, C.; Williams, D.P.; Webb, S.D. Characterization of a functional C3A liver spheroid model. Toxicol. Res. 2016, 5, 1053–1065.

29. Takahashi, Y.; Hori, Y.; Yamamoto, T.; Urashima, T.; Ohara, Y.; Tanaka, H. 3D spheroid cultures improve the metabolic gene expression profiles of HepaRG cells. Biosci. Rep. 2015, 35.

30. Chang, T.T.; Hughes-Fulford, M. Monolayer and spheroid culture of human liver hepatocellular carcinoma cell line cells demonstrate distinct global gene expression patterns and functional phenotypes. Tissue Eng. Part A 2009, 15, 559–567.

31. Elje, E.; Hesler, M.; Rundén-Pran, E.; Mann, P.; Mariussen, E.; Wagner, S.; Dusinska, M.; Kohl, Y. The comet assay applied to HepG2 liver spheroids. Mutat. Res./Genet. Toxicol. Environ. Mutagenesis 2019, 845, 403033.

32. Štampar, M.; Sedighi Frandsen, H.; Rogowska-Wrzesinska, A.; Wrzesinski, K.; Filipič, M.; Žegura, B. Hepatocellular carcinoma (HepG2/C3A) cell-based 3D model for genotoxicity testing of chemicals. Sci. Total Environ. 2021, 755, 143255.

33. Sasaki, K.; Akagi, T.; Asaoka, T.; Eguchi, H.; Fukuda, Y.; Iwagami, Y.; Yamada, D.; Noda, T.; Wada, H.; Gotoh, K. Construction of three-dimensional vascularized functional human liver tissue using a layer-by-layer cell coating technique. Biomaterials 2017, 133, 263–274.

34. Mori, N.; Kida, Y.S. Expression of genes involved in drug metabolism differs between perfusable 3D liver tissue and conventional 2D-cultured hepatocellular carcinoma cells. FEBS Open Bio 2020, 10, 1985–2002.

35. Kang, H.K.; Sarsenova, M.; Kim, D.H.; Kim, M.S.; Lee, J.Y.; Sung, E.A.; Kook, M.G.; Kim, N.G.; Choi, S.W.; Ogay, V.; et al. Establishing a 3D In Vitro Hepatic Model Mimicking Physiologically Relevant to In Vivo State. Cells 2021, 10, 1268.

36. Gori, M.; Giannitelli, S.M.; Torre, M.; Mozetic, P.; Abbruzzese, F.; Trombetta, M.; Traversa, E.; Moroni, L.; Rainer, A. Biofabrication of Hepatic Constructs by 3D Bioprinting of a Cell-Laden Thermogel: An Effective Tool to Assess Drug-Induced Hepatotoxic Response. Adv. Healthc. Mater. 2020, 9, e2001163.

37. Taymour, R.; Kilian, D.; Ahlfeld, T.; Gelinsky, M.; Lode, A. 3D bioprinting of hepatocytes: Core-shell structured co-cultures with fibroblasts for enhanced functionality. Sci. Rep. 2021, 11, 5130.

38. Mueller, D.; Krämer, L.; Hoffmann, E.; Klein, S.; Noor, F. 3D organotypic HepaRG cultures as in vitro model for acute and repeated dose toxicity studies. Toxicology In Vitro 2014, 28, 104–112.

39. Cuvellier, M.; Ezan, F.; Oliveira, H.; Rose, S.; Fricain, J.C.; Langouët, S.; Legagneux, V.; Baffet, G. 3D culture of HepaRG cells in GelMa and its application to bioprinting of a multicellular hepatic model. Biomaterials 2021, 269, 120611.

40. Ott, L.M.; Ramachandran, K.; Stehno-Bittel, L. An Automated Multiplexed Hepatotoxicity and CYP Induction Assay Using HepaRG Cells in 2D and 3D. SLAS DISCOVERY Adv. Sci. Drug Discov. 2017, 22, 614–625.

41. Wang, Z.; Luo, X.; Anene-Nzelu, C.; Yu, Y.; Hong, X.; Singh, N.H.; Xia, L.; Liu, S.; Yu, H. HepaRG culture in tethered sp heroids as an in vitro three-dimensional model for drug safety screening. J. Appl. Toxicol. 2015, 35, 909–917.

42. Gunness, P.; Mueller, D.; Shevchenko, V.; Heinzle, E.; Ingelman-Sundberg, M.; Noor, F. 3D Organotypic Cultures of Hu man HepaRG Cells: A Tool for In Vitro Toxicity Studies. Toxicol. Sci. 2013, 133, 67–78.

43. Leite, S.B.; Wilk-Zasadna, I.; Zaldivar, J.M.; Airola, E.; Reis-Fernandes, M.A.; Mennecozzi, M.; Guguen-Guillouzo, C.; C hesne, C.; Guillou, C.; Alves, P.M.; et al. Three-Dimensional HepaRG Model As An Attractive Tool for Toxicity Testing. T oxicol. Sci. 2012, 130, 106–116.

44. Nelson, L.J.; Morgan, K.; Treskes, P.; Samuel, K.; Henderson, C.J.; LeBled, C.; Homer, N.; Grant, M.H.; Hayes, P.C.; Pl evris, J.N. Human Hepatic HepaRG Cells Maintain an Organotypic Phenotype with High Intrinsic CYP450 Activity/Meta bolism and Significantly Outperform Standard HepG2/C3A Cells for Pharmaceutical and Therapeutic Applications. Basi c Clin. Pharmacol. Toxicol. 2017, 120, 30–37.

45. Mandon, M.; Huet, S.; Dubreil, E.; Fessard, V.; Le Hégarat, L. Three-dimensional HepaRG spheroids as a liver model t o study human genotoxicity in vitro with the single cell gel electrophoresis assay. Sci. Rep. 2019, 9, 1–9.

46. Zhang, C.; Zhang, Q.; Li, J.; Yu, L.; Li, F.; Li, W.; Li, Y.; Peng, H.; Zhao, J.; Carmichael, P.L.; et al. Integration of in vitro data from three dimensionally cultured HepaRG cells and physiologically based pharmacokinetic modeling for assessm ent of acetaminophen hepatotoxicity. Regul. Toxicol. Pharmacol. 2020, 114, 104661.

47. Basharat, A.; Rollison, H.E.; Williams, D.P.; Ivanov, D.P. HepG2 (C3A) spheroids show higher sensitivity compared to H epaRG spheroids for drug-induced liver injury (DILI). Toxicol. Appl. Pharmacol. 2020, 408, 115279.

48. Weltin, A.; Hammer, S.; Noor, F.; Kaminski, Y.; Kieninger, J.; Urban, G.A. Accessing 3D microtissue metabolism: Lactat e and oxygen monitoring in hepatocyte spheroids. Biosens. Bioelectron. 2017, 87, 941–948.

49. Leite, S.B.; Roosens, T.; El Taghdouini, A.; Mannaerts, I.; Smout, A.J.; Najimi, M.; Sokal, E.; Noor, F.; Chesne, C.; van Grunsven, L.A. Novel human hepatic organoid model enables testing of drug-induced liver fibrosis in vitro. Biomaterials 2016, 78, 1–10.

50. Rashidi, H.; Luu, N.-T.; Alwahsh, S.M.; Ginai, M.; Alhaque, S.; Dong, H.; Tomaz, R.A.; Vernay, B.; Vigneswara, V.; Halle tt, J.M. 3D human liver tissue from pluripotent stem cells displays stable phenotype in vitro and supports compromised l iver function in vivo. Arch. Toxicol. 2018, 92, 3117–3129.

51. Meier, F.; Freyer, N.; Brzeszczynska, J.; Knöspel, F.; Armstrong, L.; Lako, M.; Greuel, S.; Damm, G.; Ludwig-Schwelling er, E.; Deschl, U.; et al. Hepatic differentiation of human iPSCs in different 3D models: A comparative study. Int. J. Mol. Med. 2017, 40, 1759–1771.

52. Takayama, K.; Kawabata, K.; Nagamoto, Y.; Kishimoto, K.; Tashiro, K.; Sakurai, F.; Tachibana, M.; Kanda, K.; Hayakaw a, T.; Furue, M.K.; et al. 3D spheroid culture of hESC/hiPSC-derived hepatocyte-like cells for drug toxicity testing. Biom aterials 2013, 34, 1781–1789.

53. Lee, G.; Kim, H.; Park, J.Y.; Kim, G.; Han, J.; Chung, S.; Yang, J.H.; Jeon, J.S.; Woo, D.H.; Han, C.; et al. Generation o f uniform liver spheroids from human pluripotent stem cells for imaging-based drug toxicity analysis. Biomaterials 2021, 269, 120529.

54. Qosa, H.; Ribeiro, A.J.S.; Hartman, N.R.; Volpe, D.A. Characterization of a commercially available line of iPSC hepatoc ytes as models of hepatocyte function and toxicity for regulatory purposes. J. Pharmacol. Toxicol. Methods 2021, 110, 107083.

55. Goulart, E.; de Caires-Junior, L.C.; Telles-Silva, K.A.; Araujo, B.H.S.; Rocco, S.A.; Sforca, M.; de Sousa, I.L.; Kobayash i, G.S.; Musso, C.M.; Assoni, A.F.; et al. 3D bioprinting of liver spheroids derived from human induced pluripotent stem cells sustain liver function and viability in vitro. Biofabrication 2019, 12, 015010.

56. Ardalani, H.; Sengupta, S.; Harms, V.; Vickerman, V.; Thomson, J.A.; Murphy, W.L. 3-D culture and endothelial cells im prove maturity of human pluripotent stem cell-derived hepatocytes. Acta Biomater. 2019, 95, 371–381.

57. Sirenko, O.; Hancock, M.K.; Hesley, J.; Hong, D.; Cohen, A.; Gentry, J.; Carlson, C.B.; Mann, D.A. Phenotypic characte rization of toxic compound effects on liver spheroids derived from iPSC using confocal imaging and three-dimensional i mage analysis. Assay Drug Dev. Technol. 2016, 14, 381–394.

58. Herzog, N.; Hansen, M.; Miethbauer, S.; Schmidtke, K.U.; Anderer, U.; Lupp, A.; Sperling, S.; Seehofer, D.; Damm, G.; Scheibner, K. Primary-like human hepatocytes genetically engineered to obtain proliferation competence display hepati c differentiation characteristics in monolayer and organotypical spheroid cultures. Cell Biol. Int. 2016, 40, 341–353.

59. Wang, Z.; Li, W.; Jing, H.; Ding, M.; Fu, G.; Yuan, T.; Huang, W.; Dai, M.; Tang, D.; Zeng, M. Generation of hepatic sph eroids using human hepatocyte-derived liver progenitor-like cells for hepatotoxicity screening. Theranostics 2019, 9, 66 90.

60. Qiao, S.; Feng, S.; Wu, Z.; He, T.; Ma, C.; Peng, Z.; Tian, E.; Pan, G. Functional Proliferating Human Hepatocytes: In Vitro Hepatocyte Model for Drug Metabolism, Excretion, and Toxicity. Drug Metab. Dispos. 2021, 49, 305–313.