3D liver models in tissue engineering and toxicology

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Introduction

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V. Starokozhko, G.M.M.Groothuis. Judging the value of “liver-on-a-chip” devices for prediction of toxicity. Expert Opinion on Drug Metabolism & Toxicology, 2016 (in press)
Introduction

Acute liver failure (ALF) is the clinical manifestation of severe and sudden hepatic injury of various origins leading to the development of hepatic encephalopathy, systemic inflammation and multi-organ failure (Bernal et al., 2010). Although rare, ALF poses an urgent problem due to the high mortality rate associated with this illness. The causes of ALF might vary from viral infections and drugs to inherited disorders. To date, liver transplantation is the only effective treatment available for ALF patients. However, orthotopic transplantation is limited by the shortage of liver donors. Several extracorporeal devices based on incorporated absorption and filtration systems have been used in the clinic as detoxification and support therapy for patients with ALF. However, even though some patients show improvement, overall these artificial liver devices are unable to significantly reduce mortality (Fuhrmann et al., 2011, Kramer and Kodras, 2011, Krisper et al., 2011). This is due to the fact that these artificial liver systems lack the proper metabolic, synthetic and detoxification functions of the liver, for which liver cells are needed. Therefore, cell-based liver therapy including creation of a bioartificial liver (BAL) have been proposed as an alternative approach to whole liver transplantation, or as a temporary solution, reducing the mortality rate of patients on the waiting list. A BAL can be designed and used as an extracorporeal device or as a transplantable construct.

Evolving progress in tissue engineering in the past decade has brought us closer to the development of a BAL. So far, several extracorporeal BAL models have been proposed and some of them have been used in the clinic (Pless, 2010, Demetriou et al., 2004, Chamuleau et al., 2006). Some of the tested BALs showed limited improvements in certain clinical parameters, such as ammonia and total bilirubin concentrations or neurological state. Unfortunately, the improvement in survival was not possible to assess due to the small patient group size and the orthotopic liver transplantation received by some of the patients.

All clinically tested BALs so far used hepatocytes of xenogeneic origin (primary porcine hepatocytes) or human hepatoma-derived cell line (C3A) (Chamuleau et al., 2006, van de Kerkhove et al., 2004, Pless, 2007). Even though no cases of zoonosis, virus transmission or tumor development have been reported, these BALs still pose risks, which might be detected only in later stages of clinical trials where hundreds to thousands of patients are involved. In addition, many challenging tasks, such as finding the ideal cell source, the implementation of a bile removal component, and inclusion of all of the various liver cell
types in a BAL, still remain. Therefore, a lot of attention has been given recently to developing 3D liver tissue constructs with improved liver functions and bioreactor support systems (McKenzie et al., 2008).

The liver is the central organ for metabolism and elimination of many endogenous and exogenous compounds. Therefore, and because of its location between the intestine and the systemic circulation, it is the organ with the highest exposure to toxicants (Lee and Senior, 2005). Drug-induced liver injury (DILI) is the second main cause of ALF worldwide and is the first main cause of liver failure in the USA and Europe (Bernal et al., 2010). Moreover, DILI is a major cause of drug failure during clinical trials or drug withdrawal from the market (Yang et al., 2013). DILI can be classified as intrinsic or idiosyncratic hepatotoxicity. The former is regarded as predictable, relatively common and dose-dependent with a short latency (Verma and Kaplowitz, 2009). These types of reactions are usually predicted in preclinical studies and during clinical trials. Idiosyncratic DILI, on the other hand, is rare, usually does not show a classical dose-dependency and the onset of the injury might vary from 1 week to 1 year (Hussaini and Farrington, 2007). It occurs only in a small fraction of the patients (<1:10,000) and, therefore, is usually not identified during clinical trials that are limited to a few thousand participants (Navarro and Senior, 2006). Intrinsic DILI usually results from direct toxicity of the drug or its metabolites, whereas idiosyncratic DILI is of an as yet undefined nature and often involves immunological responses.

DILI can manifest itself with various features and can involve different toxicological pathways, with drug-induced cholestatic injury being one of them. Drug-induced cholestasis (DIC) is characterized by an impaired bile flow, which is a result of drug effects on hepatocytes directly or on biliary epithelial cells, causing so-called ductular injury. DIC is the leading manifestation of DILI and numerous drugs have been identified as potentially cholestatic compounds (Qiu et al., 2016). One of the most common causes of DIC is the inhibition of the bile salt export pump (BSEP) on the apical side of hepatocytes (Yang et al., 2013) and an Adverse Outcome Pathway has been proposed based on this mechanism (Vinken et al., 2013). However, very often patients present a mixed pattern of injury: a combination of acute hepatitis, necrosis and cholestasis (Verma and Kaplowitz, 2009). Due to the complexity of the cholestatic injury, its low frequency, and often idiosyncratic nature, and the absence of highly predictive biomarkers to diagnose it, the occurrence of DIC is often only reported after drug approval for registration (Kaplowitz, 2005, Padda et al., 2011).

Currently available in vivo animal models have not been always powerful and predictive enough to predict liver toxicity in humans during the drug development process. In
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In general, they lack sufficient predictivity for human absorption, metabolism, distribution and excretion (ADME) and toxicity and it is often difficult to derive insight into the mechanism of the toxicity in in vivo experiments. Therefore, many efforts have been made to select reliable biomarkers and develop predictive in vitro models, preferably based on human cells to detect hepatotoxic effects as early as possible in the drug-development process. Tissue engineering technologies aim to create models that reconstruct the anatomy and physiology of the liver in vitro and which can be further used in regenerative medicine as well as in xenobiotic toxicity testing. Besides cell-based models for drug toxicity testing, tissue-based models will also be discussed in this chapter, such as precision-cut liver slices (PCLS), stressing advantages and disadvantages of these models as well as points for improvement.

3D liver models in tissue engineering

Since BAL devices aim to temporarily (extracorporeal devices) or permanently (transplantable constructs) take over the metabolic and excretory functions of the liver, the requirements for their functional capabilities are high. However, until now there is no consensus in the BAL community as to what functions or characteristics are considered to be the most important for a BAL (van Wenum et al., 2014).

Source of hepatocytes for tissue engineering

The biggest challenge in the development of the BAL is to develop a safe, readily available and highly functional source of human hepatocytes (Nibourg et al., 2012). Primary human hepatocytes (PHH) are considered to be the ideal candidate for BAL development with respect to their functions and human origin. However, their limited availability, lack of proliferation capacity in vitro and relatively rapid loss of function in in vitro culture limit their utility in BAL development (van Wenum et al., 2014, Ulvestad et al., 2013, Al Battah et al., 2011). Nevertheless, progress has been made by coculturing hepatocytes with non-parenchymal cells (NPC) in 3D culture systems for longer period of time with better maintenance of function (Kostadinova et al., 2013). Hepatocyte cell lines such as HepaRG and HepG2 have been used in a BAL, however, up to now they express liver functions at much lower levels than freshly isolated PHH (van Wenum et al., 2014). Stem cells are currently considered as a potential, infinite source for generation of various cell types. Moreover, they could be derived from the patient’s own cells, thereby overcoming the need
for the life-long use of immunosuppressant drugs. However, this is only possible for patients with progressive chronic liver diseases, since the generation of patient-specific iPSC-hepatocytes takes at least 6 weeks and, therefore, not suitable for patients with ALF. Hepatocyte-like cells have been derived from different stem cell sources, each of which has its advantages and limitations (Nibourg et al., 2012, Al Battah et al., 2011, Behbahan et al., 2011). Embryonic stem cells (ESCs) have the favorable property of self-renewal, and can differentiate into different lineages, including hepatocytes. However, ethical barriers associated with these cells encouraged scientists to search for alternative sources of human stem cells. Induced pluripotent stem cells (iPSCs) share many similar characteristics with ESCs, but avoid ethical issues surrounding the ESCs use. On the other hand, potential tumorigenicity is still considered an obstacle for the clinical application of these cells (Li et al., 2011, Tang et al., 2011). However, continuous advancement in the technology has substantially reduced the risk of iPSCs cells and clinical application of iPSC-derived somatic cells is expected in the near future. Adult (multipotent) stem cells were suggested to be non-oncogenic and were extensively studied with respect to their ability to differentiate into hepatocyte-like cells. Mesenchymal stem cells (MSCs) derived from adipose tissue (ADSCs) appear to be the most accessible source of adult stem cells. However, the differentiation and functionality of ADSC-derived hepatocytes still need to be improved before they can be used for BAL development (Lue et al., 2010). iPSCs and adult MSCs can be used to generate autologous therapies, minimizing or even eliminating problems associated with immune rejections after transplantation.

**Optimization of cell differentiation and culture**

Currently, there are numerous protocols available describing the generation of hepatocyte-like cells from iPSCs. However, some of these protocols were shown to be inefficient and cell differentiation appeared to be inadequate; cells were not sufficiently mature for future therapeutic application. Moreover, the majority of iPSC-derived hepatocytes are poorly characterized and often without proper benchmark (Song et al., 2009, Iwamuro et al., 2012, Takayama et al., 2012, Luni et al., 2016, Giobbe et al., 2015). Therefore, new techniques to improve the generation of hepatic cells from stem cells have been sought and applied, such as co-cultivation of hepatocytes with NPC, and culturing cells in 3D structures. For example, it was shown that 3D configuration not only promotes iPSCs hepatic differentiation, but also improves their metabolic and synthesis functions (Gieseck et al.,
NPC are known to contribute directly to various liver functions and they were also shown to improve hepatocyte function in vitro (Salerno et al., 2011, Enomoto et al., 2014, Yang et al., 2013). Though hepatocytes have been co-cultivated with different liver NPC, the introduction of all liver cell types with good functionality to the BAL remains still one of the biggest challenges. In Chapter 2 an extensive overview on the advances in the use of NPC in a BAL is presented.

In order to be incorporated into a BAL, the cells should be seeded on a scaffold that serves not only to fulfill a supportive role and promote cell functions, but also makes the use of a BAL easier. Today, numerous biomaterials for scaffold development are available on the market (synthetic, natural, decellularized matrices and hydrogels), each of which has its advantages and disadvantages (Lee and Cho, 2012).

Extracorporeal BAL devices need to be perfused with patient plasma, and therefore, should have channel-like structures to allow sufficient contact of plasma components with cells in the BAL. Flow of medium was shown to be beneficial for hepatic differentiation and maturation of stem cells. For example, ESC-derived hepatocytes exhibit higher metabolic and synthesis functions when cultured in a perfused bioreactor than in 2D static cultures (Miki et al., 2011). However, only a few studies so far have tried to differentiate iPSCs in the BAL bioreactor directly under flow. Hepatic differentiation and maturation of iPSCs directly in the 3D bioreactor may offer great advantages, such as overcoming the need to harvest the total amount of cells needed for the BAL from 2D cultures and loading into a 3D bioreactor, which is considered to be a delicate and laborious step, with a risk of losing cells and cell viability.

Resemblance of the BAL structure with in vivo liver architecture is still a main challenge in liver engineering. The use of microfluidic systems enables direct manipulation of cell loading to help overcome problems such as random positioning of the various cell types and low cell density in the device (Schütte et al., 2011, Larkin et al., 2013, Kasuya et al., 2012). For example, the application of bioprinting technologies allows the construction of 3D biomimetic liver models with precise cell positioning of hepatocytes, endothelial cells and mesenchymal cells in the scaffold, mimicking the liver architecture to a large extent. In this study, no flow was applied to the system and thus zonation was not achieved. The degree of differentiation, although somewhat better than in 2D, was not comparable to fresh tissue (Ma et al., 2016b). Moreover, it became possible to include automatically and selectively only viable cells during construction of liver units, while dead cells were not engrafted into the system (Schütte et al., 2011). This capability is particularly useful when cryopreserved cells are used for BAL development.
To conclude, achievements in the generation of hepatic-like cells from stem cells and bioreactor designs brought us one step closer to the construction of functional liver tissue for a BAL. However, the functionality of the stem-cell derived hepatocytes has not yet reached yet liver \textit{in vivo} levels and, therefore, improvements in the differentiation protocols have to be made before these cells can be successfully used in the clinic. Moreover, challenges such as finding new sources of NPC for BAL development and their incorporation into the BAL are not fully resolved yet, and should be addressed more in the future. An extracorporeal BAL that exhibits all liver functions on levels comparable to the human liver \textit{in vivo} would be useful not only in the clinic, but also in drug discovery and toxicity testing (Lin et al., 2015, Dash et al., 2009).

3D liver models in toxicology

As has been mentioned before, one of the most important characteristics that liver models for tissue engineering or toxicity testing should have is the presence of well-functioning hepatocytes. However, it has been recognized that not only the biotransformation by liver metabolic enzymes contributes to the onset and progression of drug-induced adverse effects, but also a polarized localization of liver transporters and the presence of NPC.

Ideally, \textit{in vitro} liver models should reconstruct the anatomy and physiology of the liver as closely as possible. 3D liver models better simulate \textit{in vivo} physiological environment than 2D cell cultures (Fitzgerald et al., 2015, Soldatow et al., 2013). In Table 1, we summarized the most important characteristics that an \textit{in vitro} liver model for toxicity testing should possess. So far, only two 3D liver models come close to fulfilling these criteria: the liver-on-a-chip model and PCLS.

Cell-based liver-on-a-chip models

Organ-on-a-chip technologies have been proposed as a new generation of \textit{in vitro} models for drug candidate screening in the preclinical phase of drug development. Liver-on-a-chip models, defined here as 3D microfluidic cell culture systems that aim to mimic closely the anatomy, physiology and functionality of the liver, are gaining increasing attention as \textit{in vitro} alternatives for \textit{in vivo} testing. The chips on which cells are seeded are mostly made from optically transparent polymers and usually contain channels the size of which ranges between 50-500 µm. They can contain monocultures of hepatocytes, 2D or 3D co-cultures of
### Table 1. Desirable characteristics of a liver *in vitro* model.

<table>
<thead>
<tr>
<th>Structural aspects</th>
<th>Multicellularity</th>
<th>Non-parenchymal liver cells are important for numerous hepatic functions and are involved in drug-induced adverse outcome pathways</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D structure</td>
<td>3D cell structure mimics the <em>in vivo</em> situation better and improves functions of hepatocytes</td>
<td></td>
</tr>
<tr>
<td>Flow</td>
<td>Flow mimics the <em>in vivo</em> situation better and improves functions of hepatocytes and EC <em>in vitro</em>, and in single pass mode removes waste products, and allows <em>in vivo</em>-like concentration profiles</td>
<td></td>
</tr>
<tr>
<td>Human cell source</td>
<td>Human cells predict human toxicity better</td>
<td></td>
</tr>
<tr>
<td>Integration of biosensors</td>
<td>Integration of biosensors allows precise monitoring of intracellular and extracellular events involved in drug toxicity.</td>
<td></td>
</tr>
<tr>
<td>Physiological fluid to tissue ratio</td>
<td>Physiological fluid to tissue ratio helps to establish physiological concentration gradients between cells and medium</td>
<td></td>
</tr>
</tbody>
</table>

### Functional aspects

<table>
<thead>
<tr>
<th>Drug metabolism activity</th>
<th>Metabolic activity levels similar to those in liver <em>in vivo</em> are crucial for a reliable toxicity prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transporter expression</td>
<td>Expression of transporters at similar levels and localization as <em>in vivo</em> is crucial for a reliable estimation of the actual drug exposure</td>
</tr>
<tr>
<td>Liver zonation</td>
<td>Liver zonation is important to identify primary target areas for toxicity in the liver and to reflect the balance between toxification and detoxification of drugs</td>
</tr>
<tr>
<td>Long term viability</td>
<td>Essential for drug-induced toxicities that require prolonged drug exposure</td>
</tr>
</tbody>
</table>

### Requirements for application in drug testing

<table>
<thead>
<tr>
<th>High sensitivity</th>
<th>Systems should be able to identify all the potential toxic candidates</th>
</tr>
</thead>
<tbody>
<tr>
<td>High specificity</td>
<td>Systems should preferably produce no false positive results</td>
</tr>
<tr>
<td>Affordable and easy to handle</td>
<td>In order to be used in a routine drug testing, liver chip systems should be affordable to the majority of pharmaceutical companies and CROs and not require highly specialized experts</td>
</tr>
<tr>
<td>Possibility for high throughput drug screening</td>
<td>To allow fast analysis of multiple candidates</td>
</tr>
</tbody>
</table>
hepatocytes with several other NPC or other stromal cells, hepatocyte spheroids or organoids formed by mono- or co-cultures.

Up to now, although there is consensus that liver functions and particularly biotransformation and transport functions as well as toxic responses should be reflected as close to \textit{in vivo} as possible and be stable preferably for several days up to weeks, there is still discussion on how to reach this. The presence of hepatocytes and NPC of human origin, the mimicking of liver microarchitecture, proper oxygenation and the introduction of flow of medium are generally accepted now as important features.

The main challenge is the use of fully functional human cells for toxicity testing, since animal primary cells, or human cell lines like HepG2 or HepaRG, make the extrapolation of the obtained results to the human \textit{in vivo} situation hazardous. This is due to the species-specific differences in metabolism and transport of drugs, in the case of animal cells, and the low/absent metabolic and transport activity of some of the enzymes and transporters, when using human cell lines. PHH are considered as the gold standard, although the maintenance of their metabolic competence during culture is one of the big hurdles (Godoy \textit{et al.}, 2013). However recent advantages in co-culturing with NPC and application of 3D and flow conditions have resulted in major improvements. The availability of fresh human liver tissue as source for PHH is still limited, but the quality of cryopreserved cells is currently improving. The use of hiPSC-derived hepatocytes with improved differentiation grade and hepatic functions is expected in the near future and will solve the existing problem of the shortage of human hepatocytes. There is consensus that NPC are essential for proper liver functionality, and they are known to be involved in many types of adverse effects in the liver. The source of these cells was mainly freshly isolated cells, but their differentiation from hiPSC is emerging. Standardization and validation of the hiPSC differentiation protocols for hepatocytes and NPC will help to provide a steady supply of well characterized (co)cultures.

Perfusion of the liver chips not only mimics the dynamic flow environment in the liver \textit{in vivo} and stimulates hepatocyte function, but it also may improve toxicity prediction of drugs (Prot \textit{et al.}, 2012, Prill \textit{et al.}, 2016) and provide control over exposure to the toxin and removal of waste products. The application of flow to the chip in single-pass mode also would allow time-dependent variations in the concentration of the toxic compound to be tested, thereby mimicking a repeated oral dose exposure. This represents one of the most challenging issues in safety assessment using \textit{in vitro} predictive toxicology. In cultures without flow or with recirculating flow, the concentration of the drug under study is only known at the start of the experiment, and its decrease in concentration during culture is not controlled and seldom
registered. Moreover, in those situations there is no removal of waste products and metabolites that are excreted by the liver basolaterally and cleared in vivo by the kidneys, or excreted into the bile. These compounds may accumulate in static cultures over time, possibly to toxic levels. Direct connection of LC-MS or IM-MS to the single pass flow system makes it possible to perform on-line metabolism and proteomics biomarker analysis. Changes in metabolic and signaling activities of the cells may also be tracked in time, which is of importance when unstable toxic metabolites are formed. However, nowadays non-flow-based liver models also show promising long-term viability and functionality and are technically much less complicated, which offers higher chances for application in high-throughput screening.

Liver-on-a-chip systems have been shown to be able to predict the clearance and toxicity of certain drugs in line with in vivo clearance and toxicity data, and to correctly reveal their mechanisms of action (Usta et al., 2015, Khetani et al., 2015). However, the levels of sensitivity and specificity with respect to classifying drugs as liver toxins still need to be improved. Specificity of conventional culture models used by pharmaceutical companies to test drug toxicity is usually high (>95%); the sensitivity, however, is not higher than 50%. Inclusion of NPC to the cultures significantly improves the sensitivity of toxicity, reaching 70-75% (Khetani et al., 2015). The currently achieved sensitivity of 75% might seem sufficient for initial drug screening at the early phase of preclinical studies. However, drug candidates entering late stages of preclinical trials and clinical trials should be characterized with higher sensitivity, using models with better predictivity to minimize potential health risks and unnecessary costs.

It is recognized that initiation and progression of liver injury is a dynamic process and cannot be properly characterized using endpoint measurements only. Most of the in vivo and in vitro studies provide little information on the kinetics of the biological response and therefore might miss important information on a drug’s mechanism of action. Liver-on-a-chip technologies either with or without flow, on the other hand, can implement and provide direct in situ real-time monitoring of many cell parameters. For example, incorporation of fluorescent biosensors and live-cell 3D imaging allow direct assessment of cell morphology and behavior throughout the study (Hutson et al., 2016). Furthermore, electrochemical sensors coupled to the bioreactors provide more information on the functional status of cells or specific organelles, such as mitochondria and could provide real-time monitoring of oxygen uptake (Prill et al., 2016). Overall, the readout of the existing systems can include a broad
range of techniques, such as imaging, biochemical and physical assays, which enable the detection of early signs of toxicity.

Drug-drug interaction is one of the important concerns during the drug development process, as well as in the clinic. The suitability of liver-on-a-chip models for recapitulation of the \textit{in vivo} drug-drug interactions has been shown recently (Ma \textit{et al.}, 2016a).

So far, only \textit{in vivo} animal or human models can cover all aspects of the disposition of a drug in the body, starting from absorption and metabolism in the gastrointestinal (GI) track, biotransformation in the liver, systemic exposure and elimination by the kidney and the GI track. Development of a multi-organ chip for long-term culture might open a great possibility to perform repeated dose studies \textit{in vitro}. Advances in the field of microengineering allow the construction and integration of multi-organ chips to a human-on-a-chip, stressing the importance of different organ interactions in drug-induced toxicity. Interaction between intestine and liver co-cultured in a chip was shown for nanoparticle uptake and toxicity (Esch \textit{et al.}, 2014). Maschmeyer \textit{et al.} developed a four-organ-chip that includes intestine, liver, skin and kidney tissue connected to each other in a dynamic manner. It was possible to maintain the system viable and functional for 28 days (time required for repeated dose toxicity studies according to the guidelines) (Maschmeyer \textit{et al.}, 2015). However, no toxicity studies have been performed yet to further validate this model.

The current liver-on-a-chip systems still exhibit some shortcomings. One of them is the use of polydimethylsiloxane (PDMS) scaffolds for most of the models, which is hydrophobic and adsorbs/absorbs lipophilic compounds and their metabolites, leading to the misinterpretation of the exposure and thus the test results. However, recently polymer materials, such as parylene, or polycarbonate have been proposed and used in a liver-on-a-chip production, which will eliminate the unspecific binding of molecules to silicone materials such as PDMS (Esch \textit{et al.}, 2016). In addition, high costs of the current chip models and their relative complexity limit their use in a high-throughput setting (Materne \textit{et al.}, 2013). Nevertheless, despite numerous obstacles on the way to the design of an ideal liver-on-a-chip model, significant progress has been made towards the development of non-PDMS pumpless systems based on primary human cells, which are somewhat less complicated and less expensive for a broad use in high-throughput drug screening, but are not fully characterized yet (Esch \textit{et al.}, 2016).

Even though some toxicity screening of certain model drugs have been performed, none of the systems has been fully validated with respect to toxicity prediction (Usta \textit{et al.}, 2015, Bale \textit{et al.}, 2014, Shintu \textit{et al.}, 2012). Before a liver-on-a-chip system is accepted by
the regulatory authorities as an alternative for animal experiments and can be used for routine
drug toxicity screening in pharmaceutical industry, the requirements for this in vitro model
will need to be standardized. This is because many technical aspects can influence the cell
biology and response to the drug, such as culture duration, flow rate, cell density, scaffold
material and fluid to tissue ratio (Shintu et al., 2012). It has also been recognized that in
addition to the characterization of phase I and phase II enzymatic activities, transporter
activities have to be present and fully described. This is a crucial aspect, since transporters not
only determine the actual drug exposure, but also can be a direct target of drug toxicity.

The next step is to fully characterize existing human liver-on-a-chip systems and to
determine their predictive capabilities for clinical outcomes based on the correlation between
in vitro data, and available patient information and information from clinical studies. Models
that are more predictive and efficient, widely available and less expensive would be of great
value for the prediction of toxicity, and would represent an appropriate alternative to animal
testing.

**Precision-cut liver slices**

PCLS represent a miniaturized liver that preserves its 3D architecture and cell
composition. PCLS have been proven as a functional and predictive model in many
physiological and toxicological studies (Vickers and Fisher, 2013, de Graaf et al., 2010, de
Graaf et al., 2007, Olinga and Schuppan, 2013). Freshly obtained PCLS exhibit metabolic
functions comparable to those in vivo and, therefore, can be reliably used for drug clearance
and toxicity testing (de Graaf et al., 2007, Elferink et al., 2011). Moreover, the presence of
NPC makes it possible to study pathological mechanisms that require the involvement of
these cell types. For example, Kupffer cell-mediated response to lipopolysaccharide (LPS)
exposure is found in PCLS (Olinga et al., 2001). In addition, mouse and human PCLS have
been used to study idiosyncratic DILI where inflammation is a contributing factor (Hadi et al.,
2012, Hadi et al., 2013).

Physiological, polarized transporter expression on hepatocytes in PCLS makes this
model suitable for studies where transporter function and location play a crucial role in
toxicity onset and progression. For example, the development of drug-induced cholestasis
involves BSEP inhibition on the apical side of the hepatocyte membrane and disturbances in
secretion of bile acids (BA) from the hepatocytes to the bile canaliculi. Therefore, models that
do not reconstruct liver canalicular structure and polarized expression of the transporters would not be able to reproduce this pathological process.

Recently, a perfused system for PCLS culture which mimics blood flow in vivo has been developed by van Midwoud et al. (van Midwoud et al., 2010a). Even though the flow did not seem to improve the loss of liver metabolic function, it allowed the detection of short-lived metabolites produced by the liver that were directly measured in the outflow medium using HPLC (van Midwoud et al., 2011). Moreover, coupling the perfused biochip system with an HPLC (or LC-MS) allows direct in-time measurements of metabolites or proteins produced by the liver. Furthermore, similar to the organ-on-a-chip technologies, it is possible to combine different organs together on one chip to study organ interactions. Thus, by directly connecting a microchamber with a liver slice to another microchamber with an intestinal slice, it was possible to recreate intestine-liver cross-talk (van Midwoud et al., 2010b).

The use of human PCLS opens a great possibility to test drug toxicity directly on human tissue. This not only increases the predictivity of the model, but also eliminates unnecessary animal-to-human extrapolation steps and greatly reduces animal use. For example, a newly developed non-toxic analog of acetaminophen (APAP) was shown to be non-toxic in mouse PCLS, but toxic in rat and human PCLS, stressing the importance of using human tissue for toxicity testing (Hadi et al., 2013).

One of the disadvantages of this model is the short functional viability of PCLS in culture. Even though fresh PCLS contain the whole range of phase I and phase II metabolic enzymes, as well as transporters, and their viability can be maintained for several days (Vickers et al., 2011, Vickers et al., 2004), the activity of phase I metabolic enzymes has been shown to drop already after 24 hours of incubation (de Graaf et al., 2010, Catania et al., 2003). Moreover, necrosis still occurs after 48-72h of incubation (Soldatow et al., 2013, Lerche-Langrand and Toutain, 2000). Furthermore, hardly any data are available on the maintenance of the morphological integrity and viability of various NPC cell types in the slices during prolonged incubation. Given that NPC might play a significant role in toxicity development, their presence and function in the culture are crucial for the interpretation of the data obtained.

Because the decline in metabolic function during 24 h is limited, it does not prevent the use of PCLS in acute toxicity studies. However, their utilisation in (sub)chronic toxicity studies might result in acquired data that are not fully representative of the in vivo situation, since the toxicity of a drug often directly depends on its biotransformation. Therefore, before being able to reliably use this model in prolonged toxicity testing, the maintenance of PCLS...
morphological integrity and function needs to be prolonged and fully characterized. One of
the ways to do so, is to improve culture conditions by enriching the culture media with the
growth factors and compounds necessary to support liver functions, as is already applied in
long term cultures of PHH and for maturation of stem-cell derived hepatocytes.

However, even if we could find the proper culture conditions for PCLS to represent
almost all characteristics that an in vitro liver model should possess, they cannot be used in
high-throughput settings due to the limited availability of fresh human liver tissue for
research. Moreover, human PCLS are not readily available in industrial settings and to date,
cryopreservation methods have not been successful.

Scope of the thesis

Recent development in the field of in vitro 3D liver tissue engineering and progress in
microfluidic techniques bring us closer to the goal of creating a “liver in the lab” that
represents the functions of the liver in vivo. Using human 3D liver models opens a great
possibility to study drug toxicity in the human liver without using human volunteers. It could
enable the transplantation of human-based liver constructs to patients without the need of
donors, with ultimately the possibility to make use of the patient’s own stem cells.

However, in order to improve and establish new applications for existing 3D liver
models in research, in the clinic or in pharmaceutical industry, more optimization and
validation is required. In this thesis, we aim to optimize existing 3D liver models for tissue
engineering and toxicology. We address the functionality of the optimized 3D liver models
and their suitability for application in a BAL for future clinical use as well as for acute and
(sub)chronic toxicity studies, focusing on drug-induced cholestasis. The clinical validation of
the engineered BAL described in Chapter 3 is beyond the scope of this thesis.

In Chapter 2 we discuss the recent achievements in 3D liver tissue engineering for
BAL development as well as existing challenges on the road to the development of a BAL for
transplantation. We describe such essential issues as multicellularity of the developed BAL
and recreation of vascular and biliary network structures, as well as liver zonation. We also
discuss removal of bile from the BAL system, a problem that has not been solved yet.

In Chapter 3 we describe the possibility of the hepatic differentiation of hiPSC-
derived definitive endoderm cells directly in a perfused bioreactor. This would facilitate the
use of this system in the clinic as an extracorporeal BAL, or in drug research as a model for
toxicity testing. We characterized the differentiated cells using a wide range of liver markers on the mRNA, protein and functional levels, and compared their performance with that of fresh human PCLS. The model of human PCLS is the closest model to the human liver *in vivo* with respect to functional activities, which is considered as an advantage over previously published studies. These usually have not benchmarked the observed characteristics of the differentiated cells, or have used an inadequate benchmark (e.g. cultured human hepatocytes for 48-72h) that has lost functional activity. This has lead to overestimation of the functions of stem cell-derived hepatocytes with sometimes as much as two orders of magnitude.

The research described in **Chapter 4** and **5** aimed to prolong the viability and functionality of rat and human PCLS up to 5 days using different types of media, which would extend the application of PCLS for subchronic toxicity testing. Moreover, we characterize the morphological changes that occur in slices during prolonged incubation and describe changes in tissue architecture that have never been reported before. We assess the stability of metabolic enzyme activities, synthesis functions and the expression of genes responsible for xenobiotic metabolism and transport in human PCLS during 5 days of incubation.

In **Chapter 6**, we tested whether PCLS can be used to study drug-induced cholestasis. The relevance and predictivity of this model was assessed by evaluating the potential of known cholestatic compounds to cause cholestasis by disturbing the BA homeostasis *ex vivo*. The cholestatic injury is evaluated using several biomarkers of cholestatic liver injury.

In **Chapter 7** we summarize and discuss the findings of the research presented in this thesis. We present future perspectives on the use of 3D liver models not only as models for drug toxicity screening or in the BAL setting, but also as biological liver models that can be used to unravel physiological mechanisms involved in liver function or pathological mechanisms involved in liver injury development and progression.
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References


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Part A
3D liver models in tissue engineering