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REVIEW ARTICLE

Technological advancements for the development of stem cell-based models for hepatotoxicity testing

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Abstract

Stem cells are characterized by their self-renewal capacity and their ability to differentiate into multiple cell types of the human body. Using directed differentiation strategies, stem cells can now be converted into hepatocyte-like cells (HLCs) and therefore, represent a unique cell source for toxicological applications in vitro. However, the acquired hepatic functionality of stem cell-derived HLCs is still significantly inferior to primary human hepatocytes. One of the main reasons for this is that most in vitro models use traditional two-dimensional (2D) setups where the flat substrata cannot properly mimic the physiology of the human liver. Therefore, 2D-setups are progressively being replaced by more advanced culture systems, which attempt to replicate the natural liver microenvironment, in which stem cells can better differentiate towards HLCs. This review highlights the most recent cell culture systems, including scaffold-free and scaffold-based three-dimensional (3D) technologies and microfluidics that can be employed for culture and hepatic differentiation of stem cells intended for hepatotoxicity testing. These methodologies have shown to improve in vitro liver cell functionality according to the in vivo liver physiology and allow to establish stem cell-based hepatic in vitro platforms for the accurate evaluation of xenobiotics.

Keywords Stem cells · Hepatocyte-like cells · 2D- and 3D-culture systems · In vitro toxicity · Drug screening · Microfluidics

Abbreviations

2D	Two-dimensional
2PP	Two-photon polymerization
3D	Three-dimensional
ADMSCs	Adipose-derived mesenchymal stem cells
ASCs	Adult human stem cells
BMMSCs	Bone marrow mesenchymal stem cells
BMP	Bone morphogenetic protein
BSEP	Bile salt export pump
CFOS	Proto-oncogene c-fos
CYP	Cytochrome P450
ECM	Extracellular matrix
EGR	Early growth response
FGF	Fibroblast growth factor
Gel-MOD	Methacrylamide-gelatin
Gel-MOD-AEMA	Gelatin-methacrylamide-aminoethyl-methacrylate
Gel-NB	Gel-Norbornene
hESCs	Human embryonic stem cells
HGF	Hepatic growth factor
hiPSCs	Human induced pluripotent stem cells
HLCs	Hepatocyte-like cells
hMSCs	Human mesenchymal stem/stromal cells
hSKP	Human skin-derived precursors

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IEG	Immediate early genes
MenSCs	Menstrual blood stem cells
MPCCs	Micropatterned co-cultures
NTCP	Sodium-taurocholate cotransporting polypeptide
OSM	Oncostatin M
PAI	Plasminogen activator inhibitor
PDMSCs	Placenta-derived mesenchymal stem cells
PHH	Primary human hepatocytes
PRL	Phosphatase of regenerating liver
UCMSCs	Umbilical cord mesenchymal stem cells
Wnt	Wingless-type MMTV integration site

Introduction

Over the years, experimentation using animal models has significantly contributed to the understanding of toxicological properties of harmful agents (Denayer et al. 2014). Yet, besides ethical and financial constraints, animal testing often fails to identify human hepatotoxic compounds during risk assessment due to considerable interspecies differences (Hartung 2009; Doke and Dhawale 2015). In particular, differences in hepatic phase I and II enzymes are likely the major cause of differential interspecies liver susceptibility to toxins (Hartung 2009). Compounds, potentially causing human hepatotoxicity can also be evaluated using in vitro platforms based on human cells. Primary human hepatocytes (PHH) remain the leading model for hepatotoxicity testing. However, their use is hindered by their scarce availability and dedifferentiation in culture leading to a downregulation of drug-metabolizing enzymes, hereby reducing their pharmaco-toxicological relevance (Guguen-guillouzo et al. 2010). Hepatic cell lines such as HepG2 are also widely employed, but they lack diverse metabolic capabilities, exhibit a limited sensitivity and do not represent the population diversity due to their single-donor origin (Castell et al. 2006). With these factors in mind, stem cells hold great promise to overcome the limitations of the current in vitro models. Stem cells, including human embryonic stems cells (hESCs), induced pluripotent stem cells (hiPSCs) and mesenchymal stem/stromal cells (hMSCs), have a self-renewable ability, a high proliferative potential and display either pluripotent or multipotent competences. This innate plasticity enables

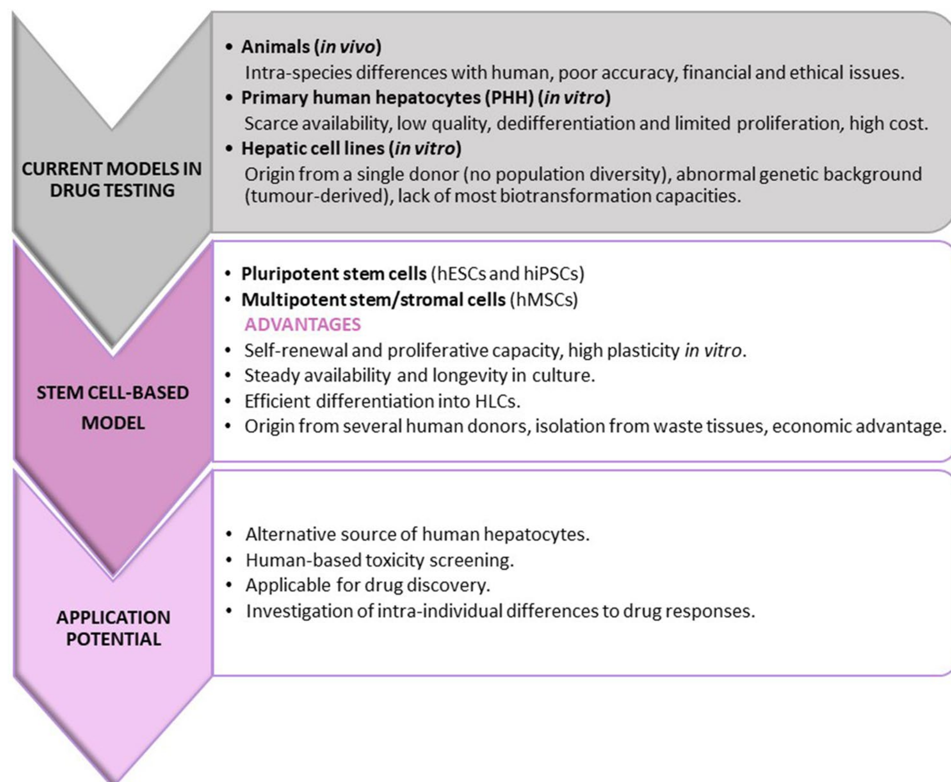

Fig. 1 Schematic summary of potential disadvantages of current in vitro models and the advantages of stem cells as a new tool for hepatotoxicity testing. *hESCs* human embryonic stem cells, *hiPSCs* human-induced pluripotent stem cells, *hMSC* human mesenchymal stem/stromal cells, *HLCs* hepatocyte-like cells

differentiation towards multiple cell types including hepatocyte-like cells (HLCs) (Davila et al. 2004; Snykers et al. 2009; Damania et al. 2014) (Fig. 1). Also, stem cell-derived hepatocytes are associated with a continuous cell supply and longevity in culture rendering them an ideal cell type for human-based toxicological studies.

The differentiation of stem cells towards HLCs is mainly carried out in two-dimensional (2D) culture systems (Baxter et al. 2010; Behbahan et al. 2011; Szkolnicka and Hay 2016). In these cultures, cells are housed in an unnatural microenvironment in which the native morphology is gradually lost and the deprivation of tight cell–cell junctions causes a reduction in metabolic activity and hepatocyte functionalities (Horvath et al. 2016; Duval et al. 2017). Three-dimensional (3D) culture systems are thought to overcome these limitations. Indeed, the unique features of 3D-systems allow physical and spatial organization of cells, which facilitate cell–cell interactions and configurations of cell receptors, improving effective signal transduction. As a result, cell behaviour in 3D-cultures can better mimic in vivo liver functionality (Rimann and Graf-Hausner 2012; Fang and Eglen 2017). Application of 3D-culture models can substantially contribute to the establishment of robust cell-based assays with increased specificity and sensitivity to drug responses (Godoy et al. 2013; Hay et al. 2014; Horvath et al. 2016).

In the present review, we describe the technological advancements in cell culture methods that improve human stem cell differentiation towards HLCs, illustrating their manufacturing processes, biological relevance and potential application in drug toxicity testing.

Hepatic differentiation of stem cells in vitro

Human pluripotent stem cells include hESCs and hiPSCs, the latter being obtained by reprogramming somatic cells to a pluripotent state by gene transduction (Meissner et al. 2007; Takahashi et al. 2007). Additionally, adult tissues and organs contain niches that harbour several types of adult stem cells (ASCs), which maintain part of their regenerative ability throughout the adult life (Caplan 2015). Among ASCs, hMSCs retain the ability to differentiate in multiple cell lineages in vitro, although they posses lower expansion capacity in comparison to pluripotent stem cells (Ullah et al. 2015). In the presence of specific culture media, hMSCs from several sources, including bone marrow mesenchymal stem cells (BMMSCs), adipose-derived mesenchymal stem cells (ADMSCs), placental-derived mesenchymal stem cells (PDMSCs), menstrual blood stem cells (MenSCs), skin-derived precursors stem cells (hSKP), umbilical cord mesenchymal stem cells (UCMSCs), can differentiate into human HLCs (Zuk et al. 2002; Divya et al. 2012; He et al. 2013; Mou et al. 2013; Rodrigues et al. 2014; Yu et al. 2015; Chang et al. 2016; Chen et al. 2016).

Although challenges remain for the generation of mature stem cell-derived hepatocytes that share the same functionality of hepatocytes of the human liver, great progress has been made in exploring differentiation strategies inspired by in vivo liver embryogenesis. In this respect, Snykers et al. (2006) were the first to develop a differentiation strategy with in vivo relevance that closely resembles the pattern of cytokine secretion during liver embryogenesis in vivo.

Indeed, in vivo, hepatocyte differentiation depends on the presence of non-parenchymal cells which are responsible for the secretion of cytokines all along the onset of hepatogenesis (Si-Tayeb et al. 2010). During the process, activated Kupffer cells secrete among others, tumor necrosis factor, interleukin 6 and oncostatin M (OSM). In addition, stellate and endothelial cells release fibroblast growth factor (FGF), bone morphogenetic protein (BMP) and hepatic growth factor (HGF) (Si-Tayeb et al. 2010).

Current protocols attempt to promote differentiation of stem cells by mimicking in vivo liver embryogenesis in three steps where FGF, HGF and OSM predominantly govern different phases of liver development (Snykers et al. 2006; Behbahan et al. 2011; Zhang et al. 2014). The first step includes the endoderm commitment induced by activin A, FGF- and BMP-families. FGF is required for endoderm specification and formation of hepatic progenitors via the transient suppression of the wingless-type MMTV integration site (Wnt) signalling pathway. FGF-mediated activation of Wnt antagonists repress the expression of *Hhex*, an essential regulator of the hepatic commitment (Twaroski et al. 2015). The second step involves the specification towards the hepatic phenotype which is mediated by the cooperation of HGF with FGF and BMP. Here, HGF plays an important role in stimulating proliferation of hepatoblast-like cells (Suzuki 2003; Ye et al. 2015). The last step implies the generation of mature hepatocytes via OSM, dexamethasone and HGF (Si-Tayeb et al. 2010). OSM, an interleukin 6 subfamily member, is required for the maturation from fetal towards adult liver. Studies showed that while both OSM and HGF upregulate albumin expression, only OSM induces its secretion (Kamiya et al. 2001). In addition, OSM induces urea production and glycogen storage (Lysy et al. 2008). These findings suggest that the interplay of HGF and OSM is crucial for the acquisition of metabolic functions in maturing hepatocytes. To replace the use of cytokines that require prolonged differentiation periods and high cost, cell-permeable compounds known as small molecules are often used in differentiation protocols (e.g. dihexa, a HGF mimetic, and CHIR99021, a Wnt agonist) (Siller et al. 2015; Mathapati et al. 2016). The addition of small molecules in culture act as agonists or inhibitors of specific cell signalling pathways and target genes proved to efficiently drive the commitment

of stem cells towards definitive endoderm and maturation of HLCs (Siller et al. 2015; Mathapati et al. 2016; Du et al. 2018). This method is cost-effective and reproducible, enabling the generation of scalable HLCs (Tasnim et al. 2015).

In addition, the generation of HLCs can be achieved by direct reprogramming of somatic cells using viral (e.g. lentiviral and adenoviral vectors) as well as non-viral approaches (e.g. recombinant proteins, micro RNAs, synthetic modified mRNA and episomal vectors) (Huang et al. 2014; Simeonov and Uppal 2014; Nakamori et al. 2017). These strategies allow for the insertion of defined lineage-specific transcription factors into the cell, thereby inducing its reprogramming to hepatic cells. The differentiation of hESCs and hiPSCs towards HLCs could also be obtained by viral transduction of SOX17, HEX and HNF4A (Inamura et al. 2011; Takayama et al. 2012).

Stem cell-based 2D-culture systems for hepatotoxicity testing

Cell cultures in monolayer, in which cells adhere to flat and rigid substrata, have been used for several decades and are well standardized. These approaches have also been widely adopted for the culture of stem cells and generation of stem cell-derived hepatocytes.

The use of matrigel-, laminin- and collagen-coated culture surfaces, which mimic somehow the biological components contained in the extracellular matrix (ECM), significantly improves cell attachment and promotes liver-specific functionalities (Wang et al. 2017). Several studies found evidence that both hESCs and hiPSCs, differentiated towards HLCs in 2D, express to some extent adult cytochromes P450 (CYP) enzymes and in some cases, transporter proteins such as sodium-taurocholate cotransporting polypeptide (NTCP) and bile salt export pump (BSEP) (Zamule et al. 2011; Ulvestad et al. 2013). Although these cells are not fully mature compared to PHH, they have also been described to own a certain sensitivity to compounds that require active metabolization (Medine et al. 2013; Sirenko et al. 2014; Szkolnicka et al. 2014). In the work of Holmgren et al., hiPSCs, differentiated towards HLCs were exposed for 14 days to repeated doses of four hepatotoxic compounds (amiodarone, aflatoxin B1, troglitazone and ximelagatran). The authors showed that hiPSCs-derived HLCs exhibited a time-dependent toxic response comparable to that of exposed HepG2 (Holmgren et al. 2014). A more recent study showed a high sensitivity of hESCs-derived HLCs for the prediction of hepatotoxicity of herbal medicines. Exposure to emodin, diosbulbin B and gallic acid resulted in responses, comparable to those obtained in PHH (Kim et al. 2018).

Advanced culture methods, using 'engineered Petri-dishes', namely 2D-micropatterned co-cultures (MPCCs), could improve the metabolic competence of hESCs/hiPSCs-derived HLCs. Khetani and Bhatia (2008), fabricated micropatterned structures in 24-multiwell format consisting of 37 hepatic microstructures of 500 µm diameter in each well in which collagen-I was absorbed to certain areas of the culture plate creating as such collagen-coated islands where hepatic cells could selectively adhere while a second cell type was seeded into the surrounding bare areas. Using this setup, the same group more recently reported remarkable improvements in drug-mediated CYP450 induction, found to be higher in MPCCs compared to conventional hiPSCs-derived HLCs monolayers (Berger et al. 2015). The robustness of the system for drug toxicity assessment was corroborated by testing a set of 47 compounds. The results showed 65–70% sensitivity and 100% specificity in hiPSCs-derived HLCs when compared to PHH. Conventional 2D-hiPSCs-derived HLCs cultures failed to detect several hepatotoxins, while both MPCCs-hiPSCs-derived HLCs and PHH cultures could correctly identify the hepatotoxins (Ware et al. 2015).

Several protocols have also been used to induce direct hepatic differentiation of hMSCs (Schwartz et al. 2002; Lee et al. 2004; Stock et al. 2008; Itaba et al. 2015; Chen et al. 2016; Xue et al. 2016). Although the obtained cells displayed high levels of hepatic markers, their metabolic enzyme activity remained, however, very low. Only a few studies report the relevance of hMSC-derived HLCs in toxicity assessment. For example, PDMSCs and BMMSCs differentiated to HLCs were exposed to *N*-nitrosodiethylamine, *N*-nitrosodimethylamine and carbon tetrachloride, resulting in different sensitivity patterns between the two cell populations. PDMSCs derived-HLCs resulted more sensitive to the hepatotoxicants than BMMSCs derived-HLCs, likely due to the expression of specific ATP-binding cassette transporters that regulates the transport of toxicants to the cells (Lee et al. 2011). Another study showed that human skin-derived precursors (hSKP) could be differentiated into cells expressing hepatic progenitor markers and adult hepatic markers, together with genes encoding for phase I and II biotransformation enzymes (Rodrigues et al. 2014). These cells were shown to be predictive for hepatic acute liver failure, steatosis and phospholipidosis upon exposure to acetaminophen, sodium-valproate and amiodarone, respectively (Rodrigues et al. 2014, 2016; Natale et al. 2017).

Altogether, these findings demonstrate that 2D-systems of stem cell-derived HLCs possess relevant hepatic functionality that is applicable for drug toxicity testing. Yet, these cells still display several characteristics of immature hepatocytes and their metabolizing potential is low. Therefore, improvement of hepatic maturation must be pursued to induce a steady expression profile of metabolizing enzymes.

From 2D- to 3D-culture systems: simulating in vivo physiology

Hepatocytes are large polygonal epithelial cells, assembled in string-like sheets that form structural units or liver lobules. Non-parenchymal cells are localized in the sinusoidal and biliary compartments of the tissue. Sheets of hepatocytes are separated from each other by a membrane formed by highly permeable liver endothelial cells. These specialized hepatic sinusoidal cells are in contact with the bloodstream that delivers nutrients and enables the formation of different oxygen gradients (Treyer and Müsch 2013).

Due to their strategic position, hepatocytes have a polarized organization that consists of (1) a lateral domain, that engages in cell–cell contacts between neighbouring hepatocytes; (2) a basal domain, that makes contact with the epithelial–blood interface in the space of Disse; (3) an apical domain, which makes up a narrow lumen between two adjacent hepatocytes forming a network of bile canaliculi (Treyer and Müsch 2013). These canaliculi extend to the bile ducts which are lined by cholangiocytes. The basal surface of cholangiocytes is associated with the basement membrane and the apical surface forms the luminal space of bile ducts surrounded by the monolayer of cholangiocytes. Apicobasal polarity is essential for the commitment of bipotential liver progenitors to cholangiocytes and for the morphogenesis of bile ducts (Tanimizu et al. 2007). The representation of this complex cellular liver architecture is not possible with 2D-systems. Conventional 2D-cultures prevent cells from forming multi-dimensional structures due to their anchorage on flat substrata, which only permits contact with neighbouring cells at the outer perimeter. Cells which are partially polarized are not able to pile up on top of one another but are restricted to expand in monolayers, which does not naturally occur in the native tissue. In this non-physiologic setup, transport of nutrients and oxygen tension is not uniform. In addition, when cells are cultured in a 2D-setup, substantial amounts of CYP enzymes become depleted, limiting the efficacy of drug metabolization. In such an environment, hepatocytes act as a single entity in which homo- and heterotypic interactions together with spatial architecture are lacking (Berger et al. 2015).

In contrast, 3D-culture techniques open up the possibility of mimicking the native ECM arrangement and can positively influence hepatic lodging, proliferation and maintenance of metabolic activities. In 3D-cultures, cells are exposed to multi-cellular contacts, thus triggering multiple stimuli resulting in higher viability and reduced apoptosis when compared to cells cultured in 2D. Also, hepatocytes growing in a 3D-pattern secrete higher levels of urea and albumin and show enhanced activity of CYP (Baharvand et al. 2006; Duval et al. 2017). Profound manipulations of

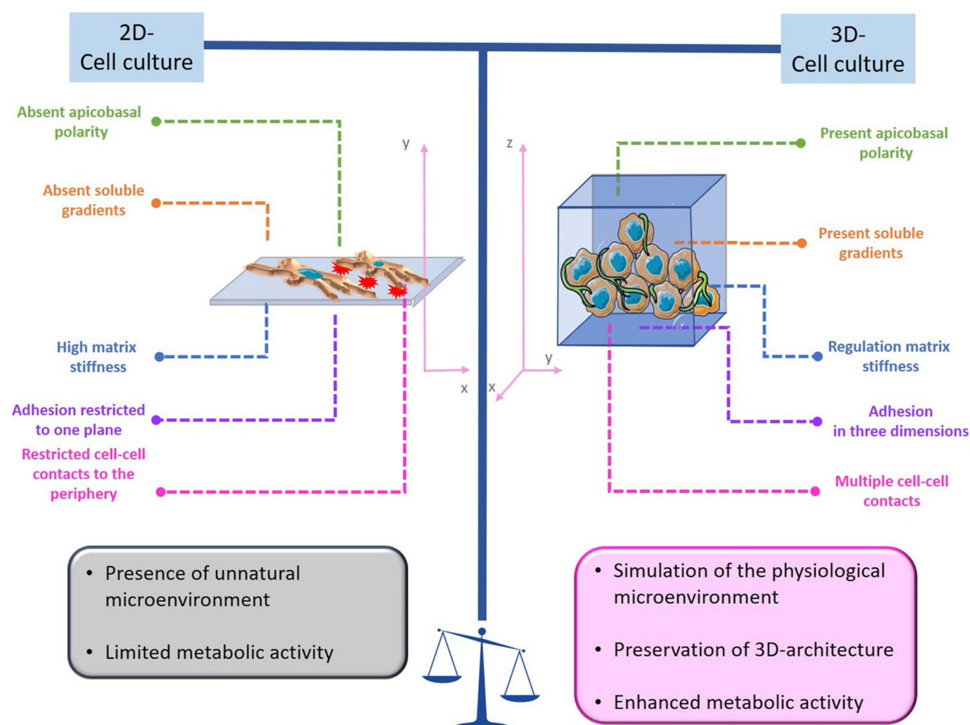

Fig. 2 Comparison of key components of 2D- and of 3D-culture systems for hepatic differentiation of stem cells. *2D* two-dimensional, *3D* three-dimensional

the ECM can be achieved in 3D-models, thereby promoting cell adhesion, survival, proliferation and differentiation of stem cells (Lin et al. 2012) (Fig. 2).

Stem cell-based 3D culture technologies

Several technological approaches aim at recreating a native-like environment for cell cultures by establishing physiologically relevant culture conditions that better represent the in vivo situation. Such systems involve growing cells in a 3D-environment and simulated vasculature. 3D-systems are commonly subdivided into scaffold-free and scaffold-based setups. In scaffold-free constructs, 3D-cell aggregates allow the formation of oxygen and nutrient gradients and develop in- and outward production of signal molecules. In turn, scaffold-based setups provide adherence cues to cells and promote migration, proliferation and cytoskeletal reorganization. The microenvironment of the native tissue can be mimicked by tailor-made 3D-scaffolds in which the material properties such as mechanical characteristics, porosity, chemical functionalization and geometry can be specifically controlled (Abbott 2003; Antoni et al. 2015; Fang and Eglen 2017; Langhans 2018). Scaffold-based constructs are meant to recreate the natural, physical and structural environment of liver tissue. These scaffolds consist of a physical support where cells can adhere, acquire their native morphology and maintain cell–cell junctions. Furthermore, 3D-structures offer a larger cultivation surface in comparison with 2D-culture systems. Cell attachment can be influenced by the selection of materials, shape, size and porosity of the backbone of the scaffold (Loh and Choong 2013).

Porous scaffolds allow cell infiltration, nutrient and oxygen transport within the 3D-construct. Pore sizes ranging from 120 to 200 µm are described to be the most promising to induce hepatogenesis of ADMSCs (Wang et al. 2010). The high porosity (65–70%) and the wide pore size enable the nutritional supply inside the scaffold. However, if the pores are too small, cell migration is limited, resulting in the formation of a cellular agglutination around the edges of the structure and limited ECM production within the scaffold. Conversely, large pore size renders the scaffold more fragile and decreases the cell growth area, limiting cell adhesion (Loh and Choong 2013).

Polymers are an attractive material for the fabrication of scaffolds, from simple to more complex matrices, as they allow the modification of porosity and mechanical properties of culture surfaces (Jain et al. 2014). The most common materials that are used for the fabrication of scaffolds are natural and synthetic polymers. Natural polymers, such as collagen, glycosaminoglycan, chitosan, starch, hyaluronic acid, cellulose and alginate are surrogates for the ECM and support cell interaction and proliferation (Gerecht et al. 2007; Li et al. 2010; Lucendo-Villarin et al. 2016). The weakness and softness of pure natural polymers have the advantage of adapting their shape to required forms. However, they have poor mechanical properties, and therefore, require further processing (Dhandayuthapani et al. 2011; Asti and Gioglio 2014). Chemically synthetized polymers, such as poly-lactic acid, poly-glycolic acid, poly lactic-*co*-glicolic acid and polycaprolactone, are gaining importance (Jain et al. 2014). For these materials several parameters such as porosity, mechanical characteristics and binding properties can be controlled, allowing the fabrication of specific scaffolds with desired characteristics. Many techniques can be used to shape polymers into complex 3D-scaffolds. An overview of these strategies, which can be used for culturing HLCs in 3D, is described in the following paragraphs.

Scaffold-free setups

Decellularized liver matrices

The liver ECM is a complex environment composed of proteoglycans, glycosaminoglycans, collagens and glycoproteins. ECM components provide binding sites for molecules and receptors which may induce significant modifications of stem cell morphology, transcriptional profile, proliferation and differentiation (Hoshiba et al. 2016). Despite the fact that the generation of sophisticated bioactive polymers is progressing, it remains challenging to reproduce the natural ECM in vitro, due to the variety of constituents produced by the various cell types present in the liver. As an alternative strategy, natural liver bioscaffolds can be obtained by decellularization of the whole organ. The process involves the removal of cellular components from the tissue, providing an empty matrix with a preserved 3D-backbone that harbours an intact vasculature and important ECM-bound growth factors (De Kock et al. 2011).

In a number of studies the potential of decellularized liver scaffolds derived from rodents and humans was explored (Uygun et al. 2010; De Kock et al. 2011; Mazza et al. 2015; Garreta et al. 2017). The decellularized matrix provides a liver-specific microenvironment, which can be used for repopulation with stem cells and promotion of hepatic differentiation (Navarro-Tableros et al. 2015). Repopulation of these matrices with human fetal hepatocytes and endothelial cells could enable their differentiation into hepatoblasts and biliary epithelial cells (Baptista et al. 2011). Also, human hepatic progenitors were engrafted in decellularized rat livers and the metabolic functionality of the generated humanized livers was investigated. After exposure to six drugs, well-known for targeting specific CYP enzymes, these reconstituted livers provided enhanced activities of metabolic enzymes compared to the 2D-culture condition (Vishwakarma et al. 2018). This already suggests that natural

bioengineered livers can support the maintenance of cell functionality, drug delivery and drug responses. Therefore, they represent valuable tools in drug testing platforms.

3D-spheroids

Scaffold-free 3D-methodology consists of medium in which cells are suspended and spontaneously aggregate into multicellular structures, known as spheroids. Culturing hepatocytes in 3D-spheroids is becoming popular since the cells maintain tight cell–cell junctions which enhance liver-specific functions. In culture, aggregation of single cells into large hepatospheres occurs either by spontaneous cell aggregation or by agitation induced in bioreactors. Also hanging-drop methods are used (Freyer et al. 2016). Cells in 3D-spheroids survive longer by producing their own ECM. They may reach sizes up to 200–300 µm. The presence of tight cellular contacts positively modifies cell complexity and physiological status. However, such compactness also has disadvantages. It hampers the transfer of oxygen, nutrients and metabolites among cells within the aggregates, leading to limited survival times (Gómez-Lechón et al. 2014). Hepatic differentiation of hESCs, hiPSCs and hMSCs in spheroid culture formats, significantly improve cell morphology and hepatic functionality (Subramanian et al. 2014; Talaei-Khozani et al. 2015). Drug toxicity testing has also been performed using 3D-spheroid cultures. 3D-aggregates of hESCs-derived HLCs exhibited enhanced enzymatic induction by omeprazole and rifampicin and were capable of the entire metabolization of these drugs. Phase II reactions, e.g. glucuronidation of acetaminophen were not displayed, possibly due to the incomplete maturation of the cells (Sengupta et al. 2014). On the other hand, Takayama et al. (2013), demonstrated an efficient hepatic differentiation method in which HLCs acquired substantial hepatic competences and were able to predict hepatotoxicity. In this study, 3D-culture spheroids were generated from hESCs and hiPSCs using nanopillar plates that consisted of microscale pillars with a pillar diameter of 160–1000 nm and a height of 280–1000 nm arrayed at the bottom of each well. The seeded cells spread uniformly at the bottom of the well and aggregated to spheroids that could be cultured for more than 20 days. hiPSC-derived HLCs, cultured in spheroids, were more susceptible to hepatotoxic drugs than HepG2-derived spheroids. However, both CYP induction and drug metabolism activity were still lower than those found in PHH cultured in monolayers. This seems to suggest that hiPSC-derived HLCs spheroids still displayed a low sensitivity. In addition, 3D-spheroids have also been combined with specific hydrogel-based materials to support the formation of HLCs in a 3D-configuration. Tasnim et al., report the development of a 3D-cellulosic scaffold containing conjugated galactose ligands. These promote the formation of spheroids in a macroporous network, increase cell–cell contacts and maintain a constant spheroid size. In this particular study, hESCs and hiPSCs were first differentiated towards HLCs in monolayer cultures for 20 days and subsequently seeded into cellulosic scaffolds for 12 days during which 3D-spheroids were formed. The obtained spheroids showed an increased urea production and albumin expression. When exposed to hepatotoxicants, including acetaminophen, troglitazone and methotrexate, these cells showed drug responses comparable to those of PHH. A slightly higher or lower sensitivity to acetaminophen and troglitazone, respectively, was observed (Tasnim et al. 2016). A recent study demonstrated that hepatospheroids differentiated from UCMSCs on gelatin-vinyl-acetate-copolymer 3D-scaffolds acquired enhanced CYP activities compared to 2D cultures. In addition, when exposed to ethanol and acetaminophen, these hepatospheroids exhibited comparable effects to HepG2 and PHH exposed to the same compounds, demonstrating their relevance in metabolism and drug toxicity studies (Chitrangi et al. 2017).

Organoids

Organoids consist of cells that spontaneously form structures that can expand in 3D. Organoid cultures display an indefinite clonal expansion and a differentiation capacity for prolonged periods (Hindley et al. 2016). All stem cell types, including hESCs, hiPSCs and hMSCs, were shown to be able to form organoids together with epithelial cells (Kretzschmar and Clevers 2016). These organoids, composed of multiple cell types, undergo multilineage differentiation commitment, mimicking in vivo embryogenesis, giving rise to a heterogeneous cell population. Heterotypic cell–cell interactions and paracrine signals govern the specification of liver progenitors and recreate stem cell niches that trigger the hepatic commitment. It has been documented that hiPSCs cultured with surrounding endothelial cells and hMSCs without direct contact between the different cell types, were able to differentiate into liver organoids that secreted albumin and expressed a hepatocyte-enriched gene profile and canalicular proteins (Asai et al. 2017).

Human liver stem cell-based organoids can be generated from single donors, holding great potential in the development of preclinical pharmacological platforms (Nantasanti et al. 2016; Shinozawa et al. 2016; Nie et al. 2018). A proof-of-concept study demonstrated that in vitro hepatic organoids derived from hiPSCs and treated with acetaminophen, exhibited comparable responses to those observed in exposed PHH (Sgodda et al. 2017).

Furthermore, the unlimited proliferation capacity of liver-derived organoids is compatible with a stable chromosomal integrity for months in culture, allowing scaling up

production without compromising the quality of the organoids (Huch et al. 2015). Several efforts have been made to improve in vitro efficiency of hepatic differentiation of HLCs-derived organoids. Scalability and reproducibility of hiPSCs-derived organoid platform could be improved using microwell arrays (Takebe et al. 2017).

Liver organoids from different cell sources already demonstrated to be suitable for drug screening and for the development of new therapies for liver diseases and cancer. Biopsy specimens can be isolated from patients and may be used for drug testing, disease modeling and prognosis. Organoid technology opens up the opportunity for the development of patient-derived mini-livers which can be further applied for personalised medicine or for the assessment of individual-specific toxicity (Nantasanti et al. 2016).

Scaffold-based setups

Nanofiber scaffolds

Electrospinning is a technique capable of producing nanofiber scaffolds using high voltage tension (Dhandayuthapani et al. 2011). Fibers, ranging from 1 to 1000 nm, are obtained by processing of polymeric liquids. During the process, nanofibers are formed by the creation and elongation of an electrified fluid jet. The path of the jet, constrained by an orifice, involves the formation of a series of small electrically driven bending coils that progressively expand and increase their sizes, finally solidifying the polymers into continuous thin fibers (Lu et al. 2013). A common electrospinning apparatus consists of a collector electrode, a high voltage supply, a syringe containing the polymer solution in liquid form and a syringe nozzle. When a high voltage is applied, the jet emerging from the solution is constrained from the needle to the collector. The collector allows the formation and elongation of randomly oriented nanofibers (Nair et al. 2004) (Fig. 3). The resulting fiber scaffold is a planar substrate with fibrillary structures. Depending on the cell size, densely condensed structures, characterized by a small inter-fiber distance could prevent cell infiltration. This inconvenience may be solved by modifying either the concentration or ejection rate of the polymer solution and the distance between the needle and the collector, such that a thicker or thinner fiber diameter could be achieved (Loh and Choong 2013). The core of the fibers can also incorporate active molecules, which can be released in a regulated manner, emulating native gradients of growth factors (Buzgo et al. 2017; Rampichová et al. 2014). The fibers provide an optimal structural environment for cell attachment and guidance cues to modulate cell behaviour (Lu et al. 2013). It has been reported that nanofiber scaffolds maintain the multilineage differentiation capacity of hMSCs and cells differentiated in these scaffolds express an increased hepatic functionality (Li et al. 2005; Hashemi et al. 2009). However, a disadvantage of fiber scaffolds is the use of toxic organic solvents during fabrication. To overcome this impediment, cross-linking strategies of pure natural polymers such as collagen, laminin and hyaluronic acid are used to coat the fibers of the scaffolds (Leino et al. 2018). Synthetic polymers coated or blended with natural polymers enhance surface biocompatibility and result in biomimetic scaffolds that can induce efficient hepatic differentiation of hMSCs (Kazemnejad et al. 2009; Ghaedi et al. 2012). Bishi et al., reported the generation of hepatic spheroid-like aggregates from hMSCs on highly porous nanofiber scaffolds, composed of a mixture of synthetic polymers such as poly-lactic acid and polycaprolactone blended with collagen. These 3D-hepatospheres displayed enhanced expression of hepatic-specific markers and albumin secretion in comparison to non-blend nanofibers, collagen alone, or a 2D-condition (Bishi et al. 2013).

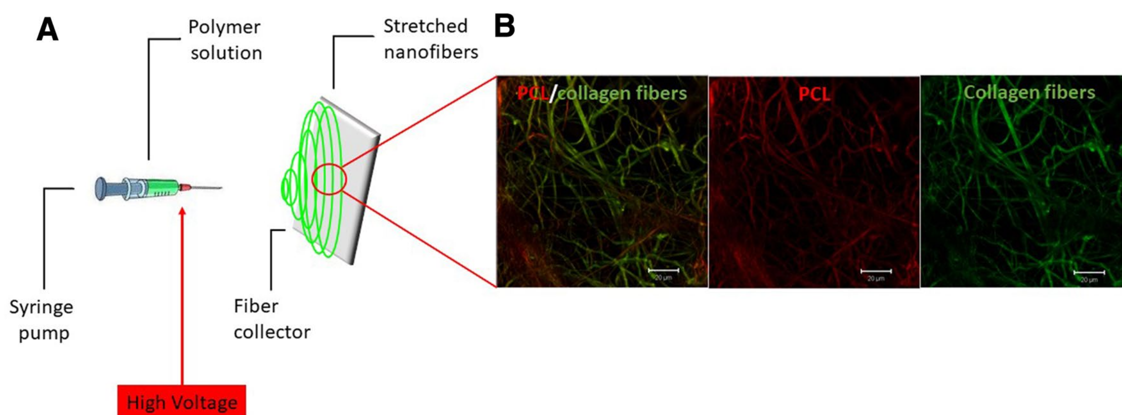

Fig. 3 **a** Schematic representation of an electrospinning setup for the production of nanofiber scaffolds. **b** Fluorescent-labelled PCL and collagen blending for the functionalization of nanofiber scaffolds. *PCL* polycaprolactone

Although efficient hepatic differentiation of stem cells has been well documented in nanofiber scaffolds, drug toxicity testing using these structures has not yet been reported.

Hydrogel-based systems

Hydrogels consist of crosslinked hydrophilic polymers with high water content that form a 3D-matrix. Natural hydrogels encompass polysaccharides and proteins that share similar protein patterns with the human ECM, thereby mimicking the natural cellular microenvironment. Once extracted from green plants, algae and animals, hydrogels can be slightly processed to enhance their endurance and compatibility for cell culture (Caliari and Burdick 2016). In contrast, synthetic hydrogels, including polyethylene glycol, polyvinyl alcohol and polyacrylate, only to mention a few, exhibit a superior mechanical strength over natural hydrogels (Upadhyay 2017). Both natural and synthetic hydrogels have physio-mechanical properties that are highly tunable, allowing the synthesis of encapsulating biomaterials and the control of matrix stiffness (Tsou et al. 2016).

Integration of cells into hydrogel networks can be best achieved via encapsulation or entrapment of living cells. Once formed, the microspheres encapsulate cells thereby forming a barrier which is permeable to oxygen, soluble molecules, metabolites and cellular waste. When high cell densities are encapsulated, cells distributed at the border will receive considerably higher nutrient amounts than cells residing internally, forming distance-dependent oxygen and nutrient gradients (Nicodemus and Bryant 2008; Gasperini et al. 2014). These spatial gradients play an important role in the regulation of the stem cell fate and functions (Jeon et al. 2013). To improve the cell expansion rate and viability, stem cells can also be encapsulated within core–shell hydrogel microfibers. Hereby, they proliferate uniformly along the fibers and retain long-term pluripotency in culture (Ikeda et al. 2017).

The maintenance of encapsulated cells within the hydrogel can be further enhanced by functionalizing the hydrogels with active peptides such as cadherin, collagen and hyaluronic acid (Parmar et al. 2015; Zhu et al. 2016). In addition, conjugation with peptides induces reorganization of the cytoskeleton resulting in stiffness changes. The latter plays a pivotal role in the determination of efficient cell attachment, cell viability, differentiation and maintenance of the cell-specific phenotype. Depending on the polymer concentration and chemical crosslinking density, hydrogel stiffness can range from very soft (<0.1 kPa) to very rigid surfaces (approximately 500 kPa) (Tsou et al. 2016). In the liver, the physiological stiffness reaches 1.5–8.5 kPa (Mueller and Sandrin 2010; Koch et al. 2011).

When PHH were seeded on hyaluronic acid hydrogels, cell survival and the organization of the cytoskeleton improved along with stiffness up to 4.6 kPa. In contrast, the maintenance of gene expression and albumin production was found to be optimal at stiffness values equal to about 1.2 kPa, which is at the limit of the physiological stiffness degree (Deegan et al. 2015). It has been observed that high matrix stiffness results in the maintenance of stem cells at the embryonic state, hampering their progression towards the hepatic phenotype (Cozzolino et al. 2016).

Several hydrogel-based systems have been developed to provide a liver-like environment that can promote the hepatic differentiation of hESCs and hMSCs. Adaptations in the hydrogel chemistry, stiffness and topography resulted in the augmentation of functional hepatic characteristics including enhanced enzymatic activities and drug metabolism (Maguire et al. 2005; Azandeh et al. 2016; Fan and Wang 2017; Lee et al. 2017; Wang et al. 2018). Hydrogels are also the most commonly used bioink materials in 3D-bioprinting. Digital bioprinting processes consist of selective deposition of different living cell types with well-defined spatial patterns (Leberfinger et al. 2017). Ma et al. (2016), were able to assemble hiPSCs-HLCs with surrounding cells from endothelial and mesenchymal origin to form proper hexagonal lobule units via bioprinting. This triculture hydrogel-based model showed advanced morphological features together with increased metabolic secretion and CYP activity as well as enhanced gene expression of hepatic markers. The metabolic activity of stem cell-derived HLCs was shown to be successfully maintained during and after the bioprinting process (Faulkner-Jones et al. 2015). Despite its great potential, the reproducibility of the generation of liver constructs by bioprinting and the preservation of their cellular functions still need to be refined. Indeed, when ejected through nozzle orifices and capillary tubes during the bioprinting process, cells experience mechanical stress that is likely to lead to cell death.

Nanoscaffolds by two-photon polymerization (2PP)

2PP is an advanced technology that allows high precision fabrication of nanoscale structures (Maruo and Fourkas 2008). A femto-second laser beam is focused onto a photosensitive liquid material and a photoinitiator, which absorbs the laser light through a two-photon absorption process, inducing the polymerization of a photopolymer. By moving the laser focus within this photosensitive material, well-defined 3D-structures in any desired geometry can be generated with sub-micrometer precision (Fig. 4a). This technique permits to accurately and specifically control architectural parameters such as pore size, shape, porosity and permeability.

Gelatin derivatives such as methacrylamide-gelatin (Gel-MOD), which belongs to the hydrogel category, can be used for manufacturing 3D-scaffolds by 2PP. Gel-MOD 3D- scaffolds provided cell anchorage and supported proliferation of adipose stem cells for a few days (Ovsianikov et al.

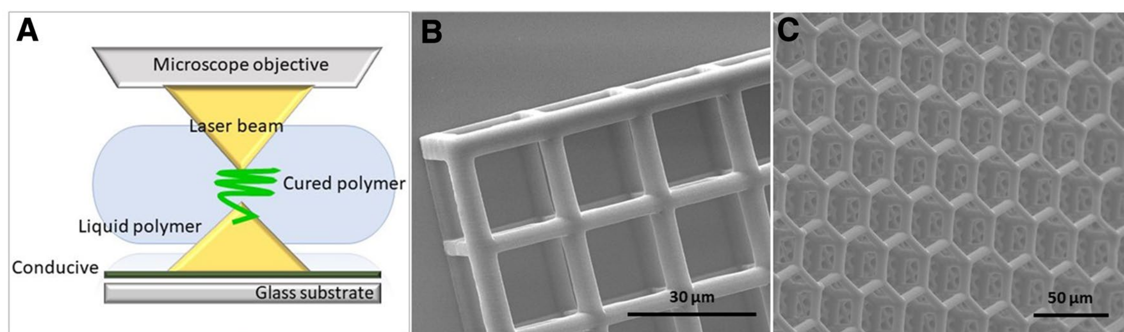

Fig. 4 **a** Schematic representation of 2PP technology used to induce high-precision polymerization of polymers for the fabrication of 3D-structures. **b** Scanning electron microscopy images of a cuboidal and **c** hexagonal 3D-nanoprinted scaffold depicted in 45° orientation. *2PP* two-photon polymerization

2011). Recently, Van Hoorick et al. (2017, 2018) reported two novel gelatin derivatives, namely gelatin-methacrylamide-aminoethylmethacrylate (Gel-MOD-AEMA) and gel-norbornene (Gel-NB), which combine superior 2PP processability and improved cellular interactivity. Besides derivatives of natural polymers, synthetic polymers can also be employed for the fabrication of microstructures due to their mechanical properties (Selimis et al. 2015).

Combination of organic and inorganic materials, such as zirconium–silicon blending is also used and was found to be appropriate for the growth of hMSCs and the development of cell networks (Koroleva et al. 2015). The inorganic component provides mechanical strength, while the organic element ensures bonding between the inorganic components and the cells. Also photosensitive hybrid materials such as ORMOCOMP® and its derived formulations (ORMOCER®, ORMOCLEAR® etc.), are gaining importance for in vitro cell culture applications (Schlie et al. 2007; Teplicky et al. 2016). Using ORMOCOMP®, a 3D-nanostructure of about $700 \times 700\ \mu m^2$ total dimension, with intercommunicating baskets of cuboidal or hexagonal shape, could be fabricated in our laboratory to mimic the 3D-backbone of the human liver (Fig. 4b).

Tailored microgeometries that mimic the native stem cell niche can be fabricated by 2PP technology and stem cell fate can be further controlled by applying biomimetic coatings (Nava et al. 2015). Although no reports have been documented yet on hepatic differentiation of stem cells using 2PP-nanoscaffolds, we believe that this technology represents a powerful engineering tool to recreate artificial stem cell niches to further control stem cell growth and differentiation.

Microfluidics

In vivo, cells are continuously exposed to the vascular network of the bloodstream, ensuring effective delivery of nutrients and activation of signalling pathways that regulate cell function. The liver holds approximately 15% of the total blood volume and, of this, only 40% is held in large vessels such as arteries and veins while 60% circulates in the sinusoids (Eipel et al. 2010). In healthy conditions, hepatocytes lining the sinusoids, experience fluid shear stress in the range of 0.1–0.5 dyn/cm^2 which is lower than the shear stress observed in larger blood vessels (Rashidi et al. 2016). In postnatal livers, hepatic stem cell progenitors are located near the Canals of Hering where hemodynamic changes of the blood flow can trigger these cells to re-enter the cell cycle and to restore the hepatocyte population (Lanzoni et al. 2016). The expression of a class of immediate early genes (IEG), including early growth response (EGR) 1, plasminogen activator inhibitor (PAI) 1, phosphatase of regenerating liver (PRL) 1 and proto-oncogene c-fos (CFOS) is triggered in response to fluid mechanical stress and is believed to be involved in the activation of proliferative-related genes to induce liver regeneration (Sato et al. 1999; Nakatsuka et al. 2006; Nishii et al. 2018).

Perfusion systems can be used to mimic the blood flow in the sinusoids, enabling transport of oxygen, nutrients and removal of waste products and as such improve hepatic differentiation in vitro (Fig. 5). Lab-on-chip devices are used as a novel strategy for the differentiation of HLCs. Microfluidic-based in vitro systems offer several advantages over conventional culture models. First, the establishment of a well-controlled gradient ensures constant addition and removal of nutrients for long-term culture. Second, microscale dimensions of the chip simulate structures of the human body and better represent the in vivo physiology. Third, the device offers a miniaturization of the system with minimal consumption of reagents and compounds, resulting in high cost-effectiveness (Gupta et al. 2016).

Several polymers such as polydimethylsiloxane, polycarbonate, polystyrene, poly-methyl methacrylate have been employed for the fabrication of lab-on-chip devices (Gupta et al. 2016). Typically, a lab-on-chip includes channels that allow cell distribution, attachment and the passage of fluid trough the grooves.

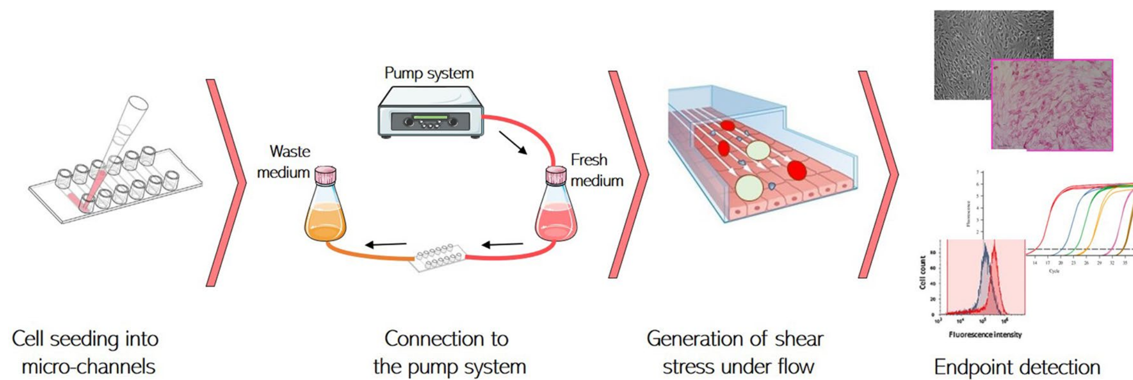

Fig. 5 Graphical representation of a microfluidic setup for the generation of cell culture medium-induced shear stress, mimicking the blood flow in liver sinusoids. Image modified from The ibidi Product and Experiment Guide (https://ibidi.com/)

There are often two ports in the device: (1) an inlet port through which fresh medium is injected to provide the essential growth factors and oxygen and (2) an outlet port which is used to remove the medium containing waste products.

At present, several microfluidic systems have been developed to achieve hepatic differentiation of human stem cells for drug assay applications (Wu et al. 2017). Increased expression of progenitor and adult hepatic markers coupled with a significant increase of hepatic functionality were observed under perfusion conditions (Ju et al. 2008; Miki et al. 2011; Wang et al. 2012; Yen et al. 2016; Ong et al. 2017). The perfusion frequency is a key parameter in the microfluidic setup which can affect the efficiency of hepatic differentiation. Giobbe et al., found that the commitment of hESCs and hiPSCs in the three germ layers and hepatic specification are directly influenced by appropriate perfusion frequencies. The authors argue that, while high perfusion frequencies could wash out endogenous secreted factors, low frequencies hold these factors into the channel to provide nutrients to the cells. When hESCs and hiPSCs were differentiated with endoderm-specific medium at an optimal perfusion frequency for 14 days, they displayed a typical polygonal shape. Furthermore, HLCs cultured under fluidics showed enhanced functional properties such as the capacity of storing glycogen, higher expression of CYP3A and albumin secretion when compared to static conditions. Exposure to acetaminophen showed also that cytotoxicity was more prominent in hESCs-HCLs differentiated in microfluidics versus hESCs-HCLs differentiated under static conditions (Giobbe et al. 2015).

More recently, the same group described the reprogramming of hiPSCs towards HLCs in a micro-scale microfluidic polydimethylsiloxane-based device. In this new setup, up to 32 independent experiments and multiple chips can be run simultaneously, increasing the throughput of the process. Hepatic functionality of mature HLCs was confirmed by high albumin secretion and glycogen intracellular storage (Luni et al. 2016).

The creation of a dynamic environment that provides multiple stimuli for stem cell differentiation paves the way for more complex systems such as an organ-on-a-chip approach (Zhang et al. 2017). This technology aims at recreating micro-tissues that mimic the functions of entire organs. For the liver, these systems attempt to reproduce the flow circulation that from the hepatic artery and portal vein is drained into the sinusoids, enabling as such the diffusion of nutrients to the hepatocytes. Ultimately, the blood flows out of the sinusoids into the central vein and removes de-oxygenated products and waste materials. Recently, Benaeiyan et al., developed a liver-lobule on a chip device, inspired by the convective-diffusive motion of the hepatic blood flow. The device consists of hexagonal chambers with a central inlet mimicking the central vein of the liver lobules and radial channels distributed around the central aperture (Banaeiyan et al. 2017). hiPSCs-derived hepatocytes differentiated in the chip for 21 days displayed considerably higher metabolic activity than the 2D-culture. In addition, the formation of a bile canaliculi network could be observed in the device (Banaeiyan et al. 2017). Overall, cell cultivation effectiveness, system automation and high throughput make the microfluidic systems appealing for further application in drug discovery. For instance, integration of microfluidic platforms with engineered 3D-scaffolds are being progressively explored to provide a more physiological system which can ultimately be applied in drug toxicity testing (Wallin et al. 2012; Gupta et al. 2016). Yet, introducing stem cell-based microfluidic platforms in drug discovery is a lengthy process that still needs to be further developed and optimised.

Conclusion

The use of human stem cell-derived hepatocytes in a drug testing platform remains a challenging task. Improvement of the hepatic functionality is necessary to match the sensitivity

Table 1 Advantages and disadvantages of advanced cell culture systems

Advanced models	Simulating in vivo physiology	Advantages	Disadvantages	References
Decellularized liver matrices	Native ECM 3D-architecture Vasculature	Cell homing Optimal metabolic activity	Inadequate for large-scale analysis	Hoshiba et al. (2016 and Vishwakarma et al. (2018)
3D-spheroids	Tight cell–cell junctions Preservation of 3D-morphology	Co-culture capacity Tight cell junctions	Heterogeneous cell population (cell size, number, shape) Simplified framework Low-throughput	Gómez-Lechón et al. (2014)
Organoids	In vivo-like architectural complexity	Patient specific Free cell spatial arrangement	Poor hepatic differentiation potential Absent vasculature Unsuitable for high-throughput	Nantasanti et al. (2016)
Nanofiber scaffolds	ECM network	Chemical surface modification Bioactive factors delivery	Fabrication involving toxic solvents Densely stacked fibers Low cell infiltration	Lu et al. (2013) and Dahlin et al. (2011)
Hydrogel-based systems	Recreation of biomimetic ECM	Biocompatible Oxygen permeability Gas exchanges Regulation of stiffness High tunability Conjugation with peptides	Degradability Optimization of appropriate mechanical properties for cell culture	Deegan et al. (2015) and Tsou et al. (2016)
Nanoscaffolds by two-photon polymerization	3D-architecture	Miniaturized structures Tailored design Engineering matrices Immobilization of molecules Biocompatibility	Expensive Sensitive detection system required	Ovsianikov et al. (2011) and Teplicky et al. (2016)
Microfluidics	Sinusoidal blood flow Recovery of apicobasal polarity and bile canaliculi	Dynamic control of culture conditions Presence of chemical gradients Suitable for high-throughput Minimal consumption of reagents	Sophisticated equipment needed Sensitive detection system required	Gupta et al. (2016), Giobbe et al. (2015) and Luni et al. (2016)

of PHH. Different technologies, providing a better representation of the physiology of human liver, can now be introduced to improve the hepatic maturity of stem cell-derived HLCs. Hence, advanced hepatic cell culture systems have been explored by mirroring key components of the in vivo microenvironment, including the 3D-architecture, necessary for correct cellular orientation and cell–cell and cell–ECM contacts in addition to the vascular network of the liver (Table 1). Several methodologies have been developed to culture cells in liver-specific microenvironments, including 3D-scaffold based and scaffold free setups and microfluidic perfusion. These methodologies not only improve the hepatic functionality of stem cell-based HLCs, but also reinforce their predictive capacity towards hepatotoxic compounds (Knight and Przyborski 2015; Lin and Khetani 2016).

Of note, stem cell-engineering models have the potential to assess patient-specific toxicity. Patient-derived cells cultured in 3D and exposed to certain drugs, have already demonstrated a significant correlation with clinical observations (Lu et al. 2017). Nevertheless, engineered stem-cell based models require further optimization to be routinely applied in drug toxicity testing. Undoubtedly, the synergy of stem cells and engineering technologies will have a great impact on the reduction of animal use as well as on the improvement of drug development strategies.

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