

We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

5,200

Open access books available

129,000

International authors and editors

150M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



Challenges for Deriving Hepatocyte-Like Cells from Umbilical Cord Mesenchymal Stem Cells for *In Vitro* Toxicology Applications

Ana Sofia Martins Serras,

Madalena Zincke dos Reis Fernandes Cipriano,

Pedro Miguel da Graça Silva and Joana Paiva Gomes Miranda

Abstract

The *in vitro* toxicology field seeks for reliable human relevant hepatic models for predicting xenobiotics metabolism and for the safety assessment of chemicals and developing drugs. The low availability and rapid loss of the phenotype or low bio-transformation activity of primary hepatocytes urged the stem cell differentiation into hepatocyte-like cells (HLCs). Umbilical cord-derived mesenchymal stem cells (UC-MSCs), in particular, offer a highly available cell source, with few ethical issues and higher genetic stability. However, the dynamic and complex microenvironment of liver development, including the cell-ECM and cell-cell interactions, pressure gradients (oxygen and nutrients) and growth factor signaling that are critical for the differentiation and maturation of hepatocytes, challenges the progress of *in vitro* hepatic models. Promising strategies like (i) cytokine and growth factor supplementation mimicking the liver development; (ii) epigenetic modification; and (iii) bioengineering techniques to recreate the liver microphysiological environment are gaining increasing importance for the development of relevant *in vitro* liver models to address the need for higher predictivity and cost efficiency. In this context, this chapter reviews the existing knowledge and recent advances on the approaches for deriving HLCs from UC-MSCs and their application for *in vitro* toxicology.

Keywords: human neonatal mesenchymal stem cells, umbilical cord mesenchymal stem cells, hepatocyte-like cells, hepatic differentiation, liver development, epigenetic modifiers, bioengineering, *in vitro* alternative models, *in vitro* toxicology

1. Introduction

The liver is a complex organ at the anatomical and physiological level, associated with numerous vital functions, including protein and urea synthesis, and regulation of the energy metabolism. It is also the main organ responsible for xenobiotics metabolism, the reason why it is often the first to contact with their metabolic

products and most of the toxins, being one of the main targets of the toxicity caused by those drugs. Indeed, drug-induced liver injury (DILI) is responsible for nearly 60% of the cases of acute liver failure [1]. Despite the increased awareness for DILI, its absolute frequency is not decreasing demonstrating the need for evaluating drugs' hepatotoxicity and for smarter *in vitro* tools to increase predictivity and to represent the patients at a population level within the drug development process [2].

Traditional *in vitro* models for hepatotoxicity studies include monolayer cell cultures (2D) and suspensions of human hepatoma cell lines or primary hepatocytes [3] (**Table 1**). The primary cultures of human hepatocytes present the most representative phenotypic and functional profile, but exhibit a short-term viability, with a quick loss of several cellular functions within the first days in culture [3], including a loss in CYP-dependent monooxygenase activities, significant downregulation of phase I and phase II enzymes, stress-related upregulation of acute-phase-response enzymes and delocalization of transporter proteins. Rat primary hepatocytes, on the other hand, have also the disadvantage of presenting interspecies differences on the biotransformation of xenobiotics [4]. To overcome such limitations, different human hepatoma cell lines have been established. These can provide a high quantity of human cells and are cost-effective. However, those benefits are often surpassed by a number of other limitations, namely their disease-like state, lower metabolizing capacity and incomplete biotransformation profile [5]. As a result, the drawbacks of the currently available models sustain the need for relevant human *in vitro* hepatotoxicity models that better resemble the *in vivo* microphysiology.

Stem cell-based hepatic models represent an important alternative to the conventional hepatic *in vitro* systems. This chapter integrates the state of the art of human umbilical cord matrix (UCM-MSCs) or blood (UCB-MSCs) hepatic differentiation and its role as an *in vitro* alternative model for biotransformation and hepatotoxicity studies.

Model	Advantages	Limitations	Ref.
Isolated hepatocytes	Obtained from whole livers or biopsies Functions close to those of hepatocytes <i>in vivo</i> Enable interspecies and pharmacogenomics studies Representative of different lobular subpopulations	Viability: 2–4 h No bile canaliculus Low availability of human tissue Interspecies differences	[6, 7]
Primary hepatocyte cultures (pHep)	Obtained from whole livers or biopsies Functions close to those of hepatocytes <i>in vivo</i> Longer viability than isolated tissue Induction/inhibition of drug-metabolizing enzymes Enable interspecies and pharmacogenomics studies	Viability: 2–4 days Early phenotypic changes Altered bile canaliculi Difficult recovery of cells and maintenance of function upon cryopreservation Low availability of human tissue Interspecies differences	[3, 7]
Hepatoma cell lines (HepG2, Huh7)	High proliferation activity and good availability Well-characterized and abundant data available	Decreased drug enzyme activities Genotype instability	[3, 5, 8, 9]

Table 1.
Advantages and limitations of traditional in vitro models for hepatotoxicity studies.

1.1 Umbilical cord-derived mesenchymal stem cells as an alternative stem cell source for generating hepatocyte-like cells

Recent developments in stem cell technology have paved the way for identifying novel candidate sources of cells as an attempt to increase the availability of functional human liver-like cells, as well as improving the reliability and the accuracy of drug screening *in vitro* [10, 11]. In fact, stem cells (SCs) are of human origin and possess the ability to self-replicate and differentiate into all cell types in the body. Regarding the liver, the differentiation strategies to derive hepatocyte-like cells (HLCs) from stem cells are mostly based on mimicking the development of hepatocytes *in vivo* and include the addition of soluble medium factors, the reconstruction of the cell-matrix and the intercellular interactions through the use of alternative cell culture strategies and the assessment of cell fate via genetic modifications and epigenetic modulation [12]. Yet, the major challenges on producing stem cell-derived HLCs *in vitro* are still the immature phenotype of the HLCs [13], the lack of defined endpoints of hepatic differentiation and maturation [14], the absence of relevant positive controls [15] and defining the best stem cell source.

Several approaches have been developed for deriving HLCs from human embryonic stem cells (hESCs) [7, 16, 17], induced pluripotent stem cells (iPSCs) [7, 18–20], bipotent liver progenitor cells [21] and mesenchymal stem cells (MSCs) [22–42]. hESCs display various advantages for clinical applications when compared to immortalized cell lines and primary cell cultures, since they are genetically normal (diploid) and do not possess the high donor-dependent variability observed in primary cells [43]. The use of these cells raises, however, various ethical, technical and legal concerns [44]. Other types of SCs, like iPSCs and adult stem cells, do not give rise to those ethical issues. Nevertheless, there is some evidence suggesting that iPSC therapy has the risk of leading to tumor formation, raising safety concerns that should be addressed by researchers to ensure the viability of this therapy [44]. iPSCs and hESCs also reveal a high risk for teratoma formation *in vivo* [45], exhibiting high genomic instability, through the accumulation of mutations [46], a concern that is not raised by neonatal MSCs [47]. MSCs, on the other hand, reveal many advantages over the other SCs, which make them suitable for toxicological and regenerative medicine applications. They can be isolated from non-controversial sources at a relatively low cost, do not require feeder layers and high serum conditions, reveal a satisfactory proliferative capacity *in vitro* and are less immunogenic [23, 26, 34, 48–51]. Interestingly enough, iPSC-derived MSCs have also been reported as less immunogenic [52].

MSC classification is still controversial, being commonly defined as adult, fetal or neonatal MSCs depending on its origin. Independently of their origin, MSCs are characterized according to the International Society for Cellular Therapy (ISCT) criteria [53]. The position paper published by the ISCT states that the isolated cells must display: (a) plastic adherence when maintained in standard two-dimensional (2D) culture; (b) specific surface protein expression, typically confirmed by flow cytometry where a minimum of 95% of the cell population must portray the expression of surface markers CD105, CD73 and CD90, whereas the markers CD45, CD34, CD14 or CD11b, CD79 α or CD19 and HLA-DR should show less than 2% expression among the isolated cells; and (c) tri-lineage differentiation capacity of the isolated cells, that is, these cells must be shown to differentiate to osteoblasts, adipocytes and chondroblasts using standard *in vitro* tissue culture-differentiating conditions [53].

MSCs are pluripotent stem cells that can be obtained from adult tissues like adipose tissue, brain, bone marrow and pancreas [54], but can also be isolated from neonatal tissues, like fetal-blood, amniotic sac and fluid, placenta [55] and

extra-embryonic tissues such as the umbilical cord (UC-MSCs) [56]. The umbilical cord, in particular, represents a source of MSCs that is readily available as it is discarded as a medical waste after birth [57]. Herein, MSCs can be isolated from the blood (UCB-MSCs) or from the matrix (UCM-MSCs) through several isolation processes, namely by enzymatic, explants or mixed enzymatic-explant digestion methods [58, 59] that result in different yields [60–62].

Depending on their origin, MSCs may present variations in morphology, proliferation potential, growth rates and differentiation capacity as well as their regenerative potential. A significant advantage of the MSCs derived from neonatal and extra-embryonic tissues over their adult counterparts is their availability, extraction using non-invasive procedures, higher isolation yields and the absence of ethical concerns [16]. Nevertheless, other advantages have been linked to those cells. Several studies have reported superior cell biological properties such as less variability resulting from the epigenetic marks related to the donor's lifestyle as well as high proliferative capacity, increased lifespan and, importantly, enhanced potency of the UC-MSCs over the other MSCs obtained from adult tissues (**Figure 1**). Indeed, in contrast to BM-MSCs, UC-MSCs maintain a significant expansion potential of 2.5 population doublings per week up to passage 22 (P22) keeping all MSC traits and genomic stability and without reaching senescence [63, 64]. Moreover, along with the adipogenic, chondrogenic and osteogenic lineages, UC-MSCs demonstrated differentiation ability into the mesodermal lineage originating from myoblasts and cardiomyocytes [65]; into the ectodermal lineage leading to neurons [66]; and into the endodermal lineage cells, giving rise to insulin-producing cells [51] and HLCs [22–42]. Besides, UC-MSCs can be obtained from donors with diverse pharmacogenetic profiles allowing for inter-individual pharmacogenomic studies and development of personalized therapies.

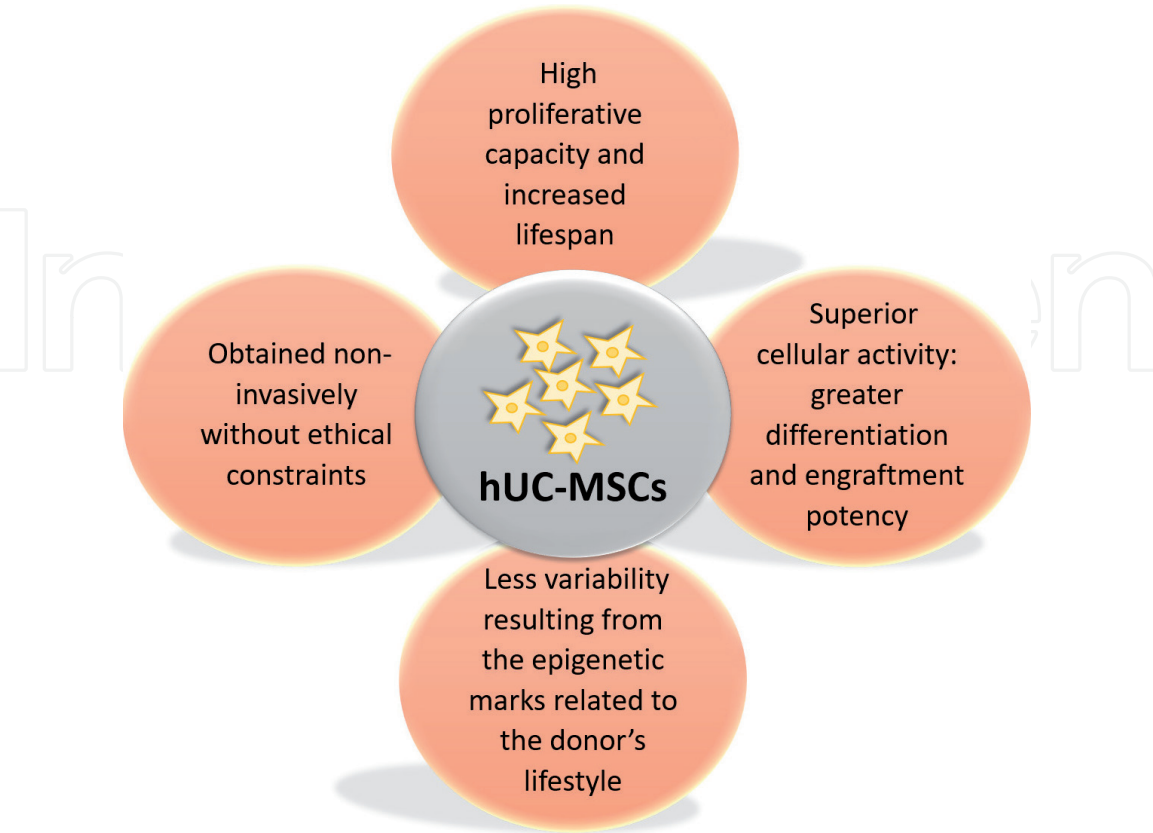


Figure 1.
Main advantages of human umbilical cord MSCs (hUC-MSCs) over other MSC sources.

2. Hepatocyte-like cell differentiation

2.1 Mimicking liver development by cytokines and growth factors supplementation

The underlying mechanisms inducing hepatocyte polarity and functional maturation *in vitro* remain largely elusive. Liver cells *in vivo* reside within a dynamic microenvironment in which biomechanical and biochemical properties of the extracellular matrix (ECM), dynamical cell-ECM and cell-cell interactions, pressure gradients (oxygen and nutrients) and growth factor signaling are critical for the differentiation and maturation of hepatocytes. The relative importance of these various factors changes during liver development and maturation. This makes developing liver models enormously challenging.

Liver development has been studied using animal models, such as mouse [67], chicken [68], zebra fish [69] and *Xenopus* [70]. The knowledge of other species' developmental biology contributed significantly to the progress and set up of protocols, which mimic the *in vivo* liver development, for deriving HLCs from human stem cells *in vitro*. As shown in **Figure 2**, the hepatogenesis process and the subsequent *in vitro* mimicking of liver development include several steps:

- i. Initially, gastrulation and endoderm specification are activated by Nodal, bone morphogenetic protein (BMP) and Wnt signaling. Signaling by Nodal initiates endoderm and mesoderm formation in a concentration-dependent manner, in which high Nodal concentrations originate the definitive endoderm [71]. The endoderm induction step has been tested *in vitro* on ESCs through cell exposure to Activin A, a growth factor from the TGF- β family, which binds the same receptors as Nodal and therefore mimics its activity [72, 73].
- ii. Gradients of fibroblast growth factor (FGF), Wnt, BMP and retinoic acid secreted from the adjacent mesoderm are responsible for patterning of endoderm [70, 74, 75] to generate the midgut, foregut and hindgut. Each domain

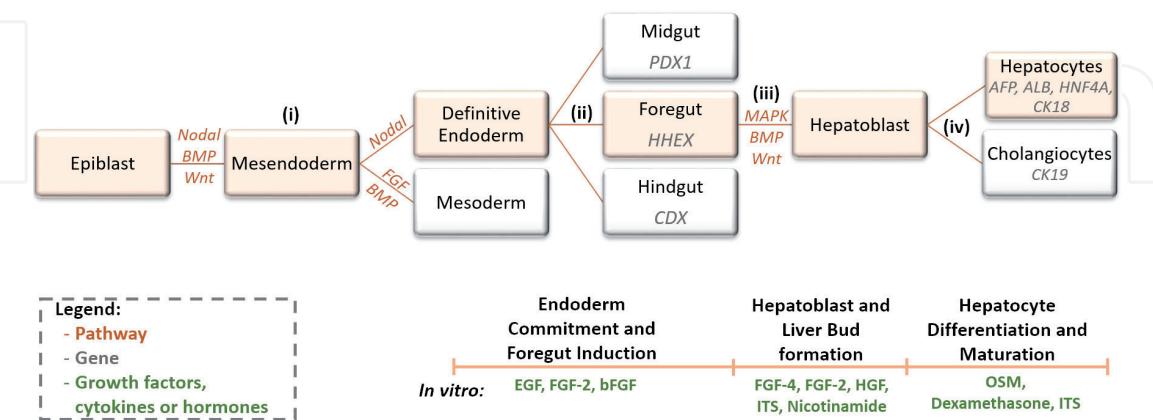


Figure 2. Hepatogenesis and the respective inducing factors for *in vitro* differentiation of MSCs into HLCs. The addition of a combination of soluble factors to the culture, at defined time points, to mimic (i) endoderm commitment and (ii) foregut induction (EGF and FGF), followed by (iii) hepatoblast and liver bud formation (FGF, HGF and ITS) and finally (iv) hepatocyte differentiation and maturation (OSM, dexamethasone and ITS) have been shown to allow hepatic differentiation to some extent, mimicking the *in vivo* ontogeny. AFP, α -fetoprotein; ALB, albumin; BMP, bone morphogenetic protein; CK, cytokeratin; EGF, epidermal growth factor; FGF, fibroblast growth factor; bFGF, basic fibroblast growth factor; HGF, hepatocyte growth factor; HNF, hepatocyte nuclear factor; ITS, insulin-transferrin-selenium; MAPK, mitogen-activated protein kinase; OSM, oncostatin M.

expresses a specific transcription factor: HHEX in the foregut, PDX1 in the midgut and CDX in the hindgut. Activation of the *HHEX* gene expression is essential to foregut formation and therefore its development into the liver. *In vitro*, this step is mimicked by exposing cells to growth factors such as FGF and EGF, once these activate the *HHEX* gene [74, 76].

- iii. After endoderm commitment and foregut induction, the foregut receives signals from the developing heart and septum transverse mesenchyme (STM), which release FGF and BMP respectively, and regulate hepatic specification to generate hepatoblasts [71, 77]. After hepatic specification, cells start expressing hepatic markers, such as α -fetoprotein (AFP), albumin (ALB), transcription factors as CEBPA and HNF4A, and change their morphology from cuboidal to pseudostratified columnar epithelium, forming the liver bud [78]. STM and hepatic mesenchyme secrete FGF, BMP, Wnt, retinoic acid and hepatocyte growth factor (HGF), which promote hepatoblast proliferation and survival [74, 75, 79, 80]. This step is generally mimicked *in vitro* using FGF to simulate the signals sent by the developing heart and STM, which induce a transformation in cell disposal and morphology [77, 79], mediating early hepatic differentiation [21]. Herein, HGF stimulates a mid-late hepatic phenotype and is commonly used to promote hepatoblast formation; however, it does not induce functional maturation [21]. FGF and HGF, as well as cell culture supplements like insulin-transferrin-sodium selenite (ITS) and nicotinamide, synergistically affect the hepatic driving pathway [34, 80].
- iv. Finally, hepatoblasts, which are bipotent cells, can differentiate into hepatocytes or biliary epithelial cells. Initially, hepatoblasts express genes associated with both adult hepatocytes (*HNF4A*, *ALB*, *CK18*) and biliary epithelial cells (*CK19*), as well as fetal liver genes such as *AFP* [71]. Additionally, these cells express *CK-14*, *DLK1*, *E-cadherin*, *EPCAM* and *CD133* and undergo proliferation and differentiation into hepatocytes and cholangiocytes [71]. One factor responsible for the induction of hepatoblast differentiation into hepatocytes and induction of metabolic maturation is oncostatin M (OSM), secreted by hematopoietic cells in the liver [81]. Indeed, several *in vitro* models for hepatocyte differentiation use dexamethasone, HGF [22, 40, 41], OSM [25, 34, 40] and TNF- α factors [21, 71] to induce hepatocyte maturation. Moreover, the use of a collagen coating [21–23, 34] improves the *in vitro* environment to promote hepatogenic differentiation by mimicking *in vivo* ontogeny.

The protocols, to differentiate MSC into functional hepatocytes, based on the *in vivo* liver development process can be categorized into two groups: cocktail and sequential. The cocktail methodology is based on one single step, whereas the sequential and time-dependent procedures are based on four, three, or two steps. Campard et al. [22] study was one of the first described studies using a three-step-based protocol for deriving UCM-MSCs into HLCs. Herein, UCM-MSCs (also designated as Wharton's jelly cells) have been isolated by an orthodox method, involving complex vein and arterial excisions, and the authors departed from a mixed, heterogeneous population of cells. Nevertheless, after the differentiation procedure, HLC derived from UCM-MSCs exhibited a hepatocyte-like morphology, the presence of several hepatic markers (CK18, ALB, AFP and connexin 32), had glycogen storage ability, produced urea and revealed an inducible CYP3A4 activity. Still, the absence of some hepatic markers in the differentiated UC-MSCs, such as HepPar1 or HNF4A, suggested that a fully mature hepatocyte phenotype

was not achieved. In another study, Zhao et al. [37], after hepatic differentiation of UC-MSC, prompted by a two-step protocol with HGF and bFGF, HLCs exhibited hepatocyte-like morphology and specific functions including albumin secretion, low-density lipoprotein uptake and urea production. In contrast, Zhang and colleagues [36], using a simpler cocktail induction protocol (with HGF and FGF-4), successfully differentiated UC-MSCs into HLCs with the same hepatic features.

Overall, those studies indicate that UC-MSCs are capable of generating hepatocyte-like cells with essential hepatic specific functions displaying an exciting potential venue toward cell-based therapeutics, human liver development studies and disease models for liver failure disorders. Yet, the weak characterization of the cells in terms of biotransformation ability has delayed their implementation for *in vitro* hepatotoxicity studies.

2.2 Epigenetic modifiers for improving HLC phenotype

Stepwise addition of factors such as EGF, FGF, HGF, nicotinamide, ITS, dexamethasone or OSM to the culture medium is used in the majority of the studies to differentiate MSCs [23, 26, 34, 35, 41]. Although the addition of these factors seems to lead to hepatic differentiation, a full mature hepatic cell has not yet been achieved. As such, the search for additional differentiation-inducing factors to induce a mature hepatic phenotype persists [34].

The normal function of cells is controlled by epigenetics, in which a combination of signaling pathways controls the balance between growth and differentiation. Therefore, besides mimicking the *in vivo* extracellular communication pathways by the use of soluble molecules, such as growth factors, cytokines, hormones and glucocorticoids, one of the strategies for controlling lineage-specific gene expression to induce a mature hepatic phenotype is by the use of chromatin remodeling agents, such as epigenetic modifiers (EM) as HDAC inhibitors (HDACi), DNMT inhibitors (DNMTi) and microRNA (miRNA).

Epigenetic modifiers change gene expression without changing the underlying DNA sequence, at the chromatin level, by modulation of its architecture between heterochromatin (transcriptionally inactive) and euchromatin (transcriptionally active) [82]. Epigenetic modulation allows to silence pluripotency transcription factors or to activate the transcription of genes of a specific lineage contributing to the improvement of the HLC phenotype [34]. These mechanisms are mainly regulated by DNA methylation, histone modifications and miRNA [82] as presented in **Figure 3**. Indeed, different strategies for hepatic differentiation based on epigenetic modification have been described so far and those include DNA methylation, histone modification and the use of microRNAs.

2.2.1 DNA methylation

DNA methyltransferases (DNMTs) introduce a methyl group, generally at CpG islands, into the DNA [82]. Decrease of DNMT1 [83] and increase of DNMT3 [84] expression have been shown to be associated with hepatic maturation. Hence, modulation of DNMTs may present a strategy for increasing liver-specific gene expression and consequently maintain a hepatic fate in HLCs [12]. 5-Azacytidine (5-AZA) is the most commonly used DNA methyltransferase inhibitor (DNMTi), whereas dimethyl sulfoxide (DMSO) emerges as a modulator of DNMTs [85].

The alteration of the DNA methylation status occurs as 5-AZA mimics the cytidine base and thus is introduced into the newly synthesized DNA strand on the S phase of the cell cycle [86]. On the subject of hepatocyte differentiation, Rothrock and colleagues [87] administered 5-AZA *in utero* to 20 days gestational age rat fetus resulting in a

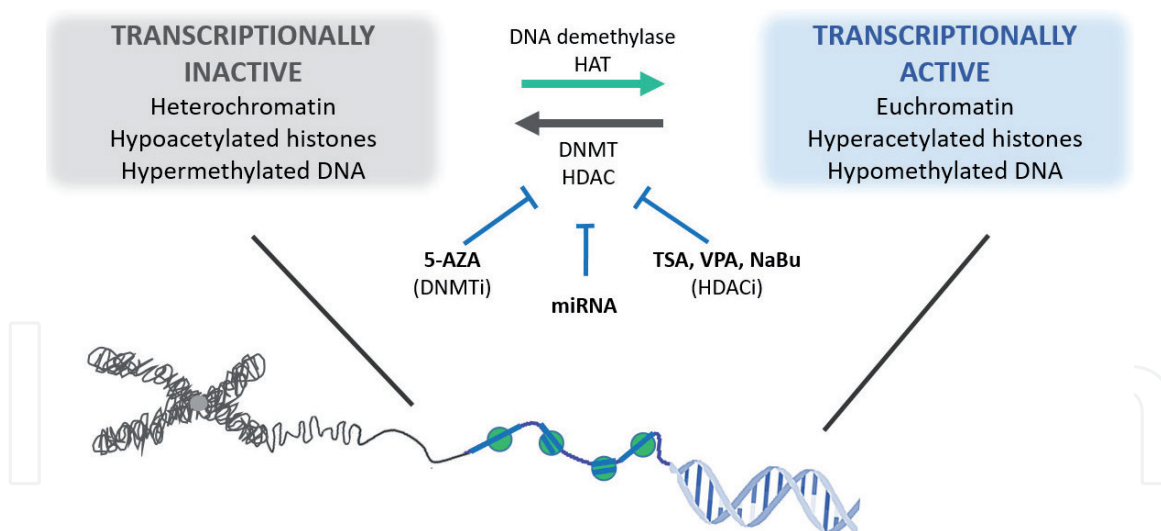


Figure 3.

Control of gene transcription by epigenetic modifiers. Hypomethylated CpG islands and hyperacetylated histone tails at the chromatin level allow gene transcription. DNMT inhibitors (DNMTi), microRNA (miRNA) and HDAC inhibitors (HDACi) modulate the chromatin structure by creating an open, transcriptionally active euchromatin. Consequently, the enhanced accessibility of transcription complexes to chromatin leads to increased transcriptional activation of several epigenetically suppressed genes. 5-AZA, 5-azacytidine; DNMT, DNA methyltransferase; HAT, histone acetyltransferase; HDAC, histone deacetylase; NaBu, sodium butyrate; TSA, trichostatin A; VPA, valproic acid.

quicker maturation of hepatocyte morphology, associated with higher activation of genes normally expressed later in liver development as *TAT* and *ALB*. *In vitro*, Yoshida et al. [25] evaluated various combinations of inducing factors, plus the addition or absence of 5-AZA, and verified that the 5-AZA combined with soluble factors was the most effective strategy for differentiating UCB-MSC that displayed a significantly higher *ALB*, *CEBPA* and *CYP1A1/2* gene expression levels, PAS positive results and urea production after 21 days of differentiation. In another study, Cipriano et al. [23] used 5-AZA as a promoter of differentiation, in the hepatoblast-like stage of differentiation, and observed a significant increase in urea production and CYP activity on HLCs.

DMSO has also been proposed as an epigenetic modifier. Although the mechanism by which DMSO induces hepatic differentiation is poorly understood, Iwatani et al. [85] suggested that it is by upregulation of the expression of DNMT3. This study was performed in mouse embryoid bodies. Yoon et al. [88], on the other hand, used trichostatin A (TSA) or DMSO treatment for the maturation steps within the hepatic differentiation procedure. Herein, TSA-treated MSCs showed higher EROD activity (human CYP450 1A1/1A2) and ammonia conversion than DMSO-treated cells. Conversely, Cipriano et al. [34] showed that, in addition to the sequential differentiation protocol, DMSO alone (in comparison to its combination with TSA) induced cellular modifications on UCM-MSCs, forming epithelial-like binucleated cells, and stimulated a homogeneous glycogen storage and improved HLC biotransformation activity. The introduction of DMSO for hepatic maturation also resulted in a significantly improved HLC phenotype and maintenance of the hepatocyte features up to 2 weeks in culture [34]. Hence, more studies are necessary in order to determine the effectiveness of DMSO on differentiation of MSCs.

2.2.2 Histone modifications

Histone deacetylases (HDACs) remove the acetyl group from the histones and modulate chromatin to regulate cell proliferation, differentiation and growth [86]. The most commonly used histone deacetylase inhibitors (HDACi) are TSA, sodium butyrate (NaBu) and valproic acid (VPA).

Similar to other HDACi, TSA reversibly and specifically inhibits HDAC leading to hyperacetylation of histones, but the exact role in hepatic differentiation and maturation is still unclear [88]. Exposure to TSA on primary rat hepatocytes culture revealed increased cell viability and albumin secretion and maintained CYP phase I enzymes' capacity by controlling the expression of liver-enriched transcription factors (LETfs) and cell cycle arrest [89]. Yoon et al. [88] found that TSA-treated MSC presented higher activity than OSM- or DMSO-treated cells, showing an epithelial-like shape right after treatment and higher urea production and ammonia removal (compared to DMSO) on day 21, as well as the expression of late hepatic markers such as *TAT* gene expression and EROD activity. Although the expression of these markers and functions indicate a mature HLC phenotype, these values are still lower than in human primary hepatocytes. Likewise, Cipriano et al. [34] tested the effect of 100, 500 and 1000 nM of TSA on UCM-MSC differentiation and observed that 1000 nM of TSA resulted in cell detachment and cell loss, whereas 100 nM did not present relevant morphological changes from a fibroblastic morphology to a more epithelial morphology during the differentiation process. Conversely, 500 nM of TSA resulted in higher EROD and UGT activities, as well as CK18 presence and epithelial morphology [34], though, as referred in the above section, TSA-treated cells could not surpass the results with DMSO-treated cells [34].

Several protocols for hepatic differentiation of SCs prime the cells with NaBu [90], in combination to Activin A [73] or bFGF and BMP4 [91], in order to induce the definitive endoderm prior to further maturation of HLC through, for instance, DMSO [92]. However, the translation of these studies to UC-MSC is still limited. To the best of our knowledge, only Panta et al. [26] showed that pre-treatment of UCM-MSCs with NaBu upregulated hepatoblast and hepatocyte markers and stimulated mature hepatic-associated functions, such as urea production, glycogen storage and G6P, CEBPA, and CYP2B6 activity, compared to non-treated differentiated cells.

Finally, VPA, an antiepileptic and anticonvulsant drug, has demonstrated to improve stem cell hepatic differentiation when administered in low doses. An et al. [27] suggested that hepatic differentiation of UC-MSC is stimulated by VPA due to upregulation of endodermal genes such as *AKT* and *ERK*. Raut and Khana [28] also verified that pre-treatment of UCM-MSC with VPA enhanced the expression of hepatocyte-specific miRNAs typically upregulated during fetal liver development, such as miR-23b cluster (miR-27b-3p, miR-24-1-5p and miR-23b-3p), miR-30a-5p, miR-26a-5p, miR-148a-3p, miR-192-5p and miR-122-5p, which contributed to a more efficient hepatic transdifferentiation.

2.2.3 microRNA (miRNA)

MicroRNAs are critical regulators during the development of liver [93]. In humans, miR-122 is the most abundant miRNA expressed in the adult liver and is known to regulate hepatocyte differentiation [28, 93].

Zhou et al. [50] validated that, besides miR-122, also miR-148a, miR-424, miR-542-5p and miR-1246 are essential for UC-MSC differentiation, given that omitting any of these five-miRNA combination prevented hepatic transdifferentiation. In addition, it was also demonstrated that HLCs transdifferentiated from those five microRNAs expressed high level of hepatic markers in only 7 days. Moreover, Khosravi et al. [94] studied the role of embryonic overexpressed miRNAs such as miR-106a, miR-574-3p and miR-451 and determined that upregulation of any of these three alone could not induce expression of hepatic genes, such as *SOX17*, *FOXA2*, *HNF4A*, *ALB*, *AFP* and *CK18*. However, the concurrent ectopic overexpression of the three miRNAs together could induce UC-MSC differentiation into functionally mature hepatocytes in an easier, faster and efficient way compared to conventional techniques [94].

In summary, these results suggest that miRNAs have a role in hepatic differentiation and can rapidly and efficiently convert stem cells into functional HLC.

2.3 Bioengineering tools for hepatic differentiation

Hepatocytes need to be exposed to the native physiology of the liver and to have cell-cell interaction similar to the *in vivo* microenvironment in order to maintain its differentiated state [95]. Engineering tools, such as microfluidics, biomaterial scaffolds and bioprinting, have enabled greater control over the cellular microenvironment and, subsequently, cell response [96]. These strategies may set the ground for producing organs or tissues on demand to be used for animal-free drug development and personalized medicine. Moreover, optimizing cell-cell interactions using different bioengineering techniques, such as 3D liver spheroids and bioprinting, would allow a better mimic of the *in vivo* physiology and thus permit to analyze cells’ response to drugs and other stimuli more accurately.

Different research groups reported various hepatic differentiation protocols by resorting to bioengineering tools. **Table 2** gathers several strategies for deriving HLCs from different umbilical cord sections both in 2D and 3D systems.

Within the umbilical cord, investigators seem to prefer using UCM-MSCs over UCB-MSCs to obtain HLCs, as shown in **Table 2**. This may be explained as

MSC source	Induction factors	Controls	Functional analysis	Hepatic markers	Ref.
Monolayer culture					
UCB	HGF, ITS, OSM, dexamethasone	MSC	LDL uptake	ALB, AFP, CK-18, CK-19, GS, TAT HGF, c-Met, PEPCK, CPS	[40]
	EGF, bFGF, HGF, OSM, ITS, nicotinamide, dexamethasone	Human Hep3B cell line	Albumin and urea production, LDL uptake, glycogen storage and CYP activity	HNF4A, CYP2B6	[41]
UCM	HGF, FGF-4	MSC	Glycogen storage, LDL uptake	ALB, AFP, CK18	[36]
	Rat-tail collagen type I coating; bFGF, HGF, nicotinamide, dexamethasone, OSM, ITS	MSC and freshly isolated liver cells	Glycogen storage, G6P and CYP3A4 activity, urea production	ALB, AFP, CK-18, CK-19, Cnx-32, TAT, TDO, CYP3A4	[22]
Monolayer culture; hypoxia					
UCM	HGF, FGF-4, nicotinamide, dexamethasone, OSM, ITS	MSC and HepG2	Albumin and urea production, LDL uptake, glycogen storage	ALB, AFP, HNF4, CK-18, AAT, G6P, CYP3A4	[42]
Monolayer culture; overexpression of TERT					
UCM	Lentiviral transfection of MSC; EGF, HGF, ITS, OSM, dexamethasone	Untransfected MSC and HepG2	Urea production, glycogen storage	ALB, AFP, CK-18	[35]

MSC source	Induction factors	Controls	Functional analysis	Hepatic markers	Ref.
Monolayer culture; epigenetic modifiers					
UCM	Rat-tail collagen; EGF, FGF-2, FGF-4, HGF, nicotinamide, dexamethasone, ITS, OSM, DMSO, 5-AZA	MSC, HepG2 and human and rat primary hepatocytes	Albumin and urea production, glycogen storage, CYP and UGT activity	CK-18, TAT, AFP, ALB, HNF4A, CEBPA, CYP1A2, CYP3A4, OATP-C, MRP-2	[34]
UCB	HGF, OSM, FGF-2, 5-AZA	Not-treated MSC	Urea production, glycogen storage	ALB, CEBPA, CEBPB, PEPCK, CYP1A1, CYP1A2	[25]
UCM	EGF, bFGF, HGF, OSM, ITS, nicotinamide, dexamethasone, NaBu	MSC not treated with NaBu, HepG2 and mouse embryonic fibroblast cell line NIH3T3	Urea production, glycogen storage	AFP, HNF3 B, ALB, CK-18, G6P, CEBPA, CYP2B6	[26]
UC	Rat-tail type I collagen coating; dexamethasone, HGF, OSM, ITS, VPA	MSC and MSC not treated with VPA	Albumin and urea production, glycogen storage, LDL uptake	Not studied	[27]
UCM	FGF-4, HGF, dexamethasone, OSM, VPA	MSC, differentiated MSC not treated with VPA and human adult liver biopsy	Albumin and urea production, glycogen storage	ALB, AFP, CK-18, G6P, TAT, AAT, HNF4A, CYP3A4, CYP1A1, miR-23b cluster, miR-26a-5p, miR-30a-5p, miR-122-5p, miR-148a-3p, miR-192-5p	[28]
3D scaffold					
UCM	Collagen/heparin coating; IGF-1, HGF, OSM dexamethasone,	MSC, HepG2; 2D culture	Albumin production, glycogen storage, G6P and CYP2B activity	ALB, CK-18, HNF4A, G6P, c-Met, CYP2B	[29]
UC	GEVAC; HGF, FGF-4, ITS, dexamethasone, OSM	MSC and HepG2; 2D culture	Albumin and urea production, CYP activity	AFP, ALB, G6P, AAT, TAT, HNF4A, CYP3A4	[30]
3D spheroids through cell pellet					
UC	HGF, bFGF, nicotinamide, dexamethasone, OSM, ITS	Small intestinal submucosa supplement in cell pellet; HepG2	Albumin and urea production, glycogen storage, CYP activity	ALB, HNF4A, CYP3A4	[31]

MSC source	Induction factors	Controls	Functional analysis	Hepatic markers	Ref.
3D spheroids through miniaturized hollow-fiber bioreactor and self-assembled suspension culture; epigenetic modifiers					
UCM	Rat-tail collagen coating; EGF, FGF-2, HGF, nicotinamide, dexamethasone, ITS, OSM, TSA, 5-AZA, DMSO	MSC and HepG2, human and rat primary hepatocytes; 2D culture	Albumin and lactate production, glycogen storage, negative glucose consumption, CYP induction and UGT activity	AFP, ALB, CK-18, TAT, HHEX, CEBPA, HNF4A, CYP1A1, CYP1B1, CYP3A4, OATP-C, MRP-2	[23]
3D spheroids through hanging drop cell culture					
UCM	IGF, HGF, OSM, dexamethasone	DMEM-treated MSC and HepG2; 2D culture	Glycogen storage	CK-18, CK-19, ALB	[32]

5-AZA, 5-azacytidine; AAT, α 1 anti-trypsin; AFP, α -fetoprotein; ALB, albumin; C-Met, HGF receptor; CEBP, CCAAT enhancer-binding protein; CK, cytokeratin; CM, conditioned medium; Cnx, Connexin; CPS, carbamoyl-phosphate synthase; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; FGF, fibroblast growth factor; G6P, glucose-6-phosphatase; GEVAC, gelatin-vinyl-acetate-copolymer; GS, glutamine synthetase; HGF, hepatocyte growth factor; HNF, hepatocyte nuclear factor; IGF, insulin-like growth factor; ITS, insulin-transferrin-selenium; LDL, low-density lipoprotein; miR, microRNA; MRP, multidrug resistance protein; MSC, mesenchymal stem cells; NaBu, sodium butyrate; OATP-C, organic anion-transporting polypeptide C; OSM, oncostatin M; PEPCK, phosphoenolpyruvate carboxykinase; TAT, tyrosine aminotransferase; TDO, tryptophan-2,3-dioxygenase; TPH2, tryptophan 2,3-dioxygenase; TSA, trichostatin A; UC, umbilical cord-derived mesenchymal stem cells, UCB-MSC, umbilical cord blood-derived MSC; UCM-MSC, umbilical cord matrix-derived mesenchymal stem cells; UGT, Uridine 5'-diphosphate glucuronosyltransferase; VPA, valproic acid.

Table 2.
Protocols for differentiation of human neonatal MSC into HLC.

UCM-MSCs are easier to isolate [97] and produce higher cell numbers with better proliferation capacities when compared to UCB-MSCs [98].

2.3.1 3D cultures of UC-MSC-derived HLCs

Animal models often fail to recapitulate human biology and are not appropriate to study tissue-specific mechanisms in a controlled fashion without the interference of other tissues [99]. Thus, creating a controlled human *in vitro* tissue using 3D culture techniques is a key strategy for producing reliable knowledge on drug toxicity and disease mechanisms [23, 29, 100]. Studies using HLCs differentiated from UC-MSC through 3D systems are still scarce. Nevertheless, **Figure 4** illustrates several strategies of bioengineering for producing functional HLCs from MSCs.

Cipriano et al. [23] reported that by resorting to 3D spheroid cultures, the HLCs obtained from UCM-MSCs exhibited a higher glycogen stain and CYP3A4 induction when compared to the correspondent 2D cultures (**Figure 5**). On the other hand, HLCs cultured in hollow-fiber bioreactors favored diclofenac conversion and albumin production [23], a function mostly associated with the perivenous phenotype [101] that is also regulated by the blood flow-mediated shear stress [100]. Alternatively, Ong et al. [31] observed that MSC-derived HLCs cultured as spheroids in pellet culture endorsed expression of a subset of hepatic genes (*CYP3A4* and *HNF4A*), secreted albumin and urea, stored glycogen and showed inducible CYP3A4 mRNA levels. Importantly, the culture conditions allowed stable cell anchorage, permitted the retention of ECM molecules produced by the cells, and

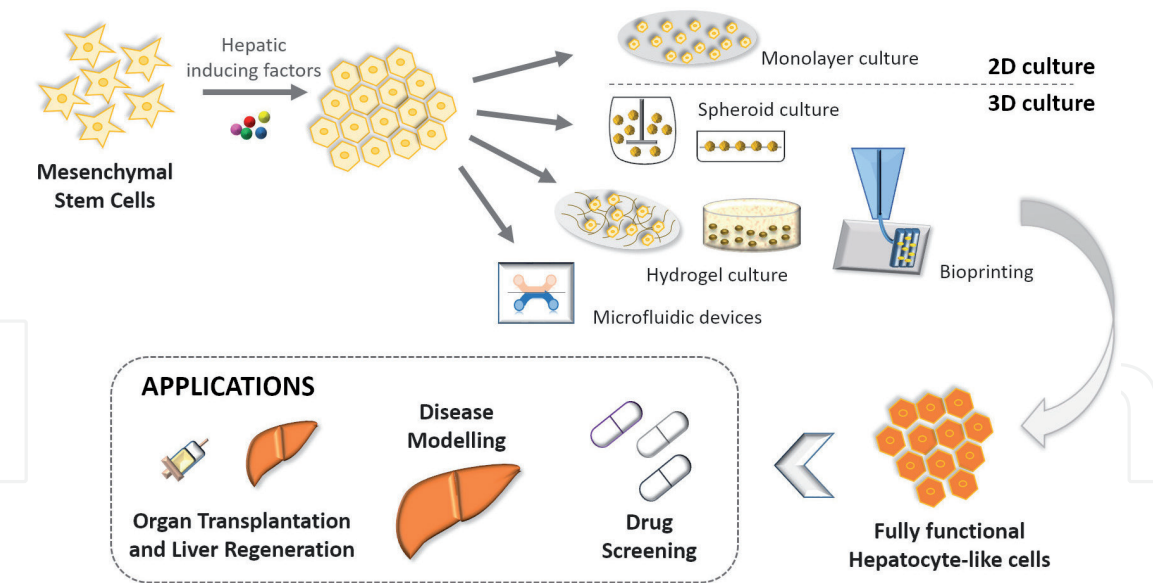


Figure 4.
2D and 3D culture strategies for the differentiation of MSCs into HLCs and potential clinical applications. Several strategies such as spheroid cultures, scaffolds, bioprinting and microfluidics have shown promissory results and represent good tools for future studies on drug screening, disease modeling and regenerative therapies using fully functional HLCs.

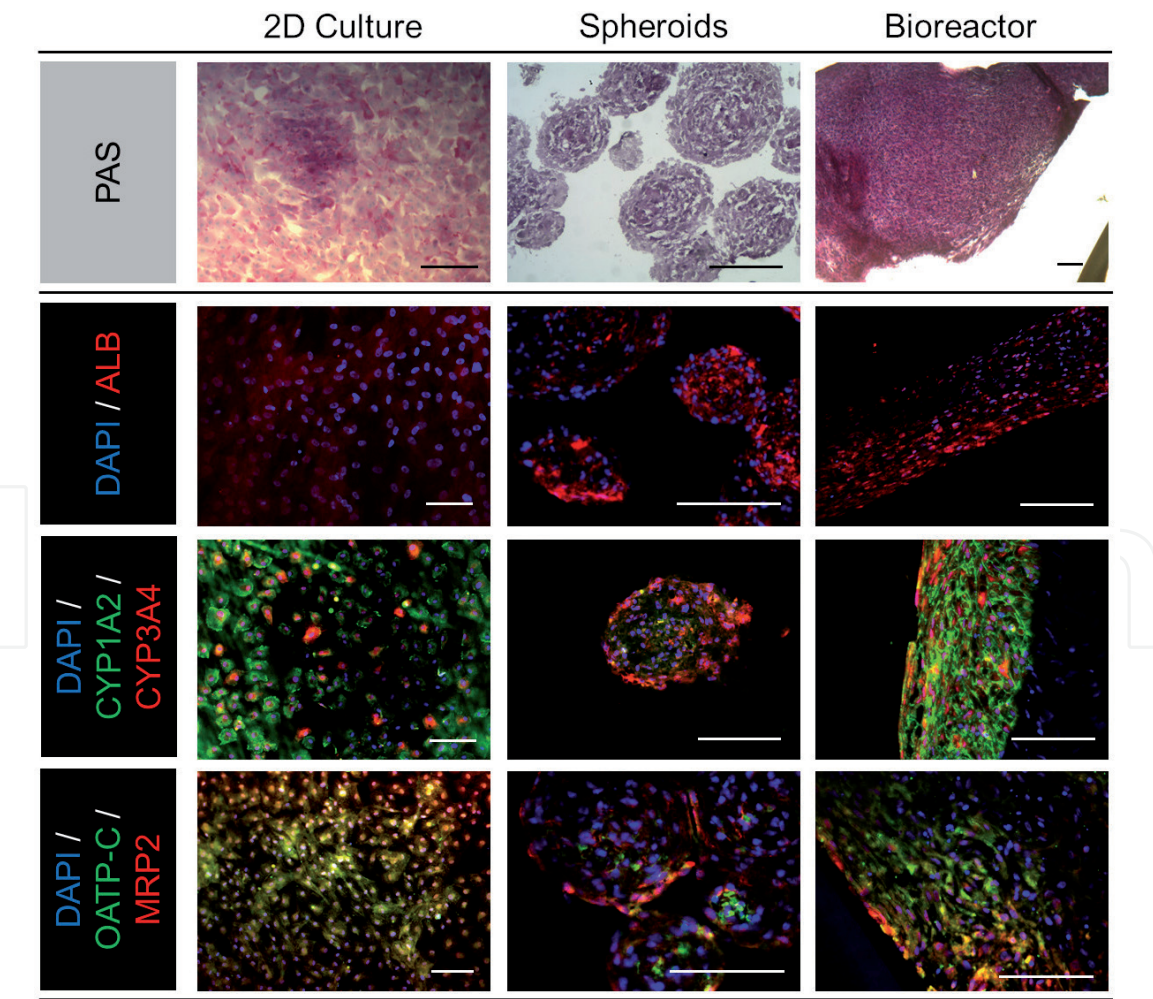


Figure 5.
Immunohistochemical and PAS analysis of HLCs-derived UCM-MSCs cultured in 2D cultures, in spheroids and in bioreactors. Representative images show the presence of the plasma protein ALB, the efflux transporter MRP-2, the uptake transporter protein OATP-C and the biotransformation enzymes CYP1A2 and CYP3A4. Cell nuclei are stained with DAPI. Scale bar: 50 μm. ALB, albumin; CYP, cytochrome P-450; HLCs, hepatocyte-like cells; MRP-2, multidrug resistance protein-2; OATP-C, organic anion-transporting polypeptide C; PAS, Periodic acid–Schiff; UCM-MSC, umbilical cord matrix mesenchymal stem cells.

when implanted into livers of hepatectomized rats also secreted human albumin into the bloodstream [31].

In the region between the blood and the hepatocytes (space of *Disse*) lies a diffuse matrix composed mostly of collagen type I and fibronectin [102]. Collagen base scaffolds have demonstrated to improve the adult hepatocyte functions as they mimic the naïve hepatocyte niche [21–23, 34]. Talaei-Khozani et al. [103] and Khodabandeh et al. [104] compared 2D and 3D cultures of UCM-MSCs in collagen films and demonstrated a better hepatogenesis and increased expression of *HNF4A* on the 3D environment. Additionally, Aleahmad et al. [29] used a 3D bioprinted collagen and heparin scaffold and verified that heparinized 2D cultures mainly expressed early liver-specific markers (e.g., *HNF4A*, *ALB*, *CK18* and *CK19*) in the presence of heparin, whereas the heparinized 3D cultures expressed both early and late liver-specific markers (e.g., *G6P*, *CYP2B*). In this study, HLCs showed a two-fold increase in albumin production compared to monolayer cultures [29]. These results infer that 3D culture conditions using collagen films can prevent loss of hepatocyte function and improve efficiency of hepatocyte differentiation.

Scaffolds other than collagen matrixes have also been proposed to direct hepatic differentiation of UC-MSCs. Chitrangi et al. [30] observed that gelatin-vinyl acetate (GEVAC) stimulates the differentiation of UC-MSCs into hepatospheroids, resulting in a better maturation, higher urea production, expression of *CYP3A4* and *CYP2C9*, higher percentage of albumin-positive cells and hepatic markers compared to 2D cultures. Hashemi et al. [33] also presented a protocol for seeding UC-MSCs on a poly(ϵ -caprolactone) (PCL) nanofiber scaffold to stimulate and then maintain hepatic differentiation.

2.3.2 Microfluidic technologies

The hepatic zonation corresponds to the different functions revealed by the hepatocytes according to their location in the hepatic lobule, which results from the gradient of concentration of the various nutrients and oxygen observed in the hepatic environment [7]. The 3D culture systems create a gradient that may stimulate the hepatic zonation and influence HLCs obtained. For instance, the 3D configuration may mimic the liver periportal environment by generating a gradient with higher oxygen, glucose and nutrients in cells closer to the capillaries in the culture system, for example, in the outer side of spheroids, leading to higher xenobiotic metabolism, urea production and glycogen synthesis [7]. The perivenous hepatocytes have less access to oxygen supplies and are characterized by a higher xenobiotic metabolism, being exposed to physiologic conditions similar to the ones observed around the inner cells [3, 7]. Moreover, hepatocytes are not the only cells present in the liver as they interact with mesenchymal cells, stellate cells, Küpffer cells, macrophages, and lymphocytes, and are exposed, *in vivo*, to a fluid perfusion [105]. Hence, *in vitro* liver function may be optimized by resorting to microfluidic technologies.

Microfluidic culture devices (MD) permit to control the microenvironment and present the ability of continued delivery of medium, drugs and soluble molecules, allowing the study of drug metabolism and interactions [96]. The effect of medium flow on inducing albumin secretion was demonstrated by Prodanov et al. [100] using a human primary hepatocyte 3D microfluidic system. Likewise, McCarty et al. [106] demonstrated the creation of spatially-controlled zonation across multiple hepatocyte metabolism levels through the application of precise concentration gradients of exogenous hormones (insulin and glucagon) and chemical (3-methylcholanthrene) induction agents in a microfluidic device, using monolayer rat

primary hepatocytes. Herein, a high concentration of insulin was directly correlated with a gradient in glycogen storage and urea production [106].

Studies reporting the hepatic differentiation of MSCs are still limited, but two studies using bone-marrow-derived MSC cultured in MDs show already a cost-effective method for HLCs production in 3 [107] to 4 weeks [108]; however, the obtained HLCs were only characterized with regard to albumin and urea quantification and showed a low metabolic performance.

3. Characterization of hepatocyte-like cells *in vitro*

A great amount of work has been developed over the past years for generating human stem cell-derived hepatocyte systems for *in vitro* toxicity testing. However, the definition of what is considered a differentiated HLC is still not unanimous and largely depends on the authors and on the purpose for which the cells are to be used. Currently, a wide and variable range of parameters is used to demonstrate the acquisition of *in vivo*-like hepatic features, which often leads to an incomplete and inconsistent cell characterization. As such, the scientific community would benefit from the harmonization and definition of the number and type of performance criteria. Indeed, Vinken and Hengstler [14] propose an optimal characterization aiming at benchmarking of hepatocyte-based *in vitro* systems for toxicity testing. This proposal comprises critical elements such as cell viability, morphology, functionality and toxicological characterization, as follows:

- Cell viability should be assessed using at least two methods that evaluate early and late key events of cytotoxicity. Moreover, a threshold of 90% viability should be adopted to discriminate between spontaneous cell death and cell death induced by toxicants.
- In terms of cellular morphology, cells must be monitored closely in order to confirm the maintenance of the hepatic polygonal shape and the detection of structural polarity markers, essential for many hepatic functions.
- Hepatocyte-specific functions, including secretion of albumin and blood coagulation factors, metabolism of carbohydrates and lipids, bile acid production and transport, as well as the detoxification of endobiotics and xenobiotics are some of the cells functionalities to be considered. This can be performed through measurement of albumin and urea production, of glycogen storage and of biotransformation enzyme activity. Other hepatic or liver-related markers, such as HNF-1/4A and PXR, should also be evaluated.
- The toxicological characterization of hepatocyte-based *in vitro* systems is important to confirm their capacity to detect prototypical types of liver toxicity. This can be achieved by using hepatotoxicants capable of replicating human *in vivo* intrinsic drug-induced liver injury, namely paracetamol dose-dependent necrosis, microvesicular steatosis induced by valproic acid and cholestasis induced by cyclosporine A.

However, a systematic interpretation of HLC-based *in vitro* systems with respect to their translation for the human *in vivo* situation remains a major challenge for future research.

4. *In vitro* toxicological applications of HLCs

Drug attrition is a major expense in the drug development process and the use of advanced *in vitro* models will likely contribute to its reduction [109]. Detection of hepatotoxicity often occurs late in the drug development process and contributes to drug attrition, withdrawn in a post-market scheme and restriction of therapeutic indications [110]. Animal-based testing is currently the base for translating *in vitro* studies to clinical trials but often do not correlate with human toxicity data [99], mainly due to interspecies differences in drug metabolism [4]. *In vitro* models cannot directly replace animal models but may occupy a new space in which, in the future, animal models will become obsolete (**Figure 6**). *In vitro* models provide tissue-specific mechanistic insights and allow to study a high number of conditions with the same cells, by means of, for example, miniaturization and higher throughput [111]. However, current *in vitro* application of differentiated HLCs in drug metabolism studies and disease modeling is still in its infancy.

Numerous studies have been published on HLCs obtained from UC-MSCs [25, 27, 34, 35, 40] as shown in **Table 2**, but few were able to demonstrate cells' biotransformation capacity, as follows: Xue et al. [49] showed that HLCs from UCM-MSCs were capable of metabolizing midazolam through CYP3A4 activity. As mentioned earlier, using a three-step protocol for UCM-MSC differentiation, Campard et al. [22] obtained HLCs that expressed important hepatic features (ALB, G6P, TDO, AAT, TAT and AFP markers, glycogen accumulation and urea production) and exhibited CYP3A4 activity. However, the absence of markers of hepatic maturation such as CYP2B6 induction after exposure to phenobarbital suggested that a relevant phenotype was not fully achieved [22]. On the other hand, Cipriano et al. [34], in contrast to the results observed when comparing HepG2 and primary human hepatocytes (PHH), obtained a comparable expression of genes involved in drug transport, amino acid metabolism and proliferation of hepatocytes between UCM-MSC-derived HLCs and PHH, indicating that HLCs are a better model for drug screening than low-metabolizing cell lines. In another study, using HLCs derived from UCM-MSC in 3D spheroids culture, Cipriano et al. [23] also observed that diclofenac was effectively converted by CYP2C9 into its hepatotoxic metabolite, 4-OH-diclofenac, and was also metabolized to a lesser extent by CYP3A4 and UGTs. Furthermore,

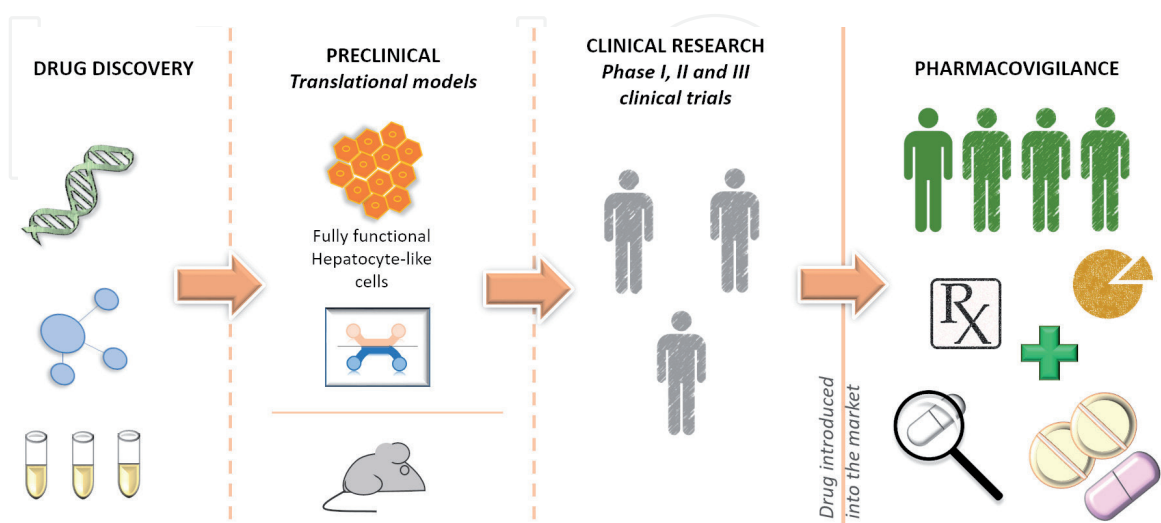


Figure 6.

Potential of mature, fully functional HLCs on drug discovery and drug development. Conventional drug development pipelines involve preclinical in vitro and in vivo research in animal models followed by clinical trials on humans. In vitro toxicology models such as fully functional HLCs may shorten the way by performing a more similar to human toxicology screen and overcome limitations associated with poor correlation, interspecies differences and ethical concerns when using animal models.

the determined IC₅₀ fell in the range of what is found on the literature for primary hepatocytes, indicating that differentiated MSCs had similar dose-response characteristics to mature primary hepatocytes for this hepatotoxicant. Diclofenac is one of the most prescribed nonsteroidal anti-inflammatory drugs (NSAIDs) worldwide [112]. As such, these results permit the future use of these HLCs on drug testing and potential hepatotoxicity screening, which is often dependent of bioactivation.

Acetaminophen (APAP) is an over-the-counter antipyretic and analgesic drug widely used in several pharmacological formulations. However, in toxic doses, APAP causes liver injury by saturation of its main inactivation pathway and shifts to the transformation, by CYP2E1, CYP2D6, CYP1A2 and CYP2A6, of APAP into its hepatotoxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) [113]. Chitrangi et al. [30] used hepatospheroids derived from UC-MSCs as an *in vitro* model for studying the metabolism and toxicity of APAP. CYP3A4 and CYP1A2 were induced in HLCs by APAP as well as reactive oxygen species (ROS) cell damage, which lead to cytoskeletal disorganization, both in HLCs and primary hepatocytes.

Hepatocytes derived from SCs may also represent a platform for drug discovery through disease modeling, in which *in vivo* cell functions and mechanisms involved in pathological processes on disease onset and progression may be analyzed [114]. There are already several liver diseases successfully modeled *in vitro* using iPSC-derived hepatocytes as, for instance, familial hypercholesterolemia [115], hemophilia A [116], hepatitis C [117] and drug-induced hepatotoxicity [118]. One of the first studies to use human UC-MSCs to define a disease was Paganelli et al. [119], who developed an *in vitro* disease model to study the mechanisms underlying hepatitis B virus (HBV) infection by differentiating UC-MSCs into HLCs and infecting them with HBV. Results showed a higher susceptibility of HBV infection on HLCs rather than on undifferentiated MSCs. Despite low replication efficiency on HLCs, viral entry was as efficient as in primary hepatocytes and mimicked appropriately the *in vivo*-restricted HBV host range [119]. These similarities between the *in vivo*, *in vitro* gold standard and UC-MSCs lead, once again, to a promising opportunity for future development of *in vitro* models for drug discovery as human UC-MSCs represent a unique, human, easily available, non-transformed *in vitro* model.

5. Conclusion and future perspectives

This chapter provides insights into the potential use of human umbilical cord MSCs for obtaining a mature HLC phenotype suitable for *in vitro* toxicological studies. As primary hepatocytes present limited capacity to expand *ex vivo*, the possibility of obtaining comparable hepatocyte-like cells from MSCs may alleviate the low cell availability of human primary hepatocytes. Moreover, the use of MSCs in a physiologically relevant microenvironment that generates fully functional HLCs would allow an integrated approach to study xenobiotics biotransformation and mechanisms of action (MoA) or toxicity (MoT).

The differentiation process of MSCs into HLCs and their potential toxicology application are still in their infancy and, in the following years, there are still major challenges to resolve before their relevant application. Firstly, improvement of the efficiency of hepatic induction *in vitro* and *in vivo* still requires further investigation on the hepatic transdifferentiation mechanisms of UC-MSCs. Secondly, the differentiation process is long and the generation and maintenance of high numbers of HLCs are still difficult to achieve. The definition of the most relevant endpoints of hepatic differentiation and maturation is of extreme importance. Thirdly, the use of relevant positive controls such as human primary hepatocytes is essential for benchmarking HLCs and its absence represents a major issue in evaluating most of the published studies.

As such, further studies will be required to allow the use of HLCs derived from UC-MSCs in the drug development process, but the strategies described in this chapter represent the first step toward the establishment of a relevant human *in vitro* hepatic model for toxicological studies. Exploring the full potential of UC-MSCs by the introduction of mechanistic models for toxicity testing, including *in vitro* disease models and hepatotoxicity models, at the pipeline of drug discovery and development will significantly reduce compound attrition rate and progressively substitute current animal models by selecting safer and more efficacious lead molecules.

Acknowledgements

This work was funded by FCT (Portugal) through the research grants TUBITAK/0003/2014 and PTDC/MED-TOX/29183/2017.

Conflict of interest

The authors declare no conflict of interest.

Appendices and Nomenclature

2D	two-dimensional
3D	three-dimensional
5-AZA	5-azacytidine
AAT	α 1 anti-trypsin
AFP	α -fetoprotein
ALB	albumin
BMP	bone morphogenetic protein
CEBP	CCAAT enhancer-binding protein
CK	cytokeratin
CYP	cytochrome P450
DILI	drug-induced liver injury
DMSO	dimethyl sulfoxide
DNMTi	DNA methyltransferase inhibitors
EGF	epidermal growth factor
EROD	7-ethoxyresorufin-O-deethylase
FGF	fibroblast growth factor
G6P	glucose-6-phosphatase
GS	glutamine synthetase
HDACi	histone deacetylase inhibitors
hESCs	human embryonic stem cells
HGF	hepatocyte growth factor
HLC	hepatocyte-like cell
HLCs	hepatocyte-like cells
HNF	hepatocyte nuclear factor
hnMSC	human neonatal mesenchymal stem cell
hnMSCs	human neonatal mesenchymal stem cells
hUCB-MSCs	human umbilical cord blood mesenchymal stem cells
hUCM-MSCs	human umbilical cord matrix mesenchymal stem cells
hUC-MSC	human umbilical cord-derived mesenchymal stem cell

IGF	insulin-like growth factor
iPSCs	induced pluripotent stem cells
ITS	insulin-transferrin-selenium
LDL	low-density lipoprotein
MD	microfluidic culture device
MiR	microRNA
MSC	mesenchymal stem cells
NaBu	sodium butyrate
OSM	oncostatin M
PAS	periodic acid Schiff's
SCs	stem cells
TAT	tyrosine aminotransferase
TDO	tryptophan-2,3-dioxygenase
TSA	trichostatin A
UGT	uridine 5'-diphosphate glucuronosyltransferase
VPA	valproic acid

Author details

Ana Sofia Martins Serras^{1†}, Madalena Zincke dos Reis Fernandes Cipriano^{1,2†},
Pedro Miguel da Graça Silva¹ and Joana Paiva Gomes Miranda^{1*}


1 Research Institute for Medicines (iMed.Ulisboa), Faculty of Pharmacy,
University of Lisbon, Lisbon, Portugal

2 Fraunhofer Institute for Interfacial Engineering and Biotechnology IGB,
Stuttgart, Germany

*Address all correspondence to: jmiranda@ff.ulisboa.pt

† Both authors equally contributed to this work.

IntechOpen

© 2020 The Author(s). Licensee IntechOpen. This chapter is distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/3.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. 

References

- [1] Lee WM. Drug-induced acute liver failure. *Clinics in Liver Disease*. 2013;**17**(4):575-586
- [2] Andrade RJ, Aithal GP, Björnsson ES, Kaplowitz N, Kullak-Ublick GA, Larrey D, et al. EASL clinical practice guidelines: Drug-induced liver injury. *Journal of Hepatology*. 2019;**70**(6):1222-1261
- [3] Gerets HHJ, Tilmant K, Gerin B, Chanteux H, Depelchin BO, Dhalluin S, et al. Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. *Cell Biology and Toxicology*. 2012;**28**(2):69-87
- [4] Martignoni M, Groothuis GMM, de Kanter R. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opinion on Drug Metabolism & Toxicology*. 2006;**2**(6):875-894
- [5] Sartipy P, Björquist P, Strehl R, Hyllner J. The application of human embryonic stem cell technologies to drug discovery. *Drug Discovery Today*. 2007;**12**(17-18):688-699
- [6] Sahu SC. *Hepatotoxicity: From Genomics to In Vitro and In Vivo Models*. Wiley; 2008. 704 p
- [7] Godoy P, Hewitt NJ, Albrecht U, Andersen ME, Ansari N, Bhattacharya S, et al. Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Archives of Toxicology*. 2013;**87**:1315-1530
- [8] Westerink WMA, Schoonen WGEJ. Cytochrome P450 enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2 cells. *Toxicology in Vitro*. 2007;**21**(8):1581-1591
- [9] Westerink WMA, Schoonen WGEJ. Phase II enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2 cells. *Toxicology in Vitro*. 2007;**21**(8):592-602
- [10] Apati A, Varga N, Berecz T, Erdei Z, Homolya L, Sarkadi B. Application of human pluripotent stem cells and pluripotent stem cell-derived cellular models for assessing drug toxicity. *Expert Opinion on Drug Metabolism & Toxicology*. 2019;**15**(1):61-75
- [11] Kim DE, Jang M-J, Kim YR, Lee J-Y, Cho EB, Kim E, et al. Prediction of drug-induced immune-mediated hepatotoxicity using hepatocyte-like cells derived from human embryonic stem cells. *Toxicology*. 2017;**387**:1-9
- [12] Snykers S, Henkens T, De Rop E, Vinken M, Fraczek J, De Kock J, et al. Role of epigenetics in liver-specific gene transcription, hepatocyte differentiation and stem cell reprogramming. *Journal of Hepatology*. 2009;**51**(1):187-211
- [13] Takayama K, Morisaki Y, Kuno S, Nagamoto Y, Harada K, Furukawa N, et al. Prediction of interindividual differences in hepatic functions and drug sensitivity by using human iPS-derived hepatocytes. *Proceedings of the National Academy of Sciences of the United States of America*. 2014;**111**(47):16772-16777
- [14] Vinken M, Hengstler JG. Characterization of hepatocyte-based in vitro systems for reliable toxicity testing. *Archives of Toxicology*. 2018;**92**:2981-2986

- [15] Kia R, Sison RLC, Heslop J, Kitteringham NR, Hanley N, Mills JS, et al. Stem cell-derived hepatocytes as a predictive model for drug-induced liver injury: Are we there yet? *British Journal of Clinical Pharmacology*. 2013;**75**(4):885-896
- [16] Anzalone R, Iacono ML, Corrao S, Magno F, Loria T, Cappello F, et al. New emerging potentials for human Wharton's jelly mesenchymal stem cells: Immunological features and hepatocyte-like differentiative capacity. *Stem Cells and Development*. 2010;**19**:423-438
- [17] Prockop DJ, Oh JY. Medical therapies with adult stem/progenitor cells (MSCs): A backward journey from dramatic results in vivo to the cellular and molecular explanations. *Journal of Cellular Biochemistry*. 2012;**113**(5):1460-1469
- [18] Freyer N, Knöspel F, Strahl N, Amini L, Schrade P, Bachmann S, et al. Hepatic differentiation of human induced pluripotent stem cells in a perfused three-dimensional multicompartiment bioreactor. *BioResearch Open Access*. 2016;**5**(1):235-248
- [19] Sjogren A-KM, Liljevald M, Glinghammar B, Sagemark J, Li X-Q, Jonebring A, et al. Critical differences in toxicity mechanisms in induced pluripotent stem cell-derived hepatocytes, hepatic cell lines and primary hepatocytes. *Archives of Toxicology*. 2014;**88**(7):1427-1437
- [20] Viiri LE, Rantapero T, Kiammehr M, Alexanova A, Oittinen M, Viiri K, et al. Extensive reprogramming of the nascent transcriptome during iPSC to hepatocyte differentiation. *Scientific Reports*. 2019;**9**:3562
- [21] Snykers S, De Kock J, Rogiers V, Vanhaecke T. In vitro differentiation of embryonic and adult stem cells into hepatocytes: State of the art. *Stem Cells*. 2009;**27**(3):577-605
- [22] Campard D, Lysy PA, Najimi M, Sokal EM. Native umbilical cord matrix stem cells express hepatic markers and differentiate into hepatocyte-like cells. *Gastroenterology*. 2008;**134**(3):833-848
- [23] Cipriano M, Freyer N, Knöspel F, Oliveira NG, Barcia R, Cruz PE, et al. Self-assembled 3D spheroids and hollow-fibre bioreactors improve MSC-derived hepatocyte-like cell maturation in vitro. *Archives of Toxicology*. 2017;**91**(4):1815-1832
- [24] De Kock J, Vanhaecke T, Biernaskie J, Rogiers V, Snykers S. Characterization and hepatic differentiation of skin-derived precursors from adult foreskin by sequential exposure to hepatogenic cytokines and growth factors reflecting liver development. *Toxicology in Vitro*. 2009;**23**(8):1522-1527
- [25] Yoshida Y, Shimomura T, Sakabe T, Ishii K, Gonda K, Matsuoka S, et al. A role of Wnt/ β -catenin signals in hepatic fate specification of human umbilical cord blood-derived mesenchymal stem cells. *American Journal of Physiology. Gastrointestinal and Liver Physiology*. 2007;**293**(5):G1089-G1098
- [26] Panta W, Imsoonthornruksa S, Yoisungnern T, Suksaweang S, Ketudat-Cairns M, Parnpai R. Enhanced hepatogenic differentiation of human Wharton's jelly-derived mesenchymal stem cells by using three-step protocol. *International Journal of Molecular Sciences*. 2019;**20**(12):1-23
- [27] An SY, Han J, Lim HJ, Park SY, Kim JH, Do BR, et al. Valproic acid promotes differentiation of hepatocyte-like cells from whole human umbilical cord-derived mesenchymal stem cells. *Tissue & Cell*. 2014;**46**(2):127-135
- [28] Raut A, Khanna A. Enhanced expression of hepatocyte-specific microRNAs in valproic acid mediated hepatic trans-differentiation of human umbilical cord derived mesenchymal

stem cells. *Experimental Cell Research*. 2016;**343**(2):237-247

[29] Aleahmad F, Ebrahimi S, Salmannezhad M, Azarnia M, Jaberipour M, Hoseini M, et al. Heparin/collagen 3D scaffold accelerates hepatocyte differentiation of Wharton's jelly-derived mesenchymal stem cells. *Tissue Engineering and Regenerative Medicine*. 2017;**14**(4):443-452

[30] Chitrangi S, Nair P, Khanna A. Three-dimensional polymer scaffolds for enhanced differentiation of human mesenchymal stem cells to hepatocyte-like cells: A comparative study. *Journal of Tissue Engineering and Regenerative Medicine*. 2017;**11**(8):2359-2372

[31] Ong SY, Dai H, Leong KW. Inducing hepatic differentiation of human mesenchymal stem cells in pellet culture. *Biomaterials*. 2006;**27**(22):4087-4097

[32] Talaei-Khozani T, Borhani-Haghighi M, Ayatollahi M, Vojdani Z. An in vitro model for hepatocyte-like cell differentiation from Wharton's jelly derived-mesenchymal stem cells by cell-base aggregates. *Gastroenterology and Hepatology from Bed to Bench*. 2015;**8**(3):188-199

[33] Hashemi SM, Soleimani M, Zargarian SS, Haddadi-Asl V, Ahmadbeigi N, Soudi S, et al. In vitro differentiation of human cord blood-derived unrestricted somatic stem cells into hepatocyte-like cells on poly(ϵ -caprolactone) nanofiber scaffolds. *Cells, Tissues, Organs*. 2009;**190**:135-149

[34] Cipriano M, Correia JC, Camões SP, Oliveira NG, Cruz P, Cruz H, et al. The role of epigenetic modifiers in extended cultures of functional hepatocyte-like cells derived from human neonatal mesenchymal stem cells. *Archives of Toxicology*. 2017;**91**(6):2469-2489

[35] Liang X, Chen X, Yang D, Huang S, Sun G, Chen Y. Differentiation of

human umbilical cord mesenchymal stem cells into hepatocyte-like cells by hTERT gene transfection in vitro. *Cell Biology International*. 2012;**36**:215-221

[36] Zhang YN, Lie PC, Wei X. Differentiation of mesenchymal stromal cells derived from umbilical cord Wharton's jelly into hepatocyte-like cells. *Cytotherapy*. 2009;**11**(5):548-558

[37] Zhao Q, Ren H, Li X, Chen Z, Zhang X, Gong W, et al. Differentiation of human umbilical cord mesenchymal stromal cells into low immunogenic hepatocyte-like cells. *Cytotherapy*. 2009;**11**(4):414-426

[38] Tsai PC, Fu TW, Chen YMA, Ko TL, Chen TH, Shih YH, et al. The therapeutic potential of human umbilical mesenchymal stem cells from Wharton's jelly in the treatment of rat liver fibrosis. *Liver Transplantation*. 2009;**15**(5):484-495

[39] Yan Y, Xu W, Qian H, Si Y, Zhu W, Cao H, et al. Mesenchymal stem cells from human umbilical cords ameliorate mouse hepatic injury in vivo. *Liver International*. 2009;**29**(3):356-365

[40] Hong SH, Gang EJ, Jeong JA, Ahn C, Hwang SH, Yang IH, et al. In vitro differentiation of human umbilical cord blood-derived mesenchymal stem cells into hepatocyte-like cells. *Biochemical and Biophysical Research Communications*. 2005;**330**(4):1153-1161

[41] Lee KD, Kuo TKC, Whang-Peng J, Chung YF, Lin CT, Chou SH, et al. In vitro hepatic differentiation of human mesenchymal stem cells. *Hepatology*. 2004;**40**(6):1275-1284

[42] Prasajak P, Leeanansaksiri W. Developing a new two-step protocol to generate functional hepatocytes from wharton's jelly-derived mesenchymal stem cells under hypoxic condition. *Stem Cells International*. 2013;**9**(29):1-10

- [43] Stummann TC, Bremer S. Embryonic stem cells in safety pharmacology and toxicology. *Advances in Experimental Medicine and Biology*. 2012;**745**:14-25
- [44] Vojnits K, Bremer S. Challenges of using pluripotent stem cells for safety assessments of substances. *Toxicology*. 2009;**270**:10-17
- [45] Herberts CA, Kwa MSG, Hermesen HPH. Risk factors in the development of stem cell therapy. *Journal of Translational Medicine*. 2011;**9**:29
- [46] Peterson SE, Loring JF. Genomic instability in pluripotent stem cells: Implications for clinical applications. *The Journal of Biological Chemistry*. 2014;**289**(8):4578-4584
- [47] Kim HJ, Park J-S. Usage of human mesenchymal stem cells in cell-based therapy: Advantages and disadvantages. *Development & Reproduction*. 2017;**21**(1):1-10
- [48] Miranda JP, Filipe E, Fernandes AS, Almeida JM, Martins JP, De La Fuente A, et al. The human umbilical cord tissue-derived MSC population UCX® promotes early motogenic effects on keratinocytes and fibroblasts and G-CSF-mediated mobilization of BM-MSCs when transplanted in vivo. *Cell Transplantation*. 2015;**24**(5):865-877
- [49] Xue G, Han X, Ma X, Wu H, Qin Y, Liu J, et al. Effect of microenvironment on differentiation of human umbilical cord mesenchymal stem cells into hepatocytes in vitro and in vivo. *BioMed Research International*. 2016;**2016**:8916534
- [50] Zhou X, Cui L, Zhou X, Yang Q, Wang L, Guo G, et al. Induction of hepatocyte-like cells from human umbilical cord-derived mesenchymal stem cells by defined microRNAs. *Journal of Cellular and Molecular Medicine*. 2017;**21**(5):881-893
- [51] Yang XF, Chen T, Ren LW, Yang L, Qi H, Li FR. Immunogenicity of insulin-producing cells derived from human umbilical cord mesenchymal stem cells. *Experimental and Therapeutic Medicine*. 2017;**13**(4):1456-1464
- [52] Khan MA, Alanazi F, Ahmed HA, Shamma T, Kelly K, Hammad MA, et al. iPSC-derived MSC therapy induces immune tolerance and supports long-term graft survival in mouse orthotopic tracheal transplants. *Stem Cell Research & Therapy*. 2019;**10**(1):1-15
- [53] Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006;**8**(4):315-317
- [54] da Silva ML, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *Journal of Cell Science*. 2006;**119**(Pt 11):2204-2213
- [55] de la Torre P, Jesús Pérez-Lorenzo M, Flores AI. Human placenta-derived mesenchymal stromal cells: A review from basic research to clinical applications. In: *Stromal Cells - Structure, Function, and Therapeutic Implications*. IntechOpen; 2019
- [56] Secunda R, Vennila R, Mohanashankar AM, Rajasundari M, Jeswanth S, Surendran R. Isolation, expansion and characterisation of mesenchymal stem cells from human bone marrow, adipose tissue, umbilical cord blood and matrix: A comparative study. *Cytotechnology*. 2015;**67**(5):793-807
- [57] Nekanti U, Mohanty L, Venugopal P, Balasubramanian S, Totey S, Ta M. Optimization and scale-up of Wharton's jelly-derived mesenchymal stem cells for clinical applications. *Stem Cell Research*. 2010;**5**(3):244-254
- [58] Varaa N, Azandeh S, Khodabandeh Z, Gharravi AM. Wharton's jelly

mesenchymal stem cell: Various protocols for isolation and differentiation of hepatocyte-like cells; narrative review. *Iranian Journal of Medical Sciences*. 2019;**44**(6):437-448

[59] Hassan G, Kasem I, Soukkarieh C, Aljamali M. A simple method to isolate and expand human umbilical cord derived mesenchymal stem cells: Using explant method and umbilical cord blood serum. *International Journal of Stem Cells*. 2017;**10**(2):184-192

[60] Zeddou M, Briquet A, Relic B, Josse C, Malaise MG, Gothot A, et al. The umbilical cord matrix is a better source of mesenchymal stem cells (MSC) than the umbilical cord blood. *Cell Biology International*. 2010;**34**(7):693-701

[61] Singh TP, Sherpa ML, Pradhan A, Singh TA. Neonatal derived mesenchymal stem cells can Be isolated from human umbilical cord Wharton's jelly but not from human umbilical cord blood. *Journal of Evolution of Medical and Dental Sciences*. 2019;**8**(12):849-854

[62] Ganchas Soares RI, Baptista Coelho MC, Silva Santos JM, Martins JP, Basto VA, Estilita Monteiro Da Cruz P, et al. Optimised and defined method for isolation and preservation of precursor cells from human umbilical cord. WO/2009/044379. Portugal; 2009

[63] Santos JM, Camões SP, Filipe E, Cipriano M, Barcia RN, Filipe M, et al. Three-dimensional spheroid cell culture of umbilical cord tissue-derived mesenchymal stromal cells leads to enhanced paracrine induction of wound healing. *Stem Cell Research & Therapy*. 2015;**6**(1):1-19

[64] Martins JP, Santos JM, Almeida JM, Filipe MA, de Almeida MVT, Almeida SCP, et al. Towards an advanced therapy medicinal product based on mesenchymal stromal cells isolated from the umbilical cord

tissue: Quality and safety data. *Stem Cell Research & Therapy*. 2014;**5**(1):9

[65] Grabowska I, Streminska W, Janczyk-Ilach K, Machaj EK, Pojda Z, Hoser G, et al. Myogenic potential of mesenchymal stem cells - the case of adhesive fraction of human umbilical cord blood cells. *Current Stem Cell Research & Therapy*. 2013;**8**(1):82-90

[66] Chen S, Zhang W, Wang JM, Duan HT, Kong JH, Wang YX, et al. Differentiation of isolated human umbilical cord mesenchymal stem cells into neural stem cells. *International Journal of Ophthalmology*. 2016;**9**(1):41-47

[67] Jung J, Zheng M, Goldfarb M, Zaret KS. Initiation of mammalian liver development from endoderm by fibroblast growth factors. *Science*. 1999;**284**(5422):1998-2003

[68] Fukuda-Taira S. Hepatic induction in the avian embryo: Specificity of reactive endoderm and inductive mesoderm. *Journal of Embryology and Experimental Morphology*. 1981;**63**:111-125

[69] Wilkins BJ, Pack M. Zebrafish models of human liver development and disease. *Comprehensive Physiology*. 2013;**3**(3):1213-1230

[70] McLin VA, Rankin SA, Zorn AM. Repression of Wnt/ β -catenin signaling in the anterior endoderm is essential for liver and pancreas development. *Development*. 2007;**134**(12):2207-2217

[71] Gordillo M, Evans T, Gouon-Evans V. Orchestrating liver development. *Development*. 2015;**142**(12):2094-2108

[72] Hay DC, Fletcher J, Payne C, Terrace JD, Gallagher RCJ, Snoeys J, et al. Highly efficient differentiation of hESCs to functional hepatic endoderm requires ActivinA and Wnt3a signaling.

Proceedings of the National Academy of Sciences of the United States of America. 2008;**105**(34):12301-12306

[73] Zhou M, Li P, Tan L, Qu S, Ying QL, Song H. Differentiation of mouse embryonic stem cells into hepatocytes induced by a combination of cytokines and sodium butyrate. *Journal of Cellular Biochemistry*. 2010;**109**(3):606-614

[74] Calmont A, Wandzioch E, Tremblay KD, Minowada G, Kaestner KH, Martin GR, et al. An FGF response pathway that mediates hepatic gene induction in embryonic endoderm cells. *Developmental Cell*. 2006;**11**(3):339-348

[75] Wang Z, Dollé P, Cardoso WV, Niederreither K. Retinoic acid regulates morphogenesis and patterning of posterior foregut derivatives. *Developmental Biology*. 2006;**297**(2):433-445

[76] Shen MM. Nodal signaling: Development roles and regulation. *Development*. 2007;**134**(6):1023-1034

[77] Ameri J, Ståhlberg A, Pedersen J, Johansson JK, Johannesson MM, Artner I, et al. FGF2 specifies hESC-derived definitive endoderm into foregut/midgut cell lineages in a concentration-dependent manner. *Stem Cells*. 2010;**28**(1):45-56

[78] Bort R, Signore M, Tremblay K, Barbera JPM, Zaret KS. Hex homeobox gene controls the transition of the endoderm to a pseudostratified, cell emergent epithelium for liver bud development. *Developmental Biology*. 2006;**290**(1):44-56

[79] Rossi JM, Dunn NR, Hogan BLM, Zaret KS. Distinct mesodermal signals, including BMPs from the septum, transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. *Genes & Development*. 2001;**15**(15):1998-2009

[80] Zorn AM. Liver Development. In: *StemBook*. Harvard Stem Cell Institute: Cambridge (MA); 2008

[81] Kamiya A, Kinoshita T, Miyajima A. Oncostatin M and hepatocyte growth factor induce hepatic maturation via distinct signaling pathways. *FEBS Letters*. 2001;**492**(1-2):90-94

[82] Mortada I, Mortada R. Epigenetic changes in mesenchymal stem cells differentiation. *European Journal of Medical Genetics*. 2017;**61**(2):114-118

[83] Kaji K, Factor VM, Andersen JB, Durkin ME, Tomokuni A, Marquardt JU, et al. DNMT1 is a required genomic regulator for murine liver histogenesis and regeneration. *Hepatology*. 2016;**64**(2):582-598

[84] Lee CW, Huang WC, Huang HD, Huang YH, Ho JH, Yang MH, et al. DNA methyltransferases modulate hepatogenic lineage plasticity of mesenchymal stromal cells. *Stem Cell Reports*. 2017;**9**(1):247-263

[85] Iwatani M, Ikegami K, Kremenska Y, Hattori N, Tanaka S, Yagi S, et al. Dimethyl sulfoxide has an impact on epigenetic profile in mouse embryoid body. *Stem Cells*. 2006;**24**(11):2549-2556

[86] El-Serafi AT. Epigenetic modifiers and stem cell differentiation. *Cancer Stem Cells*. 2012;**8**:147-154

[87] Rothrock R, Lee KL, Isham KR, Kenney FT. Changes in hepatic differentiation following treatment of rat fetuses with 5-azacytidine. *Archives of Biochemistry and Biophysics*. 1988;**263**(2):237-244

[88] Yoon HH, Jung BY, Seo YK, Song KY, Park JK. In vitro hepatic differentiation of umbilical cord-derived mesenchymal stem cell. *Process Biochemistry*. 2010;**45**(12):1857-1864

[89] Henkens T, Papeleu P, Elaut G, Vinken M, Rogiers V,

Vanhaecke T. Trichostatin A, a critical factor in maintaining the functional differentiation of primary cultured rat hepatocytes. *Toxicology and Applied Pharmacology*. 2007;**218**(1):64-71

[90] Yan Y, Zhong B, Qi W, Song Y, Wu Y, Fan Y, et al. In vitro differentiation of mouse embryonic stem cells into functional hepatocytes by sodium butyrate, hepatocyte growth factor and dexamethasone under chemically defined conditions. *African Journal of Biotechnology*. 2011;**10**(46):9493-9500

[91] Cao J, Shang CZ, Lü LH, Qiu DC, Ren M, Chen YJ, et al. Differentiation of embryonic stem cells into hepatocytes that coexpress coagulation factors VIII and IX. *Acta Pharmacologica Sinica*. 2010;**31**:1478-1486

[92] Hay DC, Zhao D, Fletcher J, Hewitt ZA, McLean D, Urruticoechea-Uriquen A, et al. Efficient differentiation of hepatocytes from human embryonic stem cells exhibiting markers recapitulating liver development in vivo. *Stem Cells*. 2008;**26**(4):894-902

[93] Deng XG, Qiu RL, Wu YH, Li ZX, Xie P, Zhang J, et al. Overexpression of miR-122 promotes the hepatic differentiation and maturation of mouse ESCs through a miR-122/FoxA1/HNF4a-positive feedback loop. *Liver International*. 2014;**34**(2):281-295

[94] Khosravi M, Azarpira N, Shamdani S, Hojjat-Assari S, Naserian S, Karimi MH. Differentiation of umbilical cord derived mesenchymal stem cells to hepatocyte cells by transfection of miR-106a, miR-574-3p, and miR-451. *Gene*. 2018;**667**:1-9

[95] Proctor WR, Foster AJ, Vogt J, Summers C, Middleton B, Pilling MA, et al. Utility of spherical human liver microtissues for prediction of clinical drug-induced liver injury. *Archives of Toxicology*. 2017;**91**(8):2849-2863

[96] Underhill GH, Khetani SR. Bioengineered liver models for drug testing and cell differentiation studies. *Cellular and Molecular Gastroenterology and Hepatology*. 2018;**5**(3):426-439.e1

[97] Reinisch A, Bartmann C, Rohde E, Schallmoser K, Bjelic-Radisic V, Lanzer G, et al. Humanized system to propagate cord blood-derived multipotent mesenchymal stromal cells for clinical application. *Regenerative Medicine*. 2007 Jul;**2**(4):371-382

[98] Araújo AB, Salton GD, Furlan JM, Schneider N, Angeli MH, Laureano AM, et al. Comparison of human mesenchymal stromal cells from four neonatal tissues: Amniotic membrane, chorionic membrane, placental decidua and umbilical cord. *Cytotherapy*. 2017;**19**(5):577-585

[99] Olson H, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, et al. Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regulatory Toxicology and Pharmacology*. 2000;**32**(1):56-67

[100] Prodanov L, Jindal R, Bale SS, Hegde M, Mccarty WJ, Golberg I, et al. Long-term maintenance of a microfluidic 3D human liver sinusoid. *Biotechnology and Bioengineering*. 2016;**113**:241-246

[101] Jungermann K, Keitzmann T. Zonation of parenchymal and nonparenchymal metabolism in liver. *Annual Review of Nutrition*. 1996;**16**:179-203

[102] Hansen LK, Wilhelm J, Fassett JT. Regulation of hepatocyte cell cycle progression and differentiation by type I collagen structure. *Current Topics in Developmental Biology*. 2006;**72**(05):205-236

[103] Talaei-Khozani T, Khodabandeh Z, Jaberipour M, Hosseini A, Bahmanpour S, Vojdani Z. Comparison of hepatic nuclear factor-4 expression in two and three-dimensional culture of

Wharton's jelly-derived cells exposed to hepatogenic medium. *Romanian Journal of Morphology and Embryology*. 2015;**56**(4):1365-1370

[104] Khodabandeh Z, Vojdani Z, Talaei-Khozani T, Jaberipour M, Hosseini A, Bahmanpour S. Comparison of the expression of hepatic genes by human Wharton's jelly mesenchymal stem cells cultured in 2D and 3D collagen culture systems. *Iranian Journal of Medical Sciences*. 2016;**41**(1):28-36

[105] Kryou C, Leva V, Chatzipetrou M, Zergioti I. Bioprinting for liver transplantation. *Bioengineering*. 2019;**6**(4):95

[106] McCarty WJ, Usta OB, Yarmush ML. A microfabricated platform for generating physiologically-relevant hepatocyte zonation. *Scientific Reports*. 2016;**6**:26868

[107] Yen MH, Wu YY, Liu YS, Rimando M, Ho JHC, Lee OKS. Efficient generation of hepatic cells from mesenchymal stromal cells by an innovative bio-microfluidic cell culture device. *Stem Cell Research & Therapy*. 2016;**7**:120

[108] Ju X, Li D, Gao N, Shi Q, Hou H. Hepatogenic differentiation of mesenchymal stem cells using microfluidic chips. *Biotechnology Journal*. 2008;**3**(3):383-391

[109] Franzen N, van Harten WH, Retèl VP, Loskill P, van den Eijnden-van Raaij J, IJzerman M. Impact of organ-on-a-chip technology on pharmaceutical R&D costs. *Drug Discovery Today*. 2019;**24**(9):1720-1724

[110] Wilke RA, Lin DW, Roden DM, Watkins PB, Flockhart D, Zineh I, et al. Identifying genetic risk factors for serious adverse drug reactions: Current progress and challenges. *Nature Reviews. Drug Discovery*. 2007;**6**(11):904-916

[111] Van Den Berg A, Mummery CL, Passier R, Van der Meer AD. Personalised

organs-on-chips: Functional testing for precision medicine. *Lab on a Chip*. 2019;**2**:198-205

[112] McGettigan P, Henry D. Use of non-steroidal anti-inflammatory drugs that elevate cardiovascular risk: An examination of sales and essential medicines lists in low-, middle-, and high-income countries. *PLoS Medicine*. 2013;**10**(2):e1001388

[113] Mazaleuskaya LL, Sangkuhl K, Thorn CF, Fitzgerald GA, Altman RB, Klein TE. PharmGKB summary: Pathways of acetaminophen metabolism at the therapeutic versus toxic doses. *Pharmacogenetics and Genomics*. 2015;**25**(8):416-426

[114] Caddeo S, Boffito M, Sartori S. Tissue engineering approaches in the design of healthy and pathological in vitro tissue models. *Frontiers in Bioengineering and Biotechnology*. 2017;**5**:40

[115] Cayo MA, Mallanna SK, Di Furio F, Jing R, Tolliver LB, Bures M, et al. A drug screen using human iPSC-derived hepatocyte-like cells reveals cardiac glycosides as a potential treatment for hypercholesterolemia. *Cell Stem Cell*. 2017;**20**(4):478-489.e5

[116] Jia B, Chen S, Zhao Z, Liu P, Cai J, Qin D, et al. Modeling of hemophilia a using patient-specific induced pluripotent stem cells derived from urine cells. *Life Sciences*. 2014;**108**(1):22-29

[117] Sa-ngiamsuntorn K, Wongkajornsilp A, Phanthong P, Borwornpinyo S, Kitiyanant N, Chantratita W, et al. A robust model of natural hepatitis C infection using hepatocyte-like cells derived from human induced pluripotent stem cells as a long-term host. *Virology Journal*. 2016;**13**(59):1-18

[118] Choudhury Y, Toh YC, Xing J, Qu Y, Poh J, Huan L, et al. Patient-specific

hepatocyte-like cells derived from
induced pluripotent stem cells model
pazopanib-mediated hepatotoxicity.
Scientific Reports. 2017;7:41238

[119] Paganelli M, Dallmeier K, Nyabi O,
Scheers I, Kabamba B, Neyts J, et al.
Differentiated umbilical cord matrix
stem cells as a new in vitro model to study
early events during hepatitis B virus
infection. Hepatology. 2013;57(1):59-69