Chapter

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

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## 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 biotransformation activity of primary hepatocytes urged the stem cell differentiation into hepatocyte-like cells (HLCs). Umbilical cord-derived mesenchymal stem cells (UC-MSC), 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-MSC 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<br>Functions close to those of hepatocytes *in vivo*<br>Enable interspecies and pharmacogenomics studies<br>Representative of different lobular subpopulations | Viability: 2–4 h<br>No bile canaliculus<br>Low availability of human tissue<br>Interspecies differences | [6, 7] |
| Primary hepatocyte cultures (pHep) | Obtained from whole livers or biopsies<br>Functions close to those of hepatocytes *in vivo*<br>Longer viability than isolated tissue<br>Induction/inhibition of drug-metabolizing enzymes<br>Enable interspecies and pharmacogenomics studies | Viability: 2–4 days<br>Early phenotypic changes<br>Altered bile canaliculi<br>Difficult recovery of cells and maintenance of function upon cryopreservation<br>Low availability of human tissue<br>Interspecies differences | [3, 7] |
| Hepatoma cell lines (HepG2, Huh7) | High proliferation activity and good availability<br>Well-characterized and abundant data available | Decreased drug enzyme activities<br>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, CD79α 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.

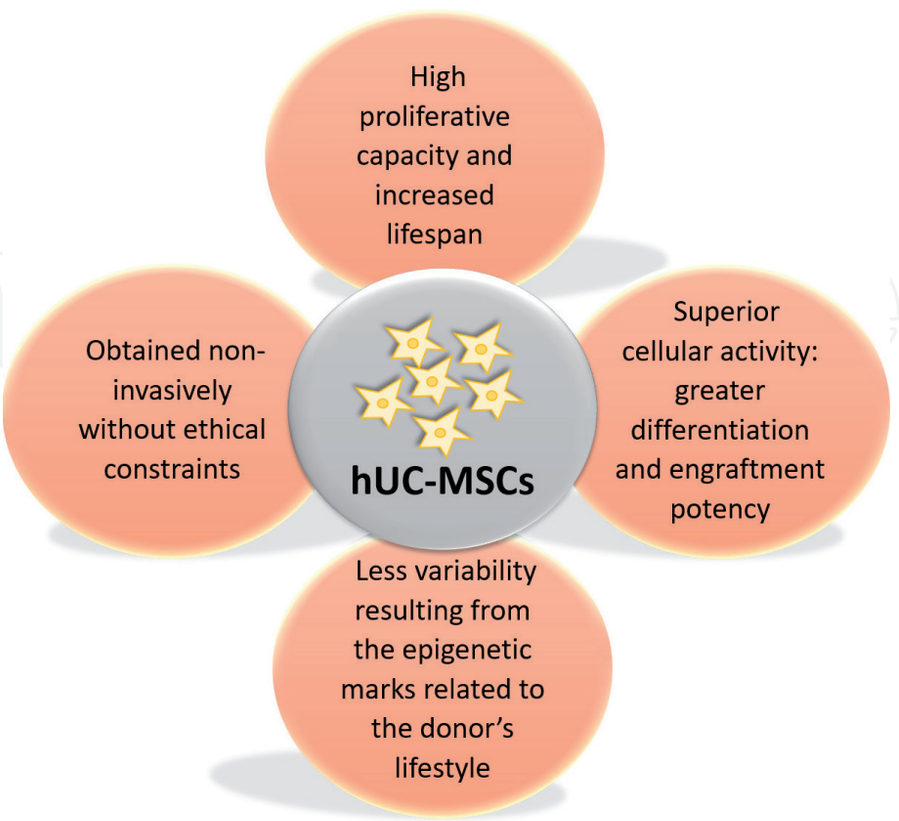


**Figure 1.**
*Main advantages of human umbilical cord MSCs (hUC-MSCs) over other MSC sources.*

## 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

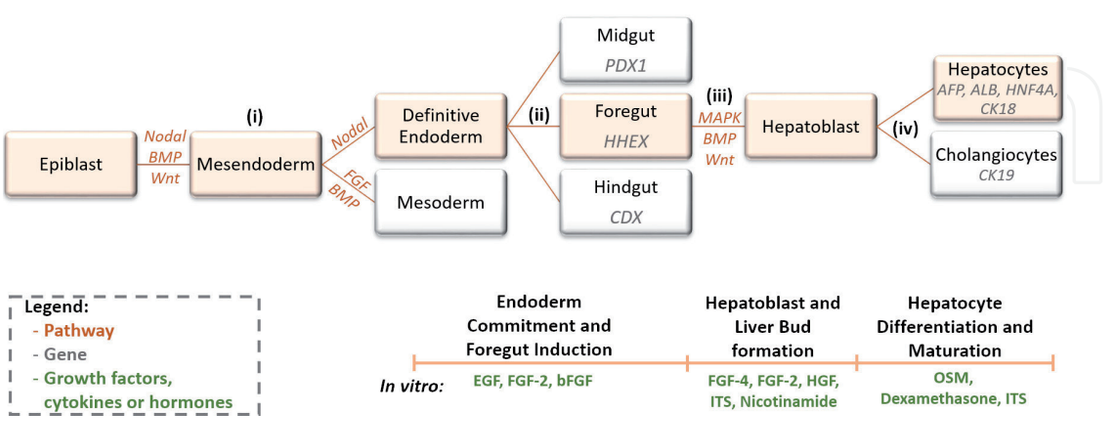


**Figure 2.**
*Hepatogenesis and the respective inducing factors for* in vitro *differentiation of MSCs into HLCs. The addition of a combination of soluble factors to the culture, at defined time points, to mimic (i) endoderm commitment and (ii) foregut induction (EGF and FGF), followed by (iii) hepatoblast 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 synthetized DNA strain 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

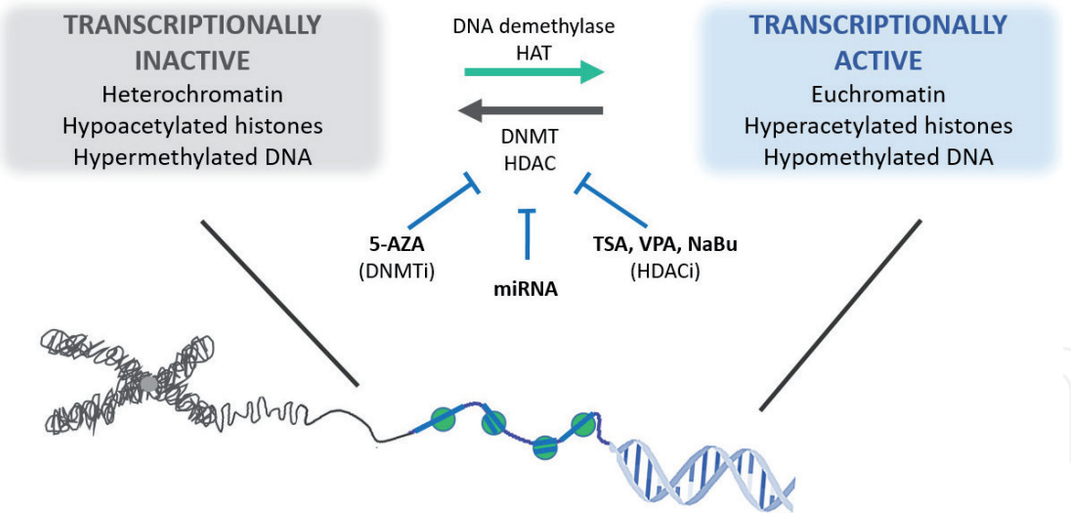


**Figure 3.**
*Control of gene transcription by epigenetic modifiers. Hypomethylated CpG islands and hyperacetylated histone tails at the chromatin level allow gene transcription. DNMT inhibitors (DNMTi), microRNA (miRNA) and HDAC inhibitors (HDACi) modulate the chromatin structure by creating an open, 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 (HDCAs) 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, α1 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

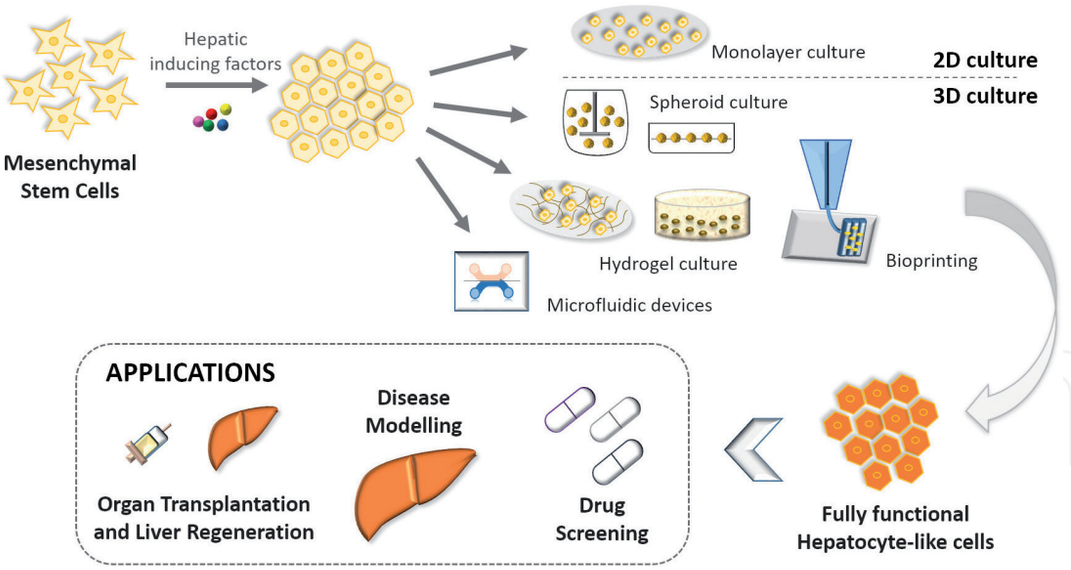


**Figure 4.**
*2D and 3D culture strategies for the differentiation of MSCs into HLCs and potential clinical applications. Several strategies such as spheroid cultures, scaffolds, bioprinting and microfluidics have shown promissory results and represent good tools for future studies on drug screening,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

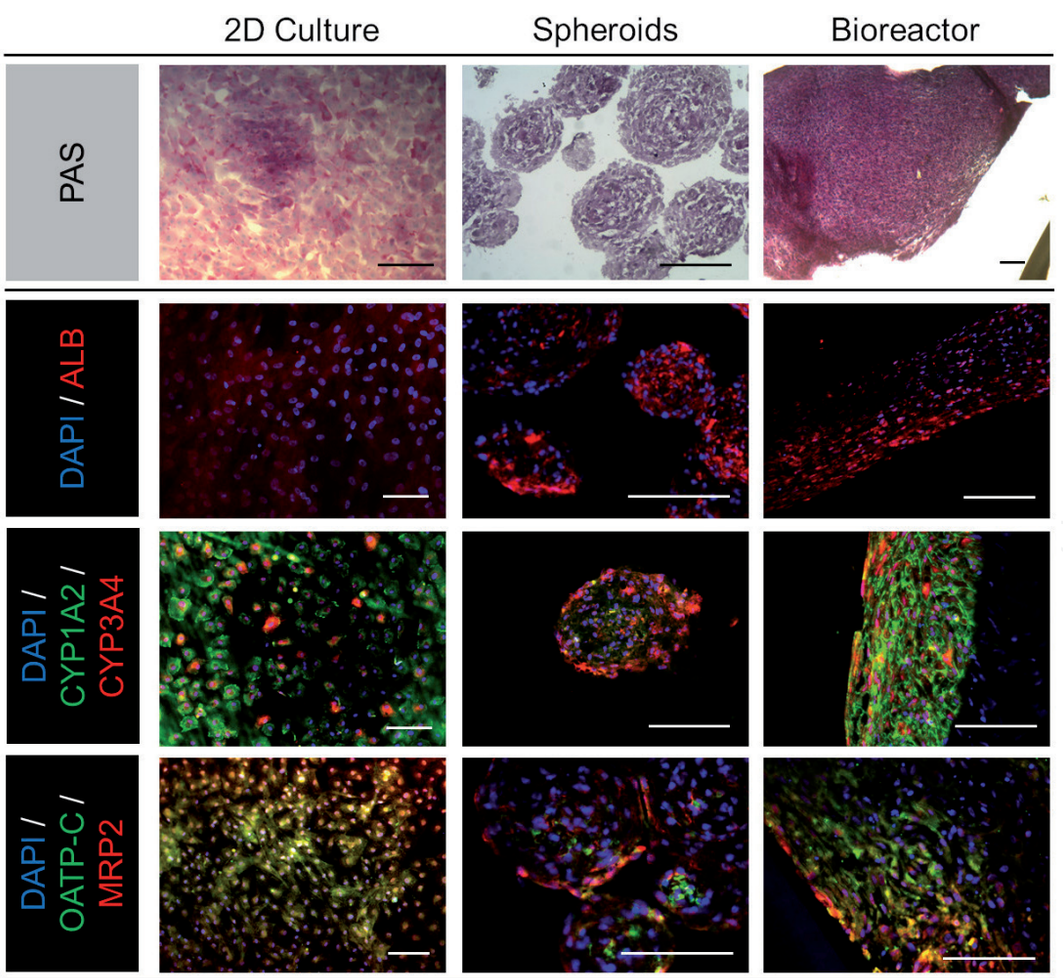


**Figure 5.**
*Immunohistochemical and PAS analysis of HLCs-derived UCM-MSCs cultured in 2D cultures, in spheroids and in bioreactors. Representative images show the presence of the plasma protein ALB, the efflux transporter MRP-2, the uptake transporter protein OATP-C and the biotransformation enzymes 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,

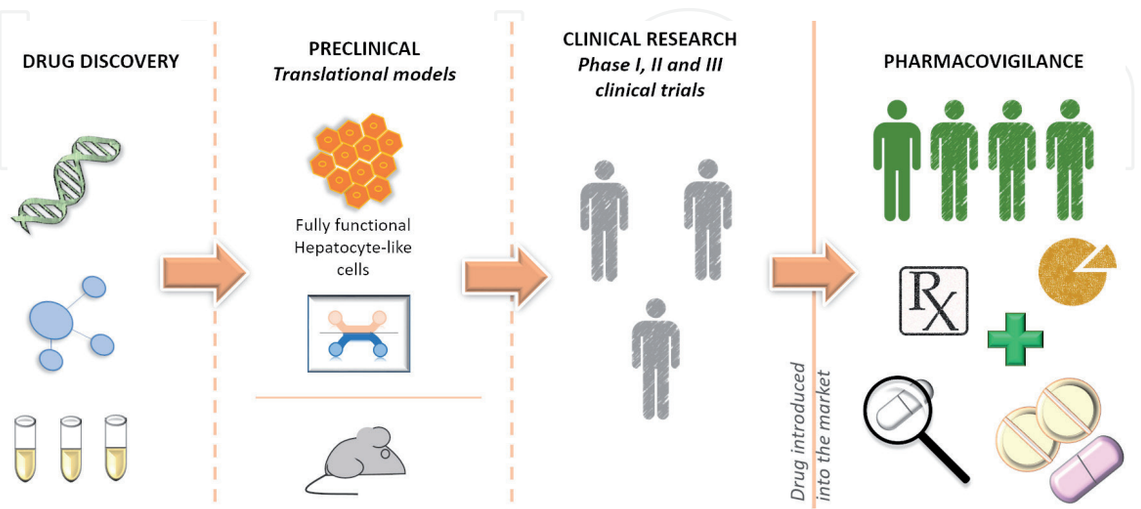


**Figure 6.**
*Potential of mature, fully functional HLCs on drug discovery and drug development. Conventional drug development pipelines involve preclinical* in vitro *and* in vivo *research in animal models followed by clinical trials on humans.* In vitro *toxicology models such as fully functional 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 felt 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 trough 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.

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## Conflict of interest

The authors declare no conflict of interest.

## Appendices and Nomenclature

| | |
|---|---|
| 2D | two-dimensional |
| 3D | three-dimensional |
| 5-AZA | 5-azacytidine |
| AAT | α1 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 |

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