Cell sources for *in vitro* human liver cell culture models

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Abstract

In vitro liver cell culture models are gaining increasing importance in pharmacological and toxicological research. The source of cells used is critical for the relevance and the predictive value of such models. Primary human hepatocytes (PHH) are currently considered to be the gold standard for hepatic *in vitro* culture models, since they directly reflect the specific metabolism and functionality of the human liver; however, the scarcity and difficult logistics of PHH have driven researchers to explore alternative cell sources, including liver cell lines and pluripotent stem cells. Liver cell lines generated from hepatomas or by genetic manipulation are widely used due to their good availability, but they are generally altered in certain metabolic functions. For the past few years, adult and pluripotent stem cells have been attracting increasing attention, due their ability to proliferate and to differentiate into hepatocyte-like cells *in vitro*. However, controlling the differentiation of these cells is still a challenge. This review gives an overview of the major human cell sources under investigation for *in vitro* liver cell culture models, including primary human liver cells, liver cell lines, and stem cells. The promises and challenges of different cell types are discussed with a focus on the complex 2D and 3D culture approaches under investigation for improving liver cell functionality *in vitro*. Finally, the specific application options of individual cell sources in pharmacological research or disease modeling are described.

Keywords: Liver cell culture, *in vitro* models, 3D culture technologies, primary human hepatocytes, liver cell lines, stem cells

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Introduction

Various *in vitro* liver cell culture models are under development to address the need for predictive models in drug development and research. Significant progress has been made within the last few years in the improvement of culture systems, leading to the enhanced stability and functionality of liver cells *in vitro*. However, a major bottleneck in the use of *in vitro* human hepatic models is the identification of suitable liver cell sources.

The main criterion for evaluation of the value of hepatic cells in basic research or pharmacological studies is the expression of typical hepatic functions and metabolic pathways. Important functions of the liver include: (i) metabolism of endogenous substrates (e.g. cell products) and exogenous compounds (e.g. drugs, chemicals); (ii) regulation of amino acids, carbohydrates, and fatty acids, (iii) synthesis of proteins, such as albumin or transferrin; and (iv) activation of inflammatory and immune reactions upon liver injury due to disease, drug, or toxin exposure.

Depending on the study aim and design, the cell type used in hepatic *in vitro* research has to fulfill each or some of those functions to reflect the situation in the native organ *in vivo*. Furthermore, preservation of hepatocyte functionality over several hours (e.g. for short-term studies on drug metabolism) or up to several days or even weeks (e.g. for long-term studies on subacute or subchronic drug toxicity) is needed to acquire relevant data. Since the stability and maintenance of the differentiated state of liver cells depends on both, the cell type used and the method of cultivation (e.g. 2D cultures or complex 3D cultures), the choice of culture model in association with a specific hepatic cell source is critical for the success of individual hepatic *in vitro* studies. In addition, standardization of experiments is required to provide reproducible and reliable results from *in vitro* hepatic cultures. Thus, a constant quality of the cells needs to be ensured and verified by appropriate quality control measures. Finally, the availability of cells is a critical factor for the usage of certain cell types in *in vitro* research. This aspect is of particular importance for studies requiring large numbers of cells and/or experiments.

In this review, the liver cell sources currently used are critically reviewed with their respective advantages and disadvantages with regard to their *in vitro* applications in drug testing and hepatic disease research. Since the predictive value of animal-derived cells is limited due to species-dependent differences in the expression of metabolic enzymes and pathways, this review focuses on

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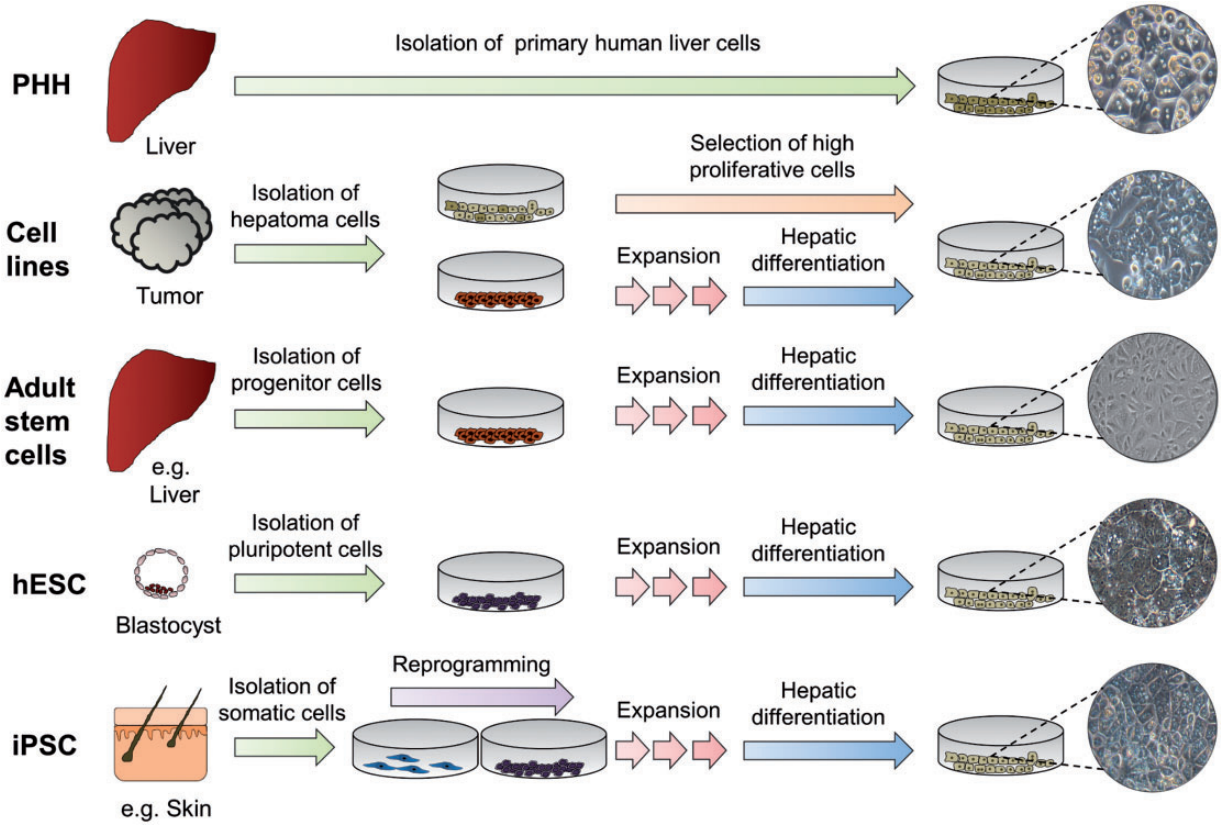

Figure 1 Processing of cells derived from different sources for the generation of human *in vitro* liver cell culture models. Major cell types include primary human hepatocytes (PHH), hepatoma cell lines, adult stem cells, human embryonic stem cells (hESC), and induced pluripotent stem cells (iPSC). Whereas PHH can be used for *in vitro* cultivation immediately after isolation, liver cell lines or stem cells need to be expanded and/or differentiated prior to their use in experiments. (A color version of this figure is available in the online journal.)

human hepatic cells from different sources, namely primary human liver cells, liver cell lines and stem cells, as shown schematically in Figure 1. In addition, approaches for increasing the functionality and stability of liver cells *in vitro* by the use of complex 2D and 3D culture models are discussed.

Primary human liver cells

General characteristics of primary human liver cells

Primary human liver cells are still considered to be the gold standard for the creation of human-relevant *in vitro* liver cell culture models. Due to their origin in native liver, they reflect the complete functionality of the human organ *in vivo* and thus provide highly predictive results in pharmacological and toxicological *in vitro* research. Furthermore, the fact that each preparation is obtained by a different donor offers the opportunity to analyze a broad range of genetic polymorphisms using individual cell isolates. On the other hand, interindividual differences and cell alterations due to the isolation procedure also cause some variations in experimental results, which make the standardization of models difficult. In addition, the scarce availability and difficult logistics of primary human liver cells prevent a larger scale use of the cells. In the following, critical aspects of the use of primary human liver cells for *in vitro* research are reviewed, including approaches to isolation, cryopreservation, and cultivation.

Cell types of the liver

The composition of the liver can be divided into two major classes of cells. Parenchymal cells of epithelial origin, namely hepatocytes and cholangiocytes, form the main liver mass[1] and represent 70 and 3–5% of the total liver cell population, respectively, as shown in rodents.[2] Non-parenchymal cells (NPC) account for approximately 25% of the total liver cell population, consisting mainly of Kupffer cells, liver endothelial cells, and hepatic stellate cells.[3]

Isolation of primary human liver cells

Primary human hepatocytes (PHH) are usually isolated from whole livers (not used for orthotopic transplantation) or resected liver tissue.[4] The development of the two-step collagenase perfusion technique introduced by Seglen and Reith[5] in 1976 was a key step and has become the centerpiece of isolation protocols for PHH. Since then, this method has been modified and optimized in various regards.[6] Recent modifications have involved adaptations for parallel isolation and separation of NPC. Supplementation of the collagenase P containing perfusion solution with 10% fetal calf serum (FCS) allows a prolonged but mild tissue digestion, resulting in a high yield and

quality of parenchymal and non-parenchymal liver cells from a single donor's tissue.[3] In another approach, density gradient separation of NPC in iodixanol and magnetic-activated cell sorting has been used for efficient separation of individual NPC populations.[7]

The cell isolation outcome depends on donor characteristics and intraoperative factors, in addition to tissue processing and cell isolation conditions.[8] Lee *et al.* investigated the PHH isolation outcome of 1034 donors.[9] The study revealed that cell viability was significantly influenced by donor characteristics, such as age, body mass index, liver fat content, liver damage (e.g. fibrosis), and the resulting changes in the clinical parameters in the blood (e.g. liver enzymes, bilirubin). In addition, the blood coagulability, warm ischemic time *in vivo* during surgery, and cold ischemic time *in vitro* during tissue transport were identified as critical factors for the success of the isolation. Surgical procedures which involve increased warm ischemia times, e.g. due to clamping during surgery, can lead to an impaired yield and viability of PHH.[8] In contrast, portal vein embolization showed no negative influence on isolation outcome,[10] and the yield of PHH was even increased with warm ischemia times *ex vivo*, and when the patients received chemotherapeutic treatment.[9] Furthermore, the type of disease was identified to have a significant impact upon the cell yield.[11] In particular, alcohol-related liver diseases were shown to cause alterations in hepatocyte function in culture.[11]

Apart from resected liver tissue, liver grafts excluded from liver transplantation or explanted diseased livers are potential sources of PHH. Human liver cells isolated from explanted livers obtained from recipients with moderate alterations in blood parameters (low labMELD score) were comparable in viability and function to cells obtained from specimens following partial liver resection.[4]

Cryopreservation of primary human liver cells

One major limitation of primary human liver cells is the availability of human tissue of good quality. The logistics and the isolation process require the staff to be experienced and to cooperate closely with surgical clinics. Therefore, a well-organized network of surgeons, biologists, and technicians is needed to obtain high quantities of high quality cells. Cryopreservation was introduced to overcome this hurdle and to provide primary human liver cells on a regular basis. A multitude of cryopreservation protocols for hepatocytes is available, which are mostly based on the use of cryoprotectants such as dimethyl sulfoxide (DMSO), albumin, FCS, and/or polyvinylpyrrolidone.[12] Successful cryopreservation critically depends on the cells being of good quality. In order to increase cell viability after freezing and thawing, preincubation with cryoprotectants like fructose and dithiothreitol or DMSO has been investigated.[13,14] Other approaches use a modified basic cryopreservation solution, e.g. HepatoZYME-SFM[15] or STEM-CELLBANKER®,[16] resulting in increased cell viability and function after thawing.

The isolation of NPC is a younger discipline and cryopreservation is still under development. Successful cryopreservation of rat and human Kupffer cells with high post-thawing cell survival and no alterations in physiological characteristics were introduced by Walbrun *et al.*,[17] using a standard cryopreservation technique based on RPMI medium with FCS and DMSO frozen down to -80°C using a cooling gradient. The cryopreservation of hepatic stellate cells requires more elaborated preservation methods due to the sensitivity of intracellular lipid droplets to freezing. A complex protocol for the storage of rat hepatic stellate cells using a precultivation step, a cryopreservation medium containing FCS and DMSO as cryoprotectants, and a complex freezing gradient was demonstrated to maintain the functional and structural integrity of the cells after cryopreservation.[18] A more simple protocol using the cryopreservation solution STEM-CELLBANKER® was applied by Nakamura *et al.*[19] showing that cryopreservation of hepatic stellate cells in different stages of activation is possible. Cryopreservation protocols specific to liver endothelial cells have not yet been described. However, the cryopreservation of endothelial cells from other tissues has been recently shown.[20] Furthermore, standardized protocols for the freezing of endothelial cells from umbilical cord veins under Good Manufacturing Practice conditions have been established,[21] which may also be tested for the cryopreservation of liver endothelial cells.

In vitro processing of primary human liver cells

PHH display the *in vivo* hepatic situation best during short-term cultivation regimes; when cultured over extended periods these cells manifest a progressive loss of the hepatocellular phenotype, both in terms of morphology and functionality. This de-differentiation process starts during the isolation process.[22] Hepatocytes are arranged in sheets of one or two cells in thickness in the liver lobule. The plasma membrane of hepatocytes is divided into different membrane areas exhibiting specific tasks, the apical membrane forming the bile canalicular network and the lateral membrane forming the cell–cell connection with adjunct hepatocytes. The basolateral membrane area is oriented toward the liver sinusoid and interacts with the collagen I-rich matrix in the space of Disse, which separates the liver sinusoidal endothelial cells from hepatocytes. During the isolation process, cell–cell connections and cell–matrix interactions are disrupted and as a consequence the hepatocytes lose their polarization. Moreover, the low-speed centrifugation of the hepatocyte cell suspension performed for removal of cell debris leads to partial loss of NPC, which are critical for the support of hepatocyte polarity *in vitro*.[6]

NPC, as well as other parenchymal cells, induce a quiescent G0 state in the hepatic cell cycle *in vivo* via cell–cell connections and soluble factors. Reperfusion injury during the isolation procedure induces oxidative stress, leading to activation of intracellular inflammatory pathways.[23] Additionally, the hepatocytes may come into contact with lipopolysaccharides (LPS) contained in collagenase P preparations from bacterial origin. Disruption of the tissue integrity as well as activation of inflammatory signaling has been reported to trigger hepatocytes to re-enter the cell cycle in the G1 phase.[24] The

proliferative priming is associated with a change in the expression pattern and consequently a loss of differentiated functions, in particular xenobiotic metabolism, urea formation, and albumin synthesis, among others.[25] This detrimental process of de-differentiation can be influenced by the cultivation strategy, as described in the following.

2D and 3D culture models for PHH

The conventional 2D culture of hepatocytes is easily achievable and thus represents the most common culture strategy. In standard 2D culture systems, PHH are directly plated on collagen-coated plastic dishes that allow cell attachment to form a confluent monolayer.[26] In comparison to hepatocytes maintained in suspension, these adherent cultures show increased enzyme stability and therefore can be used for the evaluation of metabolic stability of xenobiotics over several hours to days.[27] Recent investigations into drug transporter expression in 2D hepatocyte cultures revealed a polarized location of major sinusoidal and canalicular transporters in conjunction with corresponding transporter activities.[28] However, there is evidence that PHH cultured in conventional 2D systems for a prolonged time period display morphological alterations, provoked by an epithelial–mesenchymal transition leading to a loss of hepatocyte polarity and associated liver function.[29]

Typical strategies to prevent the de-differentiation of PHH *in vitro* aim to reestablish the microenvironment of the liver, consisting of cell–cell contacts, cell–matrix interactions, and soluble factors. In this regard, the 3D cultivation of PHH has gained increasing attention. It has been shown that 3D culture of PHH in a sandwich configuration between two layers of extracellular matrix (ECM) enables the hepatocytes to adhere three-dimensionally, leading to the formation of *in vivo* like cell–cell contacts and cell–matrix interactions.[29,30] The ECM composition appears to influence the cell shape and cell–cell contacts between hepatocytes.[31] It has been shown that the maintenance of xenobiotic metabolism in hepatocyte cultures depends primarily on the formation of proper cell–cell contacts,[32] whereas hepatic transport processes are mainly dependent on the repolarization linked to cell–matrix interactions.[22] The ECM components used for sandwich culture of hepatocytes are rat-tail collagen, Matrigel (a mixture of basal membrane components derived from mouse sarcoma) or various combinations of both matrices.[33] Transcriptional profiling revealed an improved expression of effectors of energy metabolism and bile acid metabolic pathways in hepatocytes cultured between two layers of collagen.[34] Similarly, covering hepatocytes with Matrigel has been shown to improve cellular stress responses by reducing the expression of pro-inflammatory mediators and the genes associated with integrin pathways.[35] More advanced 3D cultures mimic the liver microenvironment in cross-linked hyaluronic acid gels supplemented with soluble ECM from de-cellularized organs to improve hepatocyte attachment and viability.[36]

A scaffold-free approach to establish 3D conditions for PHH is based on the self-aggregation of the cells leading to the formation of spheroids. Such spheroid cultures can be obtained either by gravity-enforced cellular assembly in a hanging drop[37] or by seeding the cells in low-attachment culture vessels.[38,39] In both cases the obtained spheroids were shown to exhibit liver-specific functions for up to five weeks for long-term toxicity testing.[37,38] Spheroid cultures can also be used for the creation of larger constructs reflecting the physiological liver architecture. For example, PHH aggregates encapsulated in hydrogels maintained a stable hepatocyte phenotype for 21 days.[40] Such cell-laden hydrogels can be assembled by multiphase liquid–liquid systems to create 3D tissue constructs, as shown initially for mouse fibroblasts.[41] In another strategy the PHH spheroids are loaded into a bioprinter that deposits the cell aggregates simultaneously with a support structure (often hydrogel) according to a specific design template resembling the liver architecture.[42,43] Such "liver-on-a-chip" approaches provide an attractive tool to generate 3D hepatocyte cultures reflecting the *in vivo* situation at a microscale with reduced cell numbers for toxicity studies.

Co-culture of hepatocytes with NPC

Apart from hepatocyte cell–cell connections and cell–matrix interactions, the crosstalk of hepatocytes with NPC via soluble factors and cell–cell connections plays a central role in liver physiology and the maintenance of hepatocyte differentiation.[44] Recently described co-culture models of PHH and Kupffer cells enabled the evaluation of hepatocyte reactions in a pro-inflammatory environment.[45,46] A co-culture model using established cell lines for hepatocytes and stellate cells displayed remodeling processes of ECM components after the induction of hepatic steatosis.[47] Co-culture of PHH with endothelial cells was shown to support hepatocytes in the maintenance of their phenotypic morphology and in the improvement of their specific functions, in addition to the formation of capillary-like structures by co-cultured endothelial cells.[48] Recent approaches to stabilize hepatic functions of PHH are based on micropatterning techniques, which allow controlling the cell arrangement in co-culture platforms (micropatterned co-culture [MPCC]). The co-cultures in MPCC are created by seeding the hepatocytes on collagen-coated spots, which are surrounded by supporting cells, e.g. fibroblasts.[49,50] Using a number of clinically relevant drugs Khetani *et al.* could show a higher sensitivity of the MPCC as compared to conventional short-term cultures for prediction of human hepatic drug toxicity.[51]

More sophisticated tissue engineering strategies, considering the specific requirements of individual cell types, have to be conceived for the co-cultivation of more than two cell populations. For example, Kupffer cells (but also other NPC) have to undergo a recovery phase before the start of experiments to avoid activation due to the cell stress induced by the isolation procedure.[3,52] Two major approaches were attempted for the realization of complex co-culture models: One approach focuses on the use of mixtures of PHH and one or more NPC populations to allow for spontaneous re-aggregation and the formation of self-organized cell–cell contacts resulting in organoids.[37] The second approach is based on targeted engineering of

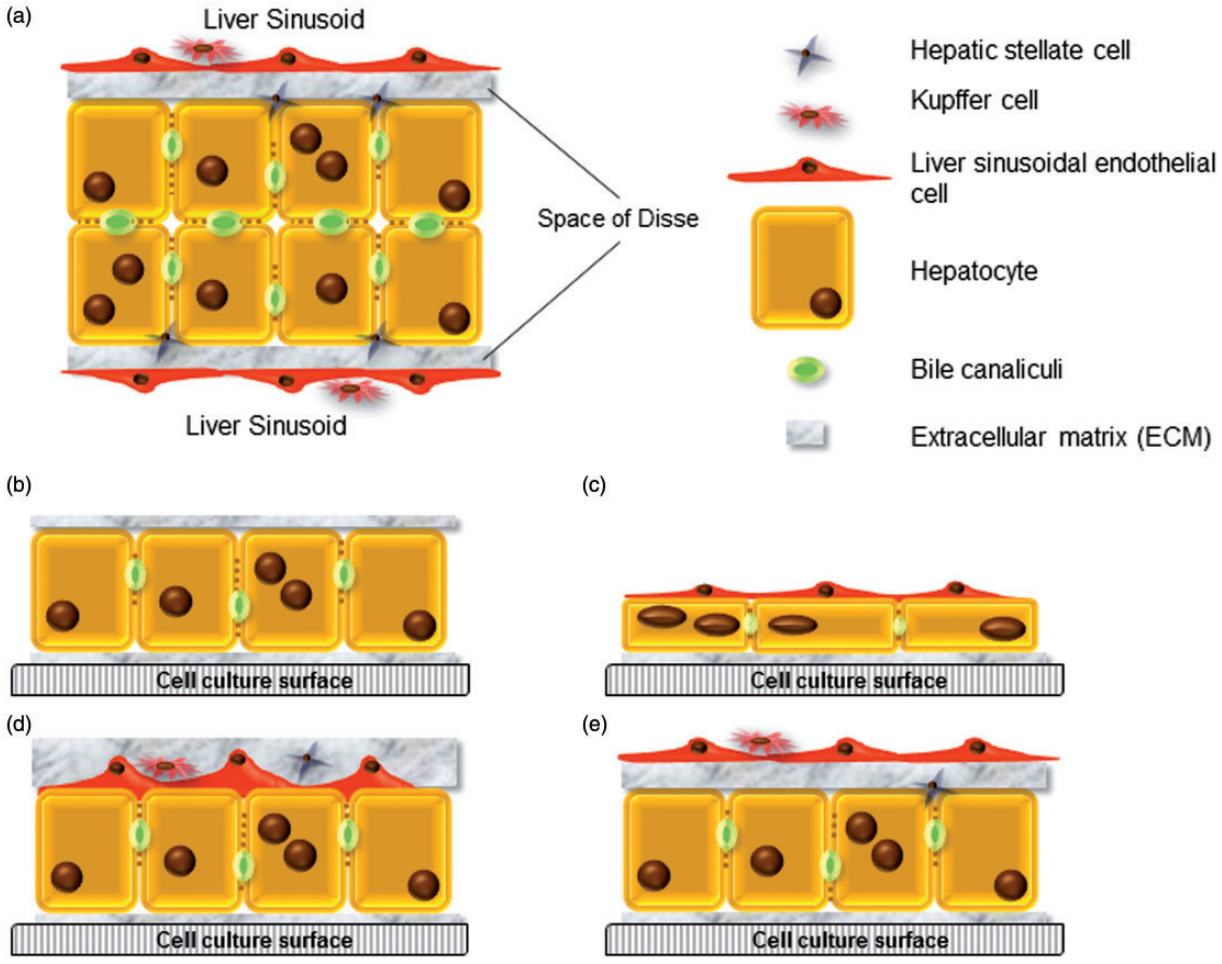

Figure 2 Approaches for improved co-culture liver models aim to approximate the structure of the native liver. (a) Schematic image of the tissue architecture in the liver sinusoid, (b) current 3D sandwich culture with primary human hepatocytes (PHH) embedded between two layers of extracellular matrix (ECM), e.g. gelled collagen,[29] (c–e) Potential concepts for 3D co-cultivation of PHH with non-parenchymal cells (NPC): (c) PHH grown on an ECM layer and overlaid with NPC, e.g. liver sinusoidal endothelial cells, (d) mixture of PHH and NPC, cultured between two layers of ECM, (e) tissue-engineered liver model consisting of PHH and hepatic stellate cells cultured in a sandwich configuration and overlaid with NPC fractions, e.g. liver sinusoidal endothelial cells and Kupffer cells. Different cell culture surfaces, such as plastic or membranes permeable for gas or solutes can be used. (A color version of this figure is available in the online journal.)

individual layers of PHH and NPC to create *in vitro* tissues reflecting the tissue architecture.[53] Concepts for the establishment of co-culture models utilizing hepatocytes and various NPC fractions are shown in Figure 2.

Dynamic 3D culture technologies and bioreactor approaches

Dynamic 3D culture models are of increasing importance in *in vitro* hepatic research, since they better reflect the perfusion conditions in the native tissue when compared to static culture systems. A number of studies have shown the potential of 3D perfusion culture to support the organoid-like cell assembly of human hepatocytes. For example, a perfused 3D microfluidic human liver model was reported to support liver-typical cell arrangement and morphology.[54] Similarly, human hepatocytes, maintained in the microfluidic LiverChip™ system showed differentiated tissue structures and cytochrome P450 (CYP)-dependent activities over seven days of culture.[55] In further approaches, microfluidic devices were used with various combinations of cell types and ECM components, such as co-culture of hepatocytes with intestinal cells for investigations of drug adsorption and metabolism,[56] hepatocyte culture in a collagen sandwich configuration with flow characteristics,[57] or using de-cellularized scaffolds as a physiological matrix for cell maintenance under dynamic conditions.[58,59]

The need for both efficient medium and oxygen transfer to the cells while allowing 3D tissue assembly is addressed by a dynamic four-compartment 3D bioreactor technology for high-density liver cell culture.[60] The bioreactor structure is illustrated in Figure 3. Cells are cultured within a 3D scaffold made of different types of hollow-fiber capillaries, which are arranged in two or more layers composed of medium and oxygen capillaries. The capillary membranes enable the transfer of solutes via hydrophilic membranes and gas exchange via hydrophobic membranes, in addition to their function as an adhesion matrix for the cells cultured between the capillaries. By this way, four compartments (two counter-currently perfused medium compartments,

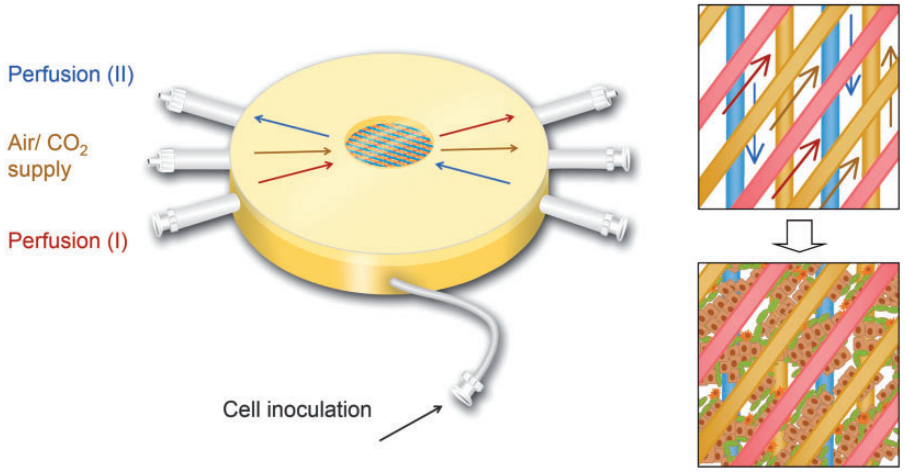

Figure 3 Miniaturized four-compartment bioreactor for high-density perfusion culture of liver cells.[66] The bioreactor technology is based on two or more layers of hollow-fiber capillaries, which serve for counter-current medium perfusion (marked in red and blue) and air/CO_2 supply (yellow). Cells are inoculated into the extra-capillary space (cell compartment). The schematic pictures on the right show the capillary structure viewed from top before and after cell seeding. (A color version of this figure is available in the online journal.)

one gas compartment and the cell compartment) are created, which form multiple repetitive units for the decentralized supply of cell aggregates with low gradients. To ensure constant culture conditions, bioreactors are operated in a perfusion device, which enables electronic control of temperature, medium perfusion rates, gas mixture, and gas supply.

Based on large-scaled versions of the technology used for clinical extracorporeal liver support therapy,[61,62] the technology was scaled down to small-scale laboratory bioreactors to reduce the cell number and amount of reagents needed for *in vitro* pharmacological studies.[63,64] Histological and immunohistochemical studies revealed the spontaneous reorganization of primary human parenchymal and non-parenchymal liver cells (biliary cells, endothelial cells, stellate cells, Kupffer cells) between the capillaries.[63] These studies also showed hepatocyte polarization, as assessed by the detection of apical membrane transporters, such as multi-drug-resistance-associated protein 2, multidrug-resistance protein 1, and breast cancer resistance protein. The stable maintenance of differentiated functions of primary human liver cells in the bioreactor under serum-free conditions was shown in miniaturized bioreactors.[65] A better preservation of drug-metabolizing enzymes was also observed in the bioreactor system when compared to static 2D cultures.[66] Thus, the technology provides suitable conditions for pharmacological studies with human hepatocytes and shows the benefit of dynamic 3D culture conditions for long-term preservation of liver-specific functions *in vitro*.

Liver cell lines

General characteristics of liver cell lines

Human hepatic cell lines generated from tumor tissue or by the genetic engineering of primary human liver cells are widely used in *in vitro* culture models due to their good availability. The high proliferation capacity and the stable metabolism of the cells make them an attractive tool for *in vitro* studies under standardized and reproducible conditions. However, the high proliferation potential of transformed cell lines generally is associated with a loss of differentiated functions, leading to some deficiencies in functional performance. Therefore, applications of liver cell lines in *in vitro* research have to take into account the specific functional properties of the cell line used.

Applications of liver cell lines in in vitro research

Tumor cell lines, such as the hepatoma cell line HepG2, are of particular interest for *in vitro* studies on cancer development and therapy. Reports focus on the investigation of specific metabolic pathways related to liver tumors or the testing of drug candidates for cancer therapy. In order to cover the different types of cancer with different sensitivities, assays are often performed in various tumor cell lines from different origins in parallel. For example, HepG2 cells and two human cholangiocarcinoma cell lines were used in a recent study to analyze the expression and regulation of cancer-related transcription factors and showed some variation in their responses.[67] In another study, liposomal C8 ceramide was shown to have a tumor-suppressive effect in several hepatocellular carcinoma cell lines, including HepG2 cells, while non-tumor hepatocytes remained unaffected.[68] The HepG2 cell line has also been used in various studies on drug metabolism and hepatotoxicity, e.g. on acetaminophen toxicity and prevention.[69] However, alterations in the hepatocyte-specific functions of the cell line due to transformation limits how closely these results relate to results for humans.

The HepaRG cell line generated from human hepatoma cells represents a promising alternative to the use of PHH for many studies on drug metabolism, disposition, and toxicity.[70,71] Differentiation of the cell line is induced by treatment with DMSO and leads to the generation of both hepatocytes and biliary cells. HepaRG cells show a high grade of differentiation and expression of typical hepatic functions, including CYP-dependent metabolism, CYP induction, and drug transporter expression.[72] Evaluation

of HepaRG cells as a model for drug toxicity testing showed a similar response to the effects of acetaminophen as PHH and a higher activation of genes related to liver damage as compared to HepG2 cells.[73] However, other studies showed a reduced sensitivity of HepaRG cells to the detection of hepatotoxic drugs.[74] These findings are in line with the discrepancy observed between HepaRG cells and PHH in the activation of apoptosis or necrosis upon exposure to acetaminophen.[75] Thus, HepaRG cells could preferably be used as a substitute to PHH in screening studies for CYP induction.

Strategies for improving the functional performance of hepatoma cell lines

To increase the functional performance of hepatoma cell lines, various strategies for genetic modification of the cells have been described. For example, transfection of HepG2 cells with human augmenter of liver regeneration resulted in increased synthesis of alpha-fetoprotein, urea, and albumin in comparison to non-transfected control cells.[76] An improvement in liver-specific functions, including expression of drug-metabolizing enzymes, was also observed after transient transfection of human hepatic cells with hepatocyte nuclear factor 4 alpha (HNF4α).[77] Using a lentiviral expression system, a range of cell lines were generated that express specific CYP enzymes individually and thus can be used to assess metabolism-associated toxicity of drugs.[78] In another strategy, transduction of PHH with selected immortalization genes was used to generate proliferating cell lines with liver-specific functionality.[79]

Further approaches to improving the functional performance of hepatoma cell lines are directed toward creating a microenvironment favoring cell differentiation and liver-typical cell assembly *in vitro*. For example, spheroid culture of HepG2 cells in a 3D matrix using different types of hydrogels (transglutaminase-cross-linked gelatin, collagen type I, and growth-factor depleted Matrigel) was associated with enhanced hepatocyte-like properties, when compared with conventional cell culture.[80] Microencapsulation of HepaRG cells in alginate spheroids was shown to support polarization of hepatocyte-like cells, which were arranged in an interconnected bile canalicular network and showed biliary transporter activity.[81] Tissue-like cell organization of HepaRG was also observed in four-compartment hollow-fiber bioreactors in conjunction with stable activities of several human-relevant CYP enzymes over several weeks.[82] These studies emphasize the importance of a physiological environment to enhance the predictivity and stability of hepatic *in vitro* models.

Stem cell-derived hepatocytes

General characteristics of stem cells

Stem cells characterized by their ability to proliferate and to differentiate into specific cell types *in vitro* offer the option to generate mature hepatic cells in large amounts with constant quality for *in vitro* investigations. In accordance to their differentiation potential and origin, stem cells can be classified into two major groups: (i) adult stem cells originating from liver or from non-hepatic cells, e.g. mesenchymal stem cells (MSC), and (ii) pluripotent stem cells, represented mainly by human embryonic stem cells (hESC) and human-induced pluripotent stem cells (hiPSC). Differences in the proliferation and differentiation properties of adult and pluripotent stem cells are responsible for their individual advantages and drawbacks in *in vitro* applications.

Adult stem cells

Adult stem cells have been described in the liver as hepatic stem cells,[83] small hepatocytes,[84] or progenitor cells,[85] based on functional and morphological criteria. Different methods for isolation, culture, and differentiation of liver stem cells from human adult or fetal liver have been described.[86–88] *In vitro* studies on adult stem cells from human or mouse liver showed that their *in vitro* behavior is influenced by cytokines, growth factors, hormones, and ECM proteins.[89–91] The importance of soluble factors is emphasized by the observation of progenitor activation in human liver cell bioreactors perfused with plasma from acute liver failure patients.[60] However, the production of larger cell numbers and standardization of the isolation and culture methods for *in vitro* applications remain a challenge.

MSC, able to differentiate into hepatocyte-like cells, have been detected in various tissues, including bone marrow,[92] adipose tissue,[93] and umbilical cord blood or tissue.[94] MSC are of particular interest in regenerative medicine, such as cell transplantation in liver diseases, since they allow autologous therapy with the patients' own cells and the safety risk is low when compared, for example, with pluripotent stem cells. However, there is some evidence that they could also be used in *in vitro* research if differentiation of these cells into fully maturated cells is achieved.[95,96] Approaches to enhance the differentiation potential of MSC *in vitro* are mainly based on improving the culture environment. For example, human umbilical cord MSC differentiated in embryonic body-like aggregates by the addition of insulin-like growth factor 1 and further growth factors showed hepatocyte-like functions, including the expression of hepatic cytokeratins and albumin, as well as glycogen storage.[97] Hepatic differentiation of mesenchymal stromal cells was also accelerated by promoting mesenchymal-to-epithelial transition through *Rac1* inhibition with small molecules.[98] However, further research is required to provide evidence of the predictive value of mesenchymal-based models in pharmacological or toxicological research.

A further promising approach is based on the transdifferentiation of pancreas-derived progenitor cells by the addition of glucocorticoids.[99] Studies using cells from rat pancreatic tissue showed stable expression of hepatic markers, including liver-typical CYP enzyme activities in 2D cultures[100] or 3D bioreactors.[101] More recently, this method was also successfully applied for hepatic transdifferentiation of acinar cells from human tissue.[102] Thus, the technology could be used to generate an easily renewable and cost-efficient source of functional human hepatocytes *in vitro* for usage in hepatic metabolism and toxicity studies.

Human pluripotent stem cells

Sources for pluripotent stem cells. Pluripotent stem cells represent a promising source to produce different cell types for application in pharmacological drug screening and toxicity testing.[103] Initial research on pluripotent stem cells was performed using hESC generated from blastocysts,[104] which raised ethical concerns about the use of hESC in research. For the past few years, hiPSC generated by reprogramming of adult cell types from various tissues[105,106] have been gaining increased importance as an ethically accepted alternative to embryonic stem cells. Similar to hESC, hiPSC are able to differentiate into all three primary germ layer derivatives, i.e. ectoderm, mesoderm, and endoderm, and show a nearly unlimited capacity for proliferation. In addition, hiPSC can be derived from various individuals and from different patient groups, making them an attractive model for studying specific diseases and therapeutic approaches, in addition to their applications in pharmacological research.

Protocols for hepatic differentiation of pluripotent stem cells. To date, a number of protocols have been established for hepatic differentiation of hESC or hiPSC. These approaches are generally based on mimicking the embryonic development of the liver by adding the different growth factors necessary for each developmental stage. Activin A and Wnt3a[107,108] are the most important cytokines for definitive endodermal commitment, although fibroblast growth factor 2 and bone morphogenetic protein 4 also play important roles.[109,110] Hepatocyte growth factor and oncostatin M are the most commonly used cytokines for hepatic maturation.[109,111,112] Although various modifications of cytokine concentration, application periods, and combinations were investigated, all of the protocols so far show that the hepatocyte-like cells (HLC) obtained still show an immature phenotype with reduced hepatic functionality when compared to PHH.[113,114]

Approaches for the improvement of hepatic maturation. Strategies to improve the hepatic differentiation of pluripotent stem cells mainly focus on 3D culture approaches, co-culture with various cell types, and the direction of lineage-specific pathways.

One field of intense research is the development of 3D culture models, which address the needs of the cells in their natural environment. In particular, the maintenance of physiological cell–cell contacts has been shown to be crucial for the preservation of the mature hepatic phenotype.[115] Under this view, hepatic differentiation was performed in differently scaled 3D culture models. Microscale approaches are mainly based on 3D cell aggregation in matrices like collagen,[116] alginate,[117] hydrogel,[118] or Matrigel.[119] Liver-specific parameters, such as albumin production, urea secretion, and CYP expression indicate a higher grade of hepatic differentiation in these models when compared to conventional 2D cultures. In a larger scale approach, hepatic differentiation of hESC was investigated in perfused 3D four-compartment hollow-fiber bioreactors with a cell compartment volume of 2–8 mL. Sivertsson *et al.*[120] were able to show a down-regulation of pathways associated to apoptosis, cancer, and proliferation in these 3D bioreactors in addition to significantly higher expression of CYP7A7, CYP2C9, and CYP3A4. In addition, formation of bile duct-like structures positive for cytokeratin (CK) 19 and CK 7 were observed after hepatic differentiation of hESC in the bioreactor system.[121]

Different co-culture models were investigated in an effort to mimic the physiological environment during embryonic development, often in combination with 3D cultures. Seeding of hiPSC-derived hepatic endodermal cells with human umbilical vein endothelial cells and human MSC in 2D culture vessels resulted in the formation of 3D cell clusters that showed a significantly increased expression of early hepatic marker genes.[122] Moreover, transplantation of these liver buds prevented drug-induced lethal liver failure in an *in vivo* mouse model. Other studies reported successful usage of fibroblasts[123–125] or human hepatic stellate cells[126] in co-culture with human pluripotent stem cells during hepatic differentiation. To simulate the *in vivo* liver architecture even more closely, co-culture of HLC with NPC and biliary cells generated from pluripotent stem cells has been investigated in some recent studies. Co-culture of hiPSC-derived hepatocytes and endothelial cells facilitated the vascularization of cell aggregates and resulted in increased albumin secretion.[127] In addition, protocols for the directed differentiation of hiPSC into cholangiocytes were described.[128–130] Sancho-Bru *et al.*[131] observed the generation of mesodermal cells that expressed genes and proteins of liver endothelial cells and stellate cells during hepatic differentiation of mouse iPSC. Since hepatic stellate cells represent the central cells responsible for liver fibrosis[132] these cells would be also of interest for the development and/or testing of specific antifibrotic drugs.

A different strategy for the improvement of hepatic maturation focuses on the inhibition of signals that drive differentiation into alternate lineage pathways, e.g. mesoderm and ectoderm, to obtain highly pure populations of differentiated endoderm.[133] In a complementary approach, overexpression of certain hepatocyte genes such as hematopoietically expressed homeobox[134] or HNF4α[135] has been shown to improve hepatic differentiation.

There are some indications that the incomplete differentiation of hiPSC is caused by a residual donor cell memory, which may impact their capacity to differentiate into the desired cell type.[136] The importance of epigenetic regulation in cell differentiation is supported by the finding that the DNA methylation of different CYP genes differs significantly between hESC-derived hepatocytes and PHH.[137] Thus, the derivation of hiPSC lines from hepatic cells could lead to enhanced hepatic differentiation, as already reported for mouse iPSC.[138] However, the so-called epigenetic memory is also influenced by the culture conditions, e.g. the number of passages.[139] Moreover, the genetic variability of individual donors was shown to influence the differentiation outcome more than the parental cell type memory, underlining the importance to select cells from a large number of donors to obtain representative results.[140]

Quality control and standardization of hiPSC-derived cell preparations. Due to the lack of standardized criteria for hepatic cells derived from pluripotent stem cells, it is difficult to compare the success of different approaches and to identify promising modifications that may enhance hepatocyte maturation. In addition, a uniform benchmark for control and evaluation of the differentiation outcome is missing. Common benchmarks are internal controls

Table 1 Comparison of available hepatic cell sources for *in vitro* liver cell culture models with respect to their properties and potential application fields

	Characteristics		
Cell type	**Advantages**	**Disadvantages**	**Application examples**
Primary human liver cells	• High functionality • Reflect human metabolism • Detection of genetic polymorphisms possible	• Limited availability • Inter-donor variability • Rapid de-differentiation *in vitro* • No proliferation *in vitro*	• Toxicity studies[51,156] • Investigation of specific human metabolism/metabolic pathways[56] • Investigation of complex drug effects or diseases in advanced culture models (e.g. co-culture systems, 3D culture, bioreactors)[29,31,45–47,52,53]
Hepatoma cell lines	• High proliferation activity • Good availability • Stable metabolic performance	• Alterations in liver specific functions (e.g. reduced activity of drug metabolizing enzymes)	• Studies on tumor biology[67] • Testing of anticancer drugs[68] • Analysis of specific metabolic/toxicity pathways according to cell line properties[69]
HepG2 cell line	• Well characterized • Abundant data available	• Differs in metabolic properties from primary human hepatocytes	
HepaRG cell line	• Retains many liver-specific functions and many cytochrome P450s • Stable karyotype • Differentiation into both hepatocyte or biliary lineages	• Derived from one donor, does not allow analyzing inter-individual variations	• Studies on human drug metabolism and toxicity (e.g. enzyme induction)[70–72,82]

Table 2 Comparison of available stem cell sources for hepatic differentiation and subsequent use in *in vitro* liver cell culture models with respect to their properties and potential application fields

	Characteristics		
Cell type	**Advantages**	**Disadvantages**	**Application examples**
Adult stem cells	• Option for patient-specific derivation	• Limited proliferation capacity	• Characterization of differentiation/maturation processes for potential *in vitro* or clinical use[92,94,95,97–99]
Liver progenitor cells	• Committed to the hepatic lineage, facilitating differentiation	• Scarce tissue availability • Low frequency in tissue	
Mesenchymal stem cells	• Good availability (e.g. from bone marrow, placenta, umbilical cord)	• Incomplete hepatic differentiation with current protocols	
Pluripotent stem cells	• Unlimited growth	• Fetal phenotype of differentiated cells with current protocols • Lack of standardized methods for cell differentiation and characterization	• Use in drug metabolism and toxicity studies[119,146,147] • Research on applications in regenerative medicine[122]
hESC	• Differentiation not affected by epigenetic memory	• Ethical considerations restrict research on hESC in dependence of national regulations	
hiPSC	• Good availability • No ethical restrictions • Option for derivation from different individuals and patient groups	• Epigenetic memory may impair hepatic differentiation	• Analysis of genetic polymorphisms • Establishment of disease models[149–153] • Drug testing with patient-specific cell lines for personalized medicine[155]

(e.g. optimized versus previous method),[118,121,127] hepatic cell lines[112] and PHH from fetal or adult origin, cultured or freshly isolated.[113,114]

It is essential that differentiation protocols are highly reproducible for *in vitro* pharmacological and toxicity studies. Most of the current differentiation protocols apply defined serum-free culture media with recombinant growth factors. However, Matrigel, which is of biological origin with batch-to-batch variations, is still commonly used for the coating of culture vessels.[117,134,137] To enable better defined culture conditions, Matrigel was replaced in a number of studies by defined matrix components such as E-cadherin,[111,141] vitronectin,[142] or laminin.[107] A further possibility to increase reproducibility is to substitute recombinant growth factors with small molecules during the hepatic differentiation of pluripotent stem cells.[143,144] To ensure a constant quality and functionality of differentiated cell preparations, cryopreservation of large batches of pluripotent stem cell-derived hepatocytes would be helpful, enabling an on-demand cell supply. To date there is little data available on freeze–thaw cycles for cryopreservation of HLC. Mandal *et al.*[145] demonstrated that the cells were highly viable after long-term cryopreservation over several months, attached to the culture surface, and displayed a range of liver-specific markers and functions.

Application of hiPSC-derived hepatocytes. An important application for hiPSC is in pharmacological research and development. For example, maintenance of hiPSC-derived hepatocytes for up to two weeks was reported, enabling repeated–dose chronic exposure to hepatotoxic compounds.[146,147] Studies on MPCC with hiPSC-derived human hepatocytes[148] indicate a similar sensitivity toward drug toxicity as observed in MPCC using PHH.[51] In addition, hepatocytes generated from pluripotent stem cells have been successfully applied in *in vitro* studies on infectious diseases like hepatitis B and C infection[149,150] or malaria pathogenesis.[151] Moreover, inherited metabolic liver diseases can be modeled by reprogramming cells from those patients and differentiating hiPSC into the affected cell type, as shown for α1-antitrypsin deficiency, familial hypercholesterolemia, and glycogen storage disease type 1a.[152,153] Another exciting field of application can be seen in testing candidate compounds in cell-based assays from a large panel of patient-derived hiPSC in a so-called *in vitro* clinical trial. More evidence could be provided to show that hiPSC-derived hepatocytes can reproduce inter-individual differences in hepatic metabolism and responses to drugs.[154,155] Thus, such strategies could be helpful to recapitulate genetic polymorphisms and to determine the range of drug responsiveness in different patients before these compounds are tested in clinical trials.

Conclusion

In order to meet the need for predictive *in vitro* hepatic models for studies in drug testing and disease research, human liver cells reflecting the functional performance of the organ *in vivo* are required. In addition, cells have to be available in large numbers and at a constant quality for use in larger scale screening studies. An ideal cell source would meet all of these criteria to be used in all kinds of studies (''one-for-all''). However, each of the currently available liver cell sources has specific advantages, but also deficiencies in one or more aspects, which implies individual scopes of application for each cell source, as summarized in Tables 1 and 2. For example, PHH exhibit liver-like functions, but their use in larger scale screening studies is restricted by their limited availability. In contrast, hepatoma cell lines and pluripotent stem cells are characterized by a high proliferation capacity and abundant availability, while their use is impeded by certain functional aberrations (hepatoma cells) or incomplete maturation (pluripotent stem cells). Thus, the choice of cell source for a specific investigation should take into account the individual characteristics of the cells to address the study requirements. Furthermore, researchers have to consider the physiological requirements of the cells in specific culture models and conditions to optimize the hepatic functionality of the cells and increase the stability of metabolic performances *in vitro*. In this regard, approaches based on complex 2D and 3D culture technologies are important to improve the predictive value and reliability of hepatic *in vitro* models in pharmacological research and disease modeling.

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