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Biotechnology Challenges to In Vitro Maturation of Hepatic Stem Cells

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Abstract

The incidence of liver disease is increasing globally. The only curative therapy for severe end-stage liver disease, liver transplantation, is limited by the shortage of organ donors. In vitro models of liver physiology have been developed and new technologies and approaches are progressing rapidly. Stem cells might be used as a source of liver tissue for development of models, therapies, and tissue-engineering applications. However, we have been unable to generate and maintain stable and mature adult liver cells *ex vivo*. We review factors that promote hepatocyte differentiation and maturation, including growth factors, transcription factors, microRNAs, small molecules, and the microenvironment. We discuss how the hepatic circulation, microbiome, and nutrition affect liver function, and the criteria for considering cells derived from stem cells to be fully mature hepatocytes. We explain the challenges to cell transplantation and consider future technologies for use in hepatic stem cell maturation, including 3-dimensional biofabrication and genome modification.

Keywords

Liver Development; Differentiation; Hepatocyte; Culture

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Conflicts of interest

This author discloses the following: A.S.-G. has a provisional patent application that describes hepatic differentiation of human pluripotent stem cells and liver repopulation. A.S.-G. is a co-founder and has a financial interest in Von Baer Wolff, Inc, a company focused on biofabrication of autologous human hepatocytes from stem cells technology. A.S.-G.'s interests are managed by the Conflict of Interest Office at the University of Pittsburgh in accordance with their policies. The remaining authors disclose no conflicts.

The only proven treatment for patients with endstage liver disease is organ transplantation, which is hampered by the lack of donors. Therefore, mortality on the waiting list can be as high as 10%.¹ Primary hepatocytes are the standard cells used for in vitro studies and are a resource for liver cell transplantation and bioartificial liver devices.² However, the complexity of the maturation process, the instability of cultured hepatocytes, and inconsistent protocols limit their application.

Hepatocytes perform essential liver functions, including plasma protein secretion, bile production, detoxification, metabolic homeostasis, and storage of vitamins and minerals. They are the most predominant parenchymal cell type, accounting for approximately 80% of the adult liver mass, and are polygonal, approximately 20–30 μm in diameter with a volume of 3000 μm^3 . Like other epithelial cell types, hepatocytes are polarized, with distinct apical, lateral, and basal membranes. Hepatocytes are polyploid; approximately 20%–30% of human hepatocytes are tetraploid and octaploid, compared with 85% in rats and mice.^{3,4}

Hepatocytes originate from definitive endoderm during embryonic development, after acquisition of hepatic competence by ventral foregut endoderm and specification of those epithelial cells into hepatic endoderm.^{5,6} Primordial liver cells transition into a non-polarized cellular phenotype (hepatoblasts), which subsequently generate liver buds. Hepatoblasts are bipotent and express fetal liver genes and genes associated with both hepatocyte and cholangiocyte lineages (cholangiocytes and epithelial cells that line the bile ducts and hepatocytes)⁷ and can differentiate into both cell types. Lineage determination relies on signaling pathways, including Notch and transforming growth factor- β , which promote biliary differentiation.^{8–10} Down-regulation of these pathways promotes specification of hepatoblasts toward hepatocyte fate.^{11–13}

Unfortunately, adult hepatocytes have limited in vitro proliferation ability and quickly de-differentiate, hampering their use ex vivo. Several cell types and in vitro techniques have been proposed for generating a potential alternative to hepatocytes, which we collectively refer to as hepatocytelike cells (HLCs). We review the available strategies for HLC production and strategies for overcoming the immature hepatic phenotypes of HLCs. We propose ways to improve the generation of mature HLCs and defined criteria for HLCs.

Cell Sources for Hepatocyte Generation

Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced PSCs (iPSCs), can differentiate into all cell types of the body, while maintaining genetic stability and are therefore a promising cell source for regenerative medicine, development of models, and drug discovery. Although the application of human ESCs is hotly debated, human iPSCs are generated by forced expression of specific pluripotency transcription factors, circumventing ethical concerns.¹⁴ Great progress has been made exploring the differentiation capacity of PSCs toward the hepatocyte lineage. Although specific culture conditions can vary, most protocols share a 3-step strategy based on ontogenetic liver development.^{15–17} The first is efficient induction of PSCs into a definitive endoderm fate (inducers include activin A, fibroblast growth factor [FGF] 2, WNT3A, BMP4, and LY294002). The second step is hepatic specification, which gives rise to hepatoblast-like

cells (inducers include hepatocyte growth factor [HGF], FGF2, FGF4, and BMP4). The final step is to induce differentiation toward hepatocytes (oncostatin M [OSM], dexamethasone, HGF, and follistatin).

A second source of HLCs are mesenchymal stromal or stem cells (MSCs), which are present in easily accessible tissues like fat, blood, and bone marrow. They have stem cell-like characteristics, such as self-renewal and multipotent differentiation capacity, including toward HLCs. In most protocols, MSCs can be directed toward hepatocyte lineage through a hepatic commitment stage (induced by FGF and HGF) and maturation stage (induced by OSM and dexamethasone).^{18,19} Besides the heterogenic population of the HLCs after differentiation, they also reflect an immature phenotype.²⁰

A third attractive cell source are hepatic progenitor/ stem cells (HPCs), which can be derived from single Lgr5⁺ (mouse) or EpCAM⁺ (human) cholangiocytes and from bile duct fragments from mouse and human livers.^{11,12,21} A 3-dimensional (3D) culture system based on laminin and collagen IV-enriched Matrigel has been established to support the long-term and genetically stable expansion of HPCs as organoids.^{11,12} Those HPCs can differentiate into the hepatocyte lineage by inhibition of Notch and Wnt signaling and activation of FGF signaling.^{11,12} This single-step differentiation process requires less effort than PSC-based methods and reaches high efficiency.¹¹ However, similar to other sources, HPC-derived HLCs also partially resemble hepatocytes.¹²

There has been no direct comparison of the maturation level of HLCs generated from these sources. Furthermore, different standards were used for characterizing HLCs in different studies. It is therefore not clear which source is optimal.

In Vivo Liver Maturation

Unfortunately, stem cell-derived HLCs do not possess all functions of mature hepatocytes. Liver maturation is complex and stage-dependent; full maturation takes as long as 2 years from birth and involves expression of several signaling pathways, such as those responsible for bile acid synthesis, drug metabolism, and amino acid transport.^{22,23} Large modifications in homeostasis can therefore alter liver maturation. Following birth, the newborn liver is affected by changes in circulation, microbiome, and nutrition that might also promote hepatic maturation (Figure 1).

Hepatic Circulation

Nutrition, circulation, and oxygen levels change radically at birth, permanently change the environment of the newborn, and result in new metabolic and homeostatic requirements. Most features of the fetal circulation, such as the umbilical cord vessels (ductus arteriosus and ductus venosus), the foramen ovale, and the placenta, are no longer needed after birth. The change from the fetal to the newborn circulation is therefore a remarkable process of homeostatic coordination. In fetal circulation, blood from the placenta returns to the fetus through the umbilical vein, which has a lower oxygen saturation compared with adult arterial circulation (approximately 80% and 98% saturation, respectively). The liver is bypassed by the ductus venosus during fetal development. To compensate for lower oxygen

conditions, fetal hemoglobin has a considerably higher affinity for oxygen than adult hemoglobin.²⁴ This observation, combined with circulation dynamics, indicates that the functional heterogeneity of the parenchymal cells in the fetal liver depend directly on microcirculation.²⁵

The right liver lobes are perfused with poorly oxygenated blood, mostly from portal blood; the left portion receives most of its blood from the umbilical vein, has a higher content of oxygen-dependent enzymes, and is more active in drug binding and metabolism.^{25,26} The hepatic artery contributes approximately 2% to fetal liver circulation and, at birth, this increases 18-fold, comprising nearly 25% of hepatic blood flow with highly oxygenated blood.²⁷ Similarly, contribution of the portal vein increases from 20%–25% to an average of 75% and with closure of the ductus venosus, its blood distribution through the liver becomes homogenous.^{26,28,29}

Oxygenation has been shown to increase albumin and urea synthesis in rat hepatocytes.³⁰ In addition, shear stress, while not usually investigated, is known to maintain or even increase function of parenchymal cells, for example, through polarization, which in turn increases liver function.^{31,32} Oxygen levels and mechanical stimulation experienced by liver cells might therefore be applicable for hepatocyte maturation. Both of these features have now been recognized in adult hepatocytes³³ and incorporated into some protocols of human ESC hepatic differentiation.^{15,34} Interestingly, other vascular mediators generated from liver circulation (eg, prostaglandins, nitric oxide, and catecholamines) could also be involved in hepatic growth and maturation, recently shown in a bioengineered liver.³⁵

The Intestinal Microbiome

During the first week after birth, the immature liver undergoes a large reorganization process.³⁶ The anatomical and functional specialization occurs among hepatic acinar units, leading to formation of 3 distinct metabolic/functional zones.^{22,23,37} Levels of hormones, oxygen, and nutrients in the periportal blood are important in this process,³⁶ but the initial stimuli that generate this metabolic patterning and the microbiome's contribution is still undetermined.

Cytochrome P450 (CYP) expression in the liver is an indication of metabolic activity, for example, the breakdown of toxic compounds. The demands of a changing environment and xenobiotic clearance are considered as some of the driving forces for CYP maturation. CYP expression is low at birth (approximately 30% of adult levels) and progressively increases, reaching adult levels by 1 year of age.²³ It was recently discovered that germ-free mice have distinct CYP expression levels compared to wild-type mice.^{38–40} This appears to correlate with the bacterial strains that colonize the gut of newborn and adult mice, which have the ability to change drug metabolism and drug response at various stages of life.⁴⁰

Studies of the link between postnatal liver maturation and bacterial colonization of the gut found that microbial-derived lithocholic acid and vitamin K2 promote maturation of PSC-derived hepatocytes and fetal hepatoblasts.⁴¹ Other bacterial-derived substances might have similar effects.

Nutrition

Nutritional input received by the fetal liver changes greatly after birth. In contrast with the fully processed nutrients received from maternal blood in utero, newborn nutrition is dependent on digestion and intestinal absorption of maternal milk. Levels of lipids, carbohydrates, proteins, and other nutrients in portal circulation are periodically increased during the postprandial period, exceeding normal levels for a newborn liver. Lipids for example, are important for liver development and homeostasis, and direct binding of free fatty acids to peroxisome proliferator-activated receptors or hepatocyte nuclear factor 4a (HNF4A), or the role of sterols, steroids, and bile salts and their nuclear receptors (eg, retinoid X receptor, farnesoid X receptor, pregnane X receptor [PXR]) might have larger effects on hepatocyte maturation than reported previously.⁴²⁻⁴⁵ Concomitantly, many hormones and growth factors are secreted during this period and most of these hepatotrophic factors (eg, insulin, glucagon, and gastrin) directly affect hepatocyte proliferation, phenotype, and metabolism.²⁵

Strategies to Promote Hepatocyte Differentiation and Maturation

The generation of HLCs, irrespective of cell source, is a complex process. Mature hepatocytes do not proliferate often, posing challenges to the in vitro generation of these cells. Components that promote hepatocyte differentiation and maturation include growth factors, transcription factors, microRNAs, small molecules, and the microenvironment (Figure 2).

Growth Factors

Growth factors regulate embryonic development. Culture media supplementation is used to remodel cell fate. We discuss the 3 important regulators of hepatocyte specification and maturation (Figure 3).

The role of OSM, an interleukin-6 family cytokine in hepatocyte maturation was well defined by Kamiya et al,⁴⁶ who demonstrated that OSM up-regulates the expression of albumin, glucose-6-phosphate dehydrogenase, and tyrosine aminotransferase in fetal hepatocytes isolated from the embryonic murine liver (embryonic day 14.5). Fetal hepatocytes incubated with OSM have a similar morphology to mature hepatocytes, such as tight intracellular contacts, highly condensed and granulated cytosol, and clear roundshaped nuclei. In addition, OSM induces hepatocytespecific functions, including glycogen synthesis, ammonia clearance, lipid synthesis, detoxification, and enhancement of homophilic cell adhesion.⁴⁷ Interestingly, OSM promotes massive proliferation and dedifferentiation of hepatocytes, dictated by maturation stage. Progenitor cells receiving OSM do not mature. In contrast, mature hepatocytes receiving OSM dedifferentiate; when OSM was withdrawn, hepatocyte functions were rescued.⁴⁸ These data indicate that OSM is important for early stages of hepatic maturation.

HGF is important throughout liver development. Knockout of HGF leads to embryonic lethality and the embryonic liver is reduced in size by loss of hepatocytes.⁴⁹ In the presence of dexamethasone, HGF up-regulates expression of several mature hepatocyte markers, such

as carbamoyl-phosphate synthase 1, glucose-6-phosphate dehydrogenase, and tyrosine aminotransferase in fetal murine hepatocytes.⁴⁷ During in vitro PSC-derived hepatocyte generation, HGF facilitates the transition into the hepatocyte specification stage by binding to its receptor (MET), which activates the STAT3 and AKT and regulates the expression of hepatocyte markers.⁵⁰

Insulin is routinely included in HLC and hepatocyte culture. Although this factor promotes survival of most cell types, insulin also preserves many hepatocyte-specific functions, including amino acid transport, protein synthesis, glycogenesis, and lipogenesis.^{51–53} Moreover, insulin has an important role in secretion of albumin by hepatocytes.⁵⁴

These growth factors are essential for hepatic specification and/or maturation of stem cells and appear to be differentiation stage-dependent. Unfortunately, growth factors alone do not induce a hepatic phenotype in HLCs comparable to freshly isolated hepatocytes.

Transcription Factors

Liver development involves the progressive activation of transcription factors. Liver-enriched transcription factors (LETFs) regulate hepatic cell fate commitment and maintenance of a mature status. LETFs include HNF4A, constitutive androstane receptor, eosinophil-associated, ribonuclease A, peroxisome proliferator-activated receptor- α , retinoid X receptor- α , farnesoid X receptor, PXR, small heterodimer partner, and liver receptor homolog 1 (Figure 3).

One of the most studied LETFs, HNF4A, is highly expressed in hepatocytes and regulates hundreds of targets involved in hepatocyte function. Using chromatin immunoprecipitation and promoter microarrays, Odom et al⁵⁵ found that 43% of actively transcribed genes in the human liver are regulated by HNF4A. Furthermore, overexpression of only HNF4A resets the hepatocyte transcription factor network in damaged hepatocytes from rats after administration of CCl₄.⁵⁶ These findings indicate that HNF4A is an important regulator of hepatocyte function.

Other LETFs control the expression of a range of effectors, including glucose and cholesterol homeostasis, urea cycle, synthesis of plasma proteins, coagulation factors, and most drug-processing proteins.^{57,58} Transcription factors regulate expression of each other.^{56,59–66} For example, HNF1A represses HNF4A-mediated activation of transcription, whereas HNF4A promotes HNF1A-mediated activation of transcription.^{65,66} In addition, HNF1A is a direct target of HNF4A⁶⁴ and vice versa,⁶⁷ so these proteins act in a positive-feedback loop. Transcription factor networks that sustain particular cell types might therefore be determined and designed.

Ectopic expression of tissue-specific transcription factors⁶⁸ can reprogram non-hepatic lineage cells into hepatocyte-like cells.^{69,70} However, all non-hepatic lineage cells reprogrammed into hepatocyte-like cells have immature features compared to freshly isolated primary hepatocytes, limiting their application. This is possibly caused by a lack of transcription factor network activators that define hepatocyte identity.

Godoy et al⁷¹ compared genome-wide gene expression profiles of ESC-derived HLCs (ES-HLCs) to primary human hepatocytes freshly isolated and cultured for 14 days. They identified groups of genes whose expression correlated with mature liver functions and shared expression levels with primary hepatocytes. These included SRY-box 11, forkhead box Q1, and Y box binding protein 3, which may be new targets for further maturation of ES-HLCs.⁷¹ Other gene products that regulate hepatic maturation were not expressed at levels observed in mature hepatocytes, leaving ES-HLCs too far removed from a bona fide hepatocyte. Notably, there are differences in messenger RNA and protein levels (up to 40%⁷²), indicating an imbalance in the ESHLC system. Therefore, transcriptome data are useful for identifying candidates, but confirmation at translational and functional levels is necessary.

Transcription factors regulate hepatocyte maturation, but measurements of their expression are not sufficient to determine hepatic maturation because most are expressed at near-adult levels in liver during mid and late gestation. Proper phenotype characterization and determination of liver-specific functions are required to accurately measure liver maturation.

MicroRNAs

MicroRNAs are a class of small non-coding RNAs that regulate cell differentiation, proliferation, and survival. Zhou et al⁷³ generated HLCs by introducing 5 microRNAs (MIR122, MIR148a, MIR424, MIR542–5p, and MIR1246) into human umbilical cord–derived MSCs. MIR122 is specifically and abundantly expressed in the liver, and accounts for 72% of all the microRNA in liver. MIR122 is required for gene expression patterns associated with maintenance of differentiation in the liver.⁷⁴ Inhibition of MIR122 reduces plasma levels of cholesterol, increases hepatic fatty acid oxidation, and decreases rates of hepatic fatty acid and cholesterol synthesis.⁷⁵ In contrast, overexpression of MIR122 in mouse ES-HLCs significantly up-regulates expression of several hepatocyte markers, including albumin and CYP3A4, which reach levels of roughly 80% of normal expression in hepatocytes.⁷⁶ Additionally, liver function, including CYP metabolism, increases in cells that overexpress MIR122. Interestingly, MIR122, FOXA1, and HNF4A form a positive feedback loop, in which MIR122 upregulates expression of FOXA1, whereas FOXA1 upregulates expression of HNF4A and HNF4A increases expression of MIR122.⁷⁶

MIR194 is also strongly up-regulated during hepatocyte differentiation. Overexpression accelerates the differentiation toward hepatocytes in HepaRG cells (a human liver progenitor cell line) and in human ESCs. Furthermore, MIR194 reduces expression of YAP1, a factor in the Hippo signaling pathway, which regulates liver development.⁷⁷ MicroRNAs regulate hepatocyte maturation and can be used as biomarkers of this process. Although their use in HLC generation is feasible, efficient and robust techniques are needed to transfer microRNAs into HLCs.

Small Molecules

Natural and synthetic small molecules, identified by cellbased phenotypic screens, are useful chemical tools for controlling and manipulating cell fate—they are safer, more efficient,

robust, and cost effective. We can separate the direct targets of small molecules during hepatocellular differentiation and maturation into 3 categories (Figure 4).

Molecules related to epigenetic modification, mainly histone acetylation and DNA methylation, contribute hepatic maturation. There have been many studies of the effects of histone deacetylase inhibitors (HDACis), such as trichostatin A and dimethylsulphoxide, on hepatocyte differentiation.^{78–84} HDACis have a broad range of effects, including up-regulating expression of LETFs, such as HNF4A, HNF1A, CEBPA, and FOXA2,⁷⁹ and increasing transcriptional activity by binding to promoter regions of *CYP* genes and glucose-6-phosphate dehydrogenase.^{80–82} Additionally, HDAC is regulate liverspecific expression of MIR122.⁷⁸

HDACi-induced differentiation is always associated with proliferation arrest,⁸⁵ which is an undesired phenotype of adult hepatocytes in vitro. Inhibitors of DNA methylation (DNMTis), such as 5-azacytidine and 5-aza-2'-deoxycytidine, also induce transcription of hepatocyte-specific genes.^{86,87} Ideally, HDACis and DNMTis can be applied together: DNMTis would be used as preconditioning agents before hepatic differentiation, whereas HDACis would be applied during or after differentiation.⁸⁸

Signaling pathway-specific antagonists and agonists including Notch, HGF and its receptor c-Met, and dexamethasone are important for the specification of hepatoblasts to either hepatocytes or cholangiocytes. Notch activation increases expression of the biliary regulator HNF1B, and reduces expression of hepatocyte regulators HNF1A, HNF4A, and CEBPA.⁸ Studies in humans, mice, and dogs confirmed that Notch inhibition induced differentiation of Lgr5⁺ liver adult stem cells toward HLCs.

A8301 inhibits transforming growth factor- β , which promotes the epithelial to mesenchymal transition and dedifferentiation of hepatocytes.⁸⁹ Siller et al⁹⁰ generated functional HLCs with the HGF receptor agonist, N-hexanoicTyr, Ile-(6) aminohexanoic amide (dihexa). In particular, dexamethasone, a synthesized glucocorticoid, induces CYP3A, which mediates drug metabolism and synthesis of cholesterol, steroids, and other lipids in hepatocytes.⁹¹ It also promotes hepatocyte morphogenesis and formation of intercellular junctions, which are required for xenobiotic biotransformation, albumin secretion, and ammonia detoxification,⁹² and up-regulates expression and transcriptional activity of LETFs, such as CEBPA, HNF1A, HNF4A, and FOXA1.⁹³ Dexamethasone also activates glucocorticoid receptor signaling; glucocorticoid receptor-dependent regulatory sequences are found within promoters of many hepatocyte-specific genes, including the albumin gene and *CYP2B*.⁹⁴ For these reasons, dexamethasone is used in almost all protocols for end-stage differentiation of hepatocytes.

The third category consists of chemicals that are known to promote hepatocyte maturation, with unknown mechanisms of action. Shan et al⁹⁵ developed a high-throughput screening platform based on albumin secretion level and proliferation of human primary hepatocytes. From a library of 12,480 small molecules, they identified 2 agents that induced proliferation and albumin secretion (FPH1 and FPH2) and 1 that induced albumin secretion alone (FH1). Further tests on iPSC-derived HLCs confirmed that FPH1 and FH1 promote hepatocyte

functions.⁹⁵ Both molecules contain the same N-phenyl-2-(Nphenylmethylsulfonamido)-acetamide core, but have different substitutions around the sulfonamide and amide phenyl rings, providing options for optimizing hepatocyte maturation.

Small molecules may be an alternative to transcription factor or growth factor–induced differentiation with improved reproducibility and reduced costs. Progress has been made replacing growth factors in the differentiation of PSCs toward definitive endoderm.^{96,97} Recently, Siller et al⁹⁰ developed a stepwise growth factor–free protocol for differentiating human PSCs toward a hepatic lineage, which reached similar levels of liver function compared to growth factor–based approaches.

Microenvironment

Hepatocytes in vivo are surrounded by extracellular matrix (ECM). Natural ECM-based hydrogels, including collagen type I and Matrigel, are routinely used for coating culture dishes for primary hepatocytes and HLCs. The collagen (type I) sandwich culture can mimic the 3D microenvironment for hepatocytes and is the standard for maintaining hepatocyte features in vitro.⁹⁸ Cell-derived matrices contain a complex but organized mixture of bioactive and biocompatible materials, and are therefore more effective than pure collagen sandwich cultures for functional HLCs maturation. Guo et al⁹⁹ demonstrated that endothelial cell–derived matrix promotes the metabolic maturation of human adipose stem cell–derived HLCs through activating hepatocyte-enriched transcription factors, FOXA2, HNF4A, and PXR. Scaffolds generated from decellularized human and rat liver ECM contain not only collagen, laminin, or fibronectin, but also ECM-bound growth factors, improving the physiology of the in vivo microenvironment.^{100,101} This also seems to be a tissue ECM–specific effect, because ECM derived from other tissues does not induce differentiation to the same extent.¹⁰² Recently, we created human liver organoids that were self-assembled in vitro from liver progenitor cells seeded onto decellularized liver ECM discs. These 3D liver organoids recapitulated several aspects of hepatobiliary organogenesis and resulted in concomitant formation of progressively more differentiated hepatocytes and bile duct structures.¹⁰³ Applications of the ECM-based 3D culture are limited by the mass transfer barriers formed by the top layer, batch-to-batch variation of ECM, and uncontrollable coating.

Many hepatocyte functions are regulated by substances released from neighboring nonparenchymal cells, such as Kupffer cells, sinusoidal endothelial cells, stellate cells, and hepatic MSCs.¹⁰⁴ Cytokines secreted by nonparenchymal cells comprise acidic FGF (stellate cells),¹⁰⁵ HGF (stellate and endothelial cells),^{105,106} tumor necrosis factor (Kupffer cells),¹⁰⁷ OSM (Kupffer cells),¹⁰⁸ interleukin-6 (Kupffer, macrophages, and endothelial cells),^{109,110} transforming growth factor- β (Kupffer cells),¹¹¹ interleukin-1 (Kupffer cells),¹¹⁰ and WNT3A (macrophages).¹¹² Of these, HGF and OSM are considered to be inducers of hepatocyte maturation.^{46,47} Co-culture systems might be better models at the tissue level. Takebe et al¹¹³ showed that co-cultured human MSCs, human umbilical vein endothelial cells, and human iPSCs self-organized into 3D liver buds with functional liver properties. In addition, the liver buds were vascularized and, when transplanted, the vasculatures connected to host vessels and demonstrated clear liver function.

Single-cell RNA sequencing can be used to investigate transcriptome variations among cells. This technology was used to determine how genetic factors and molecules interact to control liver organoid formation.¹¹⁴ Researchers used single-cell RNA sequencing analyses to determine the complex patterns of communication between the 3D microenvironment and different cell types. Interactions between vascular endothelial growth factor and its receptor induce angiogenesis in the developing liver. Although this approach has been used to study primarily early developmental processes, these tools might be used in generation of healthy and viable human liver tissue from human PSCs.

Required Functions of Adult Hepatocytes

The availability of a homogeneous source of human hepatocytes is considered the most precious tool for toxicity screening. Hepatocytes also provide a renewable, cell-based source to examine other key factors of compound attrition, including metabolism of xenobiotics by CYP enzymes, drug interactions, hepatotoxicity, the activity of drug transporters, and regenerative medicine. What are the features that define hepatocytes structurally and functionally? These hepatocyte characteristics should be taken into consideration during the generation and maturation of human HLCs and freshly isolated primary human hepatocytes should always be used as the standard for comparison (Figure 5 and Table 1).

Metabolic and Secretory Functions

The presence of hepatic enzymes with clinical implications would be useful for hepatic maturation categorization and disease modeling, for example, when studying UDPglucuronosyltransferase, glucose-6-phosphatase, or α 1-antitrypsin deficiency.¹¹⁵ Additionally, the entire hepatic drug-metabolizing enzyme system provides an in vitro model for analyzing drug metabolism and predicting hepatotoxicity. CYPs are expressed primarily in the liver and CYP3A4 is the most abundant isoenzyme in human adult liver. Evaluation of CYPs in hepatocytes classified as phase 1 metabolism may include CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP3A7, and CYP7A1. The enzymes of greatest importance for drug metabolism belong to families 1–3, and are responsible for 70%–80% of all phase 1-dependent metabolism of clinically used drugs.¹¹⁶ Finally, urea cycle-related enzymes might be important when hepatic function of HLCs is evaluated; these include the ornithine transcarbamylase gene, which is located in mitochondria, along with carbamylphosphate synthetase I and argininosuccinate synthetase.
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Hepatic transport proteins and measurement of bile acids can serve as indicators of hepatic function and maturation. However, not all hepatic functions mature simultaneously and some hepatic transporters are expressed early in development and may not be exclusive for liver.¹¹⁸ Some important hepatic transport proteins can be classified as follows: the solute carrier SLC family, including Na⁺taurocholate co-transporting polypeptides, organic anion-transporting polypeptides, organic anion transporters, and organic cation transporters; the adenosine triphosphate-binding cassette transporter family, including the multidrug resistance proteins and bile salt export pump (both belong to the ABCB family), breast

cancer resistance protein (belongs to the ABCG or White family); and the multi-drug resistance-associated proteins belonging to the ABCC family.

Chemical sensors like the aryl hydrocarbon receptor, PXR, and the constitutive androstane receptor are integral to the regulation and induction of the main CYPs and their analysis may provide strong evidence of the maturation state of HLCs. Similar to LETFs (eg, HNF4A and CEBPA),¹¹⁹ hepatic clotting factors (II, V, VII, IX, X, and fibrinogen), albumin production, urea production or ammonia metabolism, and glycogen storage may provide additional evidence of effective hepatic maturation and functional capacity in HLCs.

HLCs generated using different strategies show some degree of hepatic phenotypes, and because the features mentioned are characteristic of freshly isolated primary human hepatocytes, it is critical that they are analyzed comprehensively.

Cell Engraftment and Liver Repopulation

Patients with acute liver failure and inborn errors of hepatic metabolism are ideal candidates for cell therapy.¹²⁰ Numerous studies in rodents and few clinical trials have demonstrated the long-term safety of this procedure.^{120–122} However, hepatocyte transplants in humans have only partially restored metabolic disorders.^{121,123} The efficacy of the procedure has been limited by poor cell engraftment soon after transplantation and our inability to monitor and predict rejection episodes.¹²¹ The problems of cell engraftment and repopulation of donor hepatocytes after cell transplantation might be solved by liver-directed radiation, which would inhibit host hepatocyte proliferation and induce post-mitotic hepatocyte death, allowing donor hepatocytes to proliferate and repopulate the irradiated host liver.¹²⁴ Additional approaches to overcome engraftment and repopulation challenges involve expression of transgenes to provide cells with a repopulation advantage.¹²⁵ Nygaard et al¹²⁵ showed that hepatocytes carrying transgenes encoding coagulation factor 9 and a selection marker (a small hairpin RNA that makes cells resistant to a smallmolecule inhibitor of fumarylacetoacetate hydrolase) expressed these gene products after transplantation into mice. These experiments indicate that it is possible to genetically modify liver cells to facilitate repopulation of transplanted hepatocytes. Preclinical tests for HLCs in animal models of liver failure and/or regeneration is of great importance to demonstrate regeneration response, safety, and efficacy of HLCs after transplantation.

Immune-compromised mice with different types of liver injury have been used to study strategies to provide proliferative advantages to transplanted cells.^{126–130} The ability of transplanted cells to functionally repopulate livers of immune-deficient mice is the standard to establish that they are hepatocytes, rather than cells with hepatocyte-like features, which are unable to repopulate livers. To date, only limited repopulation of human HLCs has been reported.^{69,131–134}

The ability of human HLCs to engraft, repopulate liver, and function should be evaluated in different animal models of liver injury (for review, see Rezvani et al¹³⁵).

Fumarylacetoacetate hydrolase-deficient mice are commonly used to evaluate the regenerative and functional capacities of human HLCs. *Fah*^{-/-} mice have been crossed with *Rag*^{-/-}/*Il2rg*^{-/-} mice to produce immune-compromised triplemutant mice (FRG). Livers of

these mice can be repopulated up to 90% with transplanted human primary hepatocytes.¹²⁷ When human HLCs were transplanted into and evaluated in FRG mice, the reported achieved liver repopulation ranged from 2% to 4% after transplantation.¹³¹

Severe combined immunodeficient mice, with liver injury caused by expression of the urokinase-type plasminogen activator, are also used in studies of liver repopulation by human HLCs. In these mice, as much as 20% of the liver can be repopulated, which was quantified by immunostaining after transplantation of 4 million HLCs.¹³⁴ HLCs also repopulate livers of immune-suppressed Nagase analbuminemic rats.¹³³ These rats were given injections of retrorsine and partial (70%) hepatectomies to induce proliferation of the transplanted cells. However, repopulation efficacy was not reported and the human serum albumin levels remained low. A similar lack of HLC repopulation was reported in Gunn rats, a model for Crigler-Najjar syndrome (deficient in bilirubin metabolism).^{132,136} In these experiments, researchers irradiated approximately 30% of the liver area and regeneration was induced by expression of hepatocyte growth factor (HGF) from an adenoviral vector. Transplanted HLCs repopulated 7.5% of the irradiated median liver lobe, quantified by immunostaining, and reduced levels of bilirubin by 60%.¹³²

Future Directions

Biofabrication

Considerable effort has been dedicated to creation of hepatic tissue by 3D bioprinting.^{137–141} These novel techniques allow for the precise and simultaneous addition of cells and matrix molecules in a designed spatial configuration. With proper spatial control, bioprinters generate hepatic tissues with distinct cell types in discrete locations that resemble hepatic tissue architecture and cell distribution.¹³⁸ These bioprinted, often called mini-livers, have some features of hepatocyte function, including albumin and urea secretion and drug metabolism to a certain level.^{138,140,141} However, the low resolution of available bioprinters and methods for bioprinting the vasculature (such as sacrificial hydrogel) make it impossible to print hepatic tissue with an integrated and perfusable vascular network with complex branching in a range of diameters from large vessels to capillaries.^{142,143} The same holds true for the periportal to pericentral zonal distribution.^{142,143} The technology to transcend these challenges might be years away, but the potential of these biofabrication techniques is clear and compelling when considering whole-liver bioengineering. They allow not only the generation of organs on demand, but also a more exact and rapid 3D recreation of hepatic tissue with all of its refinements, and are only limited by our knowledge of what and where to print.

Although 3D bioprinting of whole livers lies in the future, 3D bioprinters and other biofabrication techniques might be used to generate more functional HLCs. ECM microarrays, modular assembly, rapid prototyping techniques to construct defined microarchitectures, and microchips to recreate the hepatic environment are already available.^{144–147} The biomechanical stimulation produced by culture medium perfusion in these platforms, associated with the small volumes used, produces highly efficient differentiation of iPSCs into several cell fates, including drug-metabolizing hepatocytes.¹⁴⁸ These

techniques might be used to accurately recreate the hepatic microenvironment and identify mechanisms of hepatic maturation, as well as to generate entirely functional HLCs.

Genetic Programming of Hepatic Maturation

To elucidate mechanisms of human liver maturation, we must learn more about the mechanisms that regulate hepatocyte differentiation and function. Induction of pluripotency and self-renewal in human iPSCs provides evidence for a powerful reprogramming approach. Our ability to control transcription of specific genes in human HLCs could lead to identification of factors that maintain a specific cell state or promote cell maturation. Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated Cas genes can be customized to precisely regulate gene expression and examine functions of gene products in cells. These approaches will facilitate high-throughput analyses of gene activation or repression in human HLCs and their differentiation intermediates.^{149,150}

Balboa et al¹⁵⁰ generated a chemically controllable dCas9 activator, by fusion with the dihydrofolate reductase destabilization domain. They showed that the destabilized dCas9 activator can be used to control human PSC differentiation into endodermal lineages. These CRISPR/Cas9 systems, next-generation genome sequencing, and stem cell technologies can be used in studies of liver development to identify genes that control functions of mature hepatocytes and determine how they are regulated.

Conclusions

The demand for functional liver tissue replacement options continues to grow and, despite advances in the differentiation of HLCs in vitro, generating stem cell-derived cells and tissues with primary adult hepatocyte function remains challenging. The complexity of liver tissue, together with the need for stable mature liver cells with high proliferation potential currently limit broader application of HLCs. A greater understanding of the factors involved in hepatocyte biology coupled with emerging technologies, such as biofabrication and (epi)genetic programming, may provide the tools necessary to advance the development and application of human stem cell-derived hepatocytes.

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Abbreviations used in this paper:

CRISPR	clustered regularly interspaced short palindromic repeats
CYP	cytochrome P450
3D	3-dimensional

DNMTi	DNA methylation inhibitor
ECM	extracellular matrix
ESC	embryonic stem cell
ES-HLC	embryonic stem cell-derived hepatocyte-like cell
FGF	fibroblast growth factor
HDACi	histone deacetylase inhibitor
HGF	hepatocyte growth factor
HLC	hepatocyte-like cell
HNF4A	hepatocyte nuclear factor 4a
HPC	hepatic progenitor/stem cell
iPSC	induced pluripotent stem cell
LETf	liver-enriched transcription factor
MSC	mesenchymal stromal or stem cell
OSM	oncostatin M
PSC	pluripotent stem cell
PXR	pregnane X receptor

References

1. Kim WR, Therneau TM, Benson JT, et al. Deaths on the liver transplant waiting list: an analysis of competing risks. *Hepatology* 2006;43:345–351. [PubMed: 16440361]
2. Soldatow VY, LeCluyse EL, Griffith LG, et al. In vitro models for liver toxicity testing. *Toxicol Res* 2013;2: 23–39.
3. Guidotti JE, Bregerie O, Robert A, et al. Liver cell polyploidization: a pivotal role for binuclear hepatocytes. *J Biol Chem* 2003;278:19095–19101. [PubMed: 12626502]
4. Gentric G, Desdouets C. Polyploidization in liver tissue. *Am J Pathol* 2014;184:322–331. [PubMed: 24140012]
5. Zaret KS. From endoderm to liver bud: paradigms of cell type specification and tissue morphogenesis. *Curr Top Dev Biol* 2016;117:647–669. [PubMed: 26970006]
6. Zaret KS. Genetic programming of liver and pancreas progenitors: lessons for stem-cell differentiation. *Nat Rev Genet* 2008;9:329–340. [PubMed: 18398419]
7. Schmelzer E, Zhang L, Bruce A, et al. Human hepatic stem cells from fetal and postnatal donors. *J Exp Med* 2007;204:1973–1987. [PubMed: 17664288]
8. Tanimizu N, Miyajima A. Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors. *J Cell Sci* 2004; 117:3165–3174. [PubMed: 15226394]
9. Decaens T, Godard C, de Reyniès A, et al. Stabilization of β -catenin affects mouse embryonic liver growth and hepatoblast fate. *Hepatology* 2008;47:247–258. [PubMed: 18038450]

10. Clotman F, Jacquemin P, Plumb-Rudewiez N, et al. Control of liver cell fate decision by a gradient of TGF β signaling modulated by Onecut transcription factors. *Genes Dev* 2005;19:1849–1854. [PubMed: 16103213]
11. Huch M, Dorrell C, Boj SF, et al. In vitro expansion of single Lgr5⁺ liver stem cells induced by Wnt-driven regeneration. *Nature* 2013;494:247–250. [PubMed: 23354049]
12. Huch M, Gehart H, Van Boxtel R, et al. Long-term culture of genome-stable bipotent stem cells from adult human liver. *Cell* 2015;160:299–312. [PubMed: 25533785]
13. Nantasanti S, Spee B, Kruitwagen HS, et al. Disease modeling and gene therapy of copper storage disease in canine hepatic organoids. *Stem Cell Rep* 2015;5:895–907.
14. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007;131:861–872. [PubMed: 18035408]
15. Si-Tayeb K, Noto FK, Nagaoka M, et al. Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. *Hepatology* 2010;51:297–305. [PubMed: 19998274]
16. Touboul T, Hannan NRF, Corbineau S, et al. Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. *Hepatology* 2010;51:1754–1765. [PubMed: 20301097]
17. Song Z, Cai J, Liu Y, et al. Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells. *Cell Res* 2009;19:1233–1242. [PubMed: 19736565]
18. Banas A, Teratani T, Yamamoto Y, et al. Adipose tissue-derived mesenchymal stem cells as a source of human hepatocytes. *Hepatology* 2007;46:219–228. [PubMed: 17596885]
19. Lee K-D, Kuo TK-C, Whang-Peng J, et al. In vitro hepatic differentiation of human mesenchymal stem cells. *Hepatology* 2004;40:1275–1284. [PubMed: 15562440]
20. Wu X-B, Tao R. Hepatocyte differentiation of mesenchymal stem cells. *Hepatobiliary Pancreat Dis Int* 2012; 11:360–371. [PubMed: 22893462]
21. Lu W-Y, Bird TG, Boulter L, et al. Hepatic progenitor cells of biliary origin with liver repopulation capacity. *Nat Cell Biol* 2015;17:971–983. [PubMed: 26192438]
22. Beath S Hepatic function and physiology in the newborn. *Semin Neonatol* 2003;8:337–346. [PubMed: 15001122]
23. Grijalva J, Vakili K. Neonatal liver physiology. *Semin Pediatr Surg* 2013;22:185–189. [PubMed: 24331092]
24. Lautt WW, ed. *Hepatic Circulation Physiology and Pathophysiology*. San Rafael, CA: Morgan and Claypool Life Sciences, 2009.
25. Emery JL. Functional asymmetry of the liver. *Ann N Y Acad Sci* 1963;111:37–44. [PubMed: 14085863]
26. Rudolph AM. Hepatic and ductus venosus blood flows during fetal life. *Hepatology* 1983;3:254–258. [PubMed: 6832717]
27. Lautt WW, Greenway CV. Conceptual review of the hepatic vascular bed. *Hepatology* 1987;7:952–963. [PubMed: 3308669]
28. Lind J Changes in the liver circulation at birth. *Ann N Y Acad Sci* 2006;111:110–120.
29. <Zink J. The fetal and neonatal hepatic circulation In: Lautt WW, ed. *Hepatic Circulation in Health and Disease*. New York: Raven Press, 1981:227–248.
30. Tilles AW, Baskaran H, Roy P, et al. Effects of oxygenation and flow on the viability and function of rat hepatocytes cocultured in a microchannel flat-plate bioreactor. *Biotechnol Bioeng* 2001;73:379–389. [PubMed: 11320508]
31. Tanaka Y, Yamato M, Okano T, et al. Evaluation of effects of shear stress on hepatocytes by a microchip-based system. *Meas Sci Technol* 2006;17:3167–3170.
32. Goral VN, Yuen PK. Microfluidic platforms for hepatocyte cell culture: new technologies and applications. *Ann Biomed Eng* 2012;40:1244–1254. [PubMed: 22042626]
33. Kidambi S, Yarmush RS, Novik E, et al. Oxygen-mediated enhancement of primary hepatocyte metabolism, functional polarization, gene expression, and drug clearance. *Proc Natl Acad Sci* 2009;106:15714–15719. [PubMed: 19720996]

34. <j/>Mallanna SK, Duncan SA. Differentiation of hepatocytes from pluripotent stem cells In: Schlaeger T, ed. Current Protocols in Stem Cell Biology. Vol 1 Hoboken, NJ: John Wiley & Sons, Inc, 2013:1G.4.1–1G.4.13.
35. Baptista PM, Moran EC, Vyas D, et al. Fluid flow regulation of revascularization and cellular organization in a bioengineered liver platform. *Tissue Eng Part C Methods* 2016;22:199–207. [PubMed: 26772270]
36. <j/>Monga SPS, ed. Molecular Pathology of Liver Diseases. Boston, MA: Springer, 2011.
37. Katz N, Teutsch HF, Jungermann K, et al. Heterogeneous reciprocal localization of fructose-1,6-bisphosphatase and of glucokinase in microdissected periportal and perivenous rat liver tissue. *FEBS Lett* 1977;83:272–276. [PubMed: 201494]
38. Selwyn FP, Cui JY, Klaassen CD. Special section on drug metabolism and the microbiome RNA-seq quantification of hepatic drug processing genes in germ-free mice. *Drug Metab Dispos* 2015;43:1572–1580. [PubMed: 25956306]
39. Selwyn FP, Cheng SL, Klaassen CD, et al. Regulation of hepatic drug-metabolizing enzymes in germ-free mice by conventionalization and probiotics. *Drug Metab Dispos* 2016;44:262–274. [PubMed: 26586378]
40. Selwyn FP, Cheng SL, Bammler TK, et al. Developmental regulation of drug-processing genes in livers of germfree mice. *Toxicol Sci* 2015;147:84–103. [PubMed: 26032512]
41. Avior Y, Levy G, Zimerman M, et al. Microbial-derived lithocholic acid and vitamin K 2 drive the metabolic maturation of pluripotent stem cells-derived and fetal hepatocytes. *Hepatology* 2015;62:265–278. [PubMed: 25808545]
42. Jump DB, Ren B, Clarke S, et al. Effects of fatty acids on hepatic gene expression. *Prostaglandins Leukot Essent Fatty Acids* 1995;52:107–111. [PubMed: 7784444]
43. Synold TW, Dussault I, Forman BM. The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux. *Nat Med* 2001;7:584–590. [PubMed: 11329060]
44. Laffitte BA, Chao LC, Li J, et al. Activation of liver X receptor improves glucose tolerance through coordinate regulation of glucose metabolism in liver and adipose tissue. *Proc Natl Acad Sci U S A* 2003;100:5419–5424. [PubMed: 12697904]
45. Huang W, Ma K, Zhang J, et al. Nuclear receptor-dependent bile acid signaling is required for normal liver regeneration. *Science* 2006;312:233–236. [PubMed: 16614213]
46. Kamiya A, Kinoshita T, Ito Y, et al. Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. *EMBO J* 1999;18: 2127–2136. [PubMed: 10205167]
47. Kamiya A, Kinoshita T, Miyajima A. Oncostatin M and hepatocyte growth factor induce hepatic maturation via distinct signaling pathways. *FEBS Lett* 2001;492:90–94. [PubMed: 11248243]
48. Levy G, Bomze D, Heinz S, et al. Long-term culture and expansion of primary human hepatocytes. *Nat Biotech* 2015;33:1264–1271.
49. Schmidt C, Bladt F, Goedecke S, et al. Scatter factor/ hepatocyte growth factor is essential for liver development. *Nature* 1995;373:699–702. [PubMed: 7854452]
50. Kitade M, Factor VM, Andersen JB, et al. Specific fate decisions in adult hepatic progenitor cells driven by MET and EGFR signaling. *Genes Dev* 2013;27:1706–1717. [PubMed: 23913923]
51. Ballard FJ, Wong SSC, Knowles SE, et al. Insulin inhibition of protein degradation in cell monolayers. *J Cell Physiol* 1980;105:335–346. [PubMed: 7007398]
52. Balavoine S, Feldmann G, Lardeux B. Regulation of RNA degradation in cultured rat hepatocytes: effects of specific amino acids and insulin. *J Cell Physiol* 1993;156:56–62. [PubMed: 7686166]
53. Dahn MS, Hsu CJ, Lange MP, et al. Factors affecting secretory protein production in primary cultures of rat hepatocytes. *Proc Soc Exp Biol Med* 1993;203:38–44. [PubMed: 8475137]
54. Flaim KE, Hutson SM, Lloyd CE, et al. Direct effect of insulin on albumin gene expression in primary cultures of rat hepatocytes. *Am J Physiol* 1985;249:E447–E453. [PubMed: 3904479]
55. Odom DT, Zizlsperger N, Gordon DB, et al. Control of pancreas and liver gene expression by HNF transcription factors. *Science* 2004;303:1378–1381. [PubMed: 14988562]
56. Nishikawa T, Bell A, Brooks JM, et al. Resetting the transcription factor network reverses terminal chronic hepatic failure. *J Clin Invest* 2015;125:1–12. [PubMed: 25654544]

57. Schrem H, Klempnauer J, Borlak J. Liver-enriched transcription factors in liver function and development. Part I: the hepatocyte nuclear factor network and liver-specific gene expression. *Pharmacol Rev* 2002;54:129–158. [PubMed: 11870262]
58. Schrem H, Klempnauer J, Borlak J. Liver-enriched transcription factors in liver function and development. Part II: the C/EBPs and D site-binding protein in cell cycle control, carcinogenesis, circadian gene regulation, liver regeneration, apoptosis, and liver-specific gene regulation. *Pharmacol Rev* 2004;56:291–330. [PubMed: 15169930]
59. Sumi K, Tanaka T, Uchida A, et al. Cooperative interaction between hepatocyte nuclear factor 4 alpha and GATA transcription factors regulates ATP-binding cassette sterol transporters ABCG5 and ABCG8. *Mol Cell Biol* 2007;27:4248–4260. [PubMed: 17403900]
60. Alder O, Cullum R, Lee S, et al. Hippo signaling influences HNF4A and FOXA2 enhancer switching during hepatocyte differentiation. *Cell Rep* 2014;9:261–271. [PubMed: 25263553]
61. Jackson DA, Rowader KE, Stevens K, et al. Modulation of liver-specific transcription by interactions between hepatocyte nuclear factor 3 and nuclear factor 1 binding DNA in close apposition. *Mol Cell Biol* 1993;13: 2401–2410. [PubMed: 8455618]
62. Metzger S, Halaas JL, Breslow JL, et al. Orphan receptor HNF-4 and bZip protein C/EBP alpha bind to overlapping regions of the apolipoprotein B gene promoter and synergistically activate transcription. *J Biol Chem* 1993; 268:16831–16838. [PubMed: 8344962]
63. Samadani U, Costa RH. The transcriptional activator hepatocyte nuclear factor 6 regulates liver gene expression. *Mol Cell Biol* 1996;16:6273–6284. [PubMed: 8887657]
64. Kuo CJ, Conley PB, Chen L, et al. A transcriptional hierarchy involved in mammalian cell-type specification. *Nature* 1992;355:457–461. [PubMed: 1734282]
65. Ktistaki E, Talianidis I. Modulation of hepatic gene expression by hepatocyte nuclear factor 1. *Science* 1997;277:109–112. [PubMed: 9204893]
66. Eeckhoutte J, Formstecher P, Laine B. Hepatocyte nuclear factor 4 α enhances the hepatocyte nuclear factor 1 α -mediated activation of transcription. *Nucleic Acids Res* 2004;32:2586–2593. [PubMed: 15141028]
67. Hatzis P, Talianidis I. Regulatory mechanisms controlling human hepatocyte nuclear factor 4 α gene expression. *Mol Cell Biol* 2001;21:7320–7330. [PubMed: 11585914]
68. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006;126:663–676. [PubMed: 16904174]
69. Huang P, Zhang L, Gao Y, He Z, et al. Direct reprogramming of human fibroblasts to functional and expandable hepatocytes. *Cell Stem Cell* 2014;14:370–384. [PubMed: 24582927]
70. Du Y, Wang J, Jia J, Song N, Xiang C, et al. Human hepatocytes with drug metabolic function induced from fibroblasts by lineage reprogramming. *Cell Stem Cell* 2014;14:394–403. [PubMed: 24582926]
71. Godoy P, Schmidt-Heck W, Natarajan K, et al. Gene networks and transcription factor motifs defining the differentiation of stem cells into hepatocyte-like cells. *J Hepatol* 2015;63:934–942. [PubMed: 26022688]
72. Vogel C, Marcotte EM. Insights into regulation of protein abundance from proteomics and transcriptomics analyses. *Nat Rev Genet* 2013;13:227–232.
73. Zhou X, Cui L, Zhou X, et al. Induction of hepatocyte-like cells from human umbilical cord-derived mesenchymal stem cells by defined microRNAs. *J Cell Mol Med* 2016; 21:881–893. [PubMed: 27874233]
74. Lagos-Quintana M, Rauhut R, Yalcin A, et al. Identification of tissue-specific microRNAs from mouse. *Curr Biol* 2002;12:735–739. [PubMed: 12007417]
75. Esau C, Davis S, Murray SF, et al. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab* 2006;3:87–98. [PubMed: 16459310]
76. Deng XG, Qiu RL, Wu YH, et al. Overexpression of miR122 promotes the hepatic differentiation and maturation of mouse ESCs through a miR-122/FoxA1/HNF4 α positive feedback loop. *Liver Int* 2014;34:281–295. [PubMed: 23834235]
77. Jung KH, McCarthy RL, Zhou C, et al. MicroRNA regulates hepatocytic differentiation of progenitor cells by targeting YAP1. *Stem Cells* 2016;34:1284–1296. [PubMed: 26731713]

78. Alizadeh E, Eslaminejad MB, Akbarzadeh A, et al. Upregulation of miR-122 via trichostatin A treatments in hepatocyte-like cells derived from mesenchymal stem cells. *Chem Biol Drug Des* 2016;87:296–305. [PubMed: 26360933]
79. Henkens T, Papeleu P, Elaut G, et al. Trichostatin A, a critical factor in maintaining the functional differentiation of primary cultured rat hepatocytes. *Toxicol Appl Pharmacol* 2007;218:64–71. [PubMed: 17125810]
80. Bort R, Gómez-Lechón MJ, Castell JV, et al. Role of hepatocyte nuclear factor 3 gamma in the expression of human CYP2C genes. *Arch Biochem Biophys* 2004; 426:63–72. [PubMed: 15130783]
81. Rodríguez-Antona C, Bort R, Jover R, et al. Transcriptional regulation of human CYP3A4 basal expression by CCAAT enhancer-binding protein alpha and hepatocyte nuclear factor-3 gamma. *Mol Pharmacol* 2003;63: 1180–1189. [PubMed: 12695546]
82. Massillon D, Arinze IJ, Xu C, et al. Regulation of glucose-6-phosphatase gene expression in cultured hepatocytes and H4IIE cells by short-chain fatty acids: role of hepatic nuclear factor-4alpha. *J Biol Chem* 2003;278:40694–40701. [PubMed: 12915406]
83. Soto-Gutiérrez A, Kobayashi N, Rivas-Carrillo JD, et al. Reversal of mouse hepatic failure using an implanted liver-assist device containing ES cell-derived hepatocytes. *Nat Biotechnol* 2006;24:1412–1419. [PubMed: 17086173]
84. Alizadeh E, Zarghami N, Eslaminejad MB, et al. The effect of dimethyl sulfoxide on hepatic differentiation of mesenchymal stem cells. *Artif Cells Nanomed Biotechnol* 2014:1–8.
85. Papeleu P, Loyer P, Vanhaecke T, et al. Trichostatin A induces differential cell cycle arrests but does not induce apoptosis in primary cultures of mitogen-stimulated rat hepatocytes. *J Hepatol* 2003;39:374–382. [PubMed: 12927923]
86. Jin B, Park DW, Nam KW, et al. CpG methylation of the mouse CYP1A2 promoter. *Toxicol Lett* 2004;152:11–18. [PubMed: 15294342]
87. Sgodda M, Aurich H, Kleist S, et al. Hepatocyte differentiation of mesenchymal stem cells from rat peritoneal adipose tissue in vitro and in vivo. *Exp Cell Res* 2007; 313:2875–2886. [PubMed: 17574236]
88. Snykers S, Henkens T, De Rop E, et al. Role of epigenetics in liver-specific gene transcription, hepatocyte differentiation and stem cell reprogramming. *J Hepatol* 2009;51:187–211. [PubMed: 19457566]
89. Choi SS, Diehl AM. Epithelial-to-mesenchymal transitions in the liver. *Hepatology* 2009;50:2007–2013. [PubMed: 19824076]
90. Siller R, Greenhough S, Naumovska E, et al. Small molecule-driven hepatocyte differentiation of human pluripotent stem cells. *Stem Cell Rep* 2015;4:939–952.
91. Pascussi JM, Drocourt L, Gerbal-Chaloin S, et al. Dual effect of dexamethasone on CYP3A4 gene expression in human hepatocytes. Sequential role of glucocorticoid receptor and pregnane X receptor. *Eur J Biochem* 2001; 268:6346–6358. [PubMed: 11737189]
92. Vinken M, Papeleu P, Snykers S, et al. Involvement of cell junctions in hepatocyte culture functionality. *Crit Rev Toxicol* 2006;36:299–318. [PubMed: 16809101]
93. Fraczek J, Bolleyn J, Vanhaecke T, et al. Primary hepatocyte cultures for pharmaco-toxicological studies: at the busy crossroad of various anti-dedifferentiation strategies. *Arch Toxicol* 2013;87:577–610. [PubMed: 23242478]
94. Schuetz EG, Schmid W, Schutz G, et al. The glucocorticoid receptor is essential for induction of cytochrome P-4502B by steroids but not for drug or steroid induction of CYP3A or P-450 reductase in mouse liver. *Drug Metab Dispos* 2000;28:268–278. [PubMed: 10681370]
95. Shan J, Schwartz RE, Ross NT, et al. Identification of small molecules for human hepatocyte expansion and iPS differentiation. *Nat Chem Biol* 2013;9:514–520. [PubMed: 23728495]
96. Borowiak M, Maehr R, Chen S, et al. Small molecules efficiently direct endodermal differentiation of mouse and human embryonic stem cells. *Cell Stem Cell* 2009;4: 348–358. [PubMed: 19341624]
97. Tahamtani Y, Azarnia M, Farrokhi A, et al. Treatment of human embryonic stem cells with different combinations of priming and inducing factors toward definitive endoderm. *Stem Cells Dev* 2013;22:1419–1432. [PubMed: 23249309]

98. Herrero A, Gerbal-Chaloin S, Daujat-Chavanieu M. Bioinspired liver tissue engineering In: Jabbari E, Kim DH, Lee LP, et al., eds. Handbook of Biomimetics and Bioinspiration. World Scientific Publishing Company, 2014:1177–1239.
99. Guo X, Li W, Ma M, et al. Endothelial cell-derived matrix promotes the metabolic functional maturation of hepatocyte via integrin-Src signalling. *J Cell Mol Med* 2017; 11:2809–2822.
100. Wang B, Jakus AE, Baptista PM, et al. Functional maturation of induced pluripotent stem cell hepatocytes in extracellular matrix—a comparative analysis of bioartificial liver microenvironments. *Stem Cells Transl Med* 2016;5:1257–1267. [PubMed: 27421950]
101. Soto-Gutierrez A, Zhang L, Medberry C, et al. A wholeorgan regenerative medicine approach for liver replacement. *Tissue Eng Part C Methods* 2011;17:677–686. [PubMed: 21375407]
102. Shamis Y, Hasson E, Soroker A, et al. Organ-specific scaffolds for in vitro expansion, differentiation, and organization of primary lung cells. *Tissue Eng Part C Methods* 2011;17:861–870. [PubMed: 21595544]
103. Vyas D, Baptista PM, Brovold M, et al. Self-assembled liver organoids recapitulate hepatobiliary organogenesis in vitro. *Hepatology* 2018;1:1–36.
104. Godoy P, Hewitt NJ, Albrecht U, et al. Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Arch Toxicol* 2013;87:1315–1530. [PubMed: 23974980]
105. Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev* 2008; 88:125–172. [PubMed: 18195085]
106. Bussolino F, Di Renzo MF, Ziche M, et al. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *J Cell Biol* 1992;119:629–641. [PubMed: 1383237]
107. Roberts RA, Ganey PE, Ju C, et al. Role of the Kupffer cell in mediating hepatic toxicity and carcinogenesis. *Toxicol Sci* 2007;96:2–15. [PubMed: 17122412]
108. Henkel J, Gärtner D, Dorn C, et al. Oncostatin M produced in Kupffer cells in response to PGE2: possible contributor to hepatic insulin resistance and steatosis. *Lab Invest* 2011;91:1107–1117. [PubMed: 21519329]
109. Gregory SH, Wing EJ, Danowski KL, et al. IL-6 produced by Kupffer cells induces STAT protein activation in hepatocytes early during the course of systemic listerial infections. *J Immunol* 1998;160:6056–6061. [PubMed: 9637522]
110. Feder LS, Todaro JA, Laskin DL. Characterization of interleukin-1 and interleukin-6 production by hepatic endothelial cells and macrophages. *J Leukoc Biol* 1993; 53:126–132. [PubMed: 8445325]
111. De Bleser PJ, Niki T, Rogiers V, et al. Transforming growth factor-beta gene expression in normal and fibrotic rat liver. *J Hepatol* 1997;26:886–893. [PubMed: 9126804]
112. Boulter L, Govaere O, Bird TG, et al. Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. *Nat Med* 2012;18:572–579. [PubMed: 22388089]
113. Takebe T, Zhang R-R, Koike H, et al. Generation of a vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nat Protoc* 2014;9:396–409. [PubMed: 24457331]
114. Camp JG, Sekine K, Gerber T, et al. Multilineage communication regulates human liver bud development from pluripotency. *Nature* 2017;546:533–538. [PubMed: 28614297]
115. Ghouse R, Chu A, Wang Y, et al. Mysteries of alpha-1 antitrypsin deficiency: emerging therapeutic strategies for a challenging disease. *Dis Model Mech* 2014;7:411–419. [PubMed: 24719116]
116. Sinz M, Wallace G, Sahi J. Current industrial practices in assessing CYP450 enzyme induction: preclinical and clinical. *AAPS J* 2008;10:391–400. [PubMed: 18686044]
117. Raghuvveer TS, Garg U, Graf WD. Inborn errors of metabolism in infancy and early childhood: an update. *Am Fam Physician* 2006;73:1981–1990. [PubMed: 16770930]
118. van Montfoort JE, Hagenbuch B, Groothuis GMM, et al. Drug uptake systems in liver and kidney. *Curr Drug Metab* 2003;4:185–211. [PubMed: 12769665]

119. Duncan SA, Navas MA, Dufort D, et al. Regulation of a transcription factor network required for differentiation and metabolism. *Science* 1998;281:692–695. [PubMed: 9685261]
120. Soltys KA, Soto-Gutiérrez A, Nagaya M, et al. Barriers to the successful treatment of liver disease by hepatocyte transplantation. *J Hepatol* 2010;53:769–774. [PubMed: 20667616]
121. Soltys KA, Setoyama K, Tafaleng EN, et al. Host conditioning and rejection monitoring in hepatocyte transplantation in humans. *J Hepatol* 2017;66:987–1000. [PubMed: 28027971]
122. Puppi J, Strom SC, Hughes RD, et al. Improving the techniques for human hepatocyte transplantation: report from a consensus meeting in London. *Cell Transplant* 2012;21:1–10. [PubMed: 21457616]
123. Fox IJ, Chowdhury JR, Kaufman SS, et al. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. *N Engl J Med* 1998;338:1422–1426. [PubMed: 9580649]
124. Yamanouchi K, Zhou H, Roy-Chowdhury N, et al. Hepatic irradiation augments engraftment of donor cells following hepatocyte transplantation. *Hepatology* 2009; 49:258–267. [PubMed: 19003915]
125. Nygaard S, Barzel A, Haft A, et al. A universal system to select gene-modified hepatocytes in vivo. *Sci Transl Med* 2016;8:342ra79–342ra79.
126. Tateno C, Yoshizane Y, Saito N, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* 2004;165:901–912. [PubMed: 15331414]
127. Azuma H, Paulk N, Ranade A, et al. Robust expansion of human hepatocytes in *Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}* mice. *Nat Biotechnol* 2007;25:903–910. [PubMed: 17664939]
128. Bissig K-D, Wieland SF, Tran P, et al. Human liver chimeric mice provide a model for hepatitis B and C virus infection and treatment. *J Clin Invest* 2010;120:924–930. [PubMed: 20179355]
129. Hasegawa M, Kawai K, Mitsui T, et al. The reconstituted “humanized liver” in TK-NOG mice is mature and functional. *Biochem Biophys Res Commun* 2011;405: 405–410. [PubMed: 21238430]
130. Strom SC, Davila J, Grompe M. Chimeric mice with humanized liver: tools for the study of drug metabolism, excretion, and toxicity. *Methods Mol Biol* 2010;640: 491–509. [PubMed: 20645070]
131. Zhu S, Rezvani M, Harbell J, et al. Mouse liver repopulation with hepatocytes generated from human fibroblasts. *Nature* 2014;508:93–97. [PubMed: 24572354]
132. Chen Y, Li Y, Wang X, et al. Amelioration of hyperbilirubinemia in Gunn rats after transplantation of human induced pluripotent stem cell-derived hepatocytes. *Stem Cell Reports* 2015;5:22–30. [PubMed: 26074313]
133. Basma H, Soto-Gutiérrez A, Yannam GR, et al. Differentiation and transplantation of human embryonic stem cell-derived hepatocytes. *Gastroenterology* 2009; 136:990–999. [PubMed: 19026649]
134. Carpentier A, Tesfaye A, Chu V, et al. Engrafted human stem cell-derived hepatocytes establish an infectious HCV murine model. *J Clin Invest* 2014;124:4953–4964. [PubMed: 25295540]
135. Rezvani M, Grimm AA, Willenbring H. Assessing the therapeutic potential of lab-made hepatocytes. *Hepatology* 2016;64:287–294. [PubMed: 27014802]
136. Chowdhury JR, Kondapalli R, Chowdhury NR. Gunn rat: a model for inherited deficiency of bilirubin glucuronidation. *Adv Vet Sci Comp Med* 1993;37:149–173. [PubMed: 8273513]
137. Faulkner-Jones A, Fyfe C, Cornelissen D-J, et al. Bioprinting of human pluripotent stem cells and their directed differentiation into hepatocyte-like cells for the generation of mini-livers in 3D. *Biofabrication* 2015; 7:44102.
138. Nguyen DG, Funk J, Robbins JB, et al. Bioprinted 3D primary liver tissues allow assessment of organ-level response to clinical drug induced toxicity in vitro. *PLoS One* 2016;11:e0158674. [PubMed: 27387377]
139. Ma X, Qu X, Zhu W, et al. Deterministically patterned biomimetic human iPSC-derived hepatic model via rapid 3D bioprinting. *Proc Natl Acad Sci* 2016;113:2206–2211. [PubMed: 26858399]
140. Zhong C, Xie H-Y, Zhou L, et al. Human hepatocytes loaded in 3D bioprinting generate mini-liver. *Hepatobiliary Pancreat Dis Int* 2016;15:512–518. [PubMed: 27733321]
141. Massa S, Sakr MA, Seo J, et al. Bioprinted 3D vascularized tissue model for drug toxicity analysis. *Biomicrofluidics* 2017;11:44109.

142. Ji S, Guvendiren M. Recent advances in bioink design for 3D bioprinting of tissues and organs. *Front Bioeng Biotechnol* 2017;5:23. [PubMed: 28424770]
143. Zhang YS, Yue K, Aleman J, et al. 3D bioprinting for tissue and organ fabrication. *Ann Biomed Eng* 2017; 45:148–163. [PubMed: 27126775]
144. Flaim CJ, Chien S, Bhatia SN. An extracellular matrix microarray for probing cellular differentiation. *Nat Methods* 2005;2:119–125. [PubMed: 15782209]
145. McGuigan AP, Sefton MV. Vascularized organoid engineered by modular assembly enables blood perfusion. *Proc Natl Acad Sci U S A* 2006;103:11461–11466. [PubMed: 16864785]
146. Khetani SR, Bhatia SN. Microscale culture of human liver cells for drug development. *Nat Biotechnol* 2008; 26:120–126. [PubMed: 18026090]
147. Bhatia SN, Ingber DE. Microfluidic organs-on-chips. *Nat Biotechnol* 2014;32:760–772. [PubMed: 25093883]
148. Giobbe GG, Michielin F, Luni C, et al. Functional differentiation of human pluripotent stem cells on a chip. *Nat Methods* 2015;12:637–640. [PubMed: 26030445]
149. Chavez A, Scheiman J, Vora S, et al. Highly efficient Cas9-mediated transcriptional programming. *Nat Methods* 2015;12:326–328. [PubMed: 25730490]
150. Balboa D, Weltner J, Euroola S, et al. Conditionally stabilized dCas9 activator for controlling gene expression in human cell reprogramming and differentiation. *Stem cell reports* 2015;5:448–459. [PubMed: 26352799]

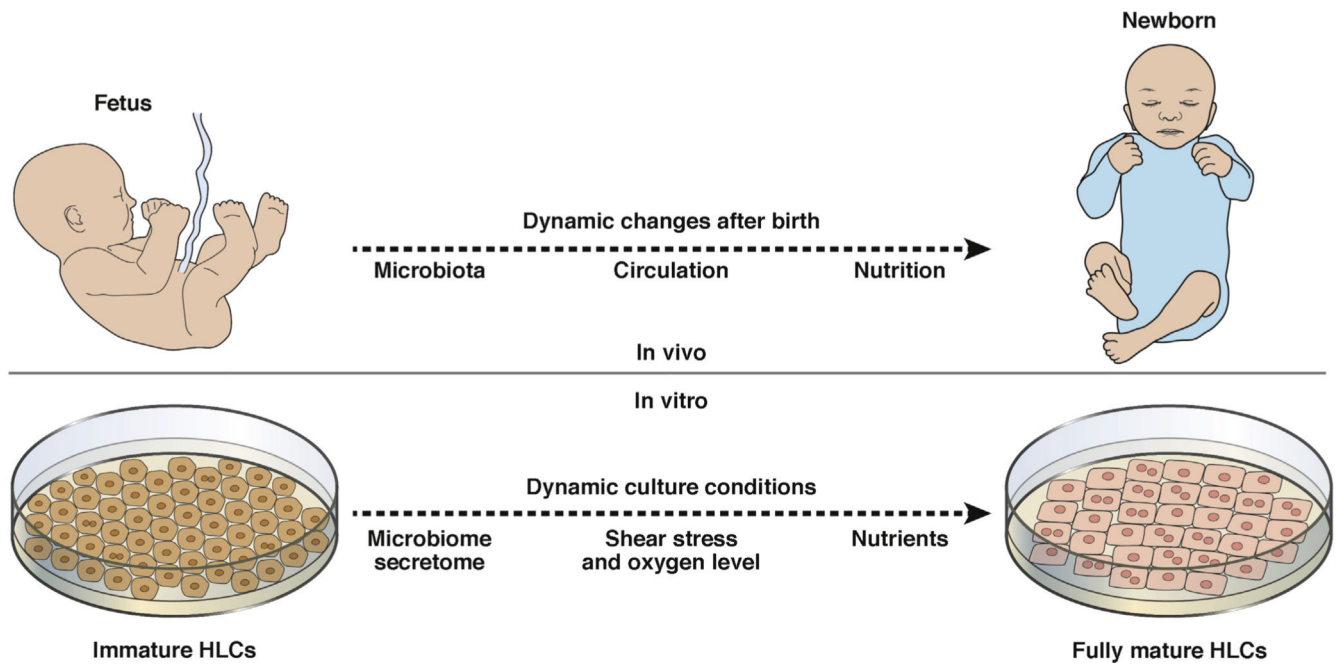


Figure 1. Changes in liver maturation from fetus to newborn. Upon birth, the human fetal liver undergoes many changes, including changes in circulation, the microbiome, and nutrition, which are required for hepatic maturation. Increasing our understanding of these changes might provide information needed to develop more efficient in vitro liver maturation methods.

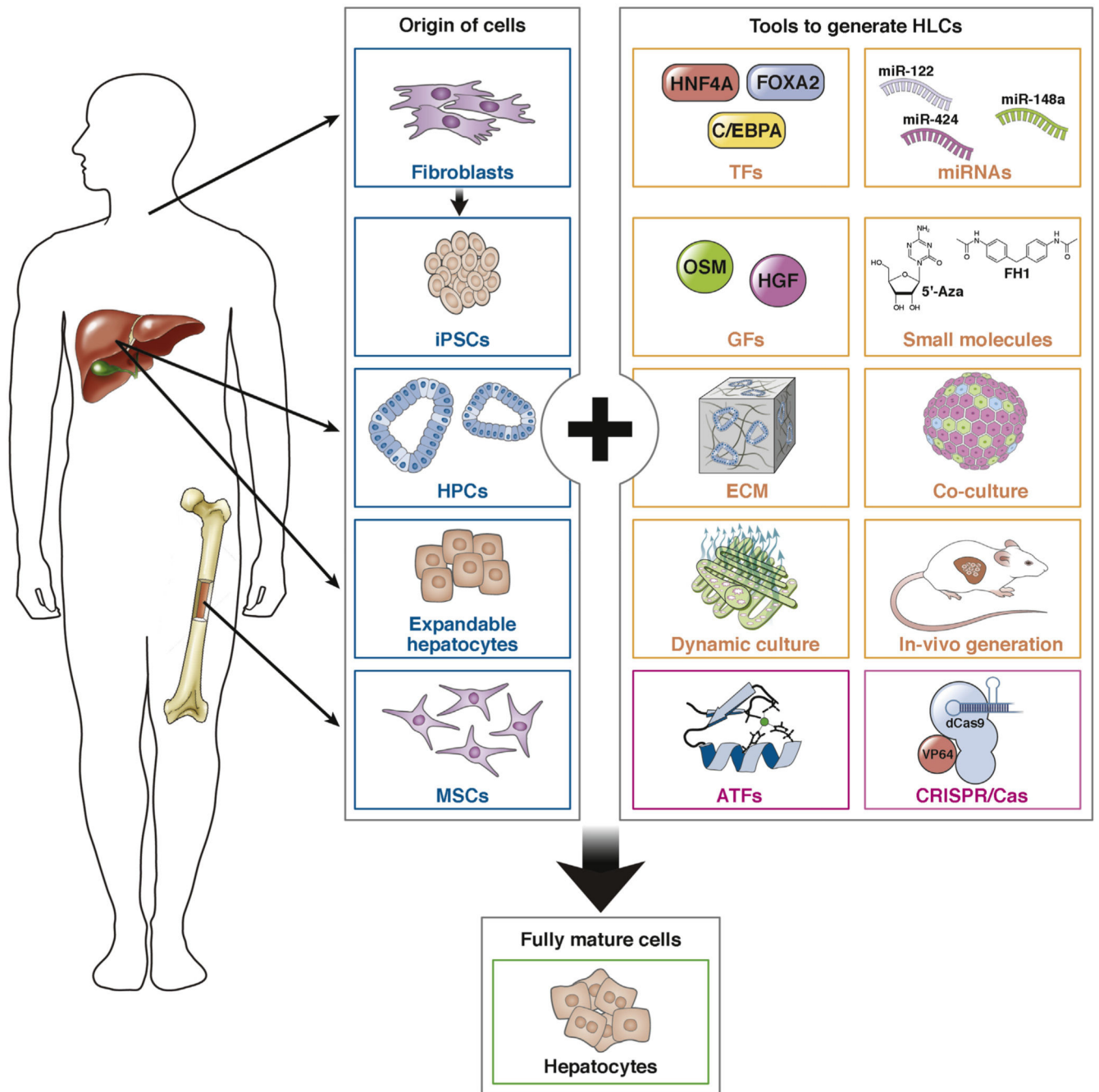


Figure 2. Cells and methods to generate HLCs. *Blue boxes* show cells, and *yellow and pink boxes* show methods, used to generate HLCs.

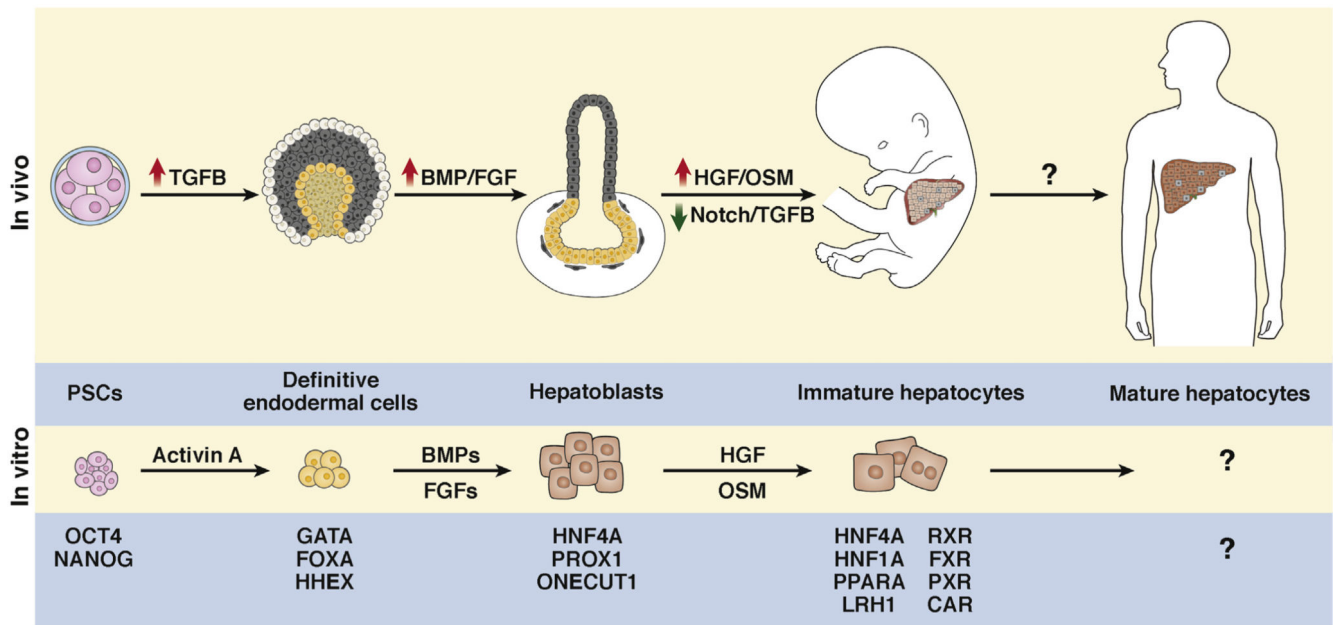


Figure 3. Molecular changes during hepatocyte differentiation. Transcription factors and signaling molecules that regulate each stage of hepatocyte differentiation. Hepatocyte-generating cells are *colored* and supporting tissue are *black* and *gray*. *Question marks* indicate pathways under investigation.

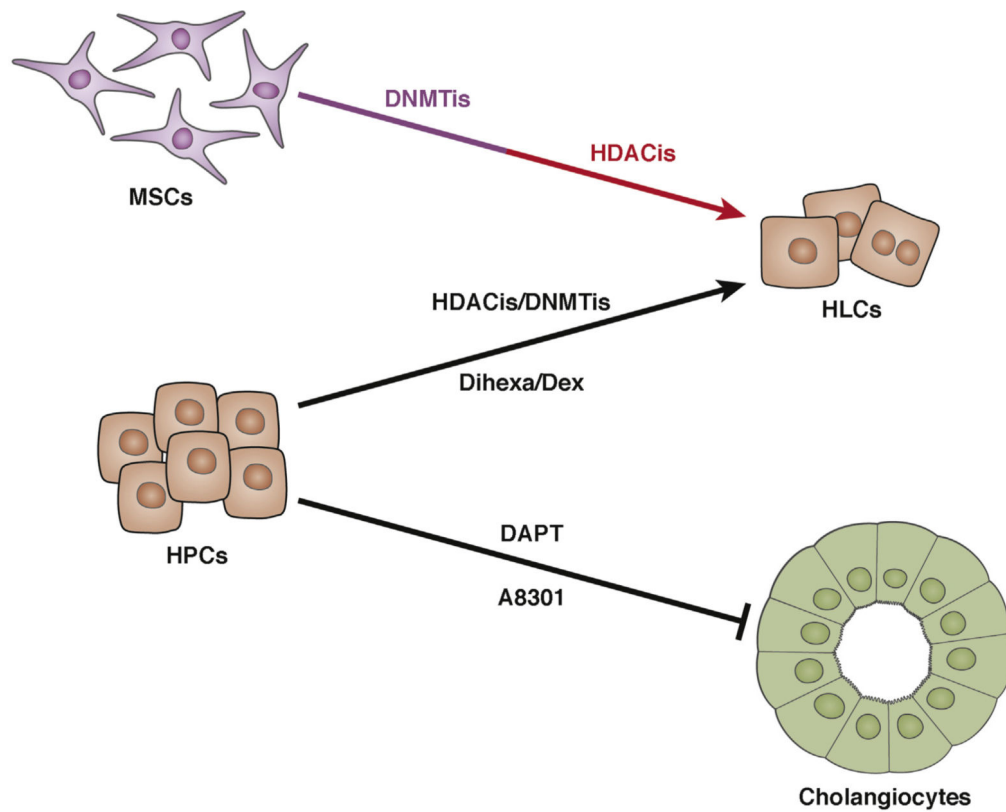


Figure 4.

Small molecules that generate HLCs or promote HLC maturation. Several small molecules can promote differentiation of HPCs and MSCs into HLCs. DNMTis prime differentiation of MSCs into HLCs. HDACis act during or after differentiation into HLCs. DAPT and A8301 block differentiation of HPCs into cholangiocytes.

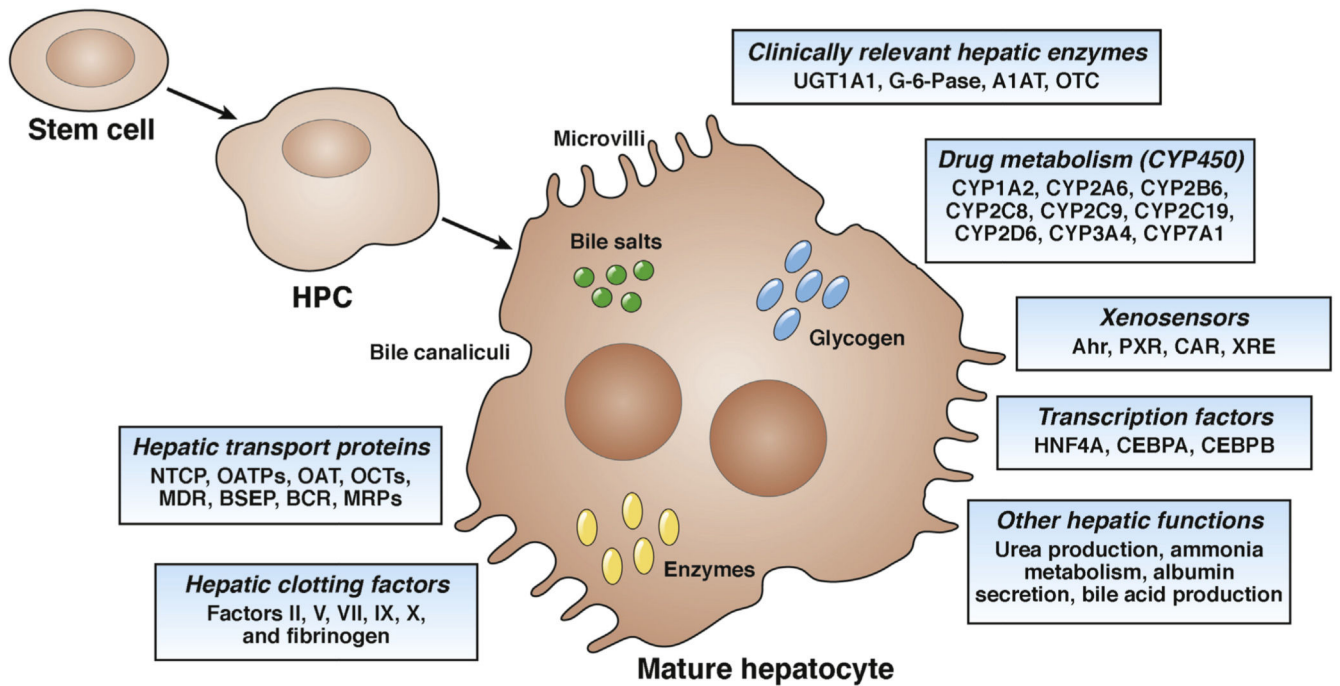


Figure 5.

Structural, metabolic, and secretory functions of mature hepatocytes. Mature hepatocytes have a typical epithelial cell structure, including polygonal shape and presence of epithelial markers, highly express genes related to drug metabolism (phase 1, 2, and 3), secrete albumin, and produce urea and bile acid.

Table 1.

Characteristics of Mature Human Hepatocytes

Characteristic	Description
Morphology	
Epithelial morphology	Polygonal in shape; microvilli on cell surface
Polarization	Tight junctions (ZO1) in apical tip of the lateral membrane; adherens junctions (E-cadherin) at lateral membrane; adaptor proteins (α - and β -catenin) at basolateral membrane
Polypliodization	Presence of 2 or more nuclei in fraction of the cells
Gene expression	
Serum proteins	<i>ALB, TF, TTR</i>
Hepatocytic TFs	<i>HNF4A, HNF1A, CEBPA/B</i>
Metabolism enzymes	<i>CYP3A4, CYP7A1, G6P</i>
Absence of biliary markers	<i>HNF1B, CK7, SOX9</i>
Absence of stem cell markers or hepatic immature markers	<i>LGR5, OCT4, CD133, AFP, CYP3A7</i>
In vitro testing	
Phase 1 CYP activities	Metabolism of phenacetin (CYP1A2), bupropion (CYP2B6), tolbutamide (CYP2C9) diclofenac (CYP2G9), diclofenac (CYP3A4)
Phase 2 transferase activities	Metabolism of bilirubin (UGT1A1), dopamine (SULT1A1), p-aminobenzoic acid (NAT1), 1-chloro-2,4-dinitrobenzene (GSTs)
Phase 3 transporter activities	Transport of indocyanine green (OATP1B1), rhodamine 123 (MDR1), 5(6)-carboxy-2, ⁰ 7 ⁰ -dichlorofluorescein (MRP2)
Bile acid synthesis	Formation of cholic acid and chenodeoxycholic acid (CYP8A1, AKR1D1, AKR1C4)
Glycogen storage	Periodic acid–Schiff staining for glycogen
Serum protein synthesis	Secretion of albumin, α -1 antitrypsin, fibronectin, transferrin, apoprotein, transthyretin, complement factors, coagulation factors
Cholesterol metabolism	Formation of 7 α -hydroxycholesterol (CYP7A1)
Lipid uptake	Transport of low-density lipoprotein
Urea metabolism	Synthesis of urea in cell extract
Coagulation factors	Factors II, V, VII, IX, X
Other clinically relevant enzymes	OCT, A1AT, BSEP, UGT1A1, G-6-Pase, FAH, ATP7B
In vivo testing	
Engraftment and repopulation	Human-specific markers or reporters to track transplanted HLCs in the liver (human-specific gene expression)
Restoration of liver function	Extended survival; presence of human proteins in the serum. Recovery of repopulating cells for genome-wide and functional metabolic studies
Tumorigenicity	Tumorigenesis in long-term transplantation experiments including several generations of re-transplantation