Hepatic and Biliary Differentiation of Somatic Cells

Chen Chen

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Hepatic and Biliary Differentiation of Somatic Cells

Lever en galweg differentiatie van somatische cellen
(met een samenvatting in het Nederlands)

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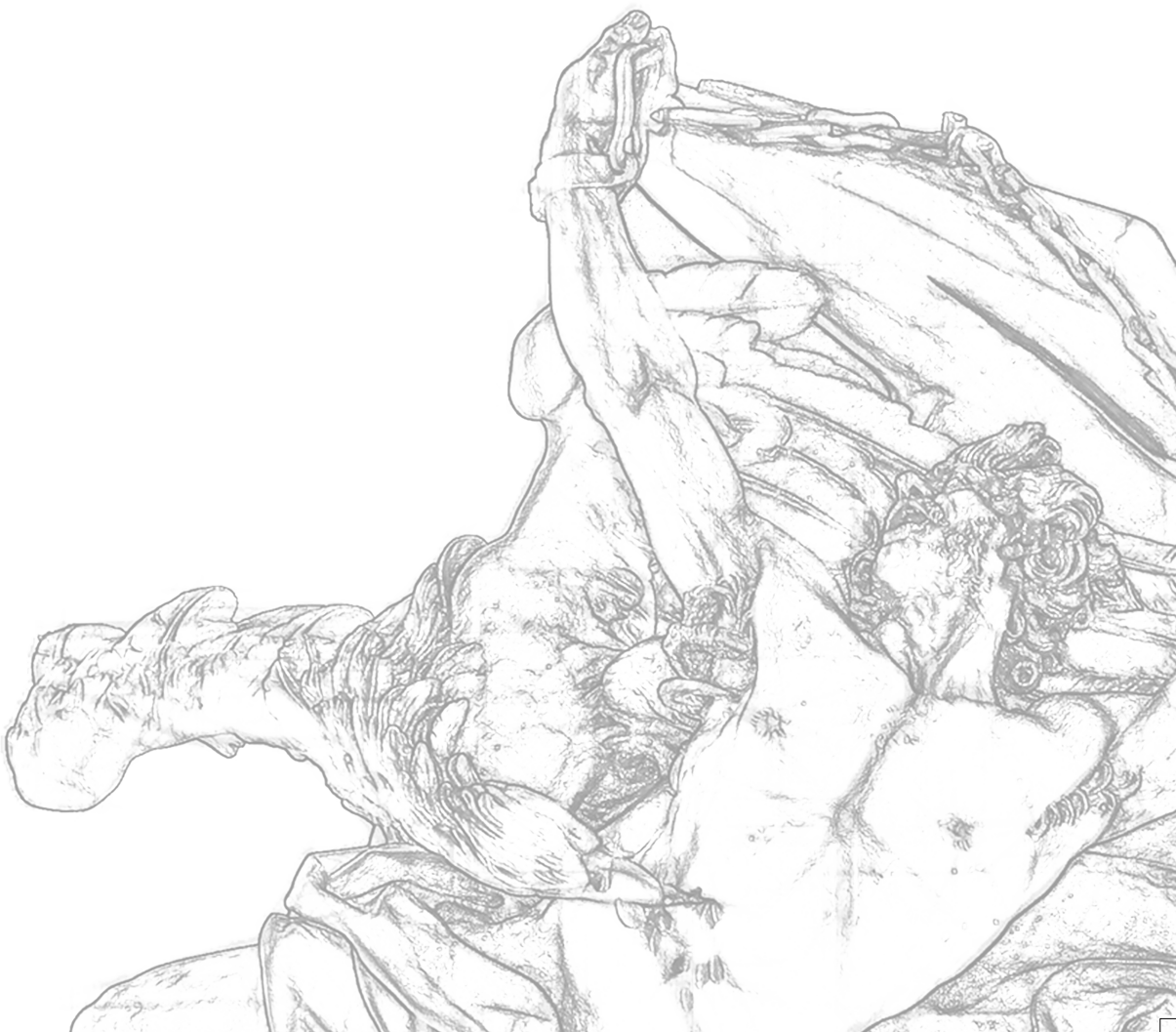


Aims and Scope

In the human body, the liver plays an important role in a variety of functions from protein and bile production to carbohydrate, cholesterol and mineral metabolism, detoxification and storage. Although already recognized by the Greeks in the ancient myth of Prometheus that the liver has powerful capacity to regenerate itself, in diseased conditions this regeneration can be severely hampered or even completely abolished. For end-stage liver disease, organ transplantation is still the most effective treatment so far. However, due to the lack of donor livers many patients die while on the waiting list. An alternative to donor organs is therefore urgently needed.

The principal aim of this thesis is to generate functional cells of the liver, which can ultimately provide the possibility to fabricate the whole organ in future. As the largest internal organ in the human body, the liver possesses a complex architecture to support its massive metabolism capacity. The smallest functional units in the liver are hepatic lobules, each of which consists of a central vein surrounded by plates of cells. These cells are generally derived from two sources. During early liver development, embryonic pluripotent stem cells generate an endoderm germ layer followed by foregut formation and then differentiate into hepatoblasts. These hepatoblasts are bi-potent somatic stem cells which can further differentiate towards either hepatocytes or cholangiocytes. Hepatocytes and cholangiocytes are the two main epithelial cell types performing most liver functions. Other supporting cells types in the liver are mainly derived from mesoderm (Figure 1).

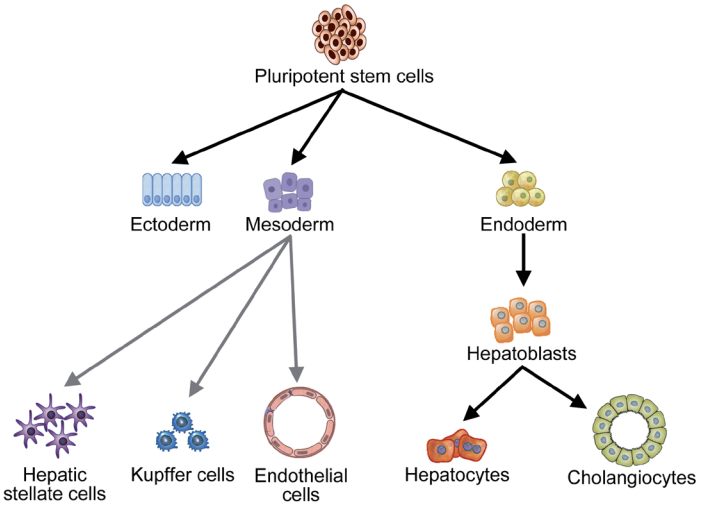


Figure 1. Schematic illustration showing the generation of hepatocytes and cholangiocytes during liver development.

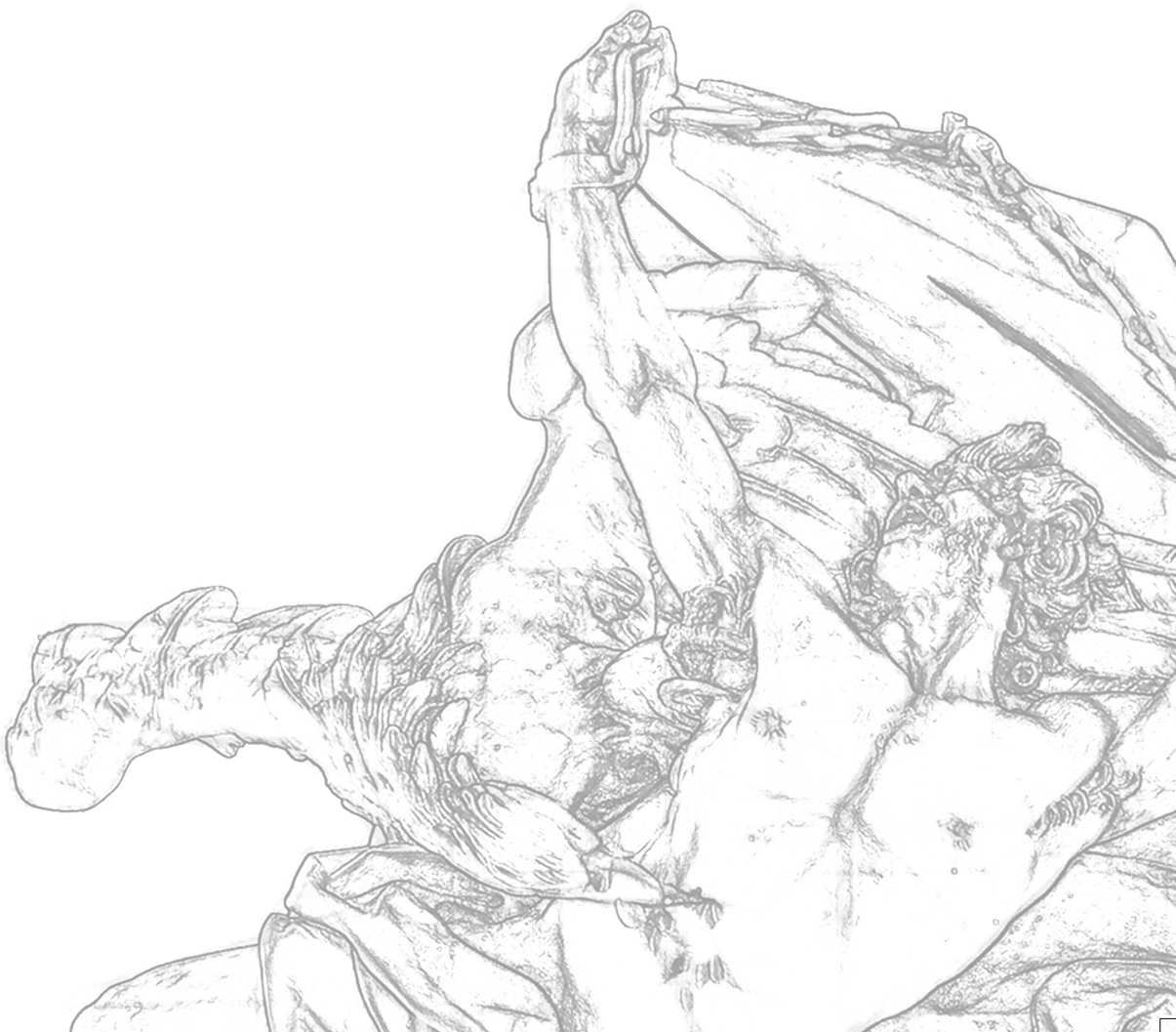
In light of the complexity of the liver, in **Chapter 1,** we systematically review the key factors directing the differentiation of somatic (liver) stem cells and the maintenance of mature functions of the liver. We propose possible strategies applying these key factors to generate functionally mature hepatocytes *in vitro* and discuss how to characterize the resulting cells.

In **Chapter 2**, we describe a method to generate hepatocyte-like cells from somatic cells through a technique called direct reprogramming. We use native extracellular matrix to induce the drug metabolism capacity of hepatocyte-like cells.

Although attempts have been made, the resulting hepatocyte-like cells still exhibit limited hepatic maturation. Therefore, in **Chapter 3**, in a dedifferentiating hepatocyte model, we describe a screening based on epigenetic modification and transcriptional analysis to identify novel transcription factors affecting this procedure. To make sure we had fully matured hepatocytes, we started with freshly isolated hepatocytes that dedifferentiate while in culture. We further apply this finding in inducing the terminal maturation of hepatocyte-like cells.

Cholangiocytes are the epithelial cells forming the intra- and extra-hepatic bile ducts. Biliary excretion is the main route to eliminate waste products and toxins from the liver. In **Chapter 4**, we establish a protocol to generate functional cholangiocyte-like cells from somatic liver stem cells. We also prove that these cholangiocyte-like cells can form artificial bile ducts which possess transportation capacity.

The findings and overall conclusion of these studies are summarized and discussed in **Chapter 5** and **6**.



Chapter 1

General introduction: 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 rapidly progressing. 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.

Introduction

The only proven treatment for patients with end-stage 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 in *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, following 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 beta (TGFB), which promote biliary differentiation [8-10]. Downregulation of these pathways promotes specification of hepatoblasts towards 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 hepatocyte-like 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.

1 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 therefore, are 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 towards 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 efficiently induction of PSCs into a definitive endoderm fate (inducers include activin A, fibroblast growth factor (FGF) 2, Wnt Family Member 3A (WNT3A), bone morphogenetic protein 4 (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 towards 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 towards 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 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 no direct comparison of the maturation level of HLCs generated from these sources. Furthermore, different standards were used for charactering 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).

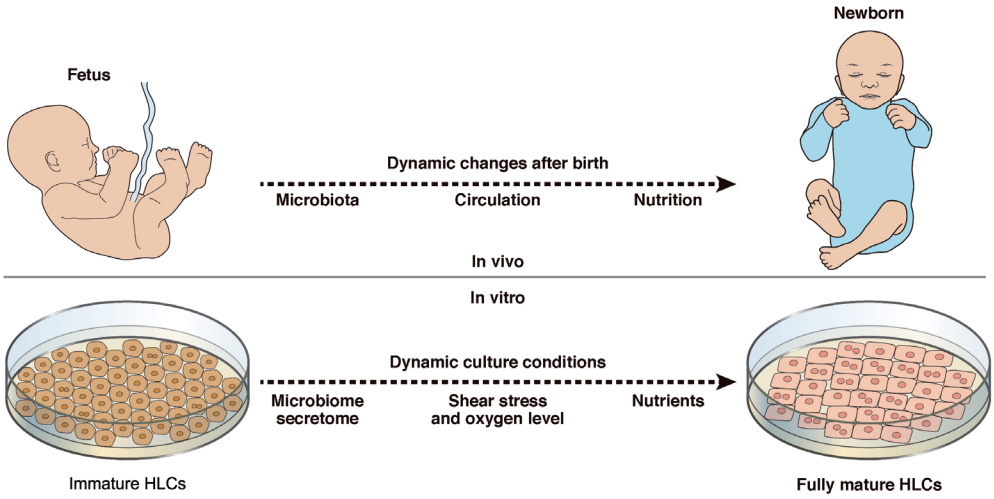

Figure 1. Changes in liver maturation from fetus to newborn. Upon birth, the human fetal liver undergoes many changes, including changes in circulation, the microbiome, and nutrition, which are required for hepatic maturation. Increasing our understanding of these changes might provide information needed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

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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 instance through polarization which in turn increases liver function [31,32]. Oxygen levels and mechanical stimulation experienced by hepatocytes might therefore be applicable for their maturation. Both features have now been recognized in adult hepatocytes [33] and incorporated into some protocols of human ESC hepatic induction [15,34]. Interestingly, other vascular mediators generated from liver circulation (prostaglandins, nitric oxide, catecholamines, etc.) 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 three 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 (~30% of adult levels) and progressively increases, reaching adult levels by one 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 post-natal 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 post-prandial 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 (PPARs) or hepatocyte nuclear factor 4 alpha (HNF4A), or the role of sterols, steroids and bile salts and their nuclear receptors (retinoid X receptor (RXR), farnesoid X receptor (FXR), pregnane X receptor (PXR), etc) might have larger effects on hepatocyte maturation than previously reported [42-45].

Concomitantly, many hormones and growth factors are secreted during this period and most of these hepatotrophic factors (insulin, glucagon, gastrin, etc) 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 including 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 three important regulators of hepatocyte specification and maturation (Figure 3).

The role of OSM, an interleukin 6 (IL6) family cytokine, in hepatocyte maturation was well defined by Kamiya et al, who demonstrated that OSM upregulates the expression of albumin, glucose-6-phosphate dehydrogenase (G6PD), and tyrosine aminotransferase in fetal hepatocytes isolated from the embryonic murine liver (embryonic day [E]14.5) [46]. Fetal hepatocytes incubated with OSM have a similar morphology to mature hepatocytes, such as tight intracellular contacts, highly condensed and granulated cytosol, and clear round-shaped nuclei. In addition, OSM induces hepatocyte-specific 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. Knock-out 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 upregulates the

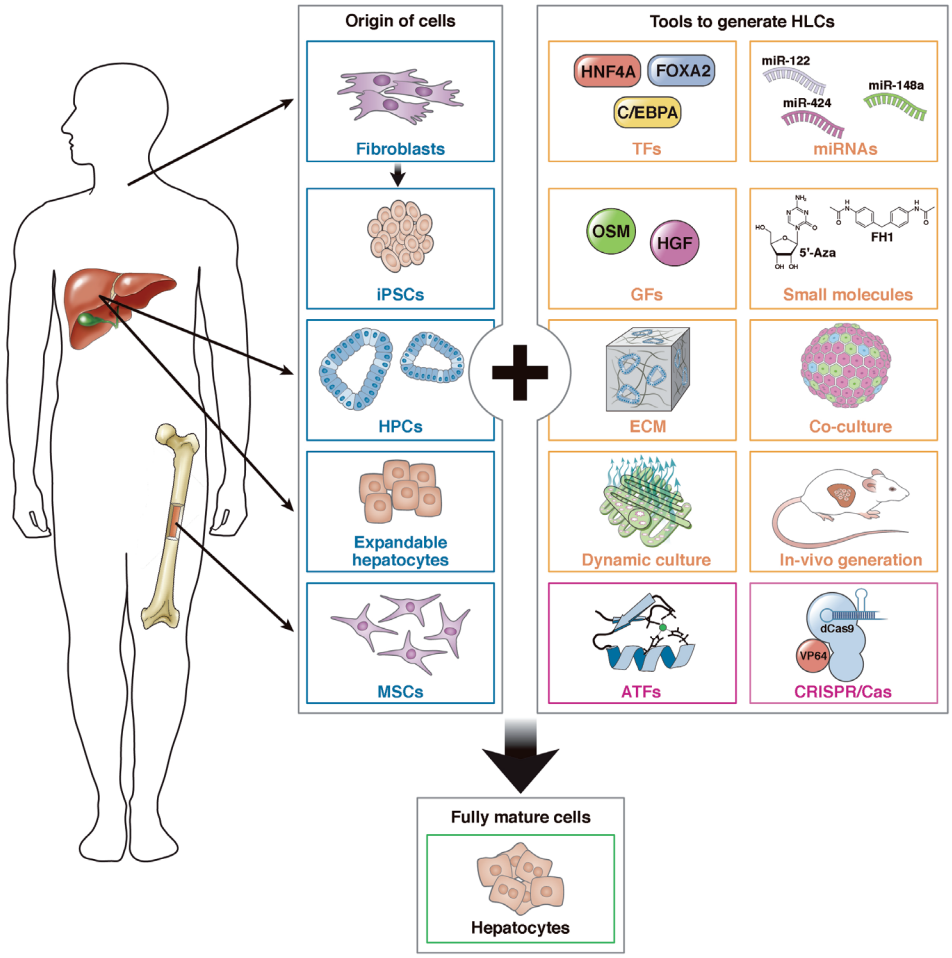

Figure 2. Cells and methods to generate HLCs. Blue boxes show cells, and yellow and pink boxes show methods, used to generate HLCs.

expression of several mature hepatocyte markers such as carbamoyl-phosphate synthase 1, G6PD, 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 (cMET), 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-

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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. LEFTs include HNF4A, constitutive androstane receptor, eosinophil-associated, ribonuclease A, PPAR alpha, RXR alpha, FXR, PXR, small heterodimer partner, and liver receptor homolog 1 (Figure 3).

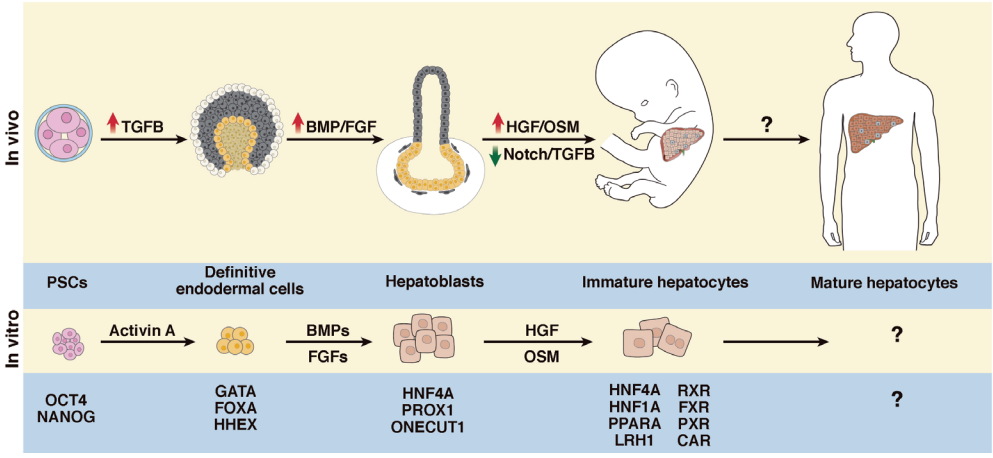

Figure 3. Molecular changes during hepatocyte differentiation. Transcription factors and signaling molecules that regulate each stage of hepatocyte differentiation. Hepatocyte-generating cells are colored and supporting tissue are black and gray. Question marks indicate pathways under investigation.

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 al found that 43% of actively transcribed genes in the human liver are regulated by HNF4A [55].

Furthermore, overexpression of only HNF4A resets the hepatocyte transcription factor network in damaged hepatocytes from rats following administration of CCl_4 [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, HNF1 homeobox A (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 HNF4A [64] 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 factors [68] can reprogram non-hepatic lineage cells into hepatocyte-like cells (iHeps) [69,70]. However, all iHeps 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 al compared genome-wide gene expression profiles of ESC-derived hepatocyte-like cells (ES-HLCs) to primary human hepatocytes freshly isolated and cultured for 14 days [71]. 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 mRNA and protein levels (up to 40% [72]) indicating an imbalance in the ES-HLC system. Therefore, transcriptome data is 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 (miRNAs) are a class of small non-coding RNAs that regulate cell differentiation, proliferation, and survival. Zhou et al generated HLCs by introducing 5 miRNAs (MIR122, MIR148a, MIR424, MIR542-5p, and MIR1246) into human umbilical cord-derived MSCs [73]. MIR122 is specifically and abundantly expressed in the liver, and accounts for 72% of all the miRNA 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 CYP family 3 subfamily A member 4 (CYP3A4), which reach levels of roughly 80% of normal expression in hepatocytes [76]. Additionally, liver function, including CYP metabolism, increases in cells overexpressing MIR122. Interestingly, MIR122, forkhead box A1 (FOXA1), and HNF4A form a positive feedback loop, in which MIR122 upregulates expression of FOXA1, whereas FOXA1 induces expression of HNF4A, and HNF4A increases expression of MIR122 [76].

MIR194 is also strongly upregulated during hepatocyte differentiation. Overexpression accelerates the differentiation towards 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 miRNAs into HLCs.

Small molecules

Natural and synthetic small molecules, identified by cell-based 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 three categories (Figure 4).

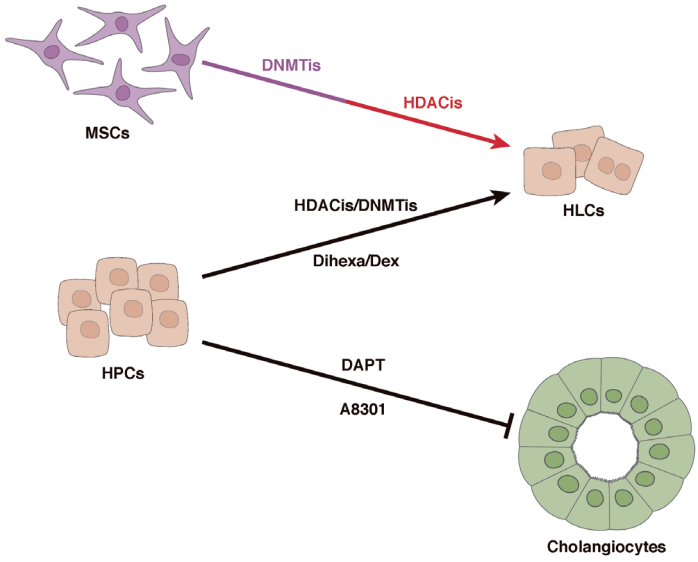

Figure 4. Small molecules that generate HLCs or promote HLC maturation. Several small molecules can promote differentiation of HPCs and MSCs into HLCs. DNMTis prime differentiation of MSCs into HLCs. HDACis act during or after differentiation into HLCs. DAPT and A8301 block differentiation of HPCs into cholangiocytes.

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 upregulating the expression of LETFs such as HNF4A, HNF1A, CEBPA and FOXA279, and increasing transcriptional activity by binding to promoter regions of CYP genes and G6PD [80–82]. Additionally, HDACis regulate liver-specific 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 [11-13].

A8301 inhibits TGFB, which promotes the epithelial to mesenchymal transition and dedifferentiation of hepatocytes [89]. Siller et al generated functional HLCs with the HGF receptor agonist, N-hexanoic-Tyr, Ile-(6) aminohexanoic amide (dihexa) [90]. 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 upregulates the expression and transcriptional activity of LETFs such as CEBPA, HNF1A, HNF4A, and FOXA1 [93]. Dexamethasone also activates glucocorticoid receptor (GR) signaling; GR-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 al 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-(N-phenylmethylsulfonamido)-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 towards definitive endoderm [96,97]. Recently, Siller et al 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 [90].

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 al 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 [99]. 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, since 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 hepato-biliary 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 non-parenchymal cells, such as Kupffer cells, sinusoidal endothelial cells, stellate cells, and hepatic mesenchymal stromal cells (MSCs) [104]. Cytokines secreted by non-parenchymal cells comprise acidic FGF (stellate cells) [105], HGF (stellate and endothelial cells) [105,106], tumor necrosis factor (Kupffer cells) [107], OSM (Kupffer cells) [108], IL6 (Kupffer, macrophages, and endothelial cells) [109,110], TGFB (Kupffer cells) [111], IL1 (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 al 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 [113].

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 patters of communication between the 3D microenvironment and different cell types. Interactions between vascular endothelial growth factor (VEGF) and its receptor induce angiogenesis in the developing liver. Although this approach has been used to primarily study 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 instance, when studying UDP-glucuronosyltransferase-, glucose-6-phosphatase-, or alpha-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 I dependent metabolism of clinically used

drugs [116]. Finally, urea cell 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 carbamyl-phosphate synthetase I and argininosuccinate synthetase [117].

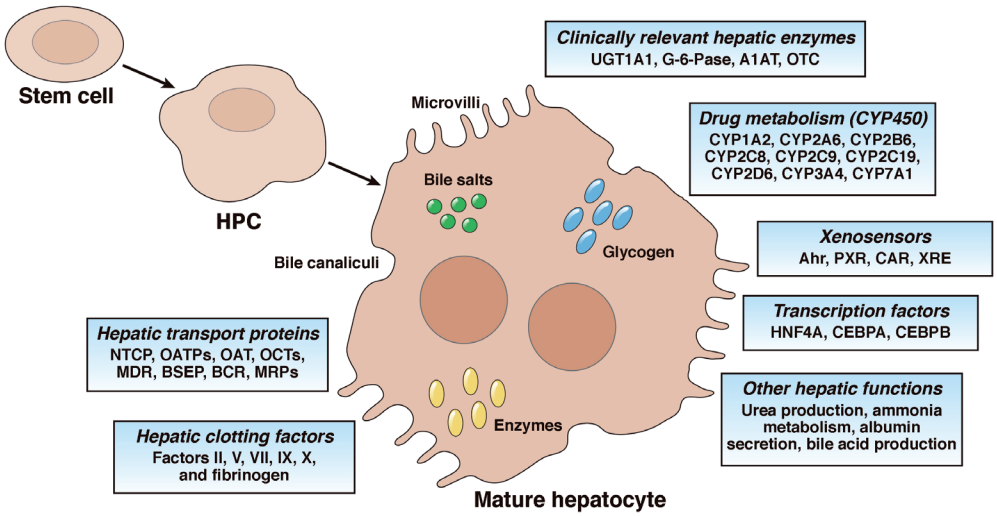

Figure 5. Structural, metabolic, and secretory functions of mature hepatocytes. Mature hepatocytes have a typical epithelial cell structure including polygonal shape and presence of epithelial markers, highly express genes related to drug metabolism (phase 1, 2 and 3), secrete albumin, produce urea and bile acid, etc.

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: a) the solute carrier SLC family, including Na+-taurocholate co-transporting polypeptides, organic anion-transporting polypeptides, organic anion transporters, and organic cation transporters; b) the ATP-binding cassette transporter family, including the multi drug 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 c) 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.

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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 (alpha- and beta-catenin) at basolateral membrane, etc.
	Polyploidization	Presence of two or more nuclei in fraction of the cells
Gene expression	Serum proteins	*ALB, TF, TTR,* etc.
	Hepatocytic TFs	*HNF4A, HNF1A, CEBPA/B,* etc.
	Metabolism enzymes	*CYP3A4, CYP7A1, G6P,* etc.
	Absence of biliary markers	*HNF1B, CK7, SOX9,* etc.
	Absence of stem cell markers or hepatic immature markers	*LGR5, OCT4, CD133,* AFP, CYP3A7, etc.
In vitro testing	Phase I CYP P450 activities	Metabolism of phenacetin (CYP1A2), bupropion (CYP2B6), tolbutamide (CYP2C9) diclofenac (CYP2G9), diclofenac (CYP3A4), etc.
	Phase II transferase activities	Metabolism of bilirubin (UGT1A1), dopamine (SULT1A1), p-aminobenzoic acid (NAT1), 1-chloro-2,4-dinitrobenzene (GSTs), etc.
	Phase III transporter activities	Transport of indocyanine green (OATP1B1), rhodamine 123 (MDR1), 5(6)-carboxy-2,'7'-dichlorofluorescein (MRP2), etc.
	Bile acid synthesis	Formation of cholic acid and chenodeoxycholic acid (CYP8A1, AKR1D1, AKR1C4), etc.
	Glycogen storage	Periodic acid–Schiff (PAS) staining for glycogen
	Serum protein synthesis	Secretion of albumin, alpha-1 antitrypsin, fibronectin, transferrin, apoprotein, transthyretin, complement factors, coagulation factors, etc.
	Cholesterol metabolism	Formation of 7α-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, etc.
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

Similar to LETFs (e.g. 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 above features are characteristic of freshly isolated primary human hepatocytes, it is critical that they are comprehensively analyzed.

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 al showed that hepatocytes carrying transgenes encoding coagulation factor 9 and a selection marker (a small hairpin RNA that makes cells resistant to a small-molecule inhibitor of fumarylacetoacetate hydrolase) expressed these gene products following transplantation into mice [125]. 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 [135]). Fumarylacetoacetate hydrolase (FAH)-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 triple-mutant mice (FRG). Livers of these mice can be repopulated by 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].

SCID mice with liver injury is caused by expression of the urokinase type plasminogen activator (uPA) 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 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 HGV 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, etc) 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 distrubition [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 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 al 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 [150]. 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.

Conclusion

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 necessary tools to further the development and application of human stem cell-derived hepatocytes.

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Conflicts of Interest

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. All the authors declare no other conflicts of interest.

Author Contribution

C.C. and B.S. drafted the manuscript, P.M.B. and A.S.-G. critically revised the manuscript for important intellectual content.

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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.

[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.

[4] Gentric G, Desdouets C. Polyploidization in liver tissue. Am J Pathol 2014;184:322–331.

[5] Zaret KS. From endoderm to liver bud: paradigms of cell type specification and tissue morphogenesis. Curr Top Dev Biol 2016;117:647–69.

[6] Zaret KS. Genetic programming of liver and pancreas progenitors: lessons for stem-cell differentiation. Nat Rev Genet 2008;9:329–340.

[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.

[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.

[9] Decaens T, Godard C, Reyniès A De, et al. Stabilization of β-catenin affects mouse embryonic liver growth and hepatoblast fate. Hepatology 2008;47:247–258.

[10] Clotman F, Jacquemin P, Plumb-Rudewiez N, et al. Control of liver cell fate decision by a gradient of TGFβ signaling modulated by Onecut transcription factors. Genes Dev 2005;19:1849–1854.

[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.

[12] Huch M, Gehart H, Boxtel R Van, et al. Long-term culture of genome-stable bipotent stem cells from adult human liver. Cell 2015;160:299–312.

[13] Nantasanti S, Spee B, Kruitwagen HS, et al. Disease modeling and gene therapy of copper storage disease in canine hepatic organoids. Stem Cell Reports 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.

[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.

[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.

[17] Song Z, Cai J, Liu Y, et al. Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells. Cell Res 2009;19:1233–42.

[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.

[19] Lee K-D, Kuo TK-C, Whang-Peng J, et al. *In vitro* hepatic differentiation of human mesenchymal stem cells. Hepatology 2004;40:1275–84.

[20] Wu X-B, Tao R. Hepatocyte differentiation of mesenchymal stem cells. Hepatobiliary Pancreat Dis Int 2012;11:360–371.

[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.

[22] Beath S. Hepatic function and physiology in the newborn. Semin Neonatol 2003;8:337–346.

[23] Grijalva J, Vakili K. Neonatal liver physiology. Semin Pediatr Surg 2013;22:185–189.

[24] Lautt WW. Hepatic Circulation Physiology and Pathophysiology. (Lautt WW, ed.). San Rafael (CA); 2009.

[25] Emery JL. Functional asymmetry of the liver. Ann N Y Acad Sci 1963;111:37–44.

[26] Rudolph AM. Hepatic and ductus venosus blood flows during fetal life. Hepatology 1983;3:254–258.

[27] Lautt WW, Greenway C V. Conceptual review of the hepatic vascular bed. Hepatology 1987;7:952–63.

[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: Hepatic Circulation in Health and Disease. Raven Press, New York; 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.

[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.

[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.

[34] Mallanna SK, Duncan SA. Differentiation of hepatocytes from pluripotent stem cells. In: Current Protocols in Stem Cell Biology.Vol 1. Hoboken, NJ, USA: 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.

[36] Satdarshan (Paul) Singh Monga. Molecular Pathology of Liver Diseases. (Monga SPS, ed.). Boston, MA: Springer US; 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;83:272–6.

[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.

[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.

[40] Selwyn FP, Cheng SL, Bammler TK, et al. Developmental regulation of drug-processing genes in livers of germ-free mice. Toxicol Sci 2015;147:84–103.

[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.

[42] Jump DB, Ren B, Clarke S, et al. Effects of fatty acids on hepatic gene expression. Prostaglandins Leukot Essent Fatty Acids 1995;52:107–11.

[43] Synold TW, Dussault I, Forman BM. The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux. Nat Med 2001;7:584–90.

[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;100:5419–24.

[45] Huang W, Ma K, Zhang J, et al. Nuclear receptor-dependent bile acid

signaling is required for normal liver regeneration. Science 2006;312:233–6.

[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;18:2127–36.

[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.

[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.

[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.

[51] Ballard FJ, Wong SSC, Knowles SE, et al. Insulin inhibition of protein degradation in cell monolayers. J Cell Physiol 1980;105:335–346.

[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.

[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.

[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:E447-53.

[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.

[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.

[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.

[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.

[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.

[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.

[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;13:2401–10.

[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;268:16831–8.

[63] Samadani U, Costa RH. The transcriptional activator hepatocyte nuclear factor 6 regulates liver gene expression. Mol Cell Biol 1996;16:6273–6284.

[64] Kuo CJ, Conley PB, Chen L, et al. A transcriptional hierarchy involved in mammalian cell-type specification. Nature 1992;355:457–461.

[65] Ktistaki E, Talianidis I. Modulation of hepatic gene expression by hepatocyte nuclear factor 1. Science 1997;277:109–12.

[66] Eeckhoute J, Formstecher P, Laine B. Hepatocyte nuclear factor 4α enhances the hepatocyte nuclear factor 1α-mediated activation of transcription. Nucleic Acids Res 2004;32:2586–2593.

[67] Hatzis P, Talianidis I. Regulatory mechanisms controlling human hepatocyte nuclear factor 4alpha gene expression. Mol Cell Biol 2001;21:7320–30.

[68] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663–76.

[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.

[70] Du Y, Wang J, Jia J, Xiang C, et al. Human hepatocytes with drug

metabolic function induced from fibroblasts by lineage reprogramming. Cell Stem Cell 2014;14:394–403.

[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.

[72] Vogel C, Marcotte EM. Insights into regulation of protein abundance from proteomics and transcriptomis 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.

[74] Lagos-Quintana M, Rauhut R, Yalcin A, et al. Identification of tissue-specific microRNAs from mouse. Curr Biol 2002;12:735–9.

[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.

[76] Deng XG, Qiu RL, Wu YH, et al. Overexpression of miR-122 promotes the hepatic differentiation and maturation of mouse ESCs through a miR-122/FoxA1/HNF4a-positive feedback loop. Liver Int 2014;34:281–295.

[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.

[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.

[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.

[80] Bort R, Gómez-Lechón MJ, Castell J V, et al. Role of hepatocyte nuclear factor 3 gamma in the expression of human CYP2C genes. Arch Biochem Biophys 2004;426:63–72.

[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;63:1180–9.

[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;278:40694–701.

[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;24:1412–9.

[84] Alizadeh E, Zarghami N, Eslaminejad MB, et al. The effect of dimethyl sulfoxide on hepatic differentiation of mesenchymal stem cells. Artif cells, nanomedicine, 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.

[86] Jin B, Park DW, Nam KW, et al. CpG methylation of the mouse CYP1A2 promoter. Toxicol Lett 2004;152:11–18.

[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.

[88] Snykers S, Henkens T, Rop E De, et al. Role of epigenetics in liver-specific gene transcription, hepatocyte differentiation and stem cell reprogrammation. J Hepatol 2009;51:187–211.

[89] Choi SS, Diehl AM. Epithelial-to-mesenchymal transitions in the liver. Hepatology 2009;50:2007–2013.

[90] Siller R, Greenhough S, Naumovska E, et al. Small-molecule-driven hepatocyte differentiation of human pluripotent stem cells. Stem Cell Reports 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.

[92] Vinken M, Papeleu P, Snykers S, et al. Involvement of cell junctions in hepatocyte culture functionality. Crit Rev Toxicol 2006;36:299–318.

[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. 2013.

[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.

[95] Shan J, Schwartz RE, Ross NT, et al. Identification of small molecules for human hepatocyte expansion and iPS differentiation. Nat Chem Biol

2013;9:514–20.

[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;4:348–58.

[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;22:1419–32.

[98] Herrero A, Gerbal-Chaloin S, Daujat-Chavanieu M. Bioinspired liver tissue engineering. In: Handbook of Biomimetics and Bioinspiration.; 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.

[101] Soto-Gutierrez A, Zhang L, Medberry C, et al. A whole-organ regenerative medicine approach for liver replacement. Tissue Eng Part C Methods 2011;17:677–686.

[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.

[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;87:1315–530.

[105] Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. Physiol Rev 2008;88:125–72.

[106] Bussolino F, Renzo MF Di, Ziche M, et al. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. J Cell Biol 1992;119:629–41.

[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.

[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;91:1107–17.

[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;160:6056–61.

[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;53:126–32.

[111] Bleser PJ De, Niki T, Rogiers V, et al. Transforming growth factor-beta gene expression in normal and fibrotic rat liver. J Hepatol 1997;26:886–93.

[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;18:572–9.

[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.

[114] Camp JG, Sekine K, Gerber T, et al. Multilineage communication regulates human liver bud development from pluripotency. Nature 2017;546:533–538.

[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.

[116] Sinz M, Wallace G, Sahi J. Current industrial practices in assessing CYP450 enzyme induction: preclinical and clinical. AAPS J 2008;10:391–400.

[117] Raghuveer TS, Garg U, Graf WD. Inborn errors of metabolism in infancy and early childhood: an update. Am Fam Physician 2006;73:1981–90.

[118] Montfoort JE van, Hagenbuch B, Groothuis GMM, et al. Drug uptake systems in liver and kidney. Curr Drug Metab 2003;4:185–211.

[119] Duncan SA, Navas MA, Dufort D, et al. Regulation of a transcription factor network required for differentiation and metabolism. Science 1998;281:692–5.

[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.

[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.

[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.

[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;338:1422–6.

[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.

[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;165:901–12.

[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.

[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.

[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.

[130] Strom SC, Davila J, Grompe M. Chimeric mice with humanized liver: tools for the study of drug metabolism, excretion, and toxicity. In: Methods in molecular biology (Clifton, N.J.).Vol 640.; 2010:491–509.

[131] Zhu S, Rezvani M, Harbell J, et al. Mouse liver repopulation with hepatocytes generated from human fibroblasts. Nature 2014;508:93–97.

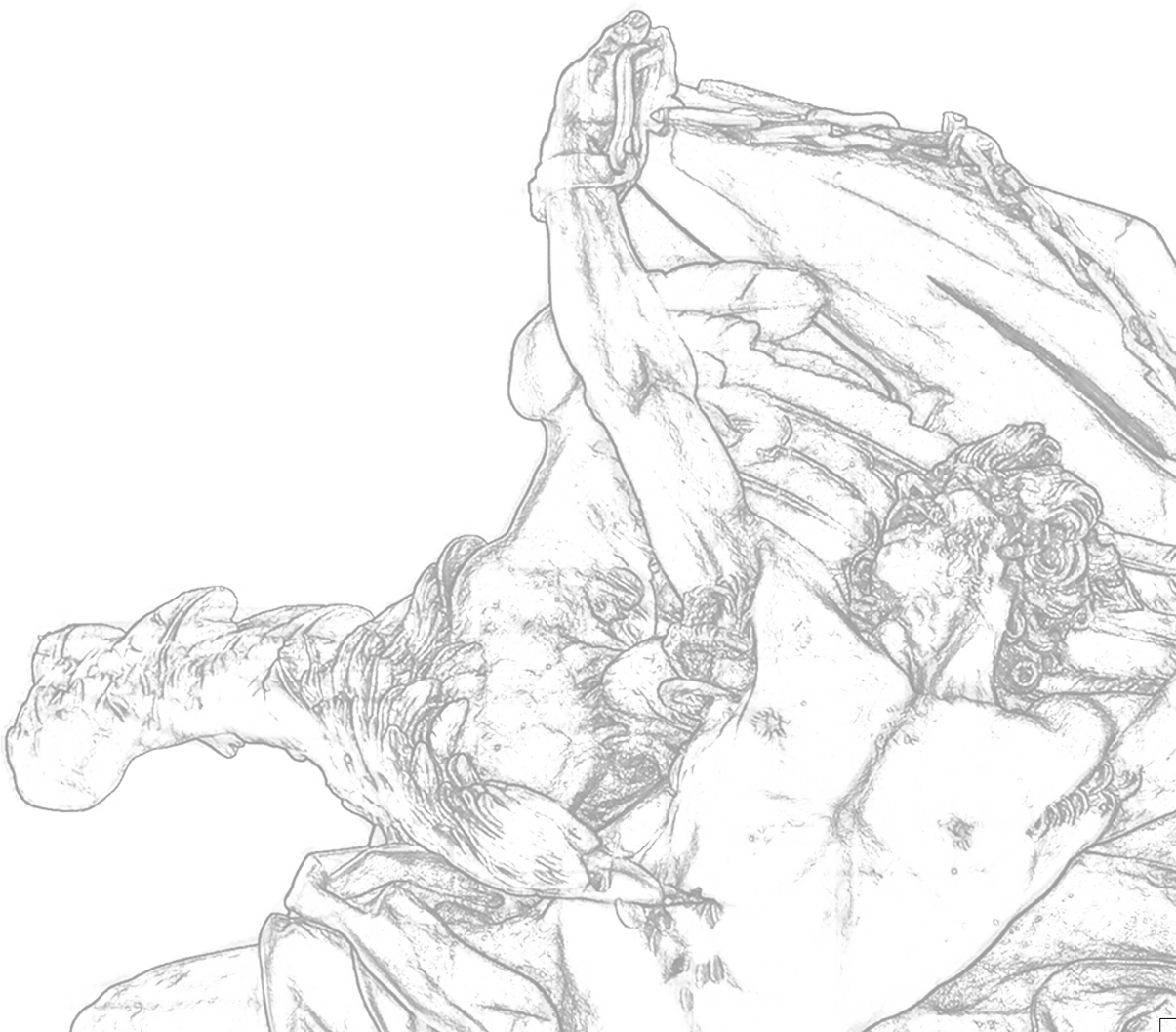
[132] Chen Y, Li Y, Wang X, et al. Amelioration of hyperbilirubinemia in Gunn rats after transplantation of human induced pluripotent stem cell-derived hepatocytes. Stem Cell Reports 2015;5:22–30.

[133] Basma H, Soto-Gutiérrez A, Yannam GR, et al. Differentiation and transplantation of human embryonic stem cell-derived hepatocytes. Gastroenterology 2009;136:990–9.

[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.
[135] Rezvani M, Grimm AA, Willenbring H. Assessing the therapeutic potential of lab-made hepatocytes. Hepatology 2016;64:287–294.
[136] Chowdhury JR, Kondapalli R, Chowdhury NR. Gunn rat: a model for inherited deficiency of bilirubin glucuronidation. Adv Vet Sci Comp Med 1993;37:149–73.
[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.
[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.
[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.
[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.
[143] Zhang YS, Yue K, Aleman J, et al. 3D bioprinting for tissue and organ fabrication. Ann Biomed Eng 2017;45:148–163.
[144] Flaim CJ, Chien S, Bhatia SN. An extracellular matrix microarray for probing cellular differentiation. Nat Methods 2005;2:119–25.
[145] McGuigan AP, Sefton M V. Vascularized organoid engineered by modular assembly enables blood perfusion. Proc Natl Acad Sci U S A 2006;103:11461–6.
[146] Khetani SR, Bhatia SN. Microscale culture of human liver cells for drug development. Nat Biotechnol 2008;26:120–6.
[147] Bhatia SN, Ingber DE. Microfluidic organs-on-chips. Nat Biotechnol 2014;32:760–72.
[148] Giobbe GG, Michielin F, Luni C, et al. Functional differentiation of human pluripotent stem cells on a chip. Nat Methods 2015;12:637–40.
[149] Chavez A, Scheiman J, Vora S, et al. Highly efficient Cas9-mediated transcriptional programming. Nat Methods 2015;12:326–328.

[150] Balboa D, Weltner J, Eurola S, et al. Conditionally stabilized dCas9 activator for controlling gene expression in human cell reprogramming and differentiation. Stem cell reports 2015;5:448–59.



Chapter 2

Hepatocyte-like cells generated by direct reprogramming from murine somatic cells can repopulate decellularized livers

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Abstract

Direct reprogramming represents an easy technique to generate hepatocyte-like cells (iHeps) from somatic cells. However, current protocols are accompanied by several drawbacks as iHeps are heterogenous and lack fully mature phenotypes of primary hepatocytes. Here, we established a polycistronic expression system to induce the direct reprogramming of mouse embryonic fibroblasts towards hepatocytes. The resulting iHeps are homogenous and display key properties of primary hepatocytes, such as expression of hepatocyte markers, albumin secretion, and presence of liver transaminases. iHeps also possess the capacity to repopulate decellularized liver tissue and exhibit enhanced hepatic maturation. As such, we present a novel strategy to generate homogenous and functional hepatocyte-like cells for applications in tissue engineering and cell therapy.

Introduction

Currently orthotopic liver transplantation is the only cure for severe acute- and chronic-liver diseases. Unfortunately, the shortage of donor livers is a growing problem. In Europe alone, twenty-two percent of the patients die while on the waiting list for a liver transplant [1]. Therefore, the development of new therapeutic strategies such as stem cell transplantation is of utmost importance. Although several types of (stem) cells show the potential to be differentiated into hepatocyte-like cells, the procurement of the stem cells as well as differentiation protocols are inefficient, incomplete, and still far from application in human medicine [2,3].

One promising alternative for generating hepatocytes is the direct reprogramming technique. As from the first direct reprogramming experiments inducing myoblasts over 30 years ago, more and more types of tissue have been generated with this technique [4]. Transcription factors together with other epigenetic modifiers coordinately play an important role in maintaining cellular identities by regulating cell-type specific gene expression programs. Based on this theory, direct reprogramming was aimed at the forced expression of these key transcription factors to activate the regulatory network supporting a specific cell fate. During direct reprogramming, one somatic cell (e.g. fibroblast) is transdifferentiated into another somatic cell (e.g. hepatocyte) without intermediate stages of pluripotency. Due to this feature, direct reprogramming represents a more reproducible and time-efficient technique compared to pluripotent stem cell-based differentiation. Direct reprogramming has been shown to allow the generation of induced hepatocytes (iHeps) from many types of somatic cells [5–8]. These iHeps acquired hepatocyte function to some extent and could extend the survival of mouse models with lethal liver disease after cell transplantation. However, full maturation of these cells *in vitro* has thus far not been achieved.

In the liver, hepatocytes are surrounded by extracellular matrix (ECM), of which the dominant ECM-components are collagens (type I, III, IV and V). Natural-based ECM, especially type IV collagen, has been routinely used for maintaining hepatocytes and iHeps [7,9]. Apart from serving as the scaffold for hepatocytes, ECM also performs signaling function by storage and release of numerous growth factors, hormones, enzymes, and cytokines. Considering the complexity of natural ECM, a single or combination of several type of collagens may not be able to mimic the genuine *in vivo* microenvironment for hepatocytes. Therefore, the decellularized

liver tissue may be a better alternative. Previously we and others have reported that decellularized liver tissue could provide an excellent environment for the *in vitro* differentiation of hepatic stem cells [10,11] as well as maintenance of primary hepatocytes [12]. However, it is still unknown whether iHeps can repopulate decellularized liver tissue and whether the maturation of iHeps can be enhanced by this more natural environment.

In this study, we report the efficient generation of iHeps from mouse embryonic fibroblasts using a polycistronic system expressing transcription factors including Forkhead Box A3 (*Foxa3*), HNF1 Homeobox A (*Hnf1A*) and GATA Binding Protein 4 (*Gata4*). Different from previously used combinations of separate vectors, each encoding one or two genes, the all-in-1 polycistronic expression system induces homogenous iHep population as well as fluorescent marker. These iHeps morphologically and functionally resemble primary hepatocytes. Furthermore, we demonstrate that iHeps can repopulate decellularized liver tissue. More importantly, iHeps cultured with decellularized liver tissue exhibit high level of maturation. This iHep-on-decellularized-liver-tissue system may serve as an optimal model for drug activity and toxicity screening.

Material and Methods

Assembly of polycistronic lentiviral vectors

To construct the lentiviral hepatocyte-generating vector, we used a third-generation lentiviral vector (pRRL-PPT-SFFV-OKSM-EGFP, kindly provided by A. Schambach, Hannover, Germany) [13]. The region encoding OKSM (Oct4, Klf4, Sox9 and Myc) was removed from the original vector by BamHI (NEB) digestion. The three modules encoding FOXA3-T2A, HNF1A-E2A and GATA4 were amplified by PCR with monocistronic vectors separately expressing *Foxa3*, *Hnf1a* and *Gata4* as templates (kindly provided by L. Hui, Shanghai, China) [7]. Each of these modules contained 15-bp overlaps at their ends. Ligation of all the modules was based on the In-Fusion HD Cloning system (Clontech), in which the In-Fusion enzyme can fuse all the modules and the backbone by recognizing 15-bp overlaps at their ends.

Cell culture, lentivirus production, and lentiviral transduction

The human embryonic kidney line HEK293T and mouse embryonic fibroblasts were

cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% v/v fetal bovine serum (FBS, Invitrogen) and 1% v/v penicillin/streptomycin. Primary hepatocytes were isolated by a 2-step perfusion procedure from C57BL/6 mice [14]. Viable hepatocytes were isolated by Percoll medium according to the manufacturer's instructions (GE Healthcare).

Virus production was performed as previously described [15]. In short, 7x106 HEK293T cells were plated 24 hours prior to transfection (day -1) in 15 cm dishes. On day 0, cells were transfected by linear polyethylenimine (PEI) (Polysciences, 1 µg DNA:5 µg PEI) with 45 µg lentiviral vector, 3.6 µg HDM-Hgpm2, 3.6 µg RC-CMV-Rev1b, 3.6 µg HDM-tat1b and 7.2 µg HDM-VSV-G. Media was refreshed after 12-16 hours. Supernatant containing virus was harvested on day 2–4. On day 4, filtered supernatant was centrifuged at 72,000 g for 2 hours at 4 °C. The pellet was resuspended in 150 µl sterile PBS with 1% w/v bovine serum albumin, and aliquots were stored at −80 °C until use.

Generation of hepatocyte-like cells

For viral transduction, on day 0, MEFs were incubated with concentrated lentivirus (1:10,000) containing the reprogramming factors and 8 µg/ml of Polybrene (Sigma-Aldrich). On day 1, MEFs were washed by HBSS (Gibco) twice and cultured with fresh MEF medium. On day 2, iHep medium, containing a 1:1 mixture of DMEM/F-12, supplemented with 10% FBS, 20 ng/ml hepatocyte growth factor, 20 ng/ml epidermal growth factor, 1 µg/ml insulin, 10^{-7} M dexamethasone, 10 mM nicotinamide, 2 mM L-glutamine, 50 µM β-mercaptoethanol and 1% v/v penicillin/streptomycin. Cells were surpassed on day 7 and day 14. The culture dishes were pre-coated with Type I collagen (Millipore). Medium was refreshed every two days.

RNA isolation, cDNA synthesis and RT-qPCR

RNA was isolated from MEFs, primary hepatocytes and 2 dimension-cultured iHeps using RNeasy Micro Kit according to the manufacturer's instructions (Qiagen). For isolating RNA from the iHeps on the discs, we firstly used PBS to wash the discs, then added lysis buffer (RLT buffer, from RNeasy micro kit, Qiagen) directly. After 5 minutes of incubation, all the lysates were collected for further procedures. cDNA was obtained using the iScript™ cDNA synthesis kit as described by the manufacturer (Bio-Rad). Relative gene expression of the selected genes was

measured using RT-qPCR. Primer design, validation, RT-qPCR conditions, and data analysis was performed as previously described [16]. Normalization was performed using the reference gene hypoxanthine phosphoribosyl transferase (*Hprt*). Details of primers are listed in Table S1.

Immunofluorescence analysis

Cells were fixed in 4% paraformaldehyde for 60 minutes and permeabilized with PBS containing 0.3% v/v Triton X-100 for 30 minutes. Cells cultured on decellularized liver discs were incubated with 0.01 g/ml $NaBH_4$ solution to reduce the background generated by the liver discs. Primary antibodies were incubated overnight. Secondary antibodies were incubated at room temperature for two hours. Tissues were then incubated with 5 μM Alexa Fluor® 488 (Life Technologies) or Alexa Fluor® 647 (for cells cultured on decellularized liver discs) according to manufacturer's instructions. Nuclei were stained with DAPI (Sigma-Aldrich). Tissues were mounted on slides with ProLong® Diamond Antifade Mounting Medium (Invitrogen). Images were acquired using Leica SPE-II confocal system. Antibody details for each protein are shown in Table S2.

Immunoblot analysis

For primary mouse hepatocytes, transduced and untransduced MEFs, whole cell lysates were prepared using RIPA lysis buffer containing 50 mM Tris–HCl, 150 mM NaCl, 1% v/v NP-40, 0.25% w/v sodium deoxycholate, 1 mM EDTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 μg/ml aprotinin and 1 mM PMSF (Sigma-Aldrich). Protein concentration was measured by DC Protein Assay (Bio-Rad). 50 μg total protein for each sample was loaded in a 10% SDS-PAGE gel, transferred to a 0.45 μm nitrocellulose membrane (Bio-Rad), and blocked with ECL Blocking agent (Amersham GE healthcare). The blots were probed with anti-Foxa3 (Thermo Fisher, PA1-813, dilution 1:500), anti-Hnf1a (Thermo Fisher, PA5-35356, dilution 1:1,000), and anti-GATA4 (Abcam, ab84593, dilution 1:1,000) overnight at 4°C and subsequently incubated with HRP-conjugated anti-rabbit (R&D systems, HAF008, dilution 1:5,000) for 1 hour at room temperature. Luminescence induced by the Amersham ECL Western Blotting Detection Reagent (GE Healthcare) was measured with a ChemiDoc XRS Imager (Bio-Rad).

Hepatocyte functional tests

For measurement of albumin secretion, iHeps, MEFs and primary hepatocytes were cultured in serum free medium. After 24 hours, culture medium was collected. Protein in the medium was concentrated using Amicon Ultra centrifugal filters (Millipore), and the amount of albumin was measured using a DxC-600 Beckman chemistry analyzer (Beckman Coulter). The values were normalized for total cell number.

For measurement of cytochrome P450 activity, iHeps, MEFs and primary hepatocytes were incubated in 50 μM luciferin-PFBE substrate (Promega) in culture medium (Gibco) containing 10% FBS for 8 hours at 37°C. Cyp3a activity was then measured with a luminometer using the P450-Glo cytochrome P450 assay kit according to the manufacturer's instructions (Promega).

For measurement of the expression of hepatic enzymes in 2 dimension-cultured iHeps, cells were lysed in distilled water. The ALP, GGT, LDH, GLDH and AST in the lysate was measured using the DxC-600 Beckman chemistry analyzer (Beckman Coulter) and values were normalized to total cell number. For measurement of the expression of hepatic enzymes in DLD-iHeps, two discs of cells were pooled together to generate enough material for each liver enzyme test as described above, the results of those tests were normalized to the value of AlamarBlue test which indicates the number of viable cells.

Decellularized liver disc preparation

Briefly, livers from 4-8 months old cadaveric rats were harvested with intact vessels (the animals were previously used by other researchers of our institution and sacrificed upon completion of their IACUC approved experiments). The livers were then cannulated through the portal vein and successively perfused at the rate of 1 ml/min with 0.5 L distilled water 5 L detergent comprising 1% v/v Triton-X 100 (Sigma-Aldrich) and 0.1% v/v ammonium hydroxide (Sigma-Aldrich), and 10 L distilled water to wash out the decellularization detergent. Decellularized livers with a level of DNA removal higher than 90% were obtained with this method.

To obtain liver discs, decellularized livers were cut into wedges and embedded in plastic molds (Sakura Finetek) with OCT and frozen at -80ºC. These cryopreserved

liver wedges were mounted onto a cryomicrotome (Leica CM 1950) to obtain liver ECM sections of 250 µm thickness. A 4-mm diameter biopsy punch was used to generate a disc from the liver sections and the discs were placed in 96-well plates. After multiple washes with PBS, the discs were sterilized by UV irradiation for 2h. Discs were stored in PBS at 4 ºC until use.

Recellularization of iHeps on decellularized liver discs

Seeding procedure was performed as previously described [10]. Briefly, $2.5x10^5$ of iHeps were suspended in 10 µl iHep medium for each disc. Discs were placed in 96-well round bottom microwell plates (Nunc). The cell suspension was slowly pipetted on top of each disc and incubated for about an hour at 37°C to allow the cells to attach. Afterwards 200 µl additional iHep medium was added to each well. As a 2-dimensional control, same number of iHeps were seeded on collagen (type I) coated 96-well tissue culture plates.

Statistical analysis

Statistical analysis and graphs were performed using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). See figure legends for details on specific statistical tests run and p values calculated for each experiment.

Results

Design of lentiviral vectors inducing hepatic reprogramming gene expression in mouse embryonic fibroblasts

Previously Warlich et al. developed a modular lentiviral vector system for generating human and mouse pluripotent stem cells (iPSCs) [13]. Apart from the region encoding the iPSC reprogramming factors (Oct4, Klf4, Sox9 and Myc, OKSM), the main modules in this vector, are spleen focus-forming virus U3 promoter (SFFV), which has been proven to mediate efficient expression in fibroblasts [17]; an internal ribosome entry site (IRES)-driven fluorescence marker, dTomato, for imaging/cell tracking studies; a post-transcriptional regulatory element derived from the woodchuck hepatitis virus (WPRE) to enhance lentiviral vector titer and expression [18]; as well as the necessary elements for lentivirus production (Figure 1A). Hepatocyte-generating factors, *Foxa3*, *Hnf1a*, and *Gata4* (FHG) [7],

were amplified independently and inserted in the vector to replace OKSM. FHG were separated by thosea asigna virus 2A (T2A) and equine rhinitis A virus 2A (E2A) sequences, which have previously been shown to mediate complete separation of recombinant proteins [19]. Mouse embryonic fibroblasts (MEFs) were transduced with FHG lentivirus and RNA and protein samples were collected after five days. Gene expression analysis, using a qPCR primer set amplifying the region on *Hnf1α* and E2A, confirmed the exogenous expression of FHG (Figure 1B). Immunoblot analysis further showed FHG were highly expressed in transduced MEFs, while they were not or very low expressed in untransduced MEFs (Figure 1C).

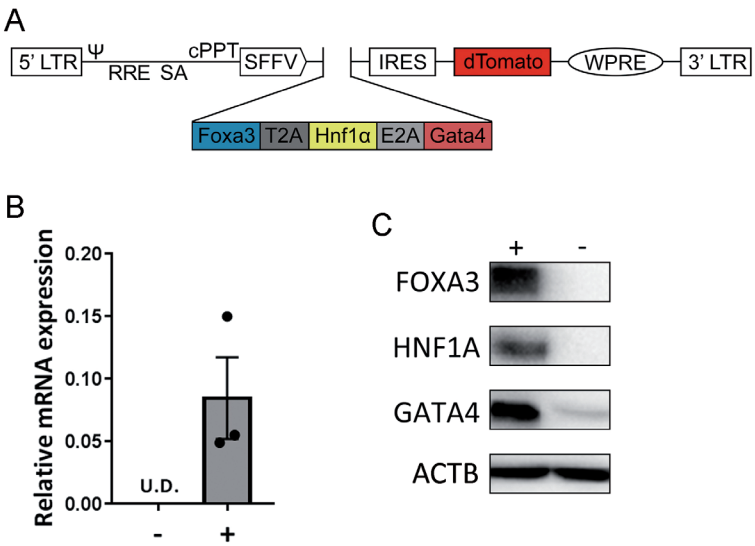


Figure 1. Design and efficacy of the polycistronic lentiviral vector. (A) Modular configuration of the self-inactivating (SIN) vector backbones for expression of the hepatocyte transcription factors Foxa3, Hnf1α and Gata4. LTR, long terminal repeat; ψ, packaging signal; RRE, rev-responsive element; SA, splice acceptor; cPPT, central polypurine tract. (B) Relative mRNA expression of the exogenous genes in MEFs without transduction (-) and with transduction of FHG lentivirus after five days (+). Data are shown as mean ± SEM of three independent experiments for each group. U.D., undetectable. (C) Protein expression of FOXA3, HNF1A, GATA4 and ACTB in untransduced (-) and transduced (+) MEFs.

Generation of mouse iHeps with polycistronic expression system

Previous studies showed that forced expression of a set of transcription factors could reprogram MEFs into hepatocyte lineage [7,8]. This system however was based on separate expression vectors, which led to uncertain transduction

of the individual genes in cells, leading to a heterogenous population. We questioned whether the all-in-1 polycistronic expression system could overcome this issue. MEFs were transduced with the all-in-1 lentivirus and fluorescence was observed after two days, which indicates the successful transduction and expression of exogenous genes (data not shown). Two days after transduction we changed the MEF culture medium to iHep culture medium (Figure 2A, medium components based on 8). After two weeks, we observed that almost 90% of the cells were dTomato positive (Figure 2B). Besides, the cell morphology changed from elongated to polygonal shape (Figure 2C), which is a typical phenotype of hepatocytes. We designated these cells induced hepatocyte-like cells (iHeps).

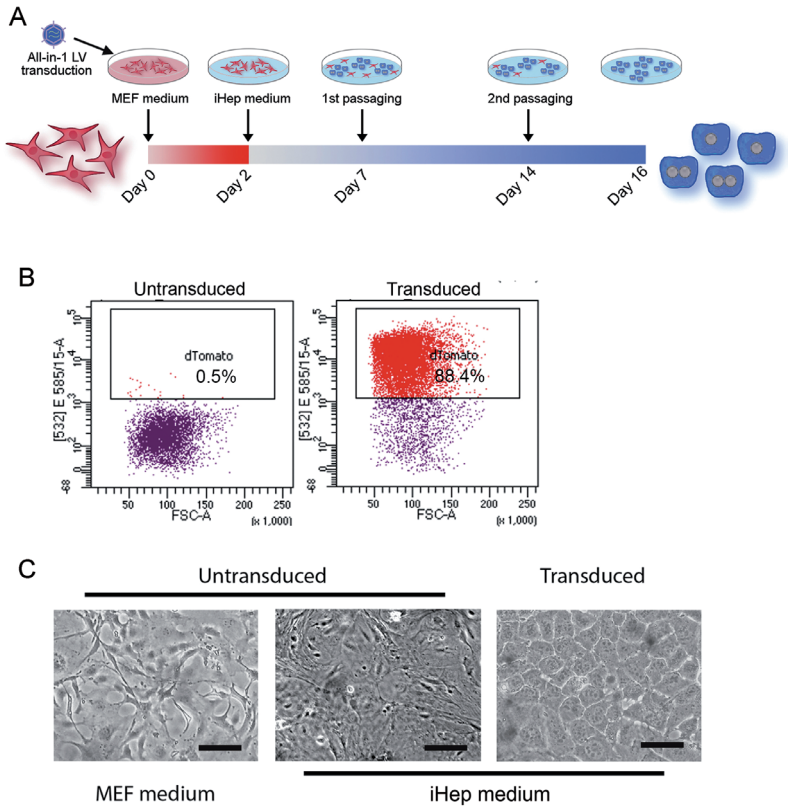
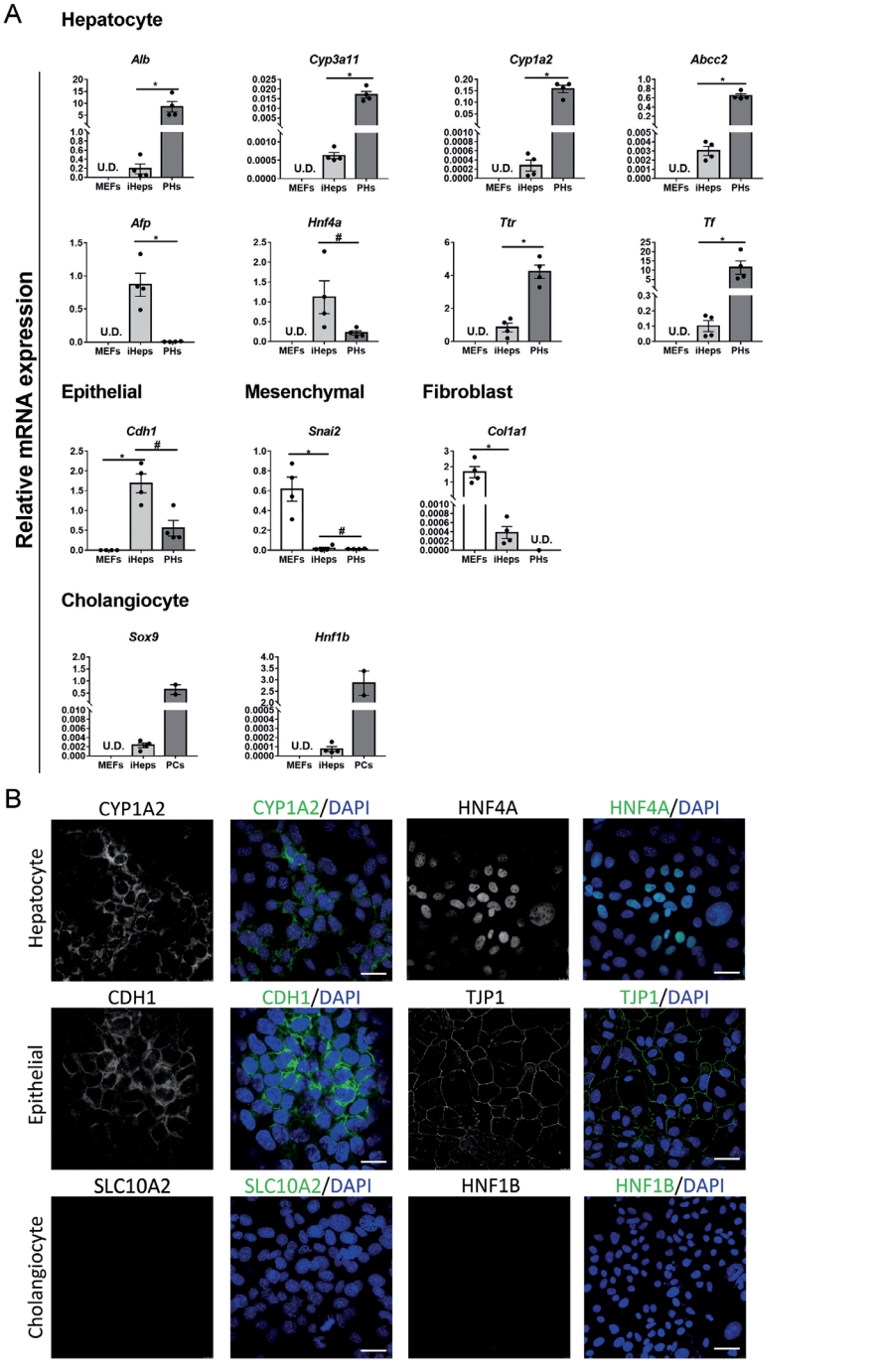


Figure 2. Generation of iHeps from MEFs and morphology and iHeps. (A) Schematic showing the generation of iHeps. Cells were cultured in MEF medium for two days after transduction. From day 2, cells were cultured in iHep medium. Cells were subcultured twice around day 7 and day 14, when reaching confluency. (B) Representative flow cytometry analysis of dTomato expression in untransduced MEFs (left) and iHeps (day 16, right). (C) Morphology of untransduced MEFs cultured in MEF medium (left) and iHep medium (middle) and iHeps (right) in iHep medium for 16 days. Scale bar = 100 μm.

Gene expression analysis demonstrated little expression of mesenchymal marker snail family transcriptional repressor 2 (*Snai2*) and fibroblast marker collagen type I alpha 1 chain (*Col1a1*) in iHeps (Figure 3A). In contrast, epithelial marker cadherin 1 (*Cdh1*, also known as E-cadherin) was highly expressed in iHeps (Figure 3A) which indicates the mesenchymal-epithelial transition of cell fate. Furthermore, iHeps displayed increased expression of the following hepatocyte markers: albumin (*Alb*), cytochrome P450 family 1 subfamily A member 2 (*Cyp1a2*), cytochrome P450 family 3 subfamily A member 11 (*Cyp3a11*), ATP binding cassette subfamily C member 2 (*Abcc2*, also known as *Mrp2*), alpha fetoprotein (*Afp*), hepatocyte nuclear factor 4 alpha (*Hnf4a*), transthyretin (*Ttr*) and transferrin (*Tf*) (Figure 3A). The cholangiocyte or hepatic progenitor markers SRY-box 9 (*Sox9*) and HNF1 homeobox B (*Hnf1b*) were expressed but at significantly lower levels compared to organoid-derived cholangiocytes generated by previous protocol (unpublished data). Immunofluorescence analysis confirmed the presence of hepatocyte markers (CYP1A2 and HNF4A) and epithelial markers (CDH1 and tight junction protein 1 (TJP1)); and the absence of cholangiocyte markers (solute carrier family 10 member 2 (SLC10A2) and HNF1B) (Figure 3B). Together, these results showed that direct reprogramming, through an all-in-1 polycistronic expression system, can induce MEFs to hepatocyte-like cells without inducing differentiation into the cholangiocyte lineage.

Functional validation of iHeps

Next, we investigated whether the iHeps generated by the all-in-1 polycistronic expression system functionally resembled primary hepatocytes. Aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and glutamate dehydrogenase (GLDH) are intracellular enzymes catalyzing important metabolism functions and they are specifically expressed in hepatocytes in the liver [20–22]. We collected lysate from MEFs, iHeps and primary hepatocytes, and measured their enzyme activities. As Figure 4 shows, the activities of AST and LDH in iHeps were upregulated (Figure 4A and B), but GLDH activity remained at similar levels compared to MEFs (Figure. 4C). LDH levels were similar as those observed in primary hepatocytes. Notably, GLDH is predominantly located in the centrilobular (Zone 3) region of the liver, while AST is more homogenously distributed [23], indicating iHeps might represent hepatocytes with non-centrilobular origin. Besides, gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP), which are related to biliary tracts, were undetectable in MEFs, iHeps and primary hepatocytes



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(data not shown), confirming the non-biliary fate of iHeps.

The cytochrome P450 enzymes (CYP) are essential in drug metabolism, in particular CYP3A4 (mouse homologous isoforms are CYP3A11, CYP3A16, CYP3A41A, CYP3A41B and CYP3A44 [24]). In iHeps, we observed the CYP3A activity was induced to almost one third of the levels compared to primary hepatocytes (Figure 4D). Another key function of hepatocytes is producing serum proteins, therefore we examined the albumin concentration in culture medium of iHeps. As Figure 4E shows, albumin was detectable in iHep culture medium reaching about half the concentration in primary hepatocyte culture medium.

Repopulation of decellularized liver discs by iHeps

Previously we and others have reported that hepatic stem cells and primary hepatocytes could repopulate decellularized liver tissue [10–12,25]. We questioned whether iHeps also possessed the repopulation capacity. When iHeps were seeded on rat decellularized liver discs (DLD), we found they survived and could repopulate DLD within one week (Figure 5A). In contrast non-reprogrammed MEFs seeded on DLD did not survive (data not shown).

Since the ECM in DLD represents a natural habitat for hepatocytes, we determined whether DLD could further enhance the maturation of iHeps. Gene expression analysis demonstrates that cytochromes P450 including Cyp1a1, Cyp1a2, Cyp2c40, Cyp3a11 and Cyp3a13, and epithelial marker Cdh1, were dramatically upregulated, while early hepatocyte marker Afp, was downregulated (Figure 5B). Interestingly, we observed that *Alb* and exogenous FHG were significantly

Figure 3. Characterization of iHeps. (A) Gene expression analysis for MEFs, iHeps, primary mouse hepatocytes (PHs) and primary cholangiocytes. Data are shown as mean ± SEM of four independent samples for each group. Asterisk represents statistical significance. *$p < 0.05$. Hash represents non-significance (two-tailed Mann–Whitney U test). Hepatocyte markers, albumin (*Alb*), cytochrome P450 3a11 (*Cyp3a11*), cytochrome P450 1a2 (*Cyp1a2*) and ATP binding cassette subfamily C member 2 (*Abcc2*); epithelial marker, caderin1 (*Cdh1*); Mesenchymal marker, snail family transcriptional repressor 2 (*Snai2*); fibroblast marker, collagen type I alpha 1 chain (*Col1a1*); cholangiocyte/hepatic progenitor markers, SRY-box 9 (*Sox9*) and HNF1 homeobox B (*Hnf1b*). (B) Immunofluorescence analysis for iHeps. Hepatocyte markers, CYP1A2 and hepatocyte nuclear factor 4 alpha (HNF4A); epithelial markers, CDH1 and tight junction protein 1 (TJP1); cholangiocyte markers, solute carrier family 10 member 2 (SLC10A2) and HNF1B. Nuclear staining with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for all conditions. Scale bar=30 μm.

downregulated (Figure 5B and D). Immunofluorescence analysis demonstrates the expression of CYP1A2 and tight junction protein 1 (TJP1) (Figure 5C). Finally, iHeps cultured with DLD exhibited increased enzyme activity of LDH, GLDH, CYP3A and CYP1A2 (Figure 5D), while GGT and ALP activity were undetectable (data not shown). Together, these results indicate that iHeps reached a high level of maturation. Although albumin expression was downregulated, the iHep-on-decellularized-liver-tissue system still represents an excellent model for phase I drug metabolism studies.

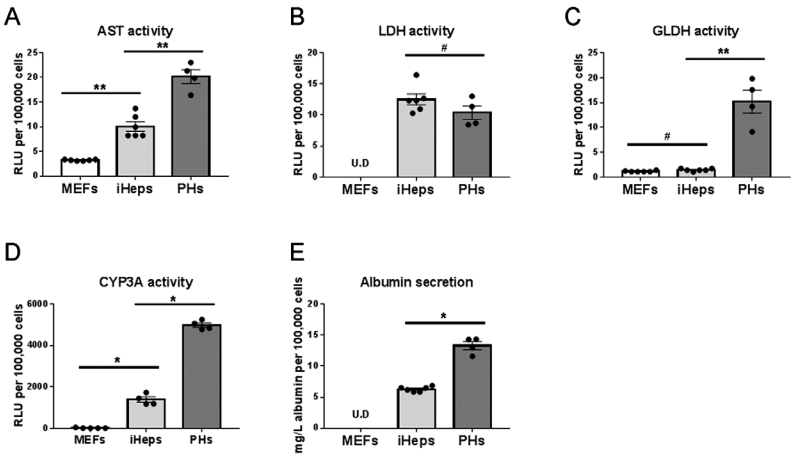

Figure 4. Functional validation of iHeps. Relative activity of hepatocyte-specific enzymes (A-C), Cyp3a activity (D) and albumin secretion (E) in MEFs, iHeps and primary hepatocytes. Results were normalized with cell number. Normalized data are shown as mean ± SEM of four independent experiments for primary hepatocytes and six independent experiments for MEFs and iHeps (P17). Asterisk represents statistical significance. *$p < 0.05$. *$p < 0.01$ Hash represents non-significance (two-tailed Mann–Whitney U test). AST, aspartate aminotransferase; LDH, lactate dehydrogenase; GLDH, glutamate dehydrogenase.

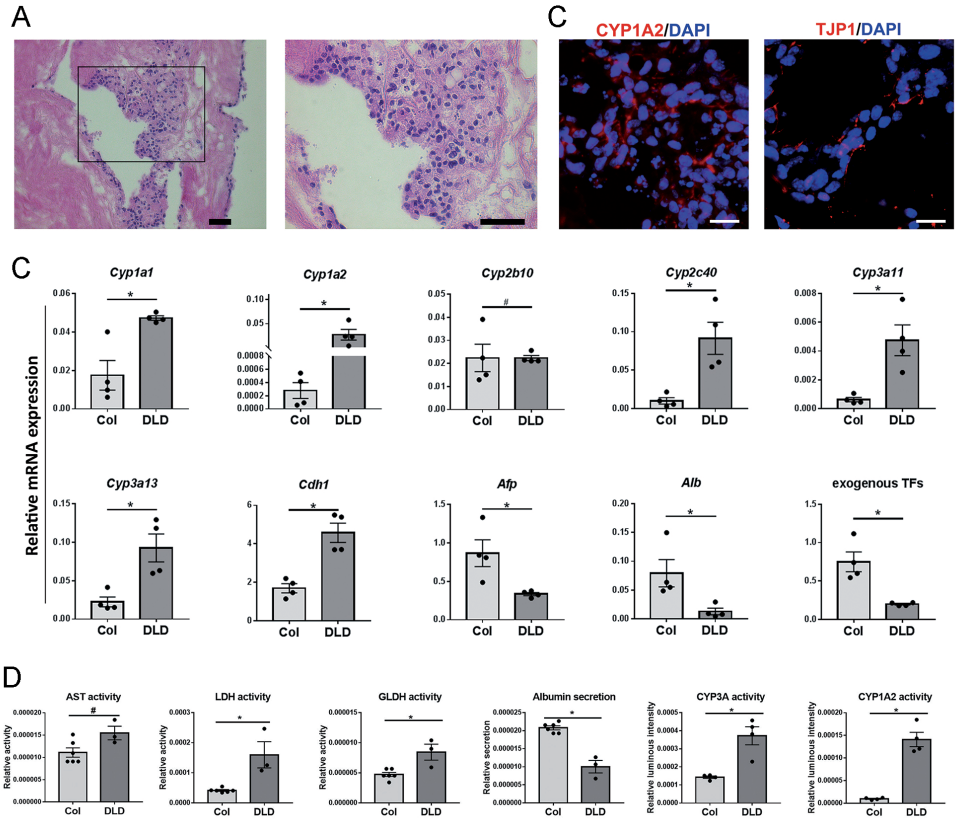

Figure 5. Repopulation of decellularized liver discs by iHeps. (a) Hematoxylin and eosin (H&E) staining showing the repopulation of decellularized liver discs by iHeps after one week. Scale bar = 50 µm. (b) Immunofluorescence analysis for iHeps cultured on decellularized liver discs (DLD). Scale bar = 20 µm. (c) Gene expression analysis for iHeps cultured on collagen (type I) coated plates (Col) and DLD. (d) Relative activity of hepatocyte-specific enzymes (AST, LDH, GLDH), CYP3A and CYP1A2 activity and albumin secretion in iHeps cultured on Col and DLD. All results were normalized with cell input with Alamar blue. Data are shown as mean ± SEM of six independent experiments for collagen 2D cultured iHeps for AST, LDH, GLDH and albumin tests, three for DLD cultured iHeps for AST, LDH, GLDH and albumin tests, four for both 2D and DLD cultured iHeps for CYP3A and CYP1A2 tests. Asterisk represents statistical significance. *$p < 0.05$. Hash represents non-significance (two-tailed Mann–Whitney U test).

Discussion

Direct reprogramming of fibroblasts into hepatocytes is time-saving and requires less effort due to the bypass of an intermediate stage of pluripotency, hence it represents an optimal way to quickly generate a large population of cells. The heterogeneity of iHeps and their lack of liver function may be due to the uncontrollable expression proportion of hepatocyte-generating factors *Foxa3*, *Hnf1a* and *Gata4* separately by means of monocistronic vectors. To overcome this issue, an all-in-1 polycistronic expression system is needed. The classical and widely used way to construct bicistronic or polycistronic vectors is to insert an internal ribosomal entry site (IRES) between genes. Because of its large molecular size and the difference in expression levels between genes before and after IRES (the gene before IRES generally has disproportional higher expression), we used the 2A peptide sequence insert in this study. This 2A peptide sequence is usually 50-60 bp comparing to around 580 bp of IRES, which enables the construction of vectors containing four or even more encoding regions. This is especially important for a lenti- and retro-viral expression system, of which the size of packaged sequence is limited [26]. Besides, genes before and after the 2A peptides are separated from each other during post-translational modification by a highly efficient cleavage event, therefore functional proteins can be obtained. The all-in-1 polycistronic expression system used in this study induced high expression of all genes at mRNA and protein level, which allowed the generation of iHeps.

During *in vitro* culture at normal conditions MEFs become senescent, or reach their Hayflick limit, after 5−7 passages [27]. To our surprise, the transduced MEFs or iHeps (transduction performed at passage 4) continued to grow for at least 30 passages over 2 months (longer-term to be determined). This growth advantage of iHeps provided us an easy way to select the positive cells over untransduced MEFs. We observed almost 90% cells were dTomato positive after two weeks and their morphology was identical after two passages (Figure 2). With further (sub)culture, this percentage kept increasing and a homogenous population was generated without the need of cell sorting. As hepatocytes do not proliferate *in vitro*, The continued proliferation of iHeps indicates that these cells are not fully mature, which is also reflected by other results, such as high expression of early hepatocyte marker *Afp* and low expression of late hepatocyte markers *Cyp3a11* and *Cyp1a2* (Figure 3). Nevertheless, this is a clear advantage when large amounts of cells are needed for tissue engineering or cell therapy approaches.

In order to improve the maturation of iHeps, we introduced iHeps into decellularized liver tissue. This natural-based extracellular matrix, mainly containing collagens, laminin and fibronectin, does not only provide mechanical and structural support for cells, but also serves as the reservoir for growth factors like HGF and bFGF [12]. After seeding on decellularized liver discs, we observed that the exogenous expression of *Foxa3*, *Hnf1a* and *Gata4* was downregulated. This explains that *Alb*, a direct downstream target of these three transcription factors [28–30], also showed lower expression. Although the precise mechanism behind the quenching of exogenous gene expression is unknown, it is a relatively common phenomenon during cell fate reprogramming [7,31] and indicates the dominance of the microenvironment. Having said this, the drug metabolism potential (CYP3A11 and CYP1A2) of iHeps were greatly enhanced and reached levels that were close to primary hepatocytes. The generation of the decellularized liver discs is relatively easy and highly reproducible [32]. The combined robust differentiation capability together with the discs results in a liver model that is more representative of the native liver tissue and has comparable hepatic features. These results indicate that the iHep-on-decellularized-liver-tissue system may represent an excellent model for phase I drug metabolism studies.

There are a few limitations in this study. The primary mouse hepatocytes were isolated by a two-step collagen perfusion method [33]. Although the majority of the isolated population were hepatocytes, the presence of some other unwanted cell types (e.g. hepatic stellate cells, cholangiocytes, etc.) is inevitable. Therefore, we used a commercial media designated for hepatocytes culture (HepatoZYME, Gibco) to select out other cell types. Since *in vitro* hepatocytes cultures could de-differentiate rapidly [34], our culture of two days before measurement could have influenced the results. This limitation could be overcome by utilizing freshly isolated pure hepatocytes (e.g. by sorting $ASGPR^+$ cells [14]) as the most optimal reference cell type. Second, the decellularized liver tissue used in this study was derived from rats as no decellularized mouse material was available. The species incompatibility of mouse cells and rat ECM might limit the full maturation potential of iHeps.

Conclusion

Here we reported an easy and efficient way to generate expandable hepatocyte-like cells. Applying decellularized liver tissue to the *in vitro* culture resulted in iHeps with enhanced hepatic features. Our finding demonstrates that hepatic ECM can induce

important functions of hepatocyte-like cells derived from somatic cells by direct reprogramming. Future research may focus on introducing supporting cell types (e.g. hepatic stellate cells, mesenchymal stromal cells and endothelial cells, etc.) to the current system to generate an even more physiological microenvironment for hepatocytes.

2

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Conflicts of Interest

The authors have declared no conflicts of interest.

Author Contribution

Chen Chen: Collection and assembly of data, data analysis and interpretation, manuscript writing;

Iris Pla-Palacín: Provision of study material or patients;

Pedro M. Baptista: Conception and design, data analysis and interpretation;

Peng Shang, Loes A. Oosterhoff and Monique E. van Wolferen: Collection and/or assembly of data;

Louis C. Penning and Niels Geijsen: Data analysis and interpretation, final approval of manuscript;

Bart Spee: Conception and design, data analysis and interpretation, final approval of manuscript.

Supplementary Figures

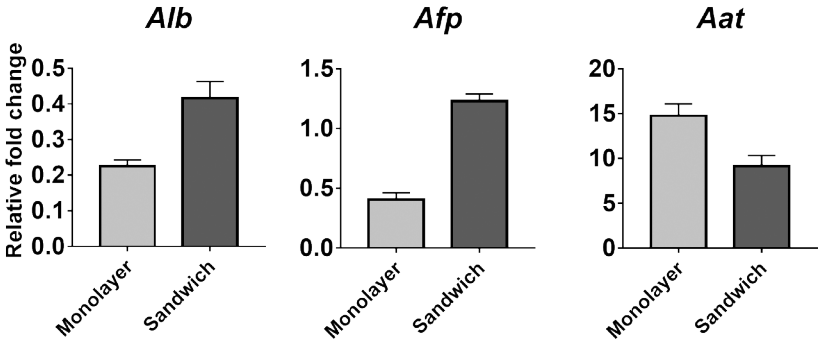


Figure S1. Comparison between monolayer and sandwich cultured iHeps. Gene expression analysis for iHeps cultured on monolayer and sandwich with collagen type I. Data are shown as mean ± SEM of two independent samples for each group.

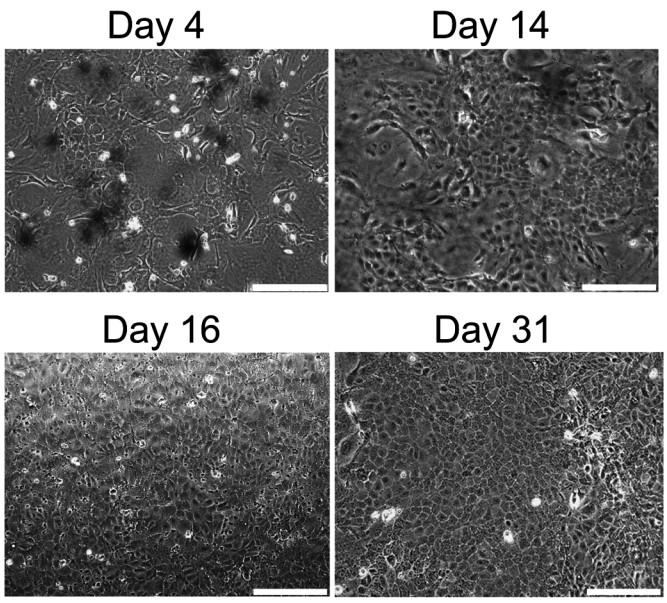


Figure S2. Morphological change during the generation of iHeps. MEFs were transduced on Day 0, photos were taken on Day 4, Day 14 (before the second passaging), Day 16 and Day 31. Scale bar = 200 μm.

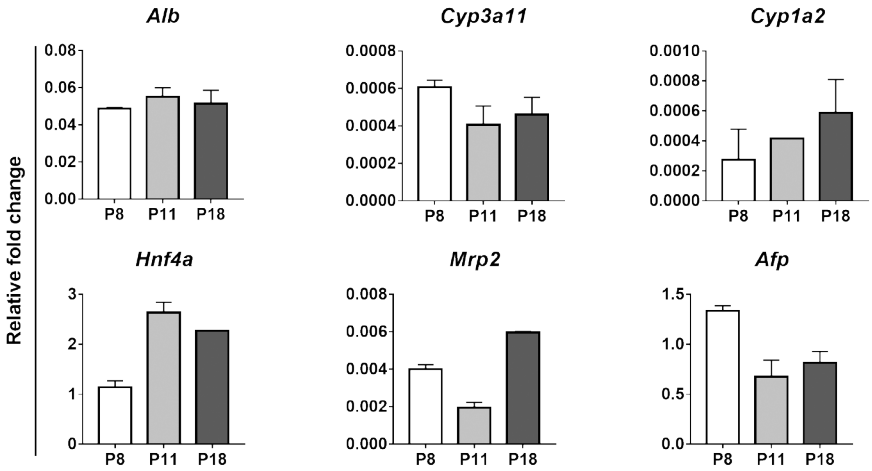


Figure S3. Comparison among iHeps at different passages. RNA samples were collected from iHeps at P8, P11 and P18. Data are shown as mean ± SEM of two independent samples for each group.

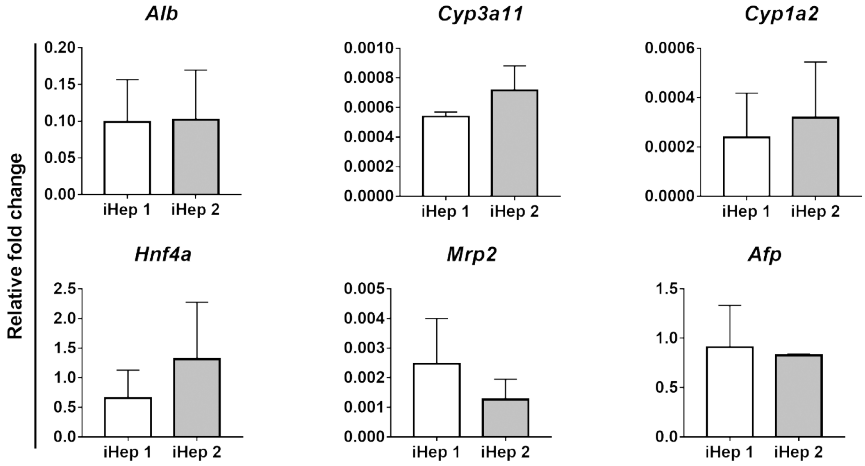


Figure S4. Comparison between iHeps generated from different donors. RNA samples were collected from iHep 1 and 2. Data are shown as mean ± SEM of two independent samples for each group.

Supplementary Tables

Supplementary Table 1. List of primers used in RT-qPCR analysis

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
Alb	GCAACACAAAGATGACAACC	CTTCATGCAAATAGTGTCCCA
Hnf1b	ACAATCCCCAGCAATCTCAGAA	GCTGCTAGCCACACTGTTAATGA
Hprt	GTGATTAGCGATGATGAACCA	CAAGTCTTTCAGTCCTGTCCA
Sox9	CCCGATTACAAGTACCAGCC	CCCTGAGATTGCCCAGAGTG
Cyp3a11	AAACTCAAGGAGATGTTCCC	TTCACTCCAAATGATGTGCT
Abcc2	GATAGCCTCATTCAGACGAC	ACCATTATCTTGTCACTGTCCA
Cyp1a2	ACCATGATGAGAAGCAGTGGA	CGAAGAGCATCACCTTCTCG
Col1a1	CTGGTTCTCCTGGTTCTCCT	CGTTGAGTCCGTCTTTGCC
Snai2	CTCCAAGAAGCCCAACTACAG	TGCCGACGATGTCCATACAG
Afp	TCGTATTCCAACAGGAGG	AGGCTTTTGCTTCACCAG
Ttr	TAGAACTGGACACCAAATCGT	CTGTAGGAGTATGGGCTGAG
Tf	CTCTTGAGAAAGCTGTGTCC	AAAGAATGGTTGAGTGGAGG
Cdh1	GAGAGAGACTGGAGTGCCACC	CTGTGTACCCTCACCATCGG
Cyp1a1	ACGAGAATGCCAATGTCCAG	CCAATCACTGTGTCTAGTTCCT
Cyp3a13	AGGCTCAAGGAGATGTTCCC	AGATGTCTTTCATGCTGGTGG
Cyp2b10	GGAACAGACACCATAAGGGA	GCAAAGATCACACCATATTCCT
Cyp2c40	TGGACTATACCATTGAACACCT	CTGGACTTTAGCTGTGATGTG

Supplementary Table 2. List of antibodies used in immunofluorescence analysis

Antibody	Species	Supplier	Cat. No.	Dilution
HNF1B	Rabbit	Sigma	HPA002083	1:400
TJP1	Rabbit	Invitrogen	40-2300	1:250
CDH1	Mouse	BD Biosciences	610181	1:100
CYP1A2	Rabbit	Abcam	Ab77795	1:600
HNF4A	Rabbit	Santa Cruz	sc-8987	1:500
SLC10A2	Rabbit	Bioss	bs-4189R	1:200

References

[1] Brangel P, Undine S. Annual Report 2016 Eurotransplant International Foundation. 2016.

[2] Wu X-B, Tao R. Hepatocyte differentiation of mesenchymal stem cells. Hepatobiliary Pancreat Dis Int 2012;11:360–371.

[3] Schwartz RE, Fleming HE, Khetani SR, et al. Pluripotent stem cell-derived hepatocyte-like cells. Biotechnol Adv 2014;32:504–513.

[4] Kelaini S, Cochrane A, Margariti A. Direct reprogramming of adult cells: Avoiding the pluripotent state. Stem Cells Cloning Adv Appl 2014;7:19–29.

[5] Huang P, Zhang L, Gao Y, et al. Direct reprogramming of human fibroblasts to functional and expandable hepatocytes. Cell Stem Cell 2014;14:370–384.

[6] Huang P, He Z, Ji S, et al. Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. Nature 2011;475:386–389.

[7] Sekiya S, Suzuki A. Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. Nature 2011;475:390–393.

[8] Du Y, Wang J, Jia J, et al. Human hepatocytes with drug metabolic function induced from fibroblasts by lineage reprogramming. Cell Stem Cell 2014;14:394–403.

[9] LeCluyse EL, Bullock PL, Parkinson A. Strategies for restoration and maintenance of normal hepatic structure and function in long-term cultures of rat hepatocytes. Adv Drug Deliv Rev 1996;22:133–186.

[10] Vyas D, Baptista PM, Brovold M, et al. Self-assembled liver organoids recapitulate hepatobiliary organogenesis *in vitro*. Hepatology 2018;1:1–36.

[11] Wang Y, Cui C Bin, Yamauchi M, et al. Lineage restriction of human hepatic stem cells to mature fates is made efficient by tissue-specific biomatrix scaffolds. Hepatology 2011;53:293–305.

[12] Soto-Gutierrez A, Zhang L, Medberry C, et al. A whole-organ regenerative medicine approach for liver replacement. Tissue Eng Part C Methods 2011;17:677–686.

[13] Warlich E, Kuehle J, Cantz T, et al. Lentiviral vector design and imaging approaches to visualize the early stages of cellular reprogramming. Mol Ther 2011;19:782–789.

[14] Severgnini M, Sherman J, Sehgal A, et al. A rapid two-step method for isolation of functional primary mouse hepatocytes: Cell characterization and asialoglycoprotein receptor based assay development. Cytotechnology 2012;64:187–195.

[15] Nantasanti S, Spee B, Kruitwagen HS, et al. Disease modeling and gene therapy of copper storage disease in canine hepatic organoids. Stem Cell Reports 2015;5:895–907.

[16] Steenbeek FG van, Spee B, Penning LC, et al. Altered Subcellular Localization of Heat Shock Protein 90 Is Associated with Impaired Expression of the Aryl Hydrocarbon Receptor Pathway in Dogs Ryffel B, ed. PLoS One 2013;8:e57973.

[17] Baum C, Hegewisch-Becker S, Eckert HG, et al. Novel retroviral vectors for efficient expression of the multidrug resistance (mdr-1) gene in early hematopoietic cells. J Virol 1995;69:7541–7.

[18] Schambach A, Bohne J, Baum C, et al. Woodchuck hepatitis virus post-transcriptional regulatory element deleted from X protein and promoter sequences enhances retroviral vector titer and expression. Gene Ther 2006;13:641–645.

[19] Kim JH, Lee SR, Li LH, et al. High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. ` PLoS One 2011;6:1–8.

[20] Gowda S, Desai PB, Hull V V, et al. A review on laboratory liver function tests. Pan Afr Med J 2009;3:17.

[21] Kotoh K, Kato M, Kohjima M, et al. Lactate dehydrogenase production in hepatocytes is increased at an early stage of acute liver failure. Exp Ther Med 2011;2:195–199.

[22] Jaeschke H, McGill MR. Serum glutamate dehydrogenase-biomarker for liver cell death or mitochondrial dysfunction? Toxicol Sci 2013;134:221–222.

[23] Dancygier H. Clinical Hepatology. Springer Berlin Heidelberg; 2010.

[24] Cui JY, Renaud HJ, Klaassen CD. Ontogeny of novel cytochrome P450 gene isoforms during postnatal liver maturation in mice. Drug Metab Dispos 2012;40:1226–1237.

[25] Baptista PM, Siddiqui MM, Lozier G, et al. The use of whole organ decellularization for the generation of a vascularized liver organoid. Hepatology 2011;53:604–617.

[26] Kumar M, Keller B, Makalou N, et al. Systematic determination of the packaging limit of lentiviral vectors. Hum Gene Ther 2001;12:1893–905.

[27] Amand MM, Hanover JA, Shiloach J. A comparison of strategies for immortalizing mouse embryonic fibroblasts. J Biol Methods 2016;3:41.

[28] Lichtsteiner S, Schibler U. A glycosylated liver-specific transcription factor

stimulates transcription of the albumin gene. Cell 1989;57:1179–1187.

[29] Kajiyama Y, Tian J, Locker J. Characterization of distant enhancers and promoters in the albumin-α-fetoprotein locus during active and silenced expression. J Biol Chem 2006;281:30122–30131.

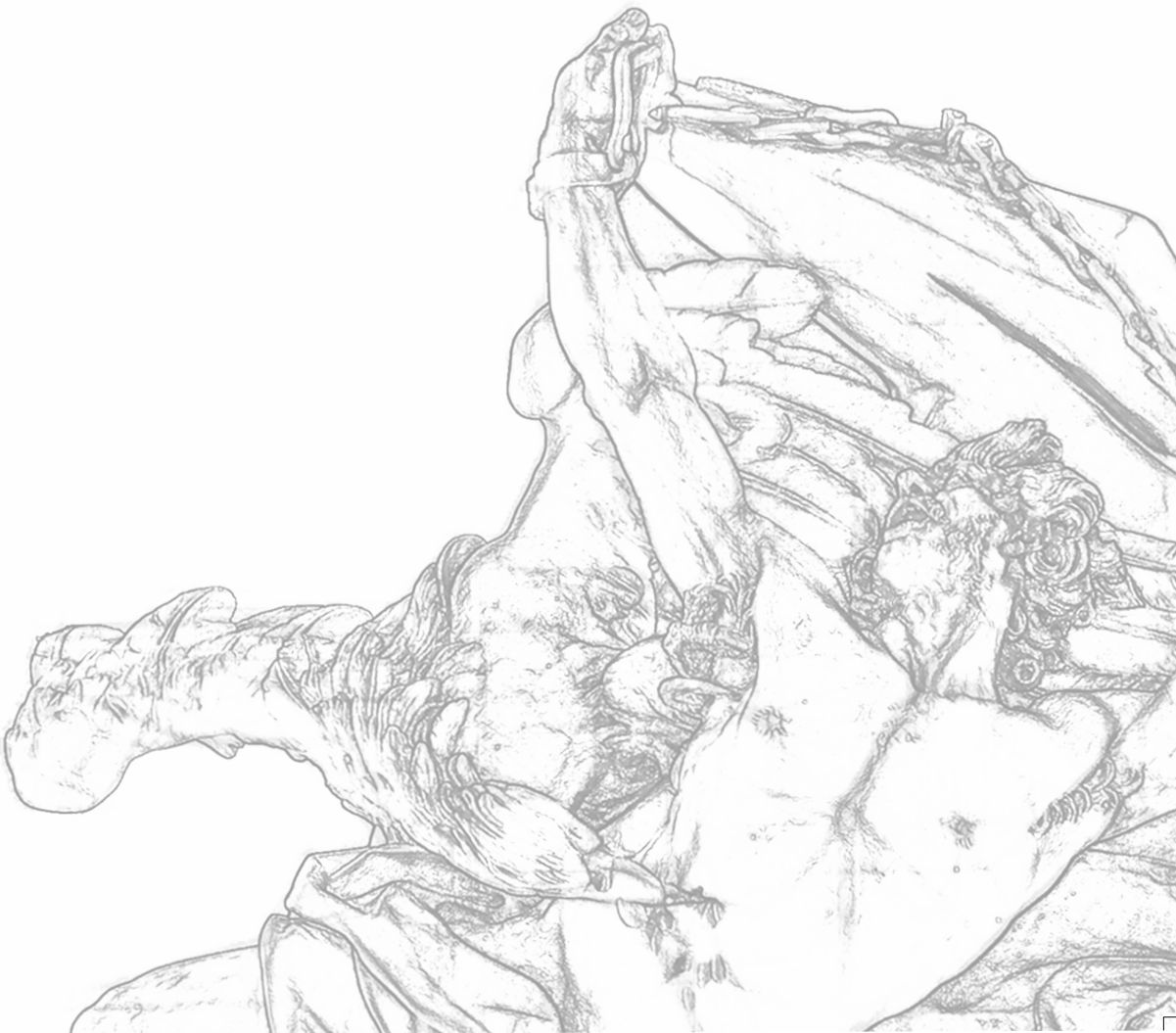
[30] Cirillo LA, Lin FR, Cuesta I, et al. Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. Mol Cell 2002;9:279–289.

[31] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663–76.

[32] Wang Y, Nicolas CT, Chen HS, et al. Recent advances in decellularization and recellularization for tissue-engineered liver grafts. Cells Tissues Organs 2017;204:125–136.

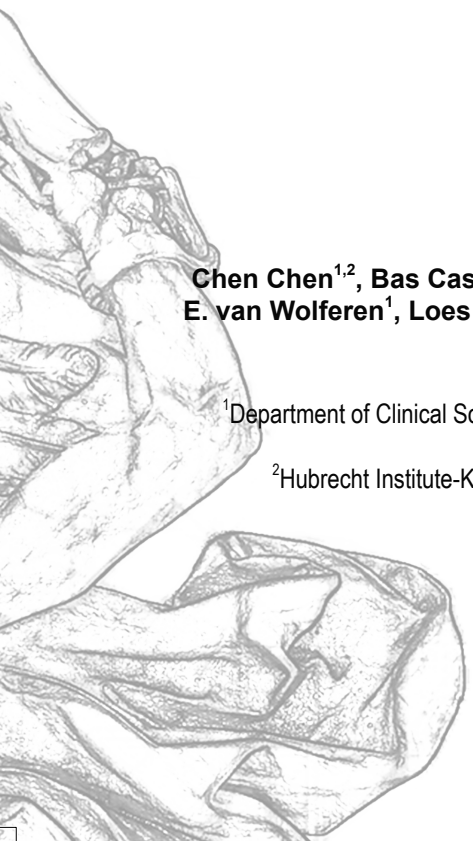
[33] Walker JM, Jackson M, Taylor a H, et al. Mouse cell culture. 2010.

[34] Chen Y, Wong PP, Sjeklocha L, et al. Mature hepatocytes exhibit unexpected plasticity by direct dedifferentiation into liver progenitor cells in culture. Hepatology 2012;55:563–574.



Chapter 3

Identification of transcription factors enhancing maturation of hepatocyte-like cells



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Submitted

Abstract

The immature features of hepatocyte-like cells (HLCs) generated from somatic cells limits their application in drug metabolism tests. In this study, we performed a three-step screening on dedifferentiating primary mouse hepatocytes based on H3K27ac chromatin immunoprecipitation followed by sequencing and RNA-seq. The screening results showed HNF4A as the master regulator for maintaining terminal maturation of hepatocytes. Forced expression of HNF4A resulted in enhanced hepatocyte features in HLCs including the upregulation of hepatocyte markers and downregulation of stem cell and cholangiocyte markers. Furthermore, hepatocyte-enriched transaminases, phase I enzyme as well as albumin also showed significantly increased expression. As such, we present a novel strategy to identify key transcription factors for specific cell types. Additionally, we provide a proof-of-concept that HNF4A plays a crucial role in hepatic maturation.

Introduction

Over the past decade, many types of stem cell derived hepatocyte-like cells have been developed, but their application in preclinical drug development is limited due to their immature hepatic phenotypes. So far, primary hepatocytes are still the optimal candidate widely used in testing compounds with unknown absorption, distribution, metabolism, excretion and toxicity (ADMET) profiles *in vitro*. However, primary hepatocytes need to be freshly isolated and preferably used soon after the isolation, since they start to lose their maturity right after they are removed from their native liver microenvironment, and exhibit stem cell-like features during *in vitro* culture [1,2]. The deterioration of mature functions is referred to as dedifferentiation. In general, activated nuclear factor kappa B (Nf-κB) and mitogen-activated protein kinase (MAPK) signaling, which respectively mediate inflammatory and proliferative responses, are believed to be the main incentives resulting in *in vitro* dedifferentiation of hepatocytes [3]. However, the clear mechanism is unknown.

Transcriptional activators, a type of transcription factors, control the most fundamental process of converting genes into functional proteins. Specifically, by binding to specific cis-regulatory DNA elements, including enhancers or promoter elements, transcriptional activators positively affect the transcription of specific genes. The importance of transcription activators on hepatocyte differentiation has been extensively studied [4–6]. Currently identification of these factors largely relies on transcriptomics and proteomics focusing on their differential expression during liver development [7,8]. Those important transcriptional activators have been used to generate hepatocyte-like cells (HLCs) from multiple sources and species [9–12]. However, the HLCs still exhibited immature characteristics, such as the expression of fetal liver marker, alpha fetal protein (*AFP*) and limited cytochrome P450 activities, which indicates the lack of transcriptional activators maintaining the identity of hepatocytes.

The transcription of a gene can be initiated only when specific transcriptional activators are present and their corresponding regulatory DNA elements related to this gene are accessible. The accessibility of regulatory DNA elements are determined by multiple epigenetic modifications, among which the commonly used are H3K4me1 defining "primed" (including inactive/poised) enhancers and H3K27ac marking active enhancers [13]. Active enhancer sequences, identified by chromatin immunoprecipitation sequencing (ChIP-seq) using antibodies specific for

H3K27ac, can further be processed by motif search analysis, which provides the possible transcription factors maintaining one specific cell type's fate.

In this study, we used a three-step selection, based on epigenome and transcriptome analysis, to identify the disappearing transcriptional activators during hepatocyte dedifferentiation. The identified hits were in agreement with previous studies [4,6,14], which proves the reliability of this method. Furthermore, we validated that this identified transcriptional activator can be used for enhancing maturation of hepatocyte-like cells generated from somatic cells.

Material and Methods

Cell isolation and culture

The human embryonic kidney line HEK293T and mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% v/v fetal bovine serum (FBS, Invitrogen) and 1% v/v penicillin/streptomycin. Primary mouse hepatocytes were isolated by a collagenase perfusion procedure [15] from mice in non-hepatocyte-related research (experiments approved by the Utrecht University's ethical committee). Isolated cells were cultured on collagen type I coated plates in HepatoZYME (Gibco) supplemented with 10% FBS for 2 hours for attaching, then cultured in HepatoZYME without serum. Media was refreshed every day for 6 days. Hepatocyte-like cells (HLCs) were generated as previously described (chapter 2). Briefly, MEFs were transduced with lentivirus co-expressing *Hnf1a*, *Foxa3* and *Gata4* and cultured in HLC medium, containing a 1:1 mixture of DMEM/F-12, supplemented with 10% FBS, 20 ng/ml hepatocyte growth factor, 20 ng/ml epidermal growth factor, 1 μg/ml insulin, 10^{-7} M dexamethasone, 10 mM nicotinamide, 2 mM L-glutamine, 50 μM β-mercaptoethanol and 1% v/v penicillin/streptomycin.

Lentivirus production and transduction

Virus production for the generation of HLCs was performed with a 3rd generation lentiviral packaging system and transduction was performed as previously described [16]. Two days after HLCs were transduced, 10 μg/ml puromycin was added in culturing medium to select cells expressing exogenous genes.

H3K27ac ChIP-seq and data analysis

About 1x10^6 mouse primary hepatocytes were fixed with 1% formaldehyde at 0, 4, 12, 36, 48, 72 and 144 hours after they were seeded on plates. H3K27ac ChIP was performed as previously described [13] and sequencing was performed on an Illumina High-seq 2000 with 1 x 40 bp configuration at the MIT BioMicro Center (http://openwetware.org/wiki/BioMicroCenter). Sequence reads were mapped to the mouse genome (mm9) using Bowtie 1.1.2 [17] and enriched regions were called using Macs2 version 2.1.1 (P=10-5, extsize=300, local lambda = 100,000) [18]. Known transcriptional start sites were subtracted from the enriched regions, the remaining regions are defined as enhancers. Enhancers from all timepoints were combined in a single non-redundant list and overlapping enhancers were merged into one. Enhancers smaller than 2kb were extended so that all enhancers were at minimal 2000bp long (peak center ± 1000bp).

For all non-redundant enhancers, the reads were counted in every timepoint using Bedtools 1.3.1 [19]. For each enhancer, the readcounts of all timepoints were pairwise compared with the readcount of the 0-hour sample with the EdgeR package [20]. Enhancers were defined as either up- or downregulated when the readcount differed at least 4-fold from the 0-hour sample.

To cluster the differentially enriched enhancers, readcount values were normalized using z-score normalization per enhancer over all timepoints. Enhancers were hierarchical clustered based on Pearson correlation and average linkage. Enhancer clusters were defined by using treecutting to create minimum of 3 and maximum of 5 groups. Motifs enrichment was performed using Homer [21].

RNA isolation, RNA-seq and data analysis

RNA was isolated from MEFs, primary hepatocytes and HLCs using RNeasy Micro Kit according to the manufacturer's instructions (Qiagen). RNA-seq was processed as described previously [22]. For the RNA-seq data analysis, the neighboring genes based on chromosomal position (genome build Mmu GRCm38.p6) within a range of 25 kbp up- or downstream of the enhancers regions of interests generated by ChIP-seq were selected. The top 200 genes with most differential expression over time within the first 48 hours based on DESeq2 analysis [23] were used for hierarchical clustering in edgeR [20]. The cluster with high expression at t=4

hours and decreasing expression over later time points were identified and their correlated enhancer regions defined in the H3K27ac ChIP-seq were used as input for HOMER Motif Analysis [21].

cDNA synthesis and RT-qPCR

cDNA was synthesized by the iScript™ cDNA synthesis kit as described by the manufacturer (Bio-Rad). Relative gene expression of the selected genes was measured using RT-qPCR. Primer design, validation, RT-qPCR conditions, and data analysis was performed as previously described [24]. Normalization was performed using the reference gene hypoxanthine phosphoribosyl transferase (*Hprt*). Details of primers are listed in Table S1.

Immunoblot analysis

Cells were lysed with RIPA buffer containing 50 mM Tris–HCl, 150 mM NaCl, 1% v/v NP-40, 0.25% w/v sodium deoxycholate, 1 mM EDTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 µg/ml aprotinin and 1 mM PMSF (Sigma-Aldrich). Protein concentration was measured by BCA Protein Assay (Bio-Rad). 10 µg total protein for each sample was loaded in a 10% SDS-PAGE gel, transferred to a 0.45 µm nitrocellulose membrane (Bio-Rad), and blocked with ECL Blocking agent (Amersham GE healthcare). The blots were probed with HNF4A antibody (Santa Cruz sc-8987, dilution 1:2000), STAT3 antibody (Abcam ab119352) and phospho-STAT3 (Tyr705) Antibody (Cell signal #9131, dilution 1:1000) overnight at 4°C and subsequently incubated with HRP-conjugated anti-rabbit (R&D systems, HAF008, dilution 1:5,000) for 1 hour at room temperature. Luminescence induced by the Amersham ECL Western Blotting Detection Reagent (GE Healthcare) was measured with a ChemiDoc XRS Imager (Bio-Rad).

Cell proliferation analysis

AlamarBlue™ Cell Viability Reagent (ThermoFisher DAL1025) was diluted 1:20 in phenol-red free DMEM/F12. 400 µl working solution was added to each well of cells in 24-well plates. Wells without cells were used to check background. After one hour of incubation in a culture stove at 37°C, fluorescence at 540/590nm were measured.

Hepatocyte functional tests

For measurement of albumin secretion, HLCs were cultured in serum free medium. After one day, culture media was collected. Micro-albumin in the media was concentrated using Amicon Ultra centrifugal filters (Millipore), and the amount of albumin was measured using a DxC-600 Beckman chemistry analyzer (Beckman Coulter). The values were normalized with AlamarBlue results.

For measurement of CYP3A and CYP1A2 activities, HLCs were incubated in 50 μM luciferin-PFBE or luciferin-1A2 (Promega) solution for 8 hours at 37°C. Activity were measured by a luminometer (Beckman Coulter).

For measurement of the expression of hepatic enzymes, HLCs were lysed in distilled water. The ALP, GGT, LDH, GLDH and AST in the lysate was measured using the DxC-600 Beckman chemistry analyzer (Beckman Coulter) and values were normalized with AlamarBlue results.

Statistical analysis

Statistical analysis and graphs were performed using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). See figure legends for details on specific statistical tests run and p values calculated for each experiment.

Results

Dedifferentiation of primary hepatocytes during in vitro culture

Primary hepatocytes were isolated from mice by a two-step collagenase perfusion procedure as previously described [25]. In order to facilitate attachment of primary mouse hepatocytes to collagen (type I) coated plates, we used the commercial hepatocyte media HepatoZYME with 10% of serum (FBS). After two hours of incubation when cells were tightly attached, the media was changed to pure HepatoZYME (Figure 1A). Although the commercial media HepatoZYME was developed to maintain hepatic maturity to the maximum degree, we still observed rapid loss of mature features of primary hepatocytes, similar to previously reported [1]. Specifically, within 24 hours, hepatocyte markers, Albumin (*Alb*), Asialoglycoprotein receptor (*Asgpr*), Cytochrome P450, Family 1 Subfamily

A Member 2 (*Cyp1a2*) and Family 3 Subfamily A Member 11 (*Cyp3a11*) were downregulated to approximately 15%, 28%, 6% and 4%, respectively, compared to freshly isolated primary hepatocytes, and their expression kept decreasing overtime. Mature hepatocyte markers, ATP Binding Cassette Subfamily C Member 2 (*Abcc2*, also known as *Mrp2*) and Prospero Homeobox 1 (*Prox1*) remained at around 5% and 20% of the expression of fresh hepatocytes, respectively. The hepatic progenitor marker alpha-fetal protein (*Afp*) and mesenchymal marker Vimentin (*Vim*) were gradually upregulated during the three days of culture (Figure 1B).

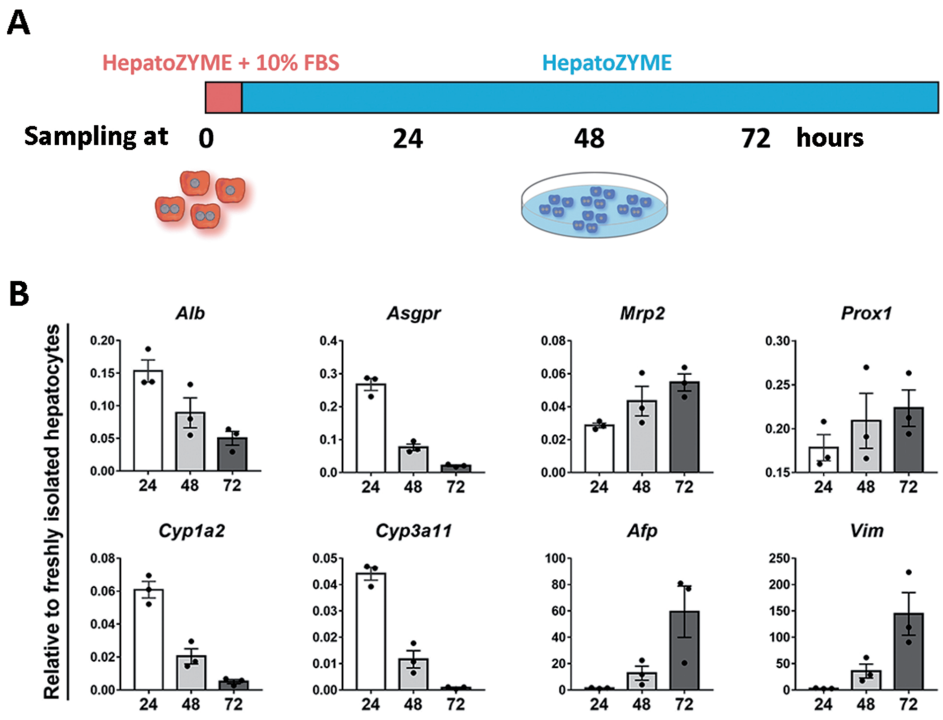


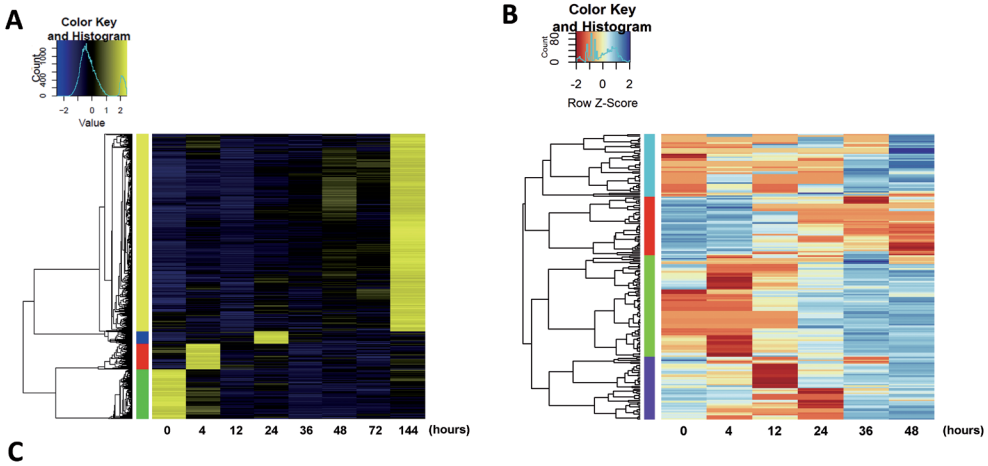
Figure 1. Dedifferentiation of primary hepatocytes during *in vitro* culture. (A) Primary hepatocytes were isolated by a two-step perfusion procedure. Cells were cultured in HepatoZYME with serum for attachment for 2 hours thereafter in HepatoZYME without serum. RNA samples were collected at 0 hour, 24 hours, 48 hours and 72 hours. (B) gene expression analysis showing the downregulation of hepatocyte markers and upregulation of mesenchymal and immature hepatocyte markers. The results of 24, 48 and 72 hours were normalized to 0 hour (freshly isolated primary hepatocytes).

Identification of transcription factors maintaining terminal maturation of primary hepatocytes

To investigate the dynamics of enhancer activity during hepatocyte dedifferentiation, we performed chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) with antibody against histone acetylation of H3K27 (H3K27ac), which is a well-established marker for active enhancer regions [13]. The identified enhancer regions were separated into four clusters based on their enrichment across the timepoints, among which the green luster, which includes 2,117 enhancers that gradually switched from an active into an inactive state (Figure 2A).

Based on chromosomal location a total of 2,760 genes were linked to the 2,117 gradually decreasing enhancer regions, of which 1,092 genes were expressed in dedifferentiating hepatocytes based on RNA-Seq. The top 200 genes with most differential expression over time within the first 48 hours were used for hierarchical clustering resulting in four groups with time specific expression patterns (Figure 2B). The cluster with high expression at t=4 hours (Figure 2B, red cluster) and decreasing expression over later time points comprises 72 genes, which correlate with a total of 118 enhancer regions defined within the cluster of early response enhancer regions based on ChIP-Seq. These enhancers were used as input for motif analysis, which generated a list of transcription factors that could potentially regulate these enhancer (Figure 2C). Among the 63 identified transcription factors, we observed that only hepatic nuclear factor 4 alpha (*Hnf4a*) had a high expression based on RNA-seq results (Figure 2D).

To validate the hit generated from epigenome and transcriptome analysis, we performed RT-qPCR and immunoblotting to determine the expression of *Hnf4a*. Both assays showed that *Hnf4a* remained at relatively high level during the first four hours of *in vitro* culture but started to decrease after that (Figure 3A and B). These results together showed that *Hnf4a* was a missing transcription factor during the dedifferentiation of hepatocytes.



C

Rank	Motif	P-value	% of Targets	% of Background	STD(Bg STD)	Best Match
1		1.00E-10	12.71%	1.32%	58.3bp (61.0bp)	GRHL1, MEF2 A/B/C, SOX 2/4/11
2		1.00E-09	6.78%	0.25%	53.4bp (55.4bp)	HNF4 A/G, ESRR A/B/G, SOX17, TEAD3
3		1.00E-09	29.66%	9.65%	57.0bp (55.8bp)	GATA3, IRF 4/5/6, NFATC 1/3, NFkB
4		1.00E-08	12.71%	1.73%	55.8bp (60.0bp)	SOX 3/6/9/10/12
5		1.00E-08	3.39%	0.02%	44.7bp (39.1bp)	ZEB1, TCF 3/4/10/21, ID4, PTF1A
6		1.00E-05	21.19%	7.31%	58.5bp (62.9bp)	SNAI2, TCF 3/4, ID4, HIF1, GCM1
7		1.00E-05	9.32%	1.44%	55.4bp (57.5bp)	STAT 1/3/4, BCL6, EHF, SPDEF
8		1.00E-05	5.08%	0.29%	45.8bp (57.8bp)	MAZ, ZFP 281/740, KLF5, ASCL2
9		1.00E-04	2.54%	0.05%	70.1bp (51.1bp)	ZFP 161/691, MTF1, E2F 2/3, ZSCAN4, HIC1
10		1.00E-03	8.47%	2.24%	41.1bp (61.3bp)	NRF1, ZFP128, ZNF264, NKX3-1
11		1.00E-02	10.17%	3.60%	50.2bp (60.3bp)	ZFP128, OCT6, SOX17, TBP1

D

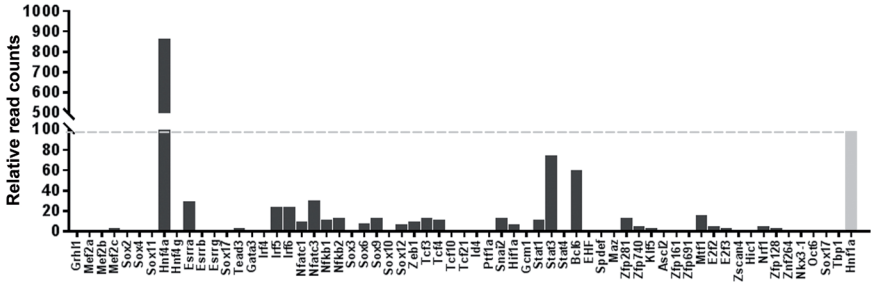


Figure 2. Screening transcription factors maintaining the maturation of hepatocytes. (A) Enhancers were clustered into four based on their differential activity identified by H3K27ac ChIP-seq. (B) Top 200 genes with most differential expression over time within the first 48 hours were used for hierarchical clustering resulting in four groups with time specific expression patterns. (C) Motifs and

(legend continued on next page)

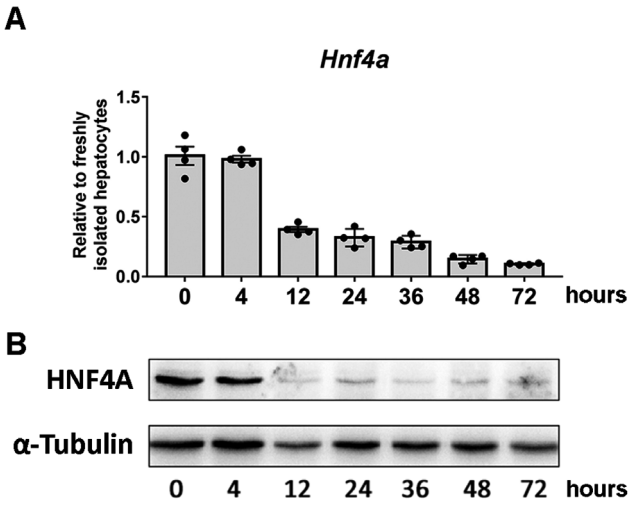


Figure 3. Expression of *Hnf4a* in dedifferentiated primary hepatocytes. (A) RT-qPCR analysis showing the expression of *Hnf4a* at mRNA level. Data are shown as mean ± SEM of four independent samples for each group. All results were normalized to freshly isolated hepatocytes. (B) Immunoblotting showing the expression of *Hnf4a* at protein level.

Induced functional maturation of hepatocyte-like cells by forced expression of Hnf4a

Previously, we used a polycistronic system co-expressing the transcription factors, including *Foxa3*, *Hnf1a* and *Gata4* (FHG), to generate murine hepatocyte-like cells (Figure 4 A and B). HLCs exhibited high homogeneity and some hepatic features, but they were still not functionally mature (chapter 2). We interrogated whether *Hnf4a* could boost the maturation of HLCs. To do so, we constructed a bicistronic vector co-expressing *Hnf4a* and *GFP* (Figure 4A). Since *Hnf4a* and *GFP* were separated by the 2A peptide sequence and these two genes had the same amount of mRNA expression [26], we used qPCR primers targeting the GFP region to indicate the exogenous expression of Hnf4a. As Figure 2B and C show, exogenous *Hnf4a* expression was highly induced compared with non-transduced mouse embryonic fibroblasts (MEFs) and it did not affect the expression of FHG. With

correlated transcription factors identified by HOMER Motif Analysis. (D) Relative read counts of genes identified by the H3K27ac ChIP-seq and RNA-seq analysis. *Hnf1a*, an HLC generating gene, was used as a control to distinguish high/low gene expression.

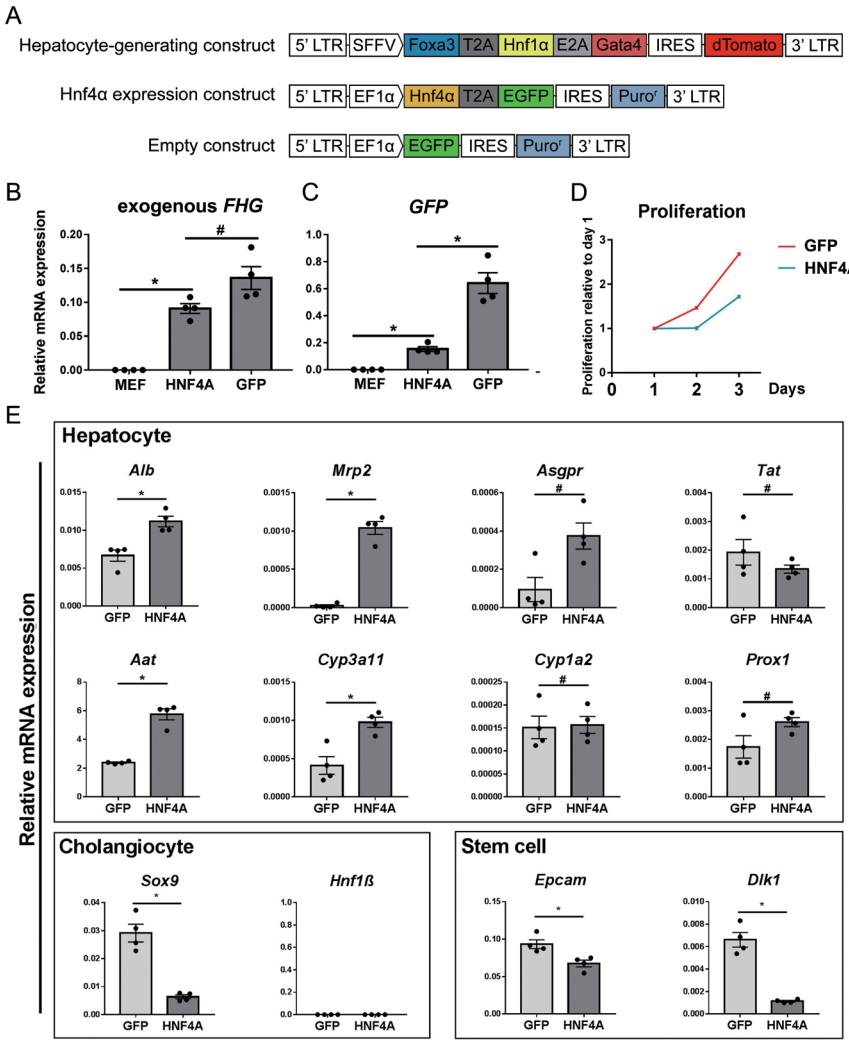


Figure 4. Forced expression of Hnf4a enhanced the maturation of hepatocyte-like cells derived from somatic cells. (A) Schematic illustration showing the constructs for hepatocyte-generating transcription factors, *Hnf4a* and empty vehicle. (B-C) qPCR analysis showing the expression of GFP and exogenous *Foxa3*, *Hnf1a* and *Gata4* (exogenous *FHG*). Data are shown as mean ± SEM of four independent samples for each group. Hash represents non-significance. Asterisk represents statistical significance. *p< 0.05 (two-tailed Mann–Whitney U test). (D) Comparison of proliferation capacity of HLCs transduced with GFP and HNF4A. (E) RT-qPCR analysis showing the expression of hepatocyte, cholangiocyte and stem cell markers in HLCs transduced with GFP and HNF4A. Data are shown as mean ± SEM of four independent samples for each group. All results were normalized to freshly isolated hepatocytes. Asterisk represents statistical significance. *p< 0.05. Hash represents non-significance (two-tailed Mann–Whitney U test).

the forced expression of *Hnf4a*, we also observed that the massive proliferating capacity of HLCs was inhibited (Figure 4D), which indicates a quiescent state similar to hepatocytes in the healthy liver [27]. RT-qPCR analysis showed that general hepatocyte marker Alb and mature hepatocyte markers *Mrp2*, *Aat* and *Cyp3a11* were significantly enhanced; while cholangiocyte markers SRY-box 9 (*Sox9*) and stem cell markers Epithelial Cell Adhesion Molecule (*Epcam*) and Delta Like Non-Canonical Notch Ligand 1 (*Dlk1*) were downregulated in the *Hnf4a*-overexpressing HLCs (Figure 4E).

We further compared the function of HLCs with or without *Hnf4a* overexpression. Functional tests showed that as one of the most important cytochrome family in the liver, activity of CYP3A was highly induced by HNF4A (Figure 5A), while CYP1A2 did not significantly change (Figure 5B). In *Hnf4a*-overexpressing HLCs, albumin secretion and hepatocyte enriched enzymes, including aspartate aminotransferase, alanine transaminase, lactate dehydrogenase and glutamate dehydrogenase, were all significantly upregulated compared to HLCs transduced only with GFP (Figure 5C-G). Cholangiocyte enriched enzyme gamma-glutamyl transferase was undetectable in both HLC groups (Figure 5H).

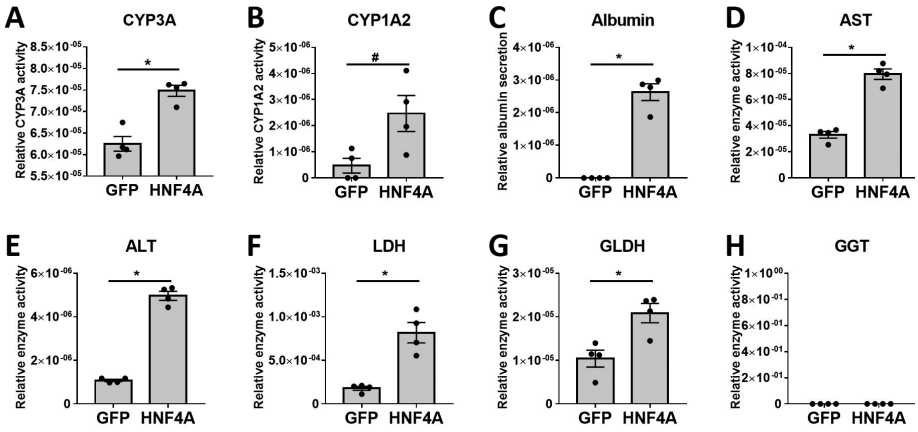


Figure 5. Functional validation of HLCs transduced with *Hnf4a*. All results were normalized with the value of Alamar blue assay. Data are shown as mean ± SEM of four independent samples for each group. Asterisk represents statistical significance. *p< 0.05. Hash represents non-significance (two-tailed Mann–Whitney U test).

Discussion

Stem and somatic cell-derived hepatocyte-like cells (HLCs) are promising alternatives for testing drug toxicity instead of isolated primary hepatocytes [28]. Nevertheless, *in vitro* generated HLCs can never achieve all the functional maturation resembling fresh primary hepatocytes [9,10,12,29]. Decryption of the gap between HLC and fully differentiated hepatocytes attracted the attention of many studies [30,31]. In our study, we aimed to identify the difference from the opposite direction, dedifferentiation of mature hepatocytes. Previously Chen and colleagues have reported that mature hepatocytes, during *in vitro* culture, underwent direct dedifferentiation into hepatic progenitor or stem cells [1], which have similar features of HLCs. To investigate the reversed procedure may provide us additional insight to understand the key players during terminal hepatic maturation. To do so, we isolated primary hepatocytes from mice by a two-step collagenase perfusion procedure which generates a homogenous population of primary hepatocytes [25]. Although serum is a known negative regulator for hepatic maturation, which induces cellular depolarization, destabilizes the bile canaliculi-like network and decreases phase I metabolism [3], it is required for facilitating the attachment of cells onto petri dishes during *in vitro* culture. Therefore, we incubated cells with serum containing medium for only 2 hours for attachment, after which serum-free medium was used. After this, primary hepatocytes were analyzed in time using H3K27ac chromatin immunoprecipitation followed by sequencing and RNA-seq. This setup allowed a thorough investigation into the epigenetic changes in dedifferentiating hepatocytes.

In previous studies, the comparison among hepatic cells of different stages was based on gene expression profiling analysis focusing on transcriptional events [30,31]. In this study, we aimed to distinguish the change by a method combining epigenome and transcriptome analysis. The initiation of transcription of a gene is controlled by two events, that is, specific transcriptional regulators needs to be present as well as their corresponding regulatory DNA elements related to this gene need to be accessible. The accessibility of regulatory DNA elements is determined by multiple epigenetic modifications including H3K4me1, H3K27ac, etc. Previous studies reported that although most H3K4me1 marked enhancer regions displayed activity when tested in reporter assays, a significant percentage was not active [32,33], which was further confirmed in another study defining H3K4me1 as a marker for active enhancers as well as those in a poised or predetermined state

[13]. Therefore, in this study, we performed epigenome analysis based on H3K27ac which has been shown to be an optimal marker for distinguishing active enhancers [13]. According to their activity across the different timepoints, enhancers could be separated into four clusters. We firstly analyzed the clusters of enhancers with increasing activity during hepatocyte dedifferentiation. As expected, this set of enhancer was enriched for binding sites belonging to transcription factors that were related to stress and inflammation (Figure S1), which are well-studied causes proven to play important roles on hepatocyte dedifferentiation [3]. Therefore, we focused on the enhancers with decreasing activity in the following experiments.

The proximal enhancers play an important role in the transcriptional regulation of genes, therefore, the enhancer regions identified to gradually lose their activity over time (green cluster in Figure 2A) were linked to their neighboring genes in the range of 25 kbps up/down-stream [34]. Surprisingly, some genes linked to the gradually inaccessible enhancers showed an upregulated expression, and that is the reason why we included gene expression profiling to exclude these unrelated hits. The remaining enhancer sequences were processed by motif search analysis, which provided a list of potential transcription factors maintaining the fate of hepatocytes. Within this list, most genes were low- or even non-expressed in primary hepatocytes, which were unlikely to function and hence excluded. One example is to reactivate the expression of STAT3 which did not rescue any dedifferentiating features (Figure S2&3). This three-step screening based on epigenome and transcriptome analysis resulted in one hit, *Hnf4a. Hnf4a* is known to be a master regulator determining the fate of hepatocytes [4–6], indicating the reliability of our screening strategy.

A limitation to this study was that this screening strategy was unable to uncover more novel transcription factors regulating the terminal maturation of hepatocytes. This may be due to three reasons. First, our epigenome analysis is only based on H3K27ac enrichment on enhancer regions, while changes on promoter regions during differentiation was overlooked. Although this restriction could be useful for identifying the regulatory elements and their associated transcription factor for specific cell types, this could also lead to false exclusion of some transcription factors, which may be only binding to the promoter regions. A method capable of overcoming this limitation may be Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq), which could identify all accessible DNA regions [35]. Second, the gap between the initiation of an epigenetic event and its resulting

transcriptional change is difficult to predict at genome-wide range, but in our study, we assumed these two events are happening sequentially at the same rate for all genes. Third, due to the complexity of adult tissue, unlike pluripotent stem cells, transcription factors may not be the only determinants for cell fates, the limited number of transcription factors may need to collaborate with other cellular and molecular components in maintaining hepatocyte features [36].

In previous studies, somatic cells were able to be transdifferentiated into hepatocyte-like cells by forced expression of transcription factors [11,12]. We also confirmed this with a polycistronic expression system resulting in HLCs with high efficiency and homogeneity (unpublished data). However, the HLCs exhibited limited mature phenotypes such as low drug metabolism capacity and unlimited proliferating capacity. In the healthy liver, mature hepatocytes remain quiescent [27], but HLCs could stably proliferate for at least 30 passages over 2 months (unpublished data), which may be due to the induced cellular stemness by HNF1A and FOXA3 [37]. By overexpressing *Hnf4a*, this phenomenon could be partially reversed. In another type of hepatic cells, the $Lgr5^+$ liver stem cells (liver organoids) [38], the inhibition of proliferation was even more significant (Figure S4). Apart from stabilizing HLCs in a relative quiescent state, HNF4A also upregulated hepatocyte markers and downregulated stem cell markers. This said, there were still hepatocyte markers like tyrosine aminotransferase (Tat), *Prox1* and *Cyp1a2* which remained at low expression level compared to freshly isolated hepatocytes. This finding is consistent with the point discussed above that although HNF4A plays a vital role in the hepatic phenotypes, a single overexpression with this factor may not be enough to induce all mature hepatocyte features. Further investigation for strategies to combine transcription factors with other molecular components such as microRNA and growth factors, and cellular components such as extracellular matrix are needed.

Acknowledgements

We thank Drs. Maya Haaker from Department of Biochemistry, Faculty of Veterinary Medicine, Utrecht University for providing primary hepatocytes for this study.

Conflicts of Interest

The authors have declared no conflicts of interest.

Supplementary Figures

Motif	P-value	log P-pvalue	% of Targets	% of Background	STD(Bg STD)	Best Match/Details
	1e-301	-6.936e+02	19.14%	3.33%	52.6bp (63.6bp)	AP-1(bZIP)/ThioMac-PU.1-ChIP-Seq(GSE21512)/Homer(0.979) More Information | Similar Motifs Found
	1e-37	-8.611e+01	8.55%	3.81%	54.1bp (59.7bp)	MA0492.1_JUND_(var.2)/Jaspar(0.913) More Information | Similar Motifs Found
	1e-20	-4.719e+01	1.71%	0.39%	57.5bp (59.5bp)	MA0462.1_BATF::JUN/Jaspar(0.706) More Information | Similar Motifs Found
	1e-19	-4.494e+01	17.93%	12.61%	55.8bp (59.0bp)	TEAD(TEA)/Fibroblast-PU.1-ChIP-Seq(Unpublished)/Homer(0.920) More Information | Similar Motifs Found
	1e-17	-4.079e+01	17.37%	12.37%	56.6bp (59.3bp)	MA0152.1_NFATC2/Jaspar(0.741) More Information | Similar Motifs Found
	1e-17	-3.933e+01	4.33%	2.03%	54.9bp (59.5bp)	MA0512.1_Rxra/Jaspar(0.836) More Information | Similar Motifs Found
	1e-15	-3.667e+01	23.66%	18.21%	56.3bp (58.9bp)	EWS:FLI1-fusion(ETS)/SK_N_MC-EWS:FLI1-ChIP-Seq(SRA014231)/Homer(0.925) More Information | Similar Motifs Found

Figure S1. Motifs identified from enhancers with increasing activity during hepatocyte dedifferentiation. The enhancers with increasing activity during hepatocyte dedifferentiation (red cluster in Fig. 2A) were analyzed. Motifs and correlated transcription factors identified by HOMER Motif Analysis.

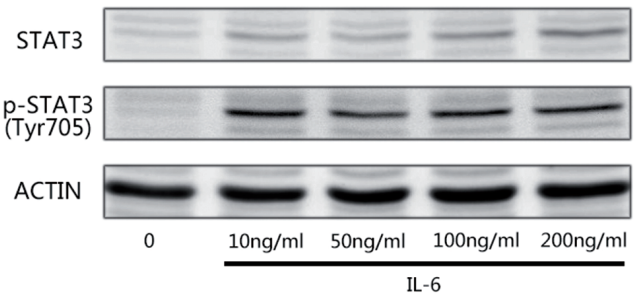


Figure S2. Immunoblotting assay showing the expression of STAT3 and phosphorylated STAT3 in hepatocytes were upregulated by IL-6. IL-6 was added to primary hepatocytes which had been cultured overnight. RNA samples were collected after 24 hours and immunoblotting was performed.

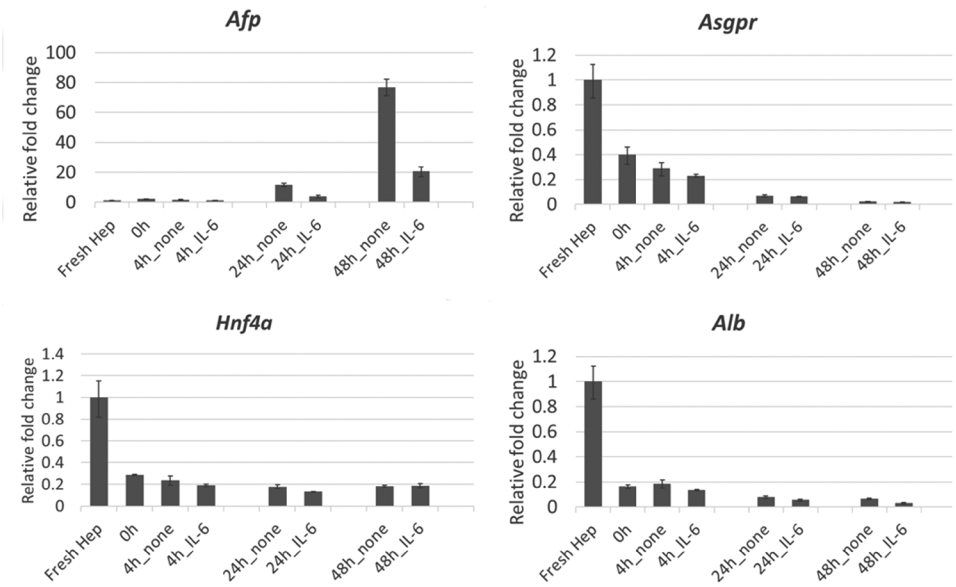


Figure S3. RT-qPCR analysis showing the expression of hepatocyte markers under the stimulation of IL-6. Data are shown as the average of two independent samples for each group. 0h represents the time point when primary hepatocytes were cultured *in vitro* for 24 hours and IL-6 was added to hepatocytes. Fetal hepatocyte marker, *Afp*, was inhibited by IL-6 while mature hepatocyte markers, *Asgpr* and *Alb* were not affected.

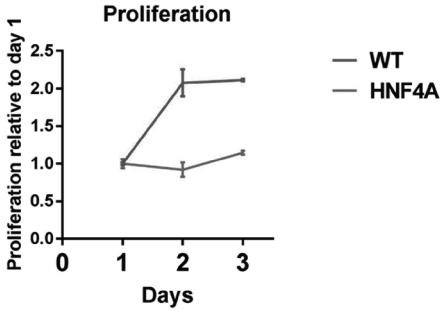


Figure S4. Comparison of proliferation capacity of wild-type liver organoids and liver organoids transduced with HNF4A.

Supplementary Table

List of primers used in RT-qPCR analysis

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
Alb	GCAACACAAAGATGACAACC	CTTCATGCAAATAGTGTCCCA
Hnf1b	ACAATCCCCAGCAATCTCAGAA	GCTGCTAGCCACACTGTTAATGA
Hprt	GTGATTAGCGATGATGAACCA	CAAGTCTTTCAGTCCTGTCCA
Sox9	CCCGATTACAAGTACCAGCC	CCCTGAGATTGCCCAGAGTG
Cyp3a11	AAACTCAAGGAGATGTTCCC	TTCACTCCAAATGATGTGCT
Mrp2	GATAGCCTCATTCAGACGAC	ACCATTATCTTGTCACTGTCCA
Cyp1a2	ACCATGATGAGAAGCAGTGGA	CGAAGAGCATCACCTTCTCG
Tat	GCTTCCTTAAGTCCAATGCG	TCAAATTCTGGGAAGTGCTC
Aat	GCTGCTGATGGATTATGCAGG	GATCTGGGCTAACCTTCTGC
Prox1	AAGGGCTATCACCCAATCAC	CATCTGTTGAACTTCACGTCC
Epcam	GCTCTTCAAAGCCAAGCA	CAATGATGATCCAGTAGGTCCTC
Dlk1	GTGTCAATGGAGTCTGCAAGG	CCCGAACGTCTATTTCGCAG

References

[1] Chen Y, Wong PP, Sjeklocha L, et al. Mature hepatocytes exhibit unexpected plasticity by direct dedifferentiation into liver progenitor cells in culture. Hepatology 2012;55:563–574.

[2] Gebhardt R, Hengstler JG, Müller D, et al. New hepatocyte *in vitro* systems for drug metabolism: metabolic capacity and recommendations for application in basic research and drug development, Standard Operation Procedures. Drug Metab Rev 2003;35:145–213.

[3] Fraczek J, Bolleyn J, Vanhaecke T, et al. Primary hepatocyte cultures for pharmaco-toxicological studies: At the busy crossroad of various anti-dedifferentiation strategies. 2013.

[4] Odom DT, Zizlsperger N, Gordon DB, et al. Control of pancreas and liver gene expression by HNF transcription factors. Science 2004;303:1378–1381.

[5] Odom DT, Dowell RD, Jacobsen ES, et al. Tissue-specific transcriptional regulation has diverged significantly between human and mouse. Nat Genet 2007;39:730–732.

[6] 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.

[7] Yamamizu K, Piao Y, Sharov AA, et al. Identification of transcription factors for lineage-specific ESC differentiation. Stem Cell Reports 2013;1:545–559.

[8] Rowe C, Gerrard DT, Jenkins R, et al. Proteome-wide analyses of human hepatocytes during differentiation and dedifferentiation. Hepatology 2013;58:799–809.

[9] Huang P, Zhang L, Gao Y, et al. Direct reprogramming of human fibroblasts to functional and expandable hepatocytes. Cell Stem Cell 2014;14:370–384.

[10] Du Y, Wang J, Jia J, et al. Human hepatocytes with drug metabolic function induced from fibroblasts by lineage reprogramming. Cell Stem Cell 2014;14:394–403.

[11] Huang P, He Z, Ji S, et al. Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. Nature 2011;475:386–389.

[12] Sekiya S, Suzuki A. Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. Nature 2011;475:390–393.

[13] Creyghton MP, Cheng AW, Welstead GG, et al. Histone H3K27ac

separates active from poised enhancers and predicts developmental state. Proc Natl Acad Sci U S A 2010;107:21931–21936.

[14] Kaestner KH. Making the liver what it is: The many targets of the transcriptional regulator HNF4α. Hepatology 2010;51:376–377.

[15] Severgnini M, Sherman J, Sehgal A, et al. A rapid two-step method for isolation of functional primary mouse hepatocytes: Cell characterization and asialoglycoprotein receptor based assay development. Cytotechnology 2012;64:187–195.

[16] Nantasanti S, Spee B, Kruitwagen HS, et al. Disease modeling and gene therapy of copper storage disease in canine hepatic organoids. Stem Cell Reports 2015;5:895–907.

[17] Langmead B, Trapnell C, Pop M, et al. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 2009;10:R25.

[18] Zhang Y, Liu T, Meyer CA, et al. Model-based analysis of ChIP-Seq (MACS). Genome Biol 2008;9:R137.

[19] Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics 2009;25:2078–2079.

[20] Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2010;26:139–140.

[21] Heinz S, Benner C, Spann N, et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol Cell 2010;38:576–589.

[22] Hashimshony T, Wagner F, Sher N, et al. CEL-Seq: Single-cell RNA-Seq by multiplexed linear amplification. Cell Rep 2012;2:666–673.

[23] Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014;15:550.

[24] Steenbeek FG van, Spee B, Penning LC, et al. Altered subcellular localization of heat shock protein 90 is associated with impaired expression of the aryl hydrocarbon receptor pathway in dogs. PLoS One 2013;8:e57973.

[25] Walker JM, Jackson M, Taylor a H, et al. Mouse cell culture. 2010.

[26] Kim JH, Lee SR, Li LH, et al. High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. PLoS One 2011;6:1–8.

[27] Berasain C, Avila MA. Regulation of hepatocyte identity and quiescence.

Cell Mol Life Sci 2015;72:3831–3851.

[28] Mann DA. Human induced pluripotent stem cell-derived hepatocytes for toxicology testing. Expert Opin Drug Metab Toxicol 2014;5255:1–5.

[29] Li X, Zuo X, Jing J, et al. Small-molecule-driven direct reprogramming of mouse fibroblasts into functional neurons. Cell Stem Cell 2015;17:195–203.

[30] 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.

[31] Camp JG, Sekine K, Gerber T, et al. Multilineage communication regulates human liver bud development from pluripotency. Nature 2017;546:533–538.

[32] Visel A, Blow MJ, Li Z, et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. Nature 2009;457:854–858.

[33] Heintzman ND, Hon GC, Hawkins RD, et al. Histone modifications at human enhancers reflect global cell-type-specific gene expression. Nature 2009;459:108–112.

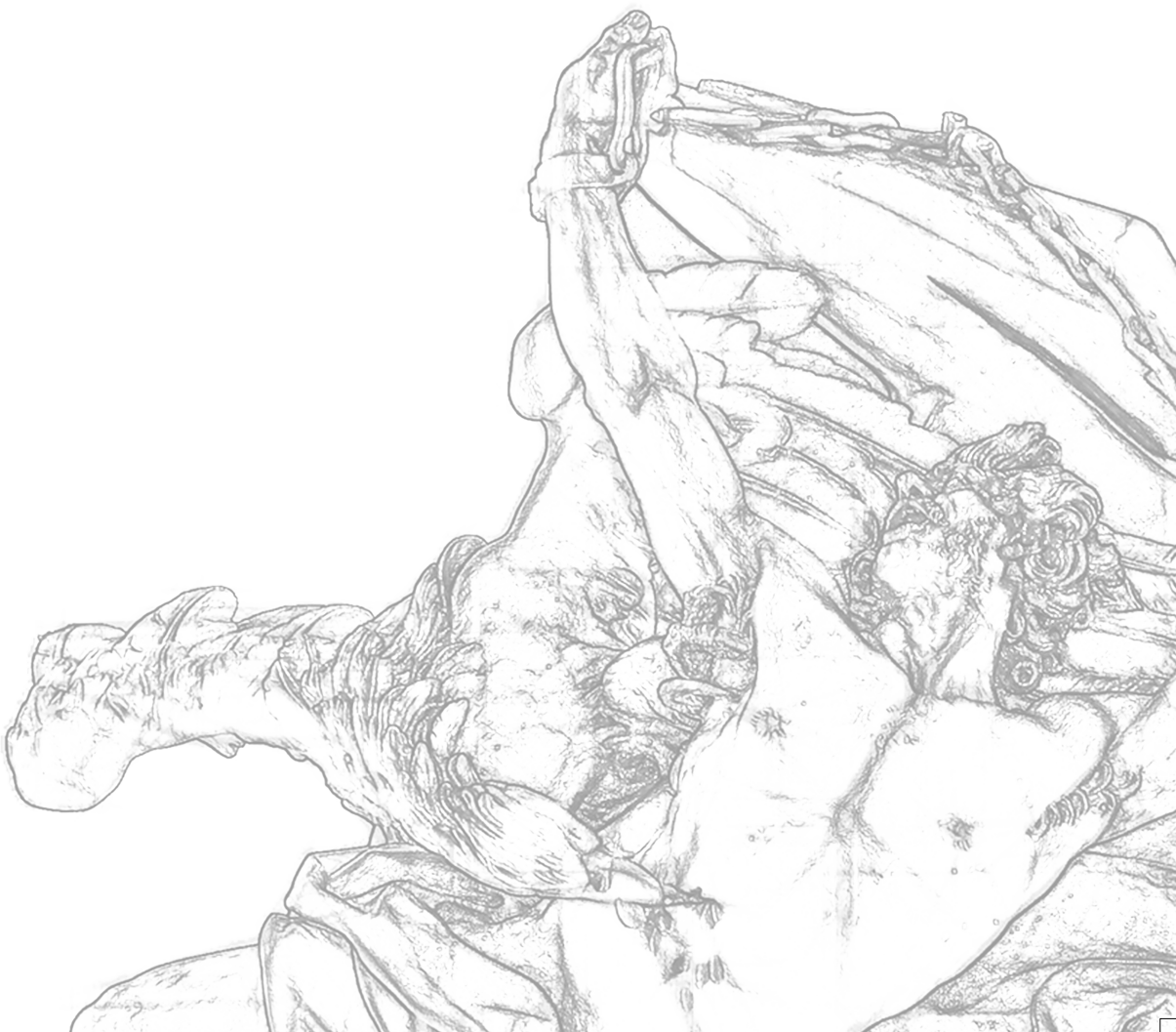
[34] Lomberk G, Blum Y, Nicolle R, et al. Distinct epigenetic landscapes underlie the pathobiology of pancreatic cancer subtypes. Nat Commun 2018;9:1978.

[35] Buenrostro JD, Giresi PG, Zaba LC, et al. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nat Methods 2013;10:1213–1218.

[36] Chen C, Soto-Gutierrez A, Baptista PM, et al. Biotechnology challenges to *in vitro* maturation of hepatic stem cells. Gastroenterology 2018;154:1258–1272.

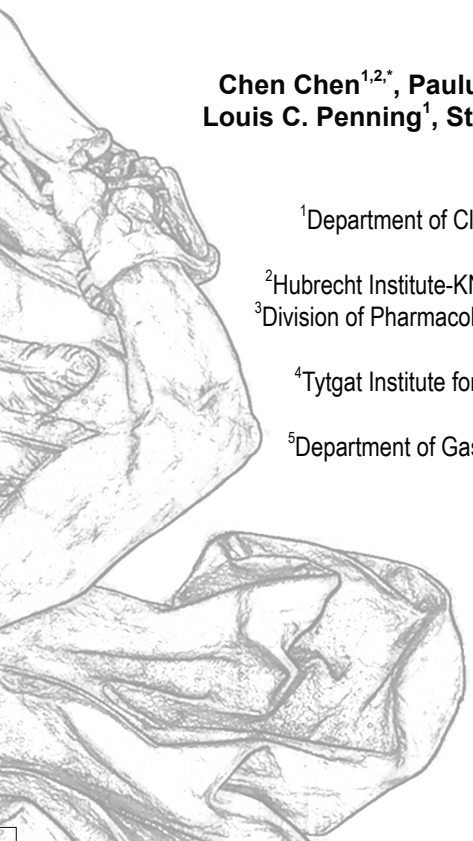
[37] Lim KT, Kim J, Hwang SI, et al. Direct conversion of mouse fibroblasts into cholangiocyte progenitor cells. Stem Cell Reports 2018;10:1522–1536.

[38] 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.



Chapter 4

Bioengineered bile ducts recapitulate key cholangiocyte functions



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Abstract

Investigation of diseases of the bile duct system and identification of potential therapeutic targets are hampered by the lack of tractable *in vitro* systems to model cholangiocyte biology. Here, we show a step-wise method for the differentiation of murine Lgr5^{+} liver stem cells (organoids) into cholangiocyte-like cells (CLCs) using a combination of growth factors and extracellular matrix components. Organoid-derived CLCs display key properties of primary cholangiocytes, such as expressing cholangiocyte markers, forming primary cilia, transporting small molecules and responding to farnesoid X receptor (FXR) agonist. Integration of organoid-derived cholangiocytes with collagen-coated polyethersulfone hollow fiber membranes (HFM) yielded bioengineered bile ducts that morphologically resembled native bile ducts and possessed polarized bile acid transport activity. As such, we present a novel *in vitro* model for studying and therapeutically modulating cholangiocyte function.

Introduction

Cholangiocytes are the epithelial cells lining the intra- and extra-hepatic biliary tree. The primary physiological function of cholangiocytes is to modify bile and transport bile constituents. The biliary excretion route is important for the elimination of waste products, such as excess cholesterol, bilirubin and hormones, as well as exogenous drugs and toxins from the liver. Cholangiocytes, like hepatocytes, can proliferate to restore damaged bile duct epithelia [1]. Impairment of the regenerative capacity of cholangiocytes can lead to a variety of biliary disorders (cholangiopathies), which together, accounted for approximately 16% of all liver transplants performed in the United States between 1988 and 2014 [2]. While most cholangiopathies in the early stage are restricted to the biliary system, their progression often results in liver cirrhosis and eventually liver failure. While it is relatively easy to culture primary cholangiocytes from multiple species, these cultures are heterogeneous and can only be maintained for 3-4 weeks [3], severely limiting their application. Moreover, in order to investigate transepithelial transport, cholangiocytes must be cultured as 3-dimensional ductular structures, however, *in vitro* models that resemble physiological bile duct epithelia are currently lacking.

To our knowledge, a source of sustainable genetically stable stem cells that can be differentiated into functional cholangiocytes has not been reported. Induced pluripotent stem cells (iPSCs) have been used to generate cholangiocytes *in vitro*, but the generating iPSCs remains low efficient, time-consuming and, in most cases, cells are genetically compromised [4]. In recent years, many internal organs, including the stomach, intestine, liver and pancreas, have been shown to contain adult stem cells that can generate into differentiated cell types of the respective organ. In the liver, the adult stem cells are marked by leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5 is a receptor for the potent Wnt agonists, R-spondins). $Lgr5^{+}$ cells can be enriched from small pieces of liver tissue and coerced to form organoids, 3-dimensional structures recapitulating organ biology, by creating conditions that mimic the stem cell niche during physiological tissue self-renewal or damage repair [5]. Liver organoids are genetically stable and can be cultured for a prolonged time; both human and murine organoid cultures are able to differentiate towards hepatocytes [6,7]. Despite this, the use of organoids as a model in cholangiocyte research is limited since key features of mature cholangiocytes, such as bile salt transporter activity, are lost [8].

Here, we report a two-step method to generate cholangiocyte-like cells (CLCs) *in vitro* from murine liver organoids. These organoid-derived CLCs are genetically stable and display essential morphological and functional features of primary cholangiocytes. Furthermore, application of CLCs onto polyethersulfone (PES) hollow fiber membranes (HFM) generate 3-dimensional ductal structures that resemble native bile ducts at the structural and functional level. These bioengineered bile ducts offer novel opportunities for studying cholangiocyte biology, modeling cholangiopathies and identification and validation of new therapeutic targets to treat these diseases.

Material and Methods

Animals

Surplus liver and gallbladder samples were obtained postmortem from 8 mice that were used in non-liver-related research (experiments approved by the Utrecht University's ethical committee); no animals were harmed or killed for this study.

Culture of murine liver organoids

Murine liver organoids were isolated and cultured as previously described [6]. Briefly, isolated ducts were mixed with Matrigel (BD Biosciences) and seeded. After gelatinization of the Matrigel, culture medium was added. The culture medium (organoids expansion medium, OEM) was based on AdvDMEM/F12 (Invitrogen) supplemented with 1% v/v B27 (Invitrogen), 1% v/v N2 (Invitrogen), 1.25 mM n-acetylcysteine (Sigma-Aldrich), 10 nM gastrin (Sigma-Aldrich), 50 ng/ml mouse EGF (Invitrogen), 5% v/v R-spondin-1-conditioned medium (the Rspo1-Fc-expressing cell line was a kind gift from Calvin J. Kuo), 100 ng/ml FGF10 (PeproTech), 10 mM nicotinamide (Sigma-Aldrich), 50 ng/ml HGF (PeproTech) and 1% v/v Penicillin/Streptomycin (Invitrogen). Organoids were split by removal from Matrigel using cold AdvDMEM/F12, mechanical dissociation into smaller fragments, and transfer into fresh Matrigel. Passage was performed weekly at a 1:4–1:8 split ratio. The medium was changed every other day.

Differentiation of murine liver organoids to CLCs

Organoids were split by removal from Matrigel using cold DMEM/F12, mechanical dissociation into smaller fragments, and transferred into mixture of Matrigel and

1.2 mg/ml rat-tail type I collagen at the ratio of 2:3. After two hours of incubation at 37°C and, when the gel mixture was solidified, CEM was added. CEM was refreshed every second day. CEM was based on DMEM/F12 supplemented with 10% v/v Fetal Bovine Serum (FBS), 1% v/v GlutaMAX (Invitrogen), 1% v/v Non-essential amino acids (Invitrogen), 50 ng/ml EGF, 50 ng/ml HGF, 0.1 μM dexamethasone and 1% Penicillin/Streptomycin. After 6 days of CEM culture, the medium was changed to CDM. CDM was based on CEM supplemented with 1.25 mM n-acetylcysteine and 10 nM gastrin. In this medium, cells were cultured for one week. CDM was refreshed every other day.

RNA isolation, cDNA synthesis and RT-qPCR

RNA was isolated from liver organoids, organoid-derived hepatocyte-like cells, M/C- and HFM-cultured CLCs using RNeasy lysis buffer directly added into the plate followed by RNA extraction according to the manufacturer's instructions (Qiagen). cDNA was obtained using the iScript™ cDNA synthesis kit as described by the manufacturer (Bio-Rad, Veenendaal, the Netherlands). A mix of random hexamers and oligo-dT primers were used. Relative gene expression of the selected genes was measured using RT-qPCR. Primer design, validation, RT-qPCR conditions, and data analysis was performed as previously described [9]. Normalization was performed using the reference genes Hypoxanthine Phosphoribosyltransferase (*Hprt*) and Hydroxymethylbilane Synthase (*Hmbs*). Details of all primers are listed in Table S1.

Cholangiocyte functional studies

For the Rhodamine123 (Rh123) transport test, cold DMEM/F12 was used to remove Matrigel and collagen from CLCs. CLCs were pretreated with DMSO or 10 μM Verapamil (Sigma-Aldrich) for 30 minutes, followed by 5 minutes of incubation with 100 μM Rh123 (Sigma-Aldrich). Then CLCs were washed 3 times with CDM. Fluorescence (excitation wavelength: 511 nm; emission wavelength: 534 nm) was visualized by Leica SPE-II confocal system after 30 minutes.

For the FXR activity test, CLCs were incubated in CDM supplemented with 10 μM GW4064 (Sigma-Aldrich) for 48 hours and RNA was isolated to determine the expression of downstream signaling of FXR target genes.

Culture of CLCs on double coated hollow fiber membranes

Murine liver organoids were removed from Matrigel using cold AdvDMEM/F12, trypsinized into single cells and small fragments and seeded in culture flasks pre-coated with rat-tail type I collagen. Cells were cultured with CEM. When cells reached confluence, they were trypsinized into single cells and seeded on hollow fiber membrane at a concentration of $1x10^6$ cells/4.5 cm fiber. The polyethersulfone HFM (SENUO Filtration Technology Co, Ltd; inner diameter: 550 ± 150 μm; outer diameter: 950 ± 350 μm; pore size: 0.2 μm) were prepared using a double coating procedure previously described [10]. Briefly, sterilized fibers were horizontally placed in 1.5 ml Eppendorf tubes and incubated with L-DOPA solution (2 mg/ml L-3,4-dihydroxyphenylalanine, 10 mM Tris buffer, pH 8.5) at 37 °C for 4 hours, during which fibers were turned 90° every hour. Similarly, the L-DOPA coated fibers were exposed to the rat-tail type I collagen solution (25 μg/ml) at 37 °C for 2 hours, during which fibers were turned 90° every 30 minutes. The collagen solution was then aspirated and the fibers were washed thoroughly in PBS prior to cell seeding. HFM-cultured cells were placed in 6-well plates and cultured in CEM for 2 weeks for expansion and CDM for 1 week for maturation.

Immunofluorescence analysis

Liver organoids, organoid-derived hepatocyte-like cells, M/C- and HFM-cultured CLCs were fixed in 4% PFA for 30 minutes and permeabilized with PBS 0.3% Triton X-100 for 30 minutes. Primary antibodies were incubated overnight. Secondary antibodies were incubated at room temperature for two hours. Tissues were then processed in a click reaction with 5 μM Alexa Fluor® 488 or 568 (Life Technologies) according to manufacturer's instructions. Nuclei were stained with DAPI (Sigma-Aldrich). Tissues were mounted on slides with ProLong® Diamond Antifade Mounting Medium (Invitrogen). Images were acquired using Leica SPE-II confocal system. Antibody details for each protein are shown in Table S2.

Trans-epithelial barrier function

Trans-epithelial barrier function was determined as previously described [11]. Briefly, HFM with or without CLCs were connected to a separated in- and outlet glass cannula assembled in a custom-made 3D printed cytocompatible polyester device. HFM were perfused (6 ml/h) with inulin-fluorescein isothiocyanate (FITC)

(0.1 mg/ml in Krebs-Henseleit buffer) for 10 min at room temperature. 100 µl sample was collected from the outer HFM compartment and its fluorescent strength was measured by a fluorometer (Labsystems).

Bile acid transport assay

HFM with CLCs were connected to a separated in- and outlet glass cannula (inner diameter 120-150 µm; DMT Trading, Aarhus, Denmark) assembled in a custom-made 3D printed cytocompatible polyester device [11] (Fig. S5) to enable a separated basolateral (inner HFM, perfusion channel) and apical compartment (outer HFM). The inlet cannula was connected to a tubing system and syringe pump, whereas the outlet cannula was connected to a tubing system and a depot to collect perfusate. During the experiment, 300 µl CDM with [^{14}C]inulin and [^{3}H] taurocholic acid (PerkinElmer) was added to the outer HFM compartment. In the inhibition group, 70 µM glycochenodeoxycholic acids were applied to the outer HFM compartment. Hank's balanced salt solution was perfused (2 ml/h) through the inner HFM for 2 hours at 37°C. The perfusate was collected at multiple time points. For each time point, 100 µl perfusate was added into 4.9 ml scintillation fluid. Radioactivity was measured by a liquid scintillation counter (Beckman Coulter). Each count was normalized to the surface area of its corresponding HFM.

Statistical analysis

Statistical analysis and graphs were performed using GraphPad Prism 7.0 (GraphPad Software Inc., USA). Data are present as box and whisker plots (minimum to maximum, Fig. 1C and 2B) and the mean ± standard error of the mean (Fig. 3C and 4C). See figure legends for details on specific statistical tests run and p values calculated for each experiment.

Data availability

All data supporting the finding of this study are included within the paper and its supplementary information.

Results

In vitro generation of cholangiocyte-like cells from murine liver organoids

We based our strategy for the generation of cholangiocytes from liver organoid cultures on established protocols for culture of human primary cholangiocytes [12] and differentiation of hepatic progenitor cells into cholangiocytes [13] (Fig. 1A). Murine liver organoids were expanded from the liver ductal compartment under previously described culture conditions [6]. Subsequently, the organoids were mechanically dissociated into small clusters and suspended in droplets of matrix, consisting of Matrigel and collagen type I. They were maintained in medium designated for biliary lineage differentiation of liver progenitor cells [12]. Under these conditions, Ki67 staining demonstrated that cells preserved their proliferation capacity (Fig. S1A); therefore, we named this medium cholangiocyte expansion medium (CEM). After 4-6 days in culture, the clusters of cells (hereafter called cholangiocyte progenitors, CPs) grew in size and formed cystic and tubular structures (Fig. 1B). Gene expression analysis (Fig. 1C) demonstrated that CPs displayed increased expression of the following cholangiocyte markers: keratin 7 (*Krt7*), keratin 19 (*Krt19*), HNF1 homeobox B (*Hnf1b*), solute carrier family 10, member 2 (*Slc10a2*, also known as *Asbt*) and solute carrier family 4, member 2 (*Slc4a2*, also known as *Ae2*). Other cholangiocyte markers, ATP-binding cassette, sub-family B, member 1B (*Abcb1b*, also known as *Mdr1b*), polycystic kidney disease 2 (*Pkd2*) and cystic fibrosis transmembrane conductance regulator (*Cftr*), remained unchanged in CPs and were also expressed in normally expanding liver organoids (OEM). The hepatocyte markers albumin (*Alb*) and cytochrome P450 family 3 subfamily A member 11 (*Cyp3a11*) were expressed in CPs but at significantly lower levels compared to organoid-derived hepatocyte-like cells. In addition, although CPs remained proliferative, the multipotency marker leucine-rich-repeat-containing G-protein-coupled receptor (*Lgr5*) was no longer detectable (Fig. 1C). Taken together, this indicated that the liver organoids differentiated towards the biliary lineage, but still exhibited some immature features.

To promote further maturation of the CPs, we tested small molecule compounds and growth factors including n-acetylcysteine (NAC), gastrin (GAS), taurocholic acid (TCA), fibroblast growth factor 10 (FGF10), and transforming growth factor beta (TGF-β), which have been reported to be directly or indirectly related to biliary functions [14-17]. Of the cholangiocyte markers with low expression in CEM

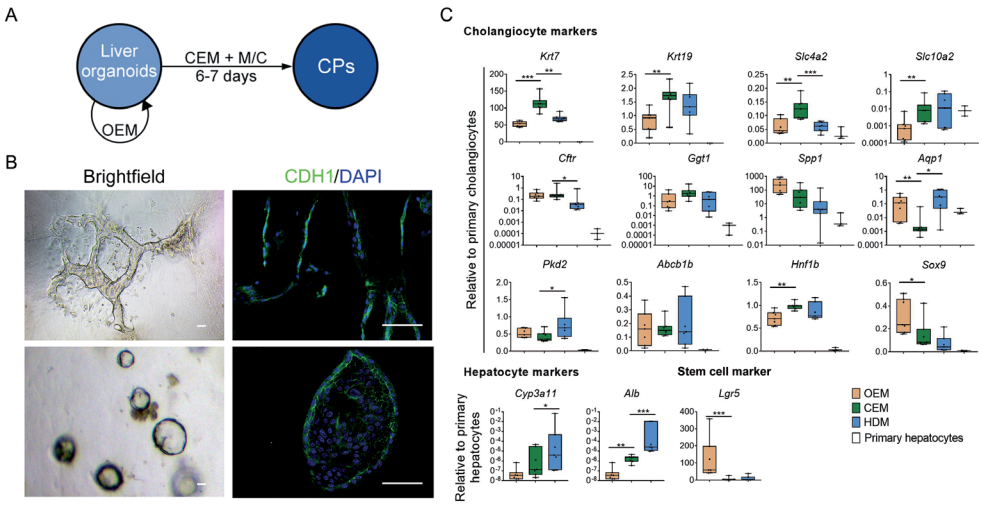


Figure 1. Differentiation of murine liver organoids towards cholangiocyte progenitors (CPs). (A) Schematic overview of the protocol for differentiation of murine liver organoids towards CPs. OEM, organoid expansion medium; CEM, cholangiocyte expansion medium; M/C, Matrigel/collagen type I mixture; CPs, cholangiocyte progenitors. (B) Tubular and cystic structures of CPs formed in CEM condition. Left, brightfield microscopy images; right, immunofluorescent staining of cadherin 1 (CDH1, also known as E-cadherin) in 3D structures formed by CPs. Nuclear staining with DAPI, 4',6-diamidino-2-phenylindole dihydrochloride for all conditions. Scale bar=120 µm. (C) Gene expression analysis for cells under OEM, CEM and HDM conditions. n=7 independent samples for OEM, CEM and HDM condition, n=3 independent samples for primary hepatocytes. Results are shown as fold change relative to mouse primary cholangiocytes for cholangiocyte markers and to mouse primary hepatocytes for hepatocyte and stem cell markers. Data are shown as box and whisker plots. Center line, median; box, interquartile range (IQR); whiskers, minimum to maximum. Asterisks represent statistical significance of differences between OEM/CEM and CEM/HDM conditions. *p<0.05, **p<0.01, ***p<0.001 (two-tailed Mann–Whitney U test). HDM, hepatocyte differentiation medium.

condition, NAC induced aquaporin 1 (*Aqp1*), osteopontin (*Spp1*) and G protein-coupled bile acid receptor 1 (*Gpbar1*) expression, while GAS induced *Spp1* expression (Fig. S2). Furthermore, as gene expression analysis showed, the combination of NAC and GAS enhanced the expression of cholangiocyte-enriched transcription factors SRY-box 9 (*Sox9*), one cut domain, family member 1 (*Onecut1*) and one cut domain, family member 2 (*Onecut2*), as well as cholangiocyte markers *Aqp1*, *Gpbar1* and *Spp1* in CPs (Fig. 2B and S3). Interestingly, media supplementation with both NAC and GAS reduced the proliferation of CPs as indicated by the quantification of RNA content (Fig. S4) and Ki67 staining (Fig. S1B). We therefore used this media composition as cholangiocyte differentiation medium (CDM) to drive the CPs towards a more mature cholangiocyte-like cell

(CLC) phenotype. Immunofluorescence analysis (Fig. 2C-H) indicated that epithelial markers, tight junction protein 1 (TJP1, also known as ZO-1) and cadherin 1 (CDH1, also known as E-cadherin), as well as cholangiocyte markers, HNF1B, KRT7, KRT19, AQP1 and gamma-glutamyl transferase 1 (GGT1) were present in CLCs. In contrast, hepatocyte markers, cytochrome P450 family 1 subfamily A member 2 (CYP1A2) and ALB were absent (Fig. 2G-H). More importantly, when we stained with acetylated-α-tubulin, we observed that CLCs acquired cilia (Fig. 2I), organelles present in mature cholangiocytes only [18].

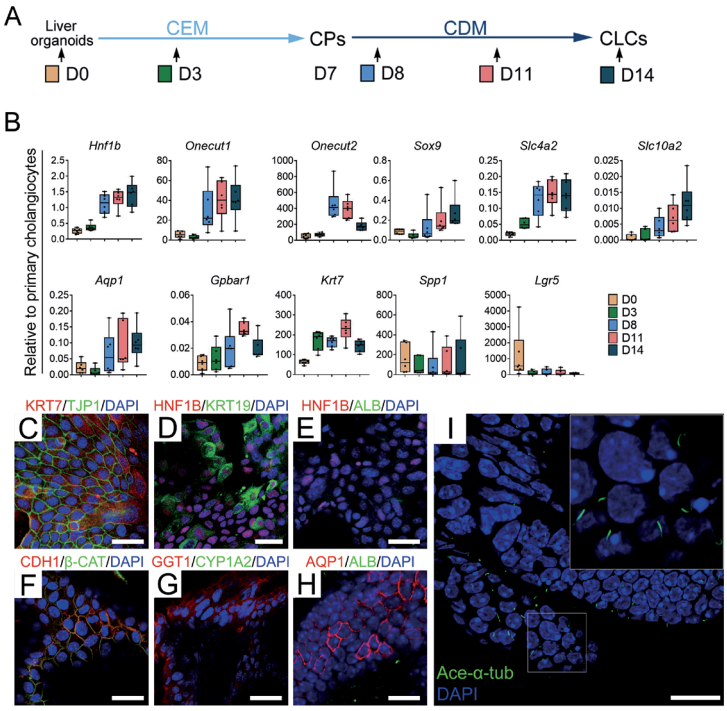


Figure 2. Differentiation of cholangiocyte progenitors (CPs) towards cholangiocyte-like cells (CLCs). (A) Liver organoids were cultured in CEM from D0 to D6, and in CDM from D7 to D14. Samples were collected on D0, D3, D8, D11 and D14. (B) Gene expression analysis during the two-step differentiation towards CLCs. n=6 independent samples for each time point. Results are shown as fold change relative to mouse primary cholangiocytes. Data are shown as box and whisker plots. Center line, median; box, interquartile range (IQR); whiskers, minimum to maximum. (C-H) Immunofluorescence analysis demonstrating the expression of key cholangiocyte markers (keratin (KRT) 7 and 19, HNF1 homeobox B (HNF1B), gamma-glutamyl transferase 1 (GGT1), aquaporin 1 (AQP1)), and epithelial markers (cadherin1 (CDH1) and tight junction protein 1 (TJP1)) and the absence of hepatocyte markers (cytochrome P450 family 1 subfamily A member 2 (CYP1A2) and albumin (ALB)). (I) CLCs acquired cilia as indicated by staining for acetylated-α-tubulin (Ace-α-tub). Nuclear staining with DAPI, 4',6-diamidino-2-phenylindole dihydrochloride for all conditions. Scale bar=20 μm.

Functional characterization of CLCs

Next, we investigated whether the *in vitro*-generated CLCs functionally resembled primary cholangiocytes. In the liver, cholangiocytes are responsible for the reabsorption of bile acids and secretion of small molecules by a series of transmembrane channel proteins including cystic fibrosis transmembrane conductance regulator (CFTR), apical sodium-dependent bile acid transporter (ASBT, encoded by *Slc10a2*), aquaporin 1 (AQP1), multidrug resistance protein 1B (MDR1B, encoded by Abcb1b), and others. To determine the secretory activity of CLCs, we incubated CLCs with Rhodamine 123 (Rh123), a fluorescent chemical compound that can be transported by MDR1B [19]. After incubation, we observed fluorescence accumulation inside both the lumen of the cysts and tubes formed by CLCs (Fig. 3A). This accumulation was blocked when CLCs were treated with the competitive MDR1B inhibitor, Verapamil (Fig. 3B), which confirmed the MDR1B-dependent transport of Rh123.

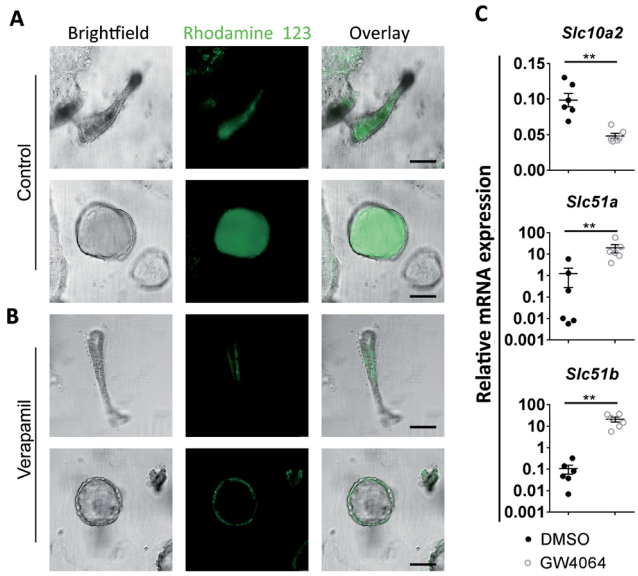
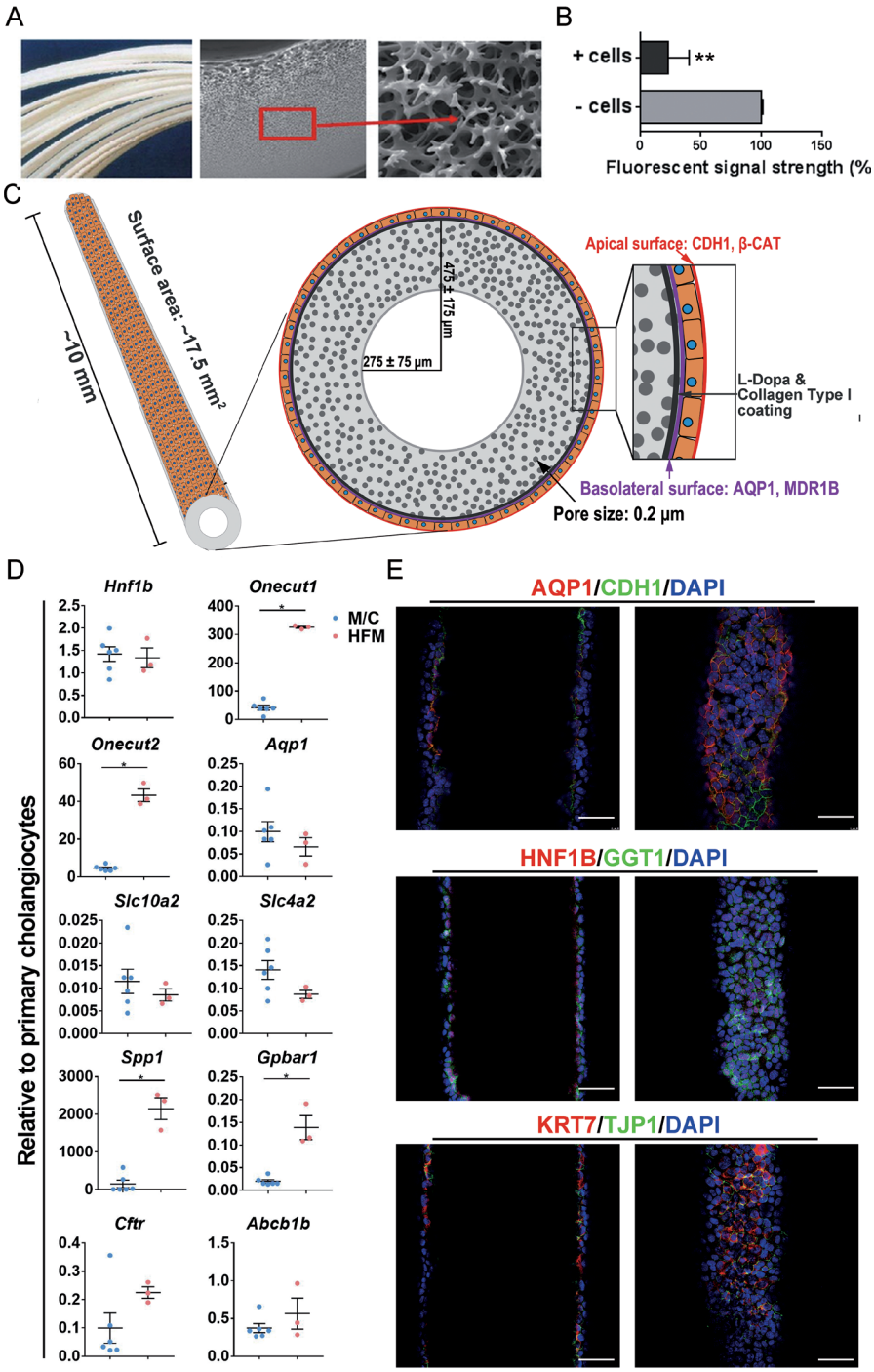


Figure 3. Functional characterization of cholangiocyte-like cells (CLCs). (A-B) Brightfield and confocal microscopic images showing MDR1B-dependent transport of fluorescent substrate Rh123 into the lumen of a tube and cyst. CLCs were pretreated with DMSO (A) and Verapamil (B) for 30 minutes. Scale bars = 75 μm. (C) Gene expression analysis showing the expression of FXR signaling downstream target genes, Slc10a2, Slc51a and Slc51b in FXR agonist (GW4064) treated group versus control (DMSO). n=6 independent samples for each group. Data are shown as mean ± SEM of six independent experiments for each group. Asterisks represent statistical significance of differences between DMSO and GW4064 treated groups. **p<0.01 (two-tailed Mann–Whitney U test).

Several studies have demonstrated the effects of farnesoid X receptor (FXR) signaling in bile acid homeostasis in the liver [20-22]. In cholangiocytes, the activation of FXR signaling leads to the excretion of bile acids, while its inactivation causes intracellular storage of bile acids [23]. To determine this physiological balance in CLCs, we treated them with GW4064, a potent FXR agonist [24]. The expression of *Slc10a2*, which encodes a transporter enhancing the apical surface uptake of bile acids in cholangiocytes, was down-regulated (Fig. 3C). In addition, the expression of solute carrier family 51, alpha and beta subunit (*Slc51a* and *Slc51b*; also known as *Ostα* and *Ostβ*), which together form a single transporter responsible for basolateral excretion of bile acids, were significantly up-regulated (Fig. 3C). Together, these results confirm that the organoid-derived CLCs possess key functionalities of primary cholangiocytes and display an adequate response to external stimuli.

HFM-cultured CLCs forming intact tube-like structures

In vivo, cholangiocytes form a monolayer that lines the lumen of the bile ducts. To investigate whether the organoid-derived CLCs could constitute advanced structures similar to native bile ducts, we used polyethersulfone (PES) hollow fiber membranes (Fig. 4A and C) as the scaffold for culturing CLCs. After two weeks of expansion, HFM-cultured CLCs formed an epithelial barrier, as confirmed by limited inulin-FITC diffusion and the dense arrangement of cells over the HFM (Fig. 4B and E). Following another week of CDM culture, gene expression analysis revealed that HFM-cultured CLCs maintained stable expression levels for most cholangiocyte markers (*Hnf1b, Aqp1, Slc10a2, Slc4a2, Cftr,* and *Abcb1b*) compared to CLCs cultured in Matrigel/collagen gel (M/C) alone. Interestingly, cholangiocyte-enriched transcription factors, *Onecut1* and *Onecut2*, and cholangiocyte markers, *Gpbar1* and *Spp1*, were significantly up-regulated in the HFM culture (Fig. 4D). Immunofluorescence analysis also confirmed that HFM-cultured CLCs maintained the expression of cholangiocyte markers (HNF1B, AQP1, GGT1 and KRT7) and epithelial markers (CDH1 and TJP1) (Fig. 4E).



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Functional characterization of HFM-cultured CLCs

To investigate whether the HFM-cultured CLCs possessed transport activities comparable to native bile ducts, we first determined their cell polarity. Immunofluorescence analysis showed that HFM-cultured CLCs acquired apical-basal polarity. More specifically, the outside of the cell reflected the apical surface (by localization of apical markers, AQP1 and MDR1B), and the inside of the cell reflected the basolateral surface (by localization of the basolateral marker, catenin beta-1 (CTNNB1, also known as β-catenin)); lateral markers (CDH1 and TJP1) were localized in the middle (Fig. 5A and B). In addition, we observed cilia on the outer surface of HFM-cultured CLCs, as indicated by acetylated-α-tubulin staining (Fig. 5C and D). This strategy facilitates access to the apical compartment which can assist transport studies, and also complements M/C culture conditions, where only the basolateral cell surface is accessible.

To measure the transport activity of HFM-cultured CLCs for bile acids, we mounted the HFM into 3D-printed bioreactors (Fig. S5) which had been tested to be non-cytotoxic [11]. *In vivo*, cholangiocytes transport bile acids from the apical surface surrounding the bile duct lumen to the basal surface facing hepatocytes. To study the reabsorption of bile acids from the apical to the basolateral compartment, we applied [^{3}H]taurocholic acid in the bioreactor outside of the HFM-cultured CLCs and determined the radioactivity in the perfusate. As a control for passive diffusion through the epithelium, we measured [^{14}C]inulin, which is not actively transported over cells. We showed that TCA transport increased rapidly in the first 10 minutes,

Figure 4. Cholangiocyte-like cells (CLCs) cultured on hollow fiber membranes (HFM). (A) HFM. Middle and right, scanning electron microscopy images, pore diameter = 0.2 μm. (B) The trans-epithelial barrier function was assessed in the presence or absence of CLCs by inulin-FITC perfusion and the flux across the HFM was quantified. Data are shown as mean ± SEM of three independent experiments. Asterisks represent statistical significance of differences between the HFM covered with and without CLCs. **$p < 0.01$ (two-tailed Mann–Whitney U test). (C) Schematic of HFM and HFM-cultured CLCs. (D) Gene expression analysis demonstrating HFM-cultured CLCs maintain cholangiocyte features. Data are shown as mean ± SEM of six independent experiments for the M/C group and three independent experiments for HFM group. Asterisks represent statistical significance of differences between HFM and M/C culture. *$p<0.05$ (Mann–Whitney U test). (E) Immunofluorescence analysis demonstrating the expression of key cholangiocyte markers (keratin 7 (KRT7), HNF1 homeobox B (HNF1B), and epithelial markers (cadherin 1 (CDH1), tight junction protein 1 (TJP1)). The left and right confocal microscopy images were acquired at different z stacks. Nuclear staining with DAPI, 4',6-diamidino-2-phenylindole dihydrochloride for all conditions. Scale bar = 80 μm.

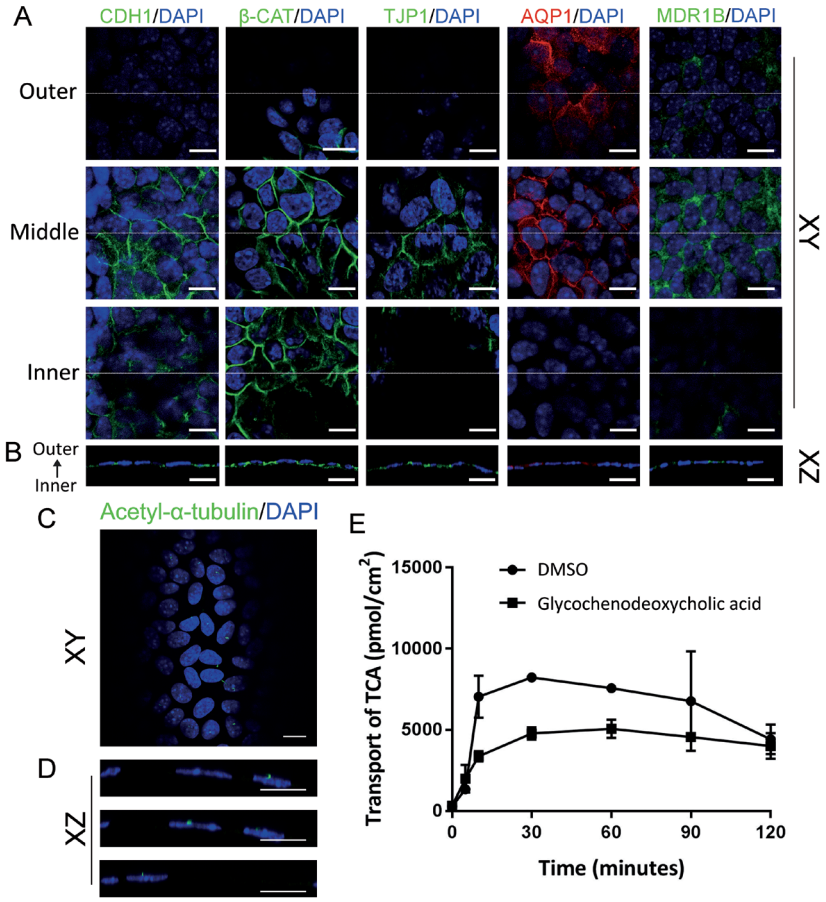

Figure 5. Characterization of HFM-cultured CLCs. (A-B) Immunofluorescence analysis demonstrating polarity of HFM-cultured CLCs by the expression of β-catenin on lateral and basolateral membrane, CDH1 and TJP1 on lateral membrane, AQP1 and MDR1B on apical and lateral membrane. XY Confocal sections (A) were acquired at the top, middle and bottom of the cells in the center. XZ Confocal sections (B) were acquired for the monolayer of CLCs on HFM. Scale bar = 10 μm. (C-D) Immunofluorescence analysis demonstrating the presence of cilium marker acetylated-α-tubulin in HFM-cultured CLCs. Confocal microscopy images were acquired at XY section (C) and XZ sections (D). Nuclear staining with DAPI, 4',6-diamidino-2-phenylindole dihydrochloride for all conditions. Scale bar = 10 μm. (E) Transport activity assays showing that HFM-cultured CLCs could transport TCA into HFM and this transport could be inhibited by 70 μM glycochenodeoxycholic acid. Each point represents means ± SEM of two independent HFM perfused with HBSS which were collected for 0, 30, 60, 90 and 120 minutes of perfusion. [^{3}H] activity (dpm) was converted to number of molecules (pmol) and normalized to the surface area of the corresponding fibers.

then remained relatively stable (Fig. 5E), while inulin diffusion remained at low levels at all time points (Fig. S6). To further confirm the active transport of TCA, we exposed CLCs to glycochenodeoxycholic acid, which has been shown to inhibit TCA uptake [25]. As Fig. 5E shows, TCA transport was reduced in HFM-cultured CLCs upon treatment with glycochenodeoxycholic acid. Furthermore, we observed accumulation of TCA in carrier-treated HFM-cultured CLCs compared to the glycochenodeoxycholic acid treated CLCs (Fig. S7). Taken together, these results indicate that HFM-cultured CLCs acquired cell polarity and bile acid transport activity, which is the key function of native bile ducts.

Discussion

Current research and applications of Lgr5$^+$ adult liver stem cells, or liver organoids have focused exclusively on their hepatocytic differentiation potential and related hepatic functions [26-28]. This study describes, for the first time, the potential of differentiating liver organoids into mature cholangiocytes. Our results demonstrate that liver organoids are truly bipotent adult stem cells as they can be differentiated towards hepatocytes [6] (Fig. S8) as well as cholangiocytes.

Functional somatic cells derived from individuals are optimal tools for personalized medicine and disease modeling. This is a valuable advantage over established cell lines, which cannot reflect genetic and potentially epigenetic variation of a broad population. Previous studies have successfully generated cholangiocytes from human induced pluripotent stem cells (iPSCs) [29-31], however, generating iPSCs remains low efficient, time-consuming and cells are genetically compromised. Hepatoblasts (the liver stem cells appearing during liver development) have also been proposed for generating cholangiocytes, but this source is difficult to access and important functional tests were lacking in the previous study [10]. Here, we present an alternative approach based on liver organoids. Organoids possess strong *in vitro* proliferation capacity which is mediated by the activation of Wnt signaling by R-spondins [6,27]. To our surprise, when organoids were exposed to CEM, which is free of Wnt activators, cholangiocyte progenitors continued proliferating for at least four passages.

To drive further maturation of CPs after CEM conditions, we tested a set of small molecule compounds and growth factors and found that a combination of gastrin and n-acetylcysteine optimally drives CP maturation toward cholangiocyte-like

cells. TGF-β has been reported to be one of the key factors controlling liver stem cells' differentiation and tubulogenesis [14,17], however, very small amounts of TGF-β (1 ng/ml) in the medium was fatal for organoids; we therefore excluded it in further studies. It is worth noting that the Growth Factor Reduced Matrigel® used in this study contains approximately 1.7 ng/ml TGF-β, which may be sufficient for morphogenesis of cholangiocytes. In the liver, cholangiocytes are exposed to bile. Although it has been demonstrated that taurocholic acid, one of the predominant bile acids in human, mice and rats, can induce the differentiation of primary cholangiocytes [16], it did not have an obvious differentiating effect on our CPs (Fig. S2).

Previous studies showed that gastrin, n-acetylcysteine and FGF10, necessary components in the organoid expansion medium, are also related to cholangiocyte proliferation and function [15,32,33]. When CEM was supplemented with gastrin and n-acetylcysteine, the expression of *Aqp1* was restored and *Gpbar1* was induced, indicating further maturation of CPs. This may be a consequence of up-regulated expression of cholangiocyte-enriched transcription factors such as *Onecut1, Onecut2* and *Sox9*, which together with *Hnf1b* play central roles on the regulatory network of biliary development [17,33,34].

In vivo, cholangiocytes form bile ducts which possess two-way transport activity, for instance, basolateral to apical transport mediated by MDR1 for broad substrate specificity (Rhodamine 123 in our study), and apical to basolateral transport mediated by ASBT or SLC10A2 for bile acids. However, in the Matrigel/collagen type I culture system, the apical membrane of CLCs is inaccessible, which makes the testing of apical-to-basolateral transport technically challenging. Therefore, we introduced the polyethersulfone hollow fiber membranes (HFM) into the culturing system. The selection of HFM was made because of its hemocompatible and biocompatible properties, allowing future clinical applications. Although this membrane was designed for low cell adhesion, the membranes appeared well suitable for bioartificial kidney tubules development after coating the membranes with extracellular matrix components. In previous research [10], we biofunctionalized the membrane using a self-polymerizing 3,4-dihydroxy-L-phenylalanine (L-DOPA) which can covalently bind collagen IV [35,36], an endogenous component in kidney basal lamina that promotes cell differentiation towards epithelial lineages [37,38]. This coating allowed selective active transport by the renal epithelial barrier and avoided loss of vital blood components when potentially used as renal replacement therapy [10,11]. By growing cells on the

outside of the membrane and applying it in a well-designed perfusion chamber compatible with microscopy, the bioengineered bile ducts can be monitored by confocal imaging allowing functional assessment [11].

In this study, using HFM coated with L-DOPA and collagen I, CLCs organized into polarized epithelial monolayers, where the apical-basolateral polarity is opposite to native bile ducts. Despite this inverted polarity, bioengineered bile ducts can still have a wide range of applications, since both apical and basolateral membrane are exposed to the external environment. Current *in vitro* models for transport studies are cell lines, such as Caco2 and (transfected) MDCK, cultured in dishes or Transwell® systems. These immortalized cells and static cultures poorly mimic the physiological situation, and could fail to predict transporter mediated changes in drug absorption/distribution [39]. The HFM-cultured CLCs can serve as bioengineered bile ducts representing a novel *in vitro* model for drug development, not only because the cells express a variety of channels and pumps, but also the ability to provide dynamic microenvironment for cells. Specifically, the HFM-cultured CLCs can be a model for intrahepatic bile ducts, since they can transport taurocholic acids, which is the key feature of intrahepatic bile ducts [40]. The opposite polarity of the bioengineered bile ducts may even be beneficial to applications in, for instance, bioartificial liver (BAL) devices. Current BAL devices are perfused bioreactors containing only hepatocytes (or hepatocyte-like cells) [41,42], in which metabolic waste is accumulated overtime. Combining our bioengineered bile ducts with BAL could acquire self-clearance capacity, which would extend the service life of each device, and hence reduce the financial burden (each device needs $3x10^9$ cells) [42].

Our current study demonstrates that murine liver organoids are bipotential and can be differentiated into functional hepatocytes and cholangiocytes. By applying them to hollow fiber membranes, these cholangiocyte-like cells polarized and possessed key cholangiocyte functions. Although there are intrinsic differences between cholangiocytes between species and even locations within the liver [1], the technology developed here may be applied to human liver organoids, and hence contribute to a better understanding of pathogenetic and therapeutic aspects of cholangiopathies.

Acknowledgements

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Author Contribution

C.C., R.M. and B.S. conceived the project and designed the experiments; C.C., P.J., S.G. and L.S. performed the experiments; H.C., K.S. and U.B. provided resources and helped write the manuscript; C.C., L.P., N.G. and B.S. wrote the paper; all authors were involved in data analysis, discussion and approved the manuscript.

Conflicts of Interest

The authors have declared no conflicts of interest.

Supplementary Figures

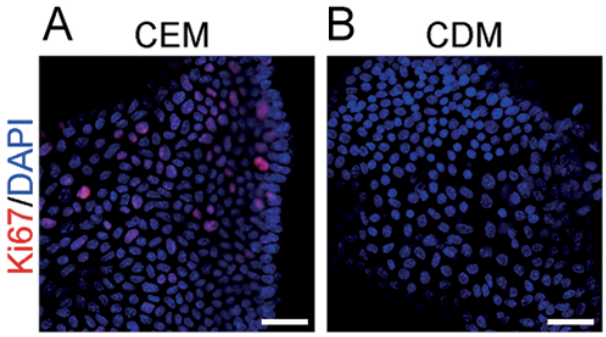


Figure S1. Reduced proliferation of CLCs upon treatment with NAC and GAS. Immunofluorescence analysis demonstrated positive cells in CEM (A) and negative cells in CDM (B) for the proliferation marker Ki67. Nuclear staining with DAPI (4',6-diamidino-2-phenylindole dihydrochloride). Scale bar = 100 µm.

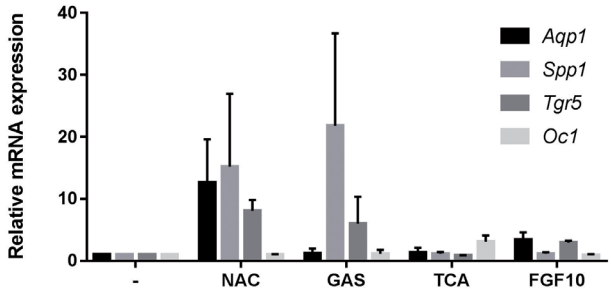


Figure S2. Optimization of cholangiocyte differentiation condition. Differentiation of murine cholangiocyte progenitors was optimized starting with CEM containing mEGF, HGF and dexamethasone. Thereafter, one of the following individual supplements was added to the CEM: 1.25 mM NAC, n-acetylcysteine; 10 nM GAS, gastrin; 10 µM TCA, taurocholic acid; 20 ng/ml FGF10, fibroblast growth factor 10. Experiments were performed on CPs derived from three independent samples.

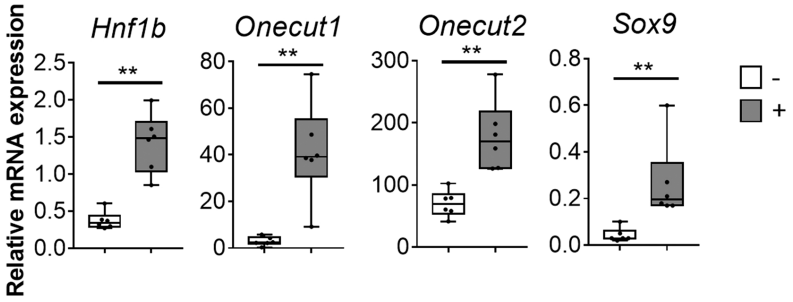


Figure S3. NAC and GAS enhanced the expression of cholangiocyte-enriched transcription factors. n=6 independent samples for each condition. "**-**" represents untreated (CEM); "+" represents NAC and GAS treated (CDM). Data are shown as box and whisker plots. Center line, median; box, interquartile range (IQR); whiskers, minimum to maximum.

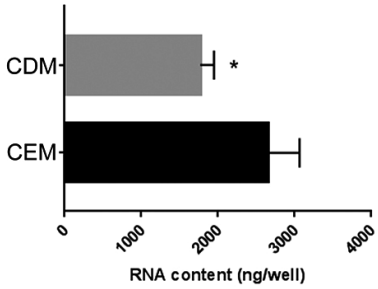


Figure S4. Reduced total RNA content of CLCs upon treatment with NAC and GAS. The same number of cells was cultured in CEM for 6 days, then in CEM or supplemented with NAC and GAS (CDM) for one week. Total RNA was isolated and quantified. The CDM group contained significantly less RNA compared to control. Experiments were performed on CPs derived from three independent donors. *p<0.01 (two tailed Mann–Whitney U test).

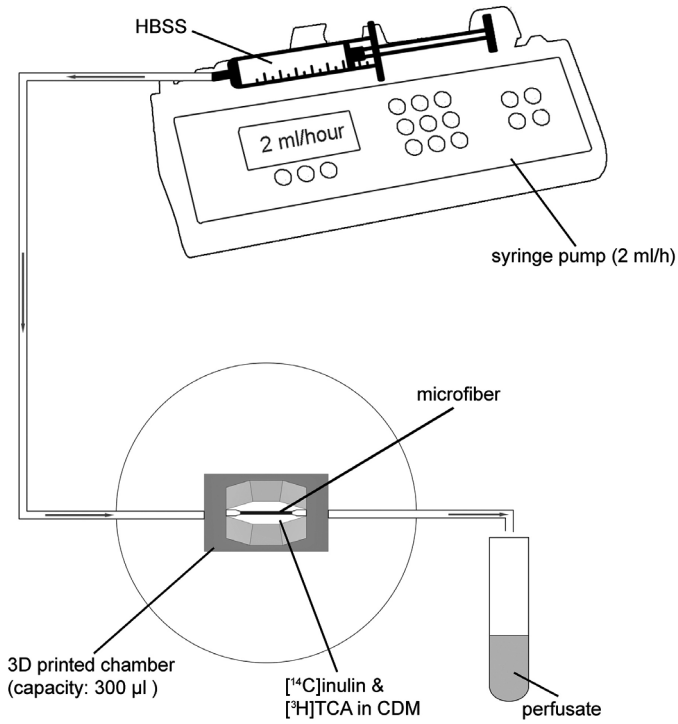


Figure S5. Schematic overview of the setup for the bile acid transport activity assay. The HFM was mounted in a 3D-printed bioreactor. HBSS was perfused by a syringe pump at a flow speed of 2 ml/hour through the HFM. The [^{14}C] and [^{3}H] signal in the perfusate was quantified.

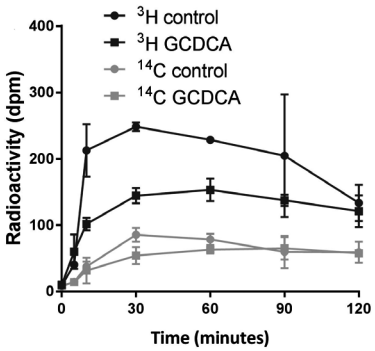


Figure S6. Transport activity assays showing that HFM-cultured CLCs could transport TCA, not inulin. Each point represents means ± SEM of two independent HFM perfused with HBSS which were collected for 0, 30, 60, 90 and 120 minutes of perfusion.

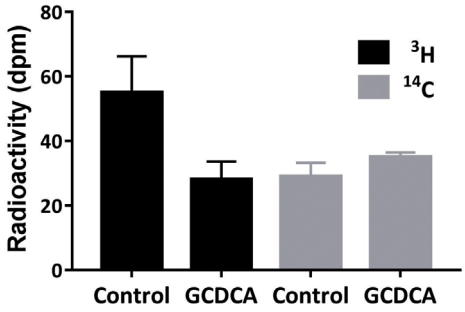


Figure S7. Active uptake of TCA by HFM-cultured CLCs. HFM-cultured CLCs were incubated with or without 70 μM glycochenodeoxycholic acid (GCDCA and Control, respectively), [^{3}H]TCA, [^{14}C]inulin, and perfused with HBSS for two hours. Cells were lysed with 2N NaOH. [^{3}H] and [^{14}C] radioactivity of the lysate was counted. Each bar represents the mean ± SEM of two independent measurements.

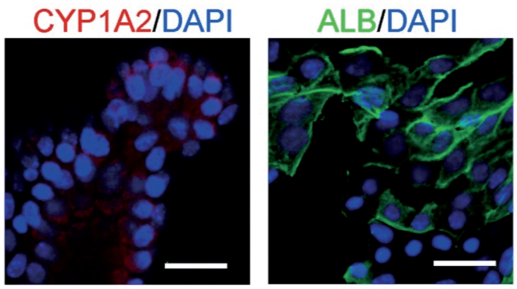


Figure S8. Differentiation of liver organoids towards hepatocyte lineage. Immunofluorescence analysis demonstrated liver organoids in HDM were positive for hepatocyte markers, CYP1A2 and ALB. Scale bar = 40 μm.

Supplementary Tables

Supplementary Table 1. List of Primers used in RT-qPCR analysis

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
Alb	GCAACACAAAGATGACAACC	CTTCATGCAAATAGTGTCCCA
Hnf1b	ACAATCCCCAGCAATCTCAGAA	GCTGCTAGCCACACTGTTAATGA
Onecut1	GAATTACAAATCACCATCTCCCAG	CTAGTCCGTGGTTCTTCCTC
Onecut2	AACAACTCGCAGAAGAAATCC	GTCATCTTGCCATTTCTCCAG
Hmbs	CTGTTCAGCAAGAAGATGGTC	TGATGCCCAGGTTCTCAG
Sox9	CCCGATTACAAGTACCAGCC	CCCTGAGATTGCCCAGAGTG
Slc51a	AGGTCTCAAGTGATGAACTGC	CAGCGAACAAGCCTCATACC
Slc51b	GAATTATTCCATCCTGGTCCTG	CTTCTTTCGATTTCTGTTTGCC
Lgr5	TGCCCATCACACTGTCACTGT	CACCCTGAGCAGCATCCTG
Spp1	AGCAAGAAACTCTTCCAAGCA	GATTCGTCAGATTCATCCGAG
Aqp1	CCGCAACTTCTCAAACCA	TGGAGTTGATGTCGTCAG
Cftr	GGATCAGGAAAGACATCACTC	GTACTCATCATAGGAAACACCA
Krt7	AGACCAAGTTTGAGACACTCCA	TTCATCTCCGCAATCTCATTCC
Pkd2	GAATGTATCTTCACCCAGTTCC	TCTGAGAGTTCCATTTCTGC
Ggt1	CCTCAAAGGATACAACTTCTC	GACACATCGACAAACTTTGG
Slc4a2	GAGAGGAAGACTTTGAATACCA	CTTGGAGAAAGAACTGAACTG
Slc10a2	GTCAGTTTGGAATCATGCCT	CAACCAGAGAAATACCAATGCT
Krt19	CAGAACCAAGTTTGAGACAG	GGCAGTAATTTCCTCCTC
Gpbar1	TGCCTCCTTCTCCACTTGAC	AGGCCATAAACTTCCAGGTAGAG
Cyp3a11	AAACTCAAGGAGATGTTCCC	TTCACTCCAAATGATGTGCT
Abcb1b	GCATTACTAATCAAAGTGGACCC	CATCAAACCAGCCTATCTCCT

Supplementary Table 2. List of antibodies used in immunofluorescent analysis

Antibody	Speicies	Supplier	Cat. No.	Dilution
Ki67	rabbit	Thermo Scientific	RM-9106-S	1:50
KRT7	mouse	Dako	M7018	1:25
KRT19	mouse	Novocastra	NCL-CK19	1:300
Albumin	mouse	Sigma	A6684	1:2000
HNF1B	rabbit	Sigma	HPA002083	1:400
TJP1	rabbit	Invitrogen	40-2300	1:250
CDH1	mouse	BD Biosciences	610181	1:100
GGT	mouse	LifeSpan Biosciences	LS-C23901	1:50
Acetyl-α-Tubulin	mouse	Thermo Scientific	32-2700	1:100
β-catenin	rabbit	Abcam	Ab6302	1:500
AQP1	rabbit	Millipore	AB2219	1:500
CYP1A2	rabbit	Abcam	Ab77795	1:600
MDR1B	mouse	Kamiya	MC-012	1:100

References

[1] Tabibian JH, Masyuk AI, Masyuk T V., et al. Physiology of cholangiocytes. Compr Physiol 2013;3:541–565.

[2] Lazaridis KN, LaRusso NF. The Cholangiopathies. Mayo Clin Proc 2015;90:791–800.

[3] Joplin R. Isolation and culture of biliary epithelial cells. Gut 1994;35:875–878.

[4] Sampaziotis F, Brito MC de, Geti I, et al. Directed differentiation of human induced pluripotent stem cells into functional cholangiocyte-like cells. Nat Protoc 2017;12:814–827.

[5] Clevers H. Modeling development and disease with organoids. Cell 2016;165:1586–1597.

[6] 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.

[7] Huch M, Gehart H, Boxtel R Van, et al. Long-term culture of genome-stable bipotent stem cells from adult human liver. Cell 2015;160:299–312.

[8] Alpini G, Glaser S, Baiocchi L, et al. Secretin activation of the apical Na^+-dependent bile acid transporter is associated with cholehepatic shunting in rats. Hepatology 2005;41:1037–1045.

[9] Steenbeek FG van, Spee B, Penning LC, et al. Altered subcellular localization of heat shock protein 90 is associated with impaired expression of the aryl hydrocarbon receptor pathway in dogs. PLoS One 2013;8:e57973.

[10] Schophuizen CMS, Napoli IE De, Jansen J, et al. Development of a living membrane comprising a functional human renal proximal tubule cell monolayer on polyethersulfone polymeric membrane. Acta Biomater 2015;14:22–32.

[11] Jansen J, Napoli IE De, Fedecostante M, et al. Human proximal tubule epithelial cells cultured on hollow fibers: living membranes that actively transport organic cations. Sci Rep 2015;5:16702.

[12] Tanimizu N, Nakamura Y, Ichinohe N, et al. Hepatic biliary epithelial cells acquire epithelial integrity but lose plasticity to differentiate into hepatocytes *in vitro* during development. J Cell Sci 2013;126:5239–46.

[13] Tanimizu N, Miyajima A, Mostov KE. Liver progenitor cells develop cholangiocyte-type epithelial polarity in three-dimensional culture. Mol Biol Cell 2007;18:1472–1479.

[14] Antoniou A, Raynaud P, Cordi S, et al. Intrahepatic bile ducts develop

according to a new mode of tubulogenesis regulated by the transcription factor SOX9. Gastroenterology 2009;136:2325–2333.

[15] Glaser S, Alvaro D, Ueno Y, et al. Gastrin reverses established cholangiocyte proliferation and enhanced secretin-stimulated ductal secretion of BDL rats by activation of apoptosis through increased expression of Ca^{2+}-dependent PKC isoforms. Liver Int 2003;23:78–88.

[16] Alpini G, Ueno Y, Glaser SS, et al. Bile acid feeding increased proliferative activity and apical bile acid transporter expression in both small and large rat cholangiocytes. Hepatology 2001;34:868–876.

[17] Clotman F, Jacquemin P, Plumb-Rudewiez N, et al. Control of liver cell fate decision by a gradient of TGFβ signaling modulated by Onecut transcription factors. Genes Dev 2005;19:1849–1854.

[18] Huang BQ, Masyuk T V, Muff MA, et al. Isolation and characterization of cholangiocyte primary cilia. Am J Physiol Gastrointest Liver Physiol 2006;291:500–509.

[19] Tang F, Ouyang H, Yang JZ, et al. Bidirectional transport of rhodamine 123 and hoechst 33342, fluorescence probes of the binding sites on p-glycoprotein, across MDCK-MDR1 cell monolayers. J Pharm Sci 2004;93:1185–1194.

[20] Li G, Guo GL. Farnesoid X receptor, the bile acid sensing nuclear receptor, in liver regeneration. Acta Pharm Sin B 2015;5:93–98.

[21] Ding L, Yang L, Wang Z, et al. Bile acid nuclear receptor FXR and digestive system diseases. Acta Pharm Sin B 2015;5:135–144.

[22] Chiang J. Bile Acid Metabolism and Signalling. Compr Physiol 2013;3:1191–1212.

[23] Jones H, Alpini G, Francis H. Bile acid signaling and biliary functions. Acta Pharm Sin B 2015;5:123–128.

[24] Maloney PR, Parks DJ, Haffner CD, et al. Identification of a chemical tool for the orphan nuclear receptor FXR. J Med Chem 2000;43:2971–2974.

[25] Balakrishnan A, Sussman DJ, Polli JE. Development of stably transfected monolayer overexpressing the Human Apical Sodium-Dependent Bile Acid Transporter (hASBT). Pharm Res 2005;22:1269–1280.

[26] Kruitwagen HS, Oosterhoff LA, Vernooij IGWH, et al. Long-term adult feline liver organoid cultures for disease modeling of hepatic steatosis. Stem Cell Reports 2017;8:822–830.

[27] Nantasanti S, Spee B, Kruitwagen HS, et al. Disease modeling and gene therapy of copper storage disease in canine hepatic organoids. Stem Cell

Reports 2015;5:895–907.

[28] Huch M, Boj SF, Clevers H. Lgr5^{+} liver stem cells, hepatic organoids and regenerative medicine. Regen Med 2013;8:385–7.

[29] Sampaziotis F, Cardoso de Brito M, Madrigal P, et al. Cholangiocytes derived from human induced pluripotent stem cells for disease modeling and drug validation. Nat Biotechnol 2015;33:845–852.

[30] Dianat N, Dubois-Pot-Schneider H, Steichen C, et al. Generation of functional cholangiocyte-like cells from human pluripotent stem cells and HepaRG cells. Hepatology 2014;60:700–714.

[31] Ogawa M, Ogawa S, Bear CE, et al. Directed differentiation of cholangiocytes from human pluripotent stem cells. Nat Biotechnol 2015;33:853–861.

[32] Zhang W, Zitron E, Homme M, et al. Aquaporin-1 channel function is positively regulated by protein kinase C. J Biol Chem 2007;282:20933–20940.

[33] Si-Tayeb K, Lemaigre FP, Duncan S a. Organogenesis and development of the liver. Dev Cell 2010;18:175–189.

[34] Clotman F, Lannoy VJ, Reber M, et al. The onecut transcription factor HNF6 is required for normal development of the biliary tract. Development 2002;129:1819–28.

[35] Oo ZY, Deng R, Hu M, et al. The performance of primary human renal cells in hollow fiber bioreactors for bioartificial kidneys. Biomaterials 2011;32:8806–15.

[36] Lee H, Rho J, Messersmith PB. Facile conjugation of biomolecu les onto surfaces via mussel adhesive protein inspired coatings. Adv Mater 2009;21:431–434.

[37] Zhang H, Tasnim F, Ying JY, et al. The impact of extracellular matrix coatings on the performance of human renal cells applied in bioartificial

[38] kidneys. Biomaterials 2009;30:2899–911.
Oliver J a, Barasch J, Yang J, et al. Metanephric mesenchyme contains embryonic renal stem cells. Am J Physiol Physiol 2002;283:F799–F809.

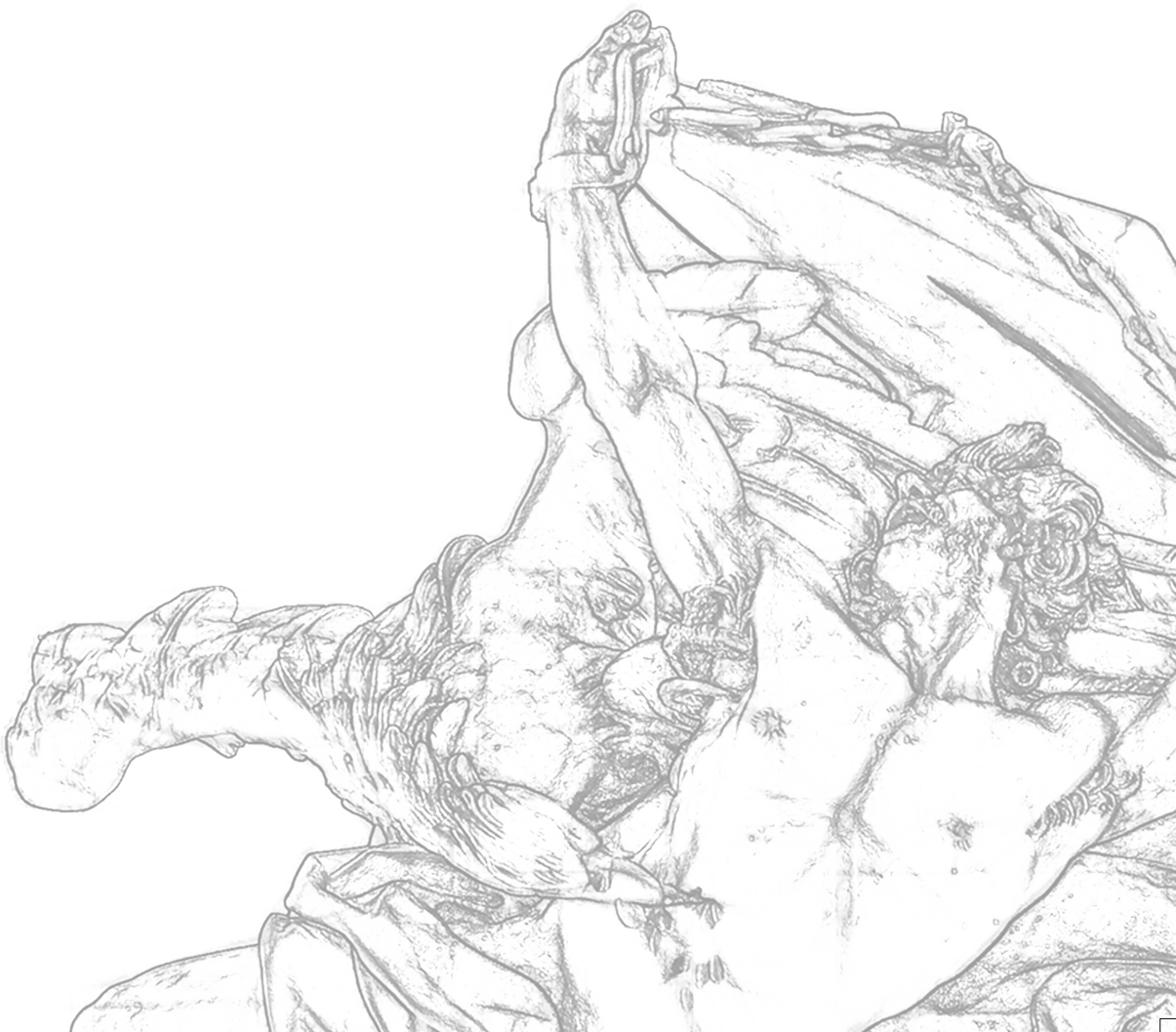
[39] Awortwe C, Fasinu PS, Rosenkranz B. Application of Caco-2 cell line in herb-drug interaction studies: current approaches and challenges. J Pharm Pharm Sci 2014;17:1–19.

[40] Cheung AC, Lorenzo Pisarello MJ, LaRusso NF. Pathobiology of biliary epithelia. Biochim Biophys Acta - Mol Basis Dis 2018;1864:1220–1231.

[41] Kerkhove MP van de, Hoekstra R, Chamuleau R a FM, et al. Clinical

application of bioartificial liver support systems. Ann Surg 2004;240:216–230.

[42] Shi X-L, Gao Y, Yan Y, et al. Improved survival of porcine acute liver failure by a bioartificial liver device implanted with induced human functional hepatocytes. Cell Res 2016;26:206–216.



Chapter 5

Summary

The liver is known as a multi-functioning blood processing “factory” in the human body. It is responsible for temporary nutrient storage, regulating nutrient and metabolite levels in the blood (sugar, fat, amino acids, nucleotides), removing old and damaged red blood cells, removing toxins from blood, and secreting bile via gallbladder into small intestines. Lastly the liver is the main organ to remove toxins form the body via biotransformation. These functions are mainly performed by hepatocytes and cholangiocytes. To generate these two epithelial cell types, in a functional state that resembles primary liver cell types, is the primary focus of this thesis.

Many cell types have been proposed as the source for producing hepatocyte-like cells (HLCs) [1-3]. Although to date fully mature HLCs still do not possess all functions of hepatocytes, emerging novel technologies and increasing knowledge of the mechanisms involved in hepatic maturation are aiding in the terminal phases of hepatic differentiation. In this quest, nature can actually provide new insights and research directions. Specifically, the rapid maturation of the neonatal liver observed within the first weeks after birth, is a great evidence of how the drastic change in environment prompts this organ to mature quickly. Hence, it can be envisioned that human fetal-to-neonatal changes such as hepatic circulation, microbiome, and nutrition can aid in hepatic maturation. In **Chapter 1**, we specified which changes might play the important role in creating fully functional HLCs.

Transcription factors may be one of the most fundamental triggers. Their roles in deciding cell fate was firstly realised thirty years ago [4] and fully confirmed in the generation of induced pluripotent stem cells (iPSCs) in this century [5,6]. In **Chapter 2**, we used a polycistronic expression system co-expressing three key hepatic transcription factors to turn mouse somatic cells into hepatocyte-like cells. Although these HLCs exhibited a homogenous phenotype and possessed some properties of primary hepatocytes, there was still a huge functional gap between HLCs and freshly isolated hepatocytes. As discussed in **Chapter 1**, one obvious difference between an *in vivo* hepatocyte and an *in vitro* hepatocyte-like cell is the microenvironment surrounding them. A recently study has shown that when the reprogramming was initiated *in vivo* the resulting cells highly resembled primary hepatocytes [7], indicating how the microenvironment can affect the maturation of HLCs. Therefore, a logic following step is to introduce native liver extracellular matrix into the *in vitro* culture system for HLCs. Indeed, the HLCs acquired increased maturation, but still at limited level as shown in **Chapter 2**.

The inability to acquire fully functional hepatocytes is disappointing. This proves the complexity and suggests that numerous factors could be taken into account. In **Chapter 3**, we investigated whether the limited maturation of HLCs was due to some missing puzzle pieces in the regulatory network of hepatic transcription factors. In order to identify these transcription factors for terminal differentiation of hepatocytes, we performed epigenetic- and transcriptional-analysis on a dedifferentiating hepatocyte model. The three-step screening results showed HNF4A as the master regulator. More importantly, it was absent during the HLC generation by direct reprogramming (by overexpressing *Hnf1a, Foxa3* and *Gata4*). Forced expression of *Hnf4a* resulted in enhanced hepatocyte features in HLCs including the upregulation of hepatocyte markers and downregulation of stem cell and cholangiocyte markers. Furthermore, hepatocyte-enriched transaminases, phase I biotransformation enzymes, as well as albumin also showed significantly increased expression. However, the gap regarding functionality between HLCs and primary hepatocytes was still not completely bridged. Unlike iPSCs, fully functional hepatocytes cannot be generated by transcription factors alone. A combination of several strategies proposed in **Chapter 1** may be the next step.

There are many cell types in the liver, some of which, such as Kupffer cells and hepatic stellate cells can secret cytokines assisting hepatocyte to function. The main epithelial cells of bile ducts, cholangiocytes, are important for maintaining a healthy environment for other cells by removing waste products and toxins out of the liver. In **Chapter 4**, we aimed at creating such an artificial bile duct model. In order to do so, we used the liver organoids, a three-dimensional system for long-term culture of hepatic progenitor cells (HPCs). Apart from their general advantages, such as their genetic stability and long-term proliferative capacity [8], we used this system mainly because the organoids have close lineage relationship with cholangiocytes. For example, during liver development, HPCs can differentiate towards cholangiocytes without any intermediate steps [9]. Moreover, liver organoids already possess some cholangiocyte functions, such as transportation activities dependent on cystic fibrosis transmembrane conductance regulator (CFTR) and permeability glycoprotein (P-gp, also known as MDR1) although to a limited degree (our unpublished data). To induce further differentiation of HPCs towards cholangiocytes, we developed a two-step protocol using growth factors and extracellular matrix to mimic the stimulation received by cholangiocytes during liver development. Organoid-derived cholangiocyte-like cells (CLCs) displayed key properties of primary cholangiocytes, such as expressing cholangiocyte markers,

forming primary cilia, transporting small molecules and responding to farnesoid X receptor (FXR) agonist. Once cultured on collagen-coated polyethersulfone hollow fiber membranes CLCs clearly resembled native bile ducts morphologically and even possessed polarized bile acid transport activity.

References

[1] Ji S, Zhang L, Hui L. Cell fate conversion: Direct induction of hepatocyte-like cells from fibroblasts. J Cell Biochem 2013;114:256–265.

[2] Schwartz RE, Fleming HE, Khetani SR, et al. Pluripotent stem cell-derived hepatocyte-like cells. Biotechnol Adv 2014;32:504–513.

[3] Aurich I, Mueller LP, Aurich H, et al. Functional integration of hepatocytes derived from human mesenchymal stem cells into mouse livers. Gut 2007;56:405–415.

[4] Lassar a B, Paterson BM, Weintraub H. Transfection of a DNA locus that mediates the conversion of 10T1/2 fibroblasts to myoblasts. Cell 1986;47:649–656.

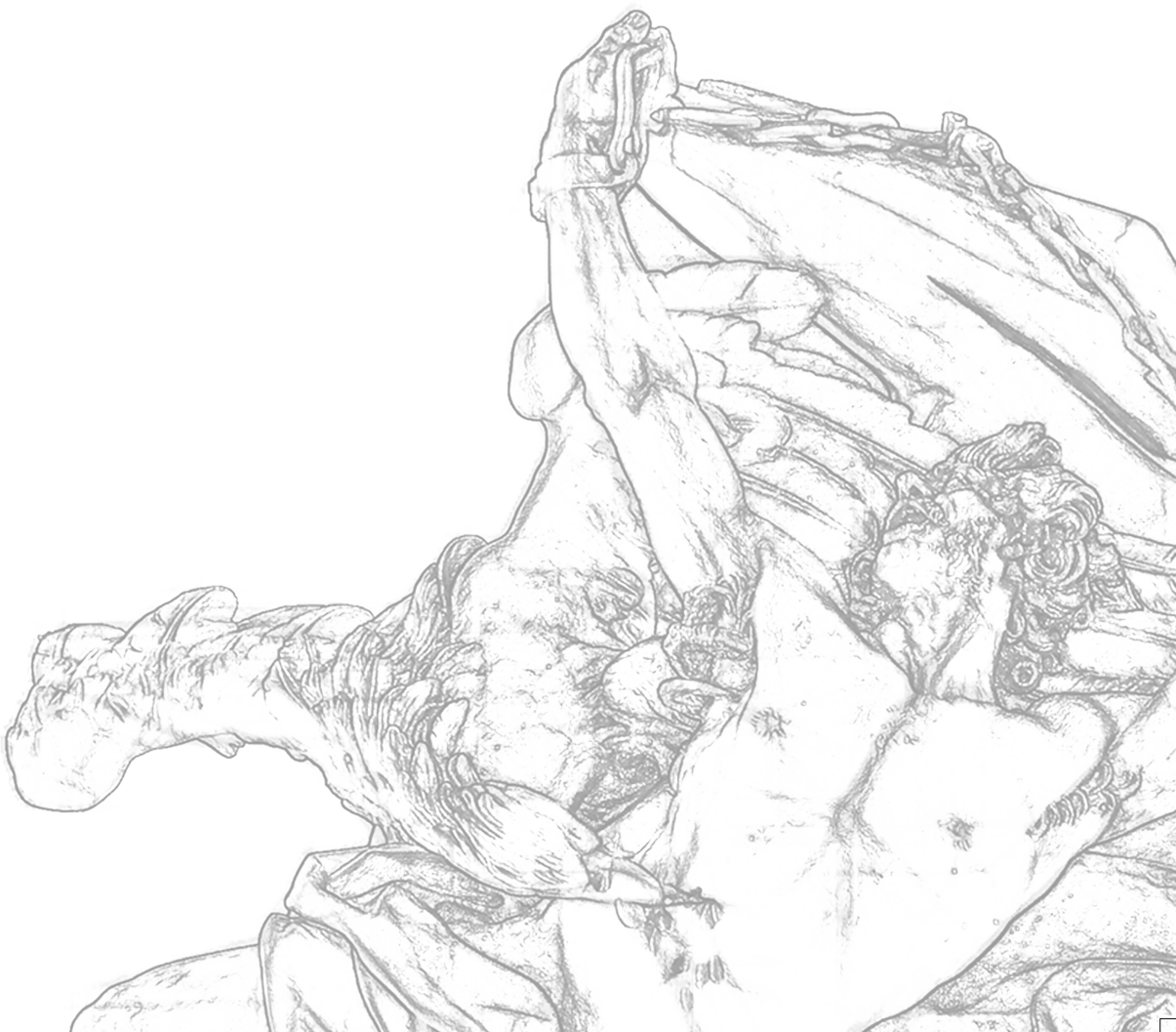
[5] Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861–872.

[6] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663–76.

[7] Song G, Pacher M, Balakrishnan A, et al. Direct reprogramming of hepatic myofibroblasts into hepatocytes *in vivo* attenuates liver fibrosis. Cell Stem Cell 2016;18:797–808.

[8] Huch M, Boj SF, Clevers H. Lgr5^{+} liver stem cells, hepatic organoids and regenerative medicine. Regen Med 2013;8:385–7.

[9] Si-Tayeb K, Lemaigre FP, Duncan S a. Organogenesis and development of the liver. Dev Cell 2010;18:175–189.



Chapter 6

Discussion

The research described in this thesis was largely accommodated in the research program Regenerative Medicine. Regenerative Medicine is a dynamic field that brings together many disciplines with the aim of developing novel therapeutic strategies for a wide variety of diseases. The multidisciplinary nature in our research is reflected by the combination of a variety of technologies, such as stem cell culture and reprogramming, gene editing, high-throughput sequencing and bioinformatics, biomaterials and bioengineering. In the following paragraphs, some of these aspects are discussed.

Transcription Factor Induced Reprogramming

Previously the dogma was that somatic cells were bound to their differentiated state, but the groundbreaking finding by Takahashi and Yamanaka in 2006 for the first time showed that this terminal state is not definitive [1]. Cells can be reprogrammed into another completely unrelated lineage under the influence of a set of specific transcription factors, this process is called direct reprogramming or trans-differentiation. Without experiencing a pluripotent stage, generation of certain cell type by direct reprogramming saves time and effort, and can avoid the tumorigenesis risks which is especially important for clinical purpose. These transcription factors are often a combination of fate-determination factors and maturation factors [2]. This strategy has successfully been applied to a number of cell types of which fibroblasts are the most common donor cell type, because of their easy access. So far, several types of functional cells have been generated from fibroblasts, including cardiomyocytes, myocytes, neurons and hepatocytes among others, and these can be used for drug screening, disease modelling or for therapeutic purposes [3–8]. The direct reprogramming has advantages over other reprogramming methods for instance through the production of induced pluripotent stem cells and subsequent differentiation (indirect reprogramming), which has a high risk of teratoma formation [9]. Besides, from a clinical point of view, direct reprogramming has the potential to be conducted in situ in the body, which can bypass the need for transplantation of differentiated cells [10].

In this regard, we aimed at looking for the key transcription factors deciding the fate of hepatocytes. Apart from the ones already discussed in Chapter 1, more transcription factors may also play important roles (Figure 1).

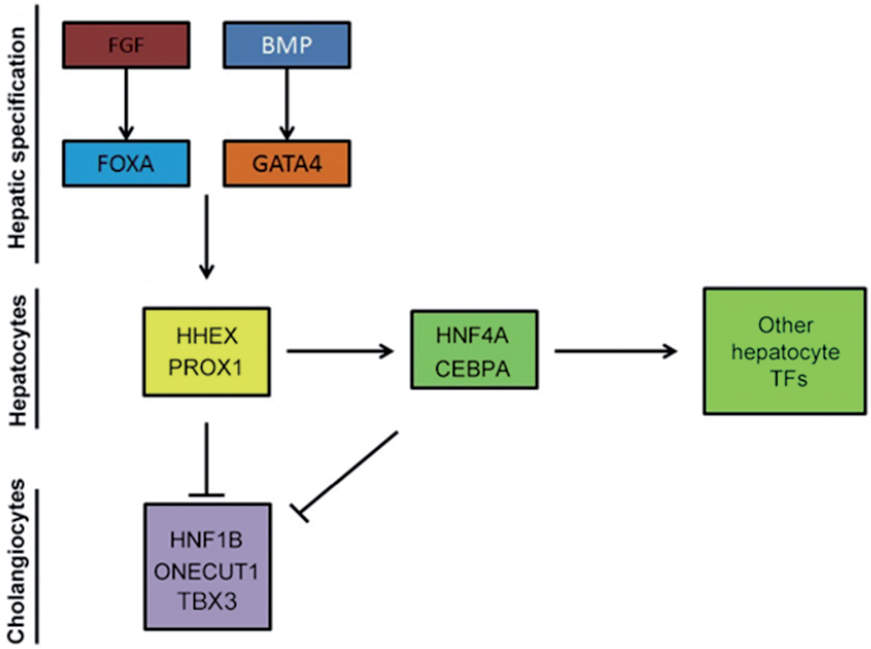


Figure 1. Transcription factors that may play a role for better direct reprogramming. FGF and BMP are growth factors inducing the expression of FOXA and GATA4. FGF, fibroblasts growth factor; BMP, bone morphogenetic protein 4; FOXA, Forkhead box protein A; GATA4, Transcription factor GATA-4.

Pioneer factors

Transcription of a gene requires the access and binding of RNA polymerase and transcription factors to the cis-regulatory elements on the DNA. The accessibility is blocked when the DNA forms a condensed state. However, there is a subset of transcription factors, termed pioneer factors, which can reverse the DNA from a closed state to an open state. Although these factors do not induce transcription on their own, they are important to initiate cell fate changes [11]. The FOXA family, including FOXA1, 2 and 3, is such an example of pioneer factors. FOXA family members have been shown to bind to the regulatory regions of hepatic genes such as albumin, transthyretin and α-1-antitrypsin [12,13]. FOXA1 and FOXA2 have been proved to cooperate in the foregut endoderm to induce hepatic specification [14]. While FOXA3 is expressed at later stages during liver development and throughout adulthood [14]. FOXA family were found have a DNA binding motif resembling

histone H5 [15], hence they could bind stably to histone bound DNA instead of free DNA and stabilize the binding of other transcription factors [16]. GATA4, which is highly expressed during hepatic specification stage of liver development, has also been shown to be able to initiate the transcription of a large number of hepatic genes [17].

CCAAT/enhancer-binding protein α (CEBPA)

CEBPA is a leucine zipper transcription factor of the CEBP family that has been shown to play a role in hepatocyte cell fate determination [18-20]. Mice with CEBPA deficiency exhibit an increase in α-fetoprotein level indicating a fetal feature of hepatocytes [18]. Hepatoblasts with low expression of CEBPA tend to differentiate to cholangiocytes [21], possibly because inhibited CEBPA leads to increased expression of HNF1B and ONECUT1, two transcription factors for cholangiocyte fate commitment [21].

Haematopoietically-expressed homeobox protein (HHEX)

HHEX is a liver enriched homeobox transcription factor important for early embryonic development of the liver [22,23]. It is regulated by the pioneer factors, FOXA2 and GATA4 [22] and can be activated by autocrine BMP signaling [24]. The deletion of HHEX in the liver results in altered expression of HNF1A, HNF1B, HNF4A and HNF6 [25,26]. Transient overexpression of HHEX has been shown to efficiently drive the differentiation of ESCs and iPSCs into hepatoblasts [27].

Prospero related homeobox 1 (PROX1)

PROX1 is another early factor in liver embryonic development. Prox1$^{-/-}$ mice are unable to migrate into the adjacent septum transversum mesenchyme resulting in 70% smaller livers compared to wild type [25]. PROX1 plays an important role in deciding the differentiation direction of hepatoblasts [28] and is highly expressed in adult hepatocytes but absent in cholangiocytes and non-parenchymal cells [29].

Forkhead box protein M1 β (FOXM1B)

After partial hepatectomy the liver has significantly increased expression of FOXM1B, specifically in cells at G1/S phase [30]. Old hepatocytes with low

proliferative capacity, when overexpressing FOXM1B, are able to repopulate the liver as efficiently as young hepatocytes [31]. Though FOXM1B is not directly related to the function of hepatocytes, when large number of cells are needed, for example for transplantation purpose, it can be a good candidate to overexpress to conquer proliferation restraint.

Although transcription factors are important for inducing many hepatic phenotypes, purely overexpressing one even a combination of several transcription factors may not be sufficient to induce all mature hepatocyte features. Hepatocytes are exposed in a highly dynamic and complex microenvironment in the liver, therefore, new strategies to combine transcription factors with other components are needed, for instance, with molecular components such as microRNA and growth factors, and cellular components such as extracellular matrix and supporting cell types.

RNA-Guided Transcriptional Activation

In the past direct reprogramming has been achieved using viral vectors to incorporate the required genes into the genome of the target cells, but for clinical application a transient induction is desirable. Although CRISP/Cas9 technology revolutionized gene-editing, a modified version of Cas9 can be used to induce gene transiently [32]. This gene induction is realized by the application of a catalytically dead Cas9 (dCas9) coupled to a transactivating domain. Together with guide RNAs, this forms a complex functionally resembling a transcription factor (artificial transcription factor, ATF). Other advantages includes, but are not limited to, the fact that this system is able to modify chromatin structure and thus able to target genes with tightly condensed promoters and enhancers [33,34].

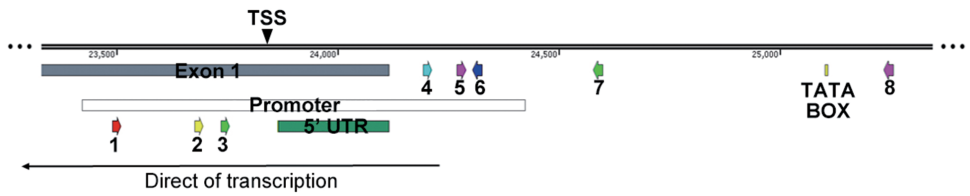


Figure 2. Schematic illustration showing the design of sgRNAs for inducing *Hnf1a*. Eight sgRNAs were designed on or around the presumptive promoter region of *Hnf1a*. TSS, transcription starting site. 5' UTR, 5' untranslated region.

With the ATF method, we have achieved preliminary success. We introduced ATFs targeting eight sites on the promoter of *Hnf1a* and its 2000-bp upstream region (Figure 2). We observed that expression of *Hnf1a* was induced in the mouse embryonic fibroblasts cell line NIH/3T3 (Figure 3). A comparison between the expression levels induced by ATF and the amount of lentivirus needed would be an interesting following step, because a high and sustainable expression of hepatocyte-generating factors is necessary for a successful direct reprogramming.

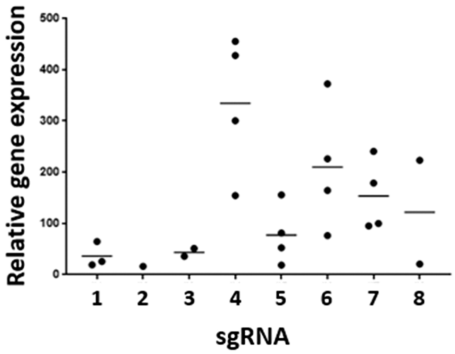


Figure 3. Relative expression of Hnf1a induced by ATFs. Eight sets of ATFs were separately transfected to NIH/3T3. RNA samples were collected and qPCR was performed after two days. Each group contained four biological replicates.

Generation of a Hepatic Maturation Reporter Cell Line

Reporter cell lines are useful tools for monitoring changes of cells under certain stimulation. In general, reporter proteins are engineered through integrating the reporter expression cassettes into cell genomes by plasmid or lentivirus vectors. The commonly used reporter proteins are either directly visible, such as fluorescent proteins (GFP, RFP, etc.), or can be quantified indirectly and mostly more sensitive, such as luciferase.

The expression level of albumin is highly correlated with the differentiation status of pluripotent stem cells towards hepatocytes during liver development. As early as the embryonic day 8.5, albumin expression starts under the stimulation from cardiac mesoderm, indicating the hepatic specification [35]. After that, its expression keeps rising and reaches the peak in the adult liver. Moreover, as indicated in Chapter 4, we also noticed that albumin decreases with dedifferentiation of hepatocyte in *in*

vitro culture. These findings suggest that the expression level of albumin can very well reflect the hepatic differentiation status of cells. Previous studies have used lentiviral vectors to introduce the exogenous *Alb* promoter followed by a fluorescent gene into the genome of targeted cells [36,37]. There are several disadvantages of this strategy. First, the copy number of the exogenous genes are difficult to measure; second, the promoter region is presumptive (normally 2000 bp upstream of transcription starting site), which means it cannot accurately reflect the broad regulation on *Alb* (for example, regulation on the enhancers of *Alb* is impossible to be measured); third, the random insertion has the potential to affect normal physiology of the targeted cells.

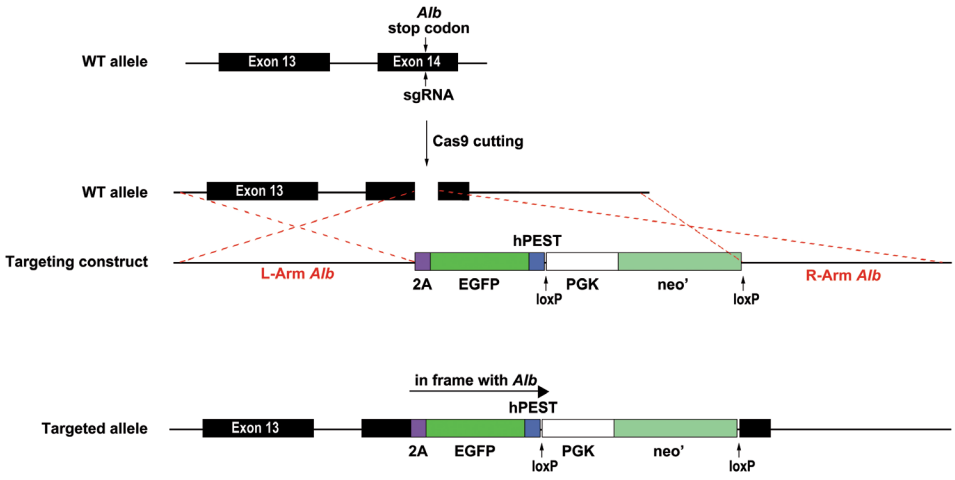


Figure 4. Gene targeting strategy to generate the *Alb* promoter-driven reporter cell line. RNA-guided Cas9 induced cutting right at the stop codon site of *Alb*. DNA double strand breaks induce highly efficient homology directed repair. The DNA template for repair includes sequences for an enhanced GFP (EGFP), a PGK promoter and a neomycin resistance gene.

To avoid these shortcomings, we established an albumin promoter-driven hepatic maturation reporter construct in mouse embryonic stem (ES) cells. The GFP gene and a selection marker were introduced right at the stop codon region of *Alb* (Figure 4). Upstream of the GFP gene is a 2A peptide sequence, to ascertain similar expression levels upstream and down steam of this 2A peptide sequence [38]. These efforts ensure that GFP and albumin are controlled by the same regulatory elements, hence will have the same amount of expression. Therefore,

the fluorescence strength of a cell is an indication of its differentiation level towards a fully mature hepatocyte. We envision that this reporter line can be used for i) the *in vitro* analysis of hepatic differentiation with superior accuracy and ii) the creation of reporter mice that can be used for monitoring *in vivo* hepatic differentiation and for tracking hepatic cells in the body.

Standardizing HLC Generation and Maturation Protocols

Because the adult liver is complex and has a wide range of functions, it is important to have a consensus on how to generate and mature HLCs, especially if they are to become an alternative for liver transplantation and cellular therapy. Hepatocyte markers in both human HLCs and human adult primary hepatocytes (albumin or cytokeratin 18, glycogen storage, and inducible cytochrome P450) [6,10,39-41] have been reported. However, proposed protocols for hepatic differentiation of human stem cells vary, giving rise to inconsistency in the definition of hepatocyte-like cells and fully matured hepatocytes. Standardization is key, and it is important to understand what properties a hepatocyte or the substitute HLC should have and how the resulting HLCs will be used. In some cases, for example, excellent secretory capabilities (e.g. albumin, alpha-1-antytripsin) are sufficient, whereas in others, high cytochrome P450s (CYPs) activity is required [41,42].

As a step towards standardization, and based on the components reviewed in Chapter 1, we propose the following analyses for stem cell-derived hepatocytes: a) gene expression analysis compared to freshly isolated hepatocytes, b) metabolism of xenobiotics and endogenous substances (hormones and ammonia); c) synthesis and secretion of albumin, clotting factors, complement factors, transporter proteins, bile, lipids and lipoproteins; and d) storage of glucose (glycogen), fat soluble vitamins A, D, E and K, folate, vitamin B12, copper and iron. Additionally, a convincing *in vivo* experiment to prove hepatocellular differentiation is to restore liver function in animal models with repopulation assays. However, repopulation experiments may only determine that a certain hepatic cell type has the capacity to generate hepatocytes *in vivo*, leaving many variables unanswered. Thus, testing a defined assortment of activities and comparing them with primary hepatocytes is the only feasible option for evaluating the *in vitro* potential of stem cell-derived hepatocyte cultures as appropriate surrogates for primary human hepatocytes.

Despite advances in the differentiation of HLCs *in vitro*, cells that functionally

recapitulate human primary adult hepatocytes and proliferate to completely replace livers for clinical applications is still elusive. Ultimately, clinical transplantation of liver cells will require the generation of high numbers of stable mature liver cells with multifunctional performance equal to that of primary human hepatocytes. Equally important, are the stringent comprehensive characterization and robust analysis of hepatocytes derived from various sources/institutes in order to ensure that the shift in phenotype of these cells towards human adult hepatocytes has truly been accomplished.

References

[1] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663–76.

[2] Xu J, Du Y, Deng H. Direct lineage reprogramming: strategies, mechanisms, and applications. Cell Stem Cell 2015;16:119–134.

[3] Sekiya S, Suzuki A. Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. Nature 2011;475:390–393.

[4] Ieda M, Fu JD, Delgado-Olguin P, et al. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. Cell 2010;142:375–386.

[5] Huang P, He Z, Ji S, et al. Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. Nature 2011;475:386–389.

[6] Huang P, Zhang L, Gao Y, et al. Direct reprogramming of human fibroblasts to functional and expandable hepatocytes. Cell Stem Cell 2014;14:370–384.

[7] Kalani MYS, Martirosyan N. Direct conversion of fibroblasts to functional neurons. World Neurosurg 2012;77:7–8.

[8] Jopling C, Boue S, Izpisua Belmonte JC. Dedifferentiation, transdifferentiation and reprogramming: three routes to regeneration. Nat Rev Mol Cell Biol 2011;12:79–89.

[9] Shi Y, Inoue H, Wu JC, et al. Induced pluripotent stem cell technology: a decade of progress. Nat Rev Drug Discov 2016;16:115–130.

[10] Song G, Pacher M, Balakrishnan A, et al. Direct reprogramming of hepatic myofibroblasts into hepatocytes *in vivo* attenuates liver fibrosis. Cell Stem Cell 2016;18:797–808.

[11] Iwafuchi-Doi M, Zaret KS. Pioneer transcription factors in cell reprogramming. Genes Dev 2014;28:2679–2692.

[12] Lai E, Prezioso VR, Tao WF, et al. Hepatocyte nuclear factor 3 alpha belongs to a gene family in mammals that is homologous to the Drosophila homeotic gene fork head. Genes Dev 1991;5:416–427.

[13] Cirillo LA, Lin FR, Cuesta I, et al. Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. Mol Cell 2002;9:279–289.

[14] Kaestner KH. The FoxA factors in organogenesis and differentiation. Curr Opin Genet Dev 2010;20:527–532.

[15] Clark KL, Halay ED, Lai E, et al. Co-crystal structure of the HNF-3/fork head DNA-recognition motif resembles histone H5. Nature 1993;364:412–

420.

[16] Cirillo LA, Zaret KS. An Early Developmental transcription factor complex that is more stable on nucleosome core particles than on free DNA. Mol Cell 1999;4:961–969.

[17] Zheng R, Rebolledo-Jaramillo B, Zong Y, et al. Function of GATA factors in the adult mouse liver. PLoS One 2013;8:1–19.

[18] Nerlov C. The C/EBP family of transcription factors: a paradigm for interaction between gene expression and proliferation control. Trends Cell Biol 2007;17:318–324.

[19] 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.

[20] 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.

[21] Shiojiri N, Takeshita K, Yamasaki H, et al. Suppression of C/EBPα expression in biliary cell differentiation from hepatoblasts during mouse liver development. J Hepatol 2004;41:790–798.

[22] Denson LA, McClure MH, Bogue CW, et al. HNF3β and GATA-4 transactivate the liver-enriched homeobox gene, Hex. Gene 2000;246:311–320.

[23] Wallace KN, Yusuff S, Sonntag JM, et al. Zebrafishhhex regulates liver development and digestive organ chirality. genesis 2001;30:141–143.

[24] Zhang W, Yatskievych TA, Cao X, et al. Regulation of Hex gene expression by a Smads-dependent signaling pathway. 2002;277:45435–45441.

[25] Sosa-Pineda B, Wigle JT, Oliver G. Hepatocyte migration during liver development requires Prox1. Nat Genet 2000;25:254–255.

[26] Tanaka H, Yamamoto T, Ban T, et al. Hex stimulates the hepatocyte nuclear factor 1 -mediated activation of transcription. 2005;442:117–124.

[27] Inamura M, Kawabata K, Takayama K, et al. Efficient generation of hepatoblasts from human ES cells and iPS cells by transient overexpression of homeobox gene HEX. Mol Ther 2011;19:400–407.

[28] Seth A, Ye J, Yu N, et al. Prox1 ablation in hepatic progenitors causes defective hepatocyte specification and increases biliary cell commitment.

Development 2014;141:538–547.

[29] Dudas J, Papoutsi M, Hecht M, et al. The homeobox transcription factor Prox1 is highly conserved in embryonic hepatoblasts and in adult and transformed hepatocytes, but is absent from bile duct epithelium. Anat Embryol (Berl) 2004;208:359–366.

[30] Wang X, Krupczak-hollis K, Tan Y, et al. Increased hepatic Forkhead Box M1B (FoxM1B) levels in old-aged mice stimulated liver regeneration through diminished $p27^{Kip1}$ protein levels and increased Cdc25B expression. 2002;277:44310–44316.

[31] Brezillon N, Lambert-Blot M, Morosan S, et al. Transplanted hepatocytes over-expressing FoxM1B efficiently repopulate chronically injured mouse liver independent of donor age. Mol Ther 2007;15:1710–1715.

[32] Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol 2014;32:347–55.

[33] Polstein LR, Perez-pinera P, Kocak DD, et al. Genome-wide specificity of DNA binding , gene regulation , and chromatin remodeling by TALE- and CRISPR/Cas9-based transcriptional activators. Genome Res 2015;25:1158–1169.

[34] Perez-Pinera P, Kocak DD, Vockley CM, et al. RNA-guided gene activation by CRISPR-Cas9-based transcription factors. Nat Methods 2013;10:973–6.

[35] Zorn. Liver Development. StemBook 2008.

[36] Tang J, Wu Q, Li Y, et al. Construction of a general albumin promoter reporter system for real-time monitoring of the differentiation status of functional hepatocytes from stem cells in mouse, rat and human. Biomed Reports 2017;6:627–632.

[37] Heo J, Factor VM, Uren T, et al. Hepatic precursors derived from murine embryonic stem cells contribute to regeneration of injured liver. Hepatology 2006;44:1478–1486.

[38] Kim JH, Lee SR, Li LH, et al. High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. PLoS One 2011;6:1–8.

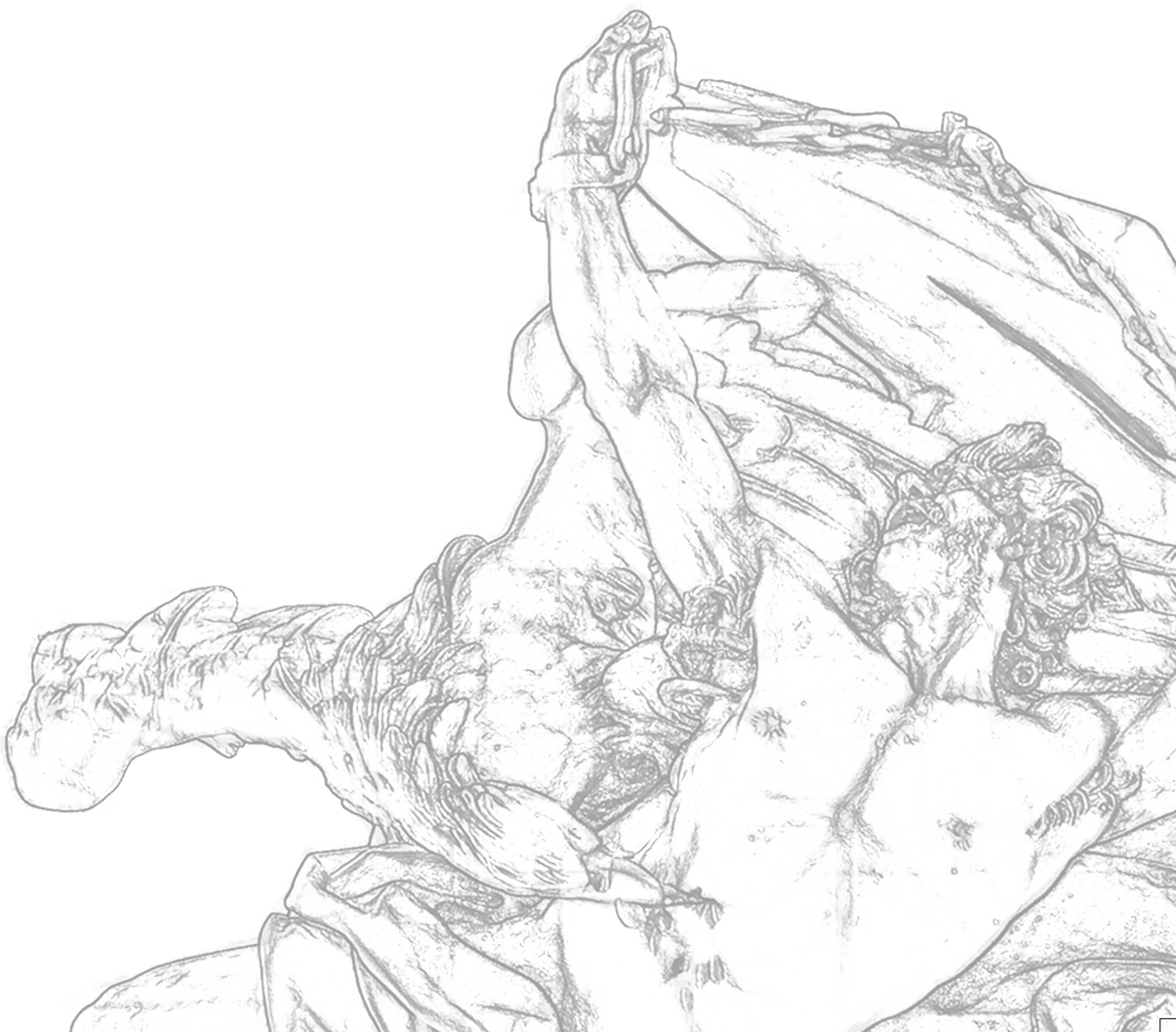
[39] Siller R, Greenhough S, Naumovska E, et al. Small-molecule-driven hepatocyte differentiation of human pluripotent stem cells. Stem Cell Reports 2015;4:939–952.

[40] Shan J, Schwartz RE, Ross NT, et al. Identification of small molecules for human hepatocyte expansion and iPS differentiation. Nat Chem Biol

2013;9:514–20.

[41] 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.

[42] Tafaleng EN, Chakraborty S, Han B, et al. Induced pluripotent stem cells model personalized variations in liver disease resulting from alpha-1-antitrypsin deficiency. Hepatology 2015;62:147–157.



Addendum

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Nederlandse Samenvatting

De lever is een multifunctioneel orgaan. Het is verantwoordelijk voor tijdelijke opslag van voedingsstoffen, reguleert bloedgehaltes van voedingsstoffen en metabolieten (suikers, vetten, aminozuren, nucleotiden), produceert verschillende stollingsfactoren, verwijdert oude en beschadigde rode bloedcellen, verwijdert toxines uit het bloed en zorgt voor excretie van gal via de galblaas naar de dunne darm. Bovendien is de lever het belangrijkste orgaan om gifstoffen uit het lichaam te inactiveren en te verwijderen via een biotransformatie proces. Al deze functies worden voornamelijk verricht door twee celtypes, te weten de hepatocyt en de cholangiocyt (galweg cel). De primaire focus van deze thesis is het genereren van deze twee epitheliale celtypes en wel zodanig dat deze functioneel identiek zijn aan primaire hepatocyten en cholangiocyten.

Meerdere cel types zijn gebruikt als bron om hepatocyt-achtige cellen (hepatocyte-like cells; HLCs) te maken. Echter de HLCs die heden ten dage beschreven zijn bezitten niet alle lever functies. De toegenomen kennis omtrent leverontwikkeling en hepatocyt rijping en de nieuwste moleculaire technieken bieden houvast om de laatste stappen tot volledig functionele hepatocyt te maken. Zo hebben nieuwe inzichten in de snelle verandering van foetaal leverweefsel naar neonataal leverweefsel, een proces dat gedurende de eerste weken na de geboorte plaatsvindt, richting gegeven aan nieuw onderzoek van hepatocyt rijping. Ook veranderingen in bloed circulatie, compositie van het microbioom en voeding lijken deze snelle veranderingen te stimuleren. In **Hoofdstuk 1** wordt beschreven welke veranderingen mogelijk van groot belang zijn voor het creëren van volledig functionele hepatocyten en worden definities gegeven waaraan deze cellen moeten voldoen.

Activiteit van specifieke lever verrijkte transcriptie factoren lijken een van de fundamentele triggers te zijn van hepatocyt rijping. In **Hoofdstuk 2** worden met een polycistronisch expressie systeem, waarin gelijktijdig drie lever specifieke transcriptie factoren tot over expressie worden gebracht, muis fibroblasten omgevormd naar HLCs. Hoewel deze HLCs fenotypisch homogeen zijn en enkele hepatocyt functies hebben, blijken ook deze cellen niet volledig functioneel identiek aan primaire hepatocyten. Zoals bediscussieerd in **Hoofdstuk 1** is er een groot verschil in micro-omgeving van gekweekte cellen (*in vitro*) en cellen in een functionele lever (*in vivo*). Een recente studie laat zien dat reprogrammering *in vivo*

kan leiden tot cellen die functioneel meer op primaire hepatocyten lijken, een sterke indicatie dat de micro-omgeving inderdaad van belang is bij de rijping van HLCs. Het lag daarom voor de hand om extracellulaire matrix van de lever te gebruiken om het *in vitro* kweeksysteem van HLCs te optimaliseren. Hiervoor werd gebruik gemaakt van gedecellulariseerde rattenlevers waar, door middel van perfusie en zeep, alle cellen uit zijn gehaald. En inderdaad, zoals beschreven in **Hoofdstuk 2**, kunnen de HLCs binnen deze micro-omgeving beter rijpen naar hepatocyten. Hoewel er sprake is van een verbetering is het echter nog steeds niet mogelijk om met deze techniek tot een vergelijkbaar functioneel niveau als primaire hepatocyten te komen.

In **Hoofdstuk 3** onderzoeken we of de beperkte hepatocyt rijping veroorzaakt wordt door het ontbreken van puzzelstukjes in het regulatie netwerk van lever transcriptie factoren. Om dit te analyseren volgen we de omgekeerde route, we doen een epigenetische- en transcriptie-analyse op dedifferentiërende hepatocyten. Deze 3-staps screening gaf aan dat HNF4A een van de meest belangrijke factoren is. Over-expressie van HNF4A resulteerde in verbeterde hepatocyt functies van HLCs. Van groot belang is hier de verhoogde expressie van hepatocyt verrijkte transaminases, fase I biotransformatie enzymen, en albumine. Echter, het functionele gat tussen HLCs en primaire hepatocyten is nog steeds niet volledig overbrugd. Zoals voorgesteld in **Hoofdstuk 1** is een combinatie strategie een vervolg stap.

Naast bovengenoemde hepatocyten zijn er nog meer celtypen in de lever. De belangrijkste epitheliale cellen van de galgangen, de cholangiocyten, spelen een cruciale rol bij het in stand houden van een niet-schadelijke omgeving doordat deze cellen afvalproducten, waaronder toxines en het agressieve gal, uit de lever verwijderen. In **Hoofdstuk 4** wordt een kunstmatig galgang model beschreven. Als bron voor cholangiocyten worden lever organoïden gebruikt; dit zijn 3-D gekweekte lever progenitor cellen die langdurig en genetisch stabiel in kweek gehouden kunnen worden en met de juiste samenstelling van groeifactoren naar hepatocyt of cholangiocyt kunnen differentiëren. Om de differentiatie naar cholangiocyt verder te sturen is een 2-staps protocol ontwikkeld met gebruik van groeifactoren en extracellulaire matrix om zo de natuurlijke leverontwikkeling na te bootsen. Deze uit organoïd verkregen cholangiocyt-achtige cellen (cholangiocyte-like cells; CLCs) vertonen gelijkenis met primaire cholangiocyten, waaronder de expressie van cholangiocyt merkers, functionele primaire cilia, specifieke transportfuncties en een

functionele farnesoid X receptor (FXR) respons. Als deze CLCs gekweekt worden op collageen-gecoate polyethersulfone hollow fiber membranen ontstaat er zelfs cellen met een galgang morfologie en een polair transport van gal zuren.

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I used to discuss with Bart how many pages I need to leave for this section, now I realize no matter how many pages there are, they are not enough to express my gratefulness. Looking back at my PhD years, it surprised me a bit that how could there be so many kind and great people around me! My dear colleagues and friends, the four years and five months staying with you was fantastic and unforgettable. It was my first time being so far from home, I was alone there, but never felt lonely.

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you for a long time, but I enjoyed the small talk with you every now and then. You have all the quality to be a good researcher, good luck! Andre, my brazilian friend, a true gentleman, it was very nice to know you and the totally different culture of Brazil. Best of luck in Austria!

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Curriculum Vitae

Chen Chen was born on the October 22nd, 1987 in Henan, China. He received his Bachelor of Science (BSc) majoring in Bioengineering from Henan University of Science and Technology, Henan, China. His research career started from 2010 when he was enrolled as a Master student in Biochemistry and Molecular Biology in the lab of Prof. Dr. Boan Li, School of Life Sciences, Xiamen University, Fujian, China. During his Master programme, he studied the role of Wnt signaling on the metastasis of colorectal cancer and obtained the Master of Science (MSc) in 2013. In October of 2013, he started the PhD training in the programme of Regenerative Medicine under the supervision of Prof. Dr. Niels Geijsen, Dr. Bart Spee and Dr. Louis C. Penning. The research described in this thesis was performed at Faculty of Veterinary Medicine, Utrecht University and the Hubrecht Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW).

List of Publications

Chen Chen, Bas Castelijns, Frank G. van Steenbeek, Peng Shang, Monique E. van Wolferen, Loes A. Oosterhoff, Louis C. Penning, Menno Creyghton, Niels Geijsen[#], Bart Spee[#]. Identification of transcription factors enhancing maturation of hepatocyte-like cells. Submitted.

Chen Chen, Iris Pla-Palacín, Pedro M. Baptista, Peng Shang, Loes A. Oosterhoff, Monique E. van Wolferen, Louis C. Penning, Niels Geijsen, Bart Spee[#]. Hepatocyte-like cells generated by direct reprogramming from murine somatic cells can repopulate decellularized livers. *Biotechnology and Bioengineering* 2018; 115:2807–2816

Chen Chen[#], Paulus G.M. Jochems, Lucia Salz, Kerstin Schneeberger, Louis C. Penning, Stan F. J. van de Graaf, Ulrich Beuers, Hans Clevers, Niels Geijsen, Rosalinde Masereeuw, Bart Spee. Bioengineered bile ducts recapitulate key cholangiocyte functions. *Biofabrication* 2018; 10:034103.

Chen Chen*, Alejandro Soto-Gutierrez*, Pedro M. Baptista, Bart Spee[#]. Biotechnology challenges to in vitro maturation of hepatic stem cells. *Gastroenterology* 2018; 154:1258–1272.

Guo-Dong Ye*, Guang-Bin Sun*, Peng Jiao*, **Chen Chen***, Qing-Feng Liu, Xiao-Li Huang, Rui Zhang, Wang-Yu Cai, Sheng-Nan Li, Jia-Fa Wu, Yun-Jia Liu, Rong-Si Wu, Yuan-Yuan Xie, Err-Cheng Chan, Yih-Cherng Liou, and Bo-An Li[#]. OVOL2, an inhibitor of WNT signaling, reduces invasive activities of human and mouse cancer cells and is down-regulated in human colorectal tumors. *Gastroenterology* 2016; 150:659–671.

Sathidpak Nantasanti, Bart Spee, Hedwig S. Kruitwagen, **Chen Chen**, Niels Geijsen, Loes A. Oosterhoff, Monique E. van Wolferen, Nicolas Pelaez, Hille Fieten, Richard W. Wubbolts, Guy C. Grinwis, Jefferson Chan, Meritxell Huch, Robert R.G. Vries, Hans Clevers, Alain de Bruin, Jan Rothuizen, Louis C. Penning, Baukje A. Schotanus[#]. Disease modeling and gene therapy of copper storage disease in canine hepatic organoids. *Stem Cell Reports* 2015; 5(5), 895–907.

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