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Modelling Disease with inducible Pluripotent Stem Cells

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Abstract.

Understanding the physiopathology of disease remains an essential step for the development of novel therapeutics. Although animal models have certainly contributed to advance this enterprise, their limitations to model all the aspects of complex human disorders is one of the major challenge faced by the biomedical research field. hiPSCs-derived from patients represent a great opportunity to overcome this deficiency since it could cover the genetic diversity needed to fully model human diseases. Here, we provided on overview of the history of hiPSCs technology and discuss common challenges and approaches that we and others have faced when using hiPSCs to modelling disease. Our emphasis is on liver disease, and consequently we review the progress of the technology to produce functional liver cells in vitro and how these systems are being used to recapitulate a diversity of developmental, metabolic, genetic, and infectious liver disorders.

INTRODUCTION

Genetics plays a key role in most common disorders. While it is estimated that there are over 10.000 different monogenic diseases affecting millions of people worldwide (1), most complex disorders involve genetic variants in multiple genes, which either trigger or increase susceptibility to disease in specific environments (2-4). Importantly, this genetic variation is known to determine whether, when, and to what extent a disease varies from person to person. This individual variability results in inconsistent and inefficient treatment strategies (5). Thus, understanding the molecular mechanisms beyond disease penetrance induced by genetic diversity

is now becoming essential for the development of personalized therapeutics. Nevertheless, the lack of suitable human experimental models has limited the study of these mechanisms. Indeed, animal models, although valuable, do not always recapitulate human pathophysiology and therefore cannot be used to elucidate detailed molecular mechanisms underlying human illness (5) especially when genetics is implicated. Therefore, generating human disease models recapitulating pathological events observed in patients is a priority.

Human induced pluripotent stem cells (6) provide new tools for disease modeling. As their in vivo counterparts (human embryonic stem cells or hESCs) (6), hiPSCs possess the ability to self-renew almost endlessly in vitro while maintaining the capacity to differentiate into virtually any cell of the human body. However, unlike hESCs, hiPSCs can be rapidly generated from patients´ biopsies providing researchers with a limitless source of patient-specific material that can be used for generating specific cell types targeted by a disease (Figure 1). Furthermore, hiPSCs also overcome the ethical drawbacks associated with the generation of hESCs from human embryos while offering an amenable system model to investigate the role of particular mutations/genes on cellular phenotype. Moreover, hiPSCs can allow the assessment of the influence of individual genetic backgrounds on the severity of a cellular phenotype related to disease (7-9). Thus hiPSCs offer an invaluable window into the complex genetic interplays underlying a pathological state, especially those related to congenital or developmental disorders (10-15). In addition, single cell analysis (16), NGS analysis (17), live imaging (18, 19), loss/gain of function experiments (20, 21), and large-scale genetic (22, 23) and chemical screenings (24-27) have enhanced our ability to study large number of molecular mechanism which are difficult to approach using model organisms. Finally, hiPSCs offer the possibility of a reasonably fast

recapitulation in vitro of pathological phenotypes, which in vivo may need up to several decades to manifest (Cancer, Alzheimer, Diabetes, among others).

It is now clear that the unique characteristics of hiPSCs enable to model a diversity of disorders but also to define the role of genetic variants in disease onset and penetrance. This knowledge will be extremely useful to predict disease risk at an individual level and to design personalized therapeutics preventing the development of pathological conditions. While this is the ultimate goal, additional technological challenges must be addressed before fulfilling the clinical promises of hiPSCs. In this review, we discuss the most relevant advances made in the field of disease modeling with hiPSCs, with particular emphasis on liver diseases and also the future technological development which could accelerate clinical development.

HISTORICAL POINT OF VIEW: DISCOVERY AND DEVELOPMENT OF IPSCS

The Train of Thoughts

Seminal studies carried out by Sir John Gurdon at the beginning of the 1960s represent the first examples of successful cellular reprogramming. By developing the technique of somatic cell nuclear transfer (SCNT), Gurdon and colleagues demonstrated that the information contained in the nucleus of differentiated cells could be reprogrammed after transplanting them into enucleated oocytes. This new hybrid cell was pluripotent and capable of progressing normally throughout embryonic development, contributing to the formation of all tissue types until forming adult individuals (28, 29). These observations established that the nucleus of a differentiated cell is genetically not different than their embryonic counterpart, and also suggested that molecular factors present in the embryonic environment are sufficient to reprogram somatic cells into an embryonic pluripotent state. Approximately four decades later, James Thompson established a

method to isolate and expand in vitro pluripotent cells from human blastocysts (30). With their capability to self-renew almost indefinitely and their potential to differentiated into virtually every cell of the body, these human embryonic stem cells (hESCs) revolutionized the biomedicine and developmental biology fields. Despite these unique characteristics, ethical concerns around the utilization of human embryos during the derivation of hESCs, have limited the clinical applications of these stem cells (31). The federal funding ban executed by the US government during the early 2000s to limit the generation of new hESCs lines is one example of a legal limitation motivated by ethical concerns associated with the production of hESCs (32, 33). Consequently, the possibility to generate pluripotent stem cells equivalent to hESCs from somatic cells using nuclear reprogramming has always been an attractive option. Proof of concept for such approach was provided by forcing the fusion of somatic cells with ESCs (34, 35). These studies confirmed that factors present in pluripotent cells were sufficient to reset somatic identity back to the pluripotent state. It was no longer after these discoveries that professor Shinya Yamanaka and Kazutoshi Takahashi reported for the first time the reprogramming of mouse fibroblasts into cells with characteristics of embryonic stem cells. These "induced pluripotent stem cells" (iPSCs) were generated by resetting the transcriptional and epigenetic program of somatic cells by overexpressing a set of transcription factors previously known for their role in pluripotency (36). Although the initial attempts required the overexpression of 24 transcription factors, Yamanaka and colleagues quickly narrowed this list to four factors: Oct3/4 (Pou5F1) and Sox2, two critical components of the core pluripotency transcriptional network (37, 38); the Krüppel-like factor 4 (KLF4), recognized for its potential to inhibiting p53-dependent repression of Nanog during differentiation, and posteriorly, for its capability to induce LIF-independent self-renewal of mouse ESCs (39); and lastly, the transcription factor cMyc, a well-known enhancer of cell proliferation

and transformation (40). Among these factors, Oct3/4, Sox2 and Klf4 were necessary for the reprogramming process, while cMyc only improved its efficiency (41).

Mouse iPSCs are indistinguishable from mESCs in terms of morphology, gene expression, teratoma formation capacity, proliferation, and the ability of differentiation into cells of the three germ layers (36). This extraordinary discovery prompted Yamanaka and other to reproduce these findings in human cells and only one year later, both Yamanaka´s and James Thompson´s groups reported almost simultaneously the generation of the first human induced pluripotent cell lines (41, 42). Interestingly, Yamanaka´s team accomplished this by using the same cocktail of transcription factors used for reprogramming mouse cells, while Thompson and colleagues generated hiPSCs by overexpressing a partially different set of factors (OCT4, SOX2, NANOG, and LIN28) (41, 42). Thomson and colleagues showed that NANOG and LIN28 were beneficial for the reprogramming process of human cells by increasing the survival of nascent hiPSCs, although LIN28 was not essential (41, 42). More importantly, they demonstrated that hiPSCs could be generated without overexpressing cMyc. This observation proved to be valuable since cMyc can induce malignant transformation (43). Currently, the Yamanka factors remain widely used, although the original cocktail of factors have been modified multiple times to increase efficacy of reprogramming often with inconsistent success (44). In addition, many modifications have been introduced to improve the delivery of the reprogramming factors such as integration-free methods (44). Episomal vectors, Sendai viruses, and synthetic mRNAs are among the most utilized methods to generate hiPSCs (45-47) without modifications of the host genome which could interfere with disease modeling or experimental outcomes.

hiPSCs as an Alternative to ESCs for Disease Modeling.

hiPSCs and hESCs display telomerase activity; specific cell surface antigens such as SSEA-3, SSEA-4, Tra-1-60, and Tra-1-81; they show similar patterns of DNA methylation on gene promoters; and can differentiate into derivatives of all three primary germ layers (41, 42, 48). Furthermore, recent analyses of gene expression patterns at the single cell level confirmed previous observation that hiPSCs and hESCs are closely related to the pluripotent stem cells of the post-implantation epiblast (49-51). Nonetheless, hiPSCs have unique advantages over hESCs. Not only do they circumvent the use of embryos, but they also allow researchers to generate patient-specific pluripotent cells that can be utilized for the study in vitro of a range of biological phenomena. These include developmental disorders; the role of genetic background on cell differentiation and disease; as well as the derivation of patient-specific platforms for drug screenings (5, 44, 52-54).

This extraordinary potential obligates constant scrutiny of the hiPSCs technology, and elements such as genomic instability and potential of differentiation are continually being evaluated (55). Indeed, as with hESCs, there has been an active debate regarding the preference of some hiPSC lines to differentiate into specific lineages. Particular attention has been given to the reprogramming process, ever since initial observations revealed that specific epigenetic signatures from the donor cells could resist the reprogramming process, contributing to the formation of limited transcriptional and epigenetic aberrations in hiPSC lines (56-60). It was suggested that this aberrant epigenetic profile could act as an "epigenetic memory" which could increase the capacity of specific hiPSCs lines to differentiate towards lineages close to their parental cells (6, 57-59, 61). Although this hypothesis has not been completely ruled out, the fact that the "epigenetic memory" tends to disappear in cells cultured for extended periods, indicates that this phenomenon may be transient and probably dependent on the method of reprogramming and culture conditions (56, 59). Interestingly, recent findings obtained by comparing genetically matched hESC and hiPSC lines

142 revealed that hPSCs are also similar in terms of transcriptome, DNA-methylome, and capacity to
143 differentiate into cells of three germ layers (48). This and other studies have reinforced the view
144 that rather than "epigenetic memory", differences in genetic background are the primary source of
145 variability in differentiation potential (48, 61-63). Accordingly, it has been recently shown, by
146 mapping expression quantitative trait loci (eQTL) and changes in copy-number variations (CNVs),
147 that divergent gene expression profiles and differentiation efficiency observed among hiPSCs lines
148 are strongly associated with changes in their genetic background (7, 22, 63). Of note, a recent
149 report showed that very few (3 out of 64) hiPSCs lines derived from different donors display
150 chromosomal abnormalities (64) suggesting that genetic instability is unlikely to affect hiPSCs
151 capacity of differentiation in vitro. Overall, the available data support the view that hiPSCs are
152 very similar to hESCs, and that most of the gene expression and epigenetic variations are
153 consequence of genetic differences amongst donors.

154 These findings are of high relevance for disease modeling. Although it is currently unclear
155 whether genetic background outweighs environmental factors, it is possible that hiPSC lines of
156 different genetic backgrounds might respond differently to extracellular cues. Accordingly, it is
157 anticipated that improvements to current protocols of differentiation will increase differentiation
158 efficiency and homogeneity of hiPSCs of different genetic background. In that context, hIPSCs
159 will represent an excellent opportunity to assess the effect of genetic background on cellular
160 phenotype during cell differentiation and disease. Nonetheless, observations made with hPSCs
161 needs to consider the impact of divergent genetic background on self-renewal and differentiation.

162 **MODELING DISEASES WITH HIPSCS**

163 In vitro disease modeling relies on the availability of specific cell types which are targeted by the

disease and display disease phenotypes. Primary cells represent the ideal solution as they are directly representative of the original tissues and organs. However, primary cells are often difficult to obtain especially from diseased tissues and they cannot be grown in vitro without losing their functional characteristics (65). Thus, the development of disease models based on human primary cells remains a very difficult enterprise despite a broad number of efforts.

hiPSCs have provided a unique opportunity to fill up this gap since they can be used to produce an almost infinite quantity of primary like cells in vitro from a diversity of patients. However, the key challenge has been to generate functional mature cells from hiPSCs. Accordingly, intense research has been carried out during the last decade to advance differentiation protocols and improve the functionality of hiPSCs-derived cells so they could resemble primary cells (32, 44, 66). Among the different approaches used to reach this objective, we can cite the combination, concentration, and timing of cytokines and growth factors used during the induction of differentiation (41-48), as well as the composition and density of extracellular substrates, spatial organization, and co-culture with other cell types (67-71). In their current state of development, however, cell types derived from hiPSCs in vitro still exhibit immature/fetal phenotypes (70, 71). Therefore, biological responses obtained from these cells need to be carefully considered in view of their fetal nature. Nonetheless, the interest of hiPSCs-derived cells for modeling adult disease has been clearly demonstrated for a number of tissues thereby establishing that these cells can be used to characterize disease phenotypes especially in the context of monogenic diseases, as well as the molecular mechanisms of response to drugs (72). Of note, an increasing number of disease-specific hiPSCs are generated on daily basis from patients afflicted with a variety of genetically inherited and complex disorders affecting virtually every organ (73). Extensive coverage has been given to those related to neurological and cardiac diseases (8, 52, 74), and therefore they will not

be described here. Instead, in this review, we will discuss some of the most significant findings regarding the use of hiPSCs to model liver diseases, which inflict a significant burden on healthcare and urgently call for effective therapies.

Modeling Liver Disease

The liver is a multifunctional organ that plays a crucial role in human physiology. It works as storage site for vitamins, minerals, and glycogen; detoxifies alcohol and drugs; synthesizes plasma proteins such as albumin and clotting factors; and produces bile necessary for lipids' digestion, among others (75). The Liver´s main functional cell type is the hepatocytes, which represent 70-80% of the organ´s mass. Hepatocyte polarization and interaction with non-parenchymal cells is essential for their proper function. Accordingly, they interact directly with sinusoidal endothelial cells through their basolateral surface, which facilitates communication between the parenchyma and the bloodstream. At the apical surface, tight junction formation between hepatocytes is required for canaliculus formation and bile acid transport (76). The liver can be attacked by a diversity of disease or injuries which can result in acute organ failure or chronic damage. In this last process, the liver gradually loses its natural organization due to inflammation and fibrosis which ultimately lead to cirrhosis (65). Liver disease constitutes a leading cause of death worldwide, and liver transplantation remains the only available therapy for end-stage liver failure (77). Hence, understanding the disease pathogenesis is not only crucial for developing new therapies, but also for improving diagnosis and prognosis.

Hepatocytes rapidly lose their metabolic activity in vitro (76) while they can only be obtained through invasive methods which are risky and often not well tolerated by patients. This aspect and their phenotypic instability drastically limit the use of primary hepatocytes to model

liver disease in a patient-specific way. Consequently, hiPSCs-derived liver cells represent an opportunity to overcome these limitations. Although producing hiPSCs-derived liver cells that can recapitulate the physiological responses observed in vivo has proven to be challenging (12, 70, 78, 79), hiPSCs-derived liver cells have already been used to model diverse aspects of the liver pathophysiology and this review will discuss some of these applications.

Current Approaches for Generation of Hepatocyte Cells from hiPSCs

To be useful for disease modeling, hiPSCs-derived hepatocytes or Hepatocyte Like Cells (HLCs) need to recapitulate the functional activities displayed by their native counterparts including the expression of hepatic markers, the acquisition of specialized structures, and specific enzymatic activity. Several groups have established protocols for directing the differentiation of hiPSCs toward HLCs and the most successful follow fundamental stages of embryonic development, such as the formation of definitive endoderm, foregut, hepatic endoderm, bipotential hepatoblasts, and hepatocyte-like cells (76, 80). Accordingly, our lab established a protocol for the direct generation of HLCs which mimics liver development in vitro. This approach involves the use of a chemically defined medium, Activin A, fibroblast growth factor 2 (FGF2), transient stimulation of the Wnt pathway, bone morphogenetic protein 4 (BMP4), and phosphoinositide 3-kinase (PI3K) inhibitor to derive definitive endoderm cells. Subsequently, the newly formed endoderm cells are grown in the presence of Activin A and B27 for induction of hepatic progenitors. Finally, maturation of the hepatic progenitors into HLCs is stimulated with hepatocyte growth factor (HGF) and Oncostatin-M (81) (Figure 2). After 25 days of differentiation, the resulting HLCs share several characteristics with primary hepatocytes: they display occasional binucleated cells, glycogen storage, apical micro-protrusions, and a prominent Golgi body (78, 81, 82). Furthermore, these cells express specific hepatocyte markers such as albumin, CK18, cytochrome P450 enzymes,

Asialoglycoprotein receptor 1 (ASGPR1), C/EBPa, and PROX1 (80). Although these observations reveal that HLCs recapitulate key features of their in vivo counterparts, the current consensus is that these cells are closer to fetal rather than adult hepatocytes (Table 1) (80). In this regard, Rashid et al. proposed that HLCs are likely to be located, from a developmental point of view, somewhere between the end of the first trimester of fetal embryonic development and adult hepatocytes (78). Consequently, HLCs exhibit the expression of the fetal markers alpha-fetoprotein (AFP) and CYP3A7 while activity of adult CYP such as CYP3A4 lag behind by several orders of magnitude in comparison to mature primary hepatocytes (52, 80, 82). Interestingly, despite this lack of complete maturation, HLCs are still able to execute hepatocyte-specific functions including: glycogen storage, bile transport, low-density lipoprotein (LDL) uptake, urea synthesis, secretion of albumin (Figure 3) and apolipoprotein B100 (ApoB100) to the extracellular medium, drug metabolism at low level, response to glucagon, and susceptibility to viral infection (76, 78, 82-84). Therefore, HLCs could be used for modeling disease in especially those concerning viral infections, lipids metabolism, and responses to hormones. However, it is important to acknowledge that in their current status, HLCs are not compatible for modeling drug toxicology or injury.

Hence, improvements to the current protocols of HLCs differentiation is a major focus and the current strategies include: co-culture with other hepatic cell types; media supplements to provide cell signaling and to compensate for metabolic changes occurring during differentiation; and specialization of the extracellular matrix. Regarding this last point, defined matrices containing either collagen, fibronectin, or vitronectin have already been used to generate HLCs effectively (80). Moreover, in recent publications, David Hay´s group reported that hiPSCs growing on Laminins could be differentiated into HLCs with very high efficiency (~90% of the cells expressed HNF4α). Interestingly, these cells demonstrated higher P450 (CYP3A) activity

than commercial frozen human primary hepatocytes or HCLs derived on Matrigel (85, 86). While this observation is of great interest, the performance of the HLCs generated using this approach remain limited when compare to freshly isolated primary hepatocytes (85, 86).

Additional efforts for improving functional maturation of HLCs have focused on reproducing the liver microenvironment more accurately (Figure 4). In one approach, researchers successfully established a three-dimensional (3D) protein-based scaffold that allows the derivation of HLCs in the presence of endothelial cells and human mesenchymal stem cells (hMSCs). Although it was reported that this method generates hepatoblast like cells and specific spatial organization, single-cell transcriptomic analyses revealed that these cells still retained their fetal status (70, 71, 79). Using a different approach, Gieseck et al. reported a method to culture HLCs using a 3D collagen-based scaffold that increased the maturation of the HLCs significantly. Accordingly, these HCLs showed improved functionality when compared to HLCs grown in 2D. Specifically, 3D-HLCs displayed higher rates of drug metabolism associated with increased cell polarization and bile canaliculi formation. Interestingly, these cells remained functional in vitro for over 75 days, which may be related to improved disposal of toxins by the polarized cells (87).

Although both of these systems represent steps forward for the development of 3D models of liver development and disease, further work is required to generate fully matured hepatocytes. It remains to be verified whether the inclusion of other non-parenchymal cells within the organoids could improve the levels of maturation, functionality, and organization of the HLCs within the organoids.

Cholangiocytes could represent the missing cell type since these biliary cells have tight functional interactions with hepatocytes. Indeed, these cells regulate bile homeostasis (12, 88),

modulate inflammatory responses and could have an essential function in liver regeneration (89, 90). Furthermore, cholangiocytes originate from the same bipotent embryonic progenitor that give rises to hepatocytes (89), which suggests a tight coordination during the formation of these two cell types in the embryonic liver. Interestingly, spatial organization of the hepatoblast during development is critical. Indeed, when the hepatoblasts localize near and around the portal vein, they create a monolayer of immature cholangiocyte progenitor called the ductal plate (91). After a series of morphogenetic rearrangements, these cells differentiate into tubular structures which ultimately form the bile ducts (92). The main functions of cholangiocytes are associated with absorption and secretion processes (91, 93). Accordingly, the polarization of these cells during differentiation is critical for reaching their maximum functionality (91). This polarization is difficult to reproduce accurately by cells organized in monolayer. Consequently, protocols that allow differentiation of hepatoblasts in 3D systems are the most effective methods to generate mature cholangiocyte-like cells (CLCs) (88, 91). Interestingly, Sampaziotis et al. recently reported a highly efficient method to generate hiPSCs-derived CLCs by using a protocol that mimics biliary development, including a final step of CLCs differentiation and functional maturation in 3D culture conditions (91). Importantly, these CLCs display functions specific to native cholangiocytes including the capacity to form branching tubular structures, the presence of primary cilia and expression of biliary markers such as CK7 (KRT7), CK19, HNF1B, GGT1, JAG1, NOTCH2, CFTR, SCR, SSTR2, AQP1, and AE2 at levels comparable to the ones found in primary cholangiocytes (88, 91). Furthermore, CLCs have the capacity to respond to acetylcholine and ATP stimuli, GGT and ALP activities, and the ability to transport bile acids. Finally, patient-specific CLCs can be used to model some aspects of polycystic and cystic fibrosis liver disease which are known to affect the biliary epithelium. Altogether, these observations show that CLCs

derived from hiPSCs could be used as a surrogate for primary cholangiocytes. However, as with HCLs, hiPSCs-derived CLCs have not reached a fully adult phenotype, and they still express the fetal biliary marker SOX9 (91). These results rise the question to whether CLCs also need to be surrounded by other liver cells in order to become fully mature in vitro. Hence, current efforts aim to reconstruct the cellular complexity of the liver using organoids technology. The resulting structure should include not only CLCs and HLCs but also Kupffer cells, stellate cells and endothelial cells to fully mimic the liver microenvironment.

Considered together, these studies demonstrate that the derivation of new and better 3D co-culture systems is feasible and could help to understand the physiopathology of a complex organ like the liver. Furthermore, to obtain in vitro–generated liver cells (hepatocytes and cholangiocytes) with maturation levels beyond the fetal stage, it will be important that new protocols of differentiation incorporate additional elements that would normally be faced by native hepatocytes during the postnatal period. These will include signals associated with the shift from placental to enteral nutrition, and gut microbiota content. This last aspect is of great importance since bacterially-derived secondary bile salts are involved in regulating the expression of cytochrome P450 isoforms, such as CYP2C9 and CYP3A4 (80).

hiPSCs-derived Hepatocytes for Modeling Infectious Disease of the Liver

A wide diversity of pathogens are known to attack the liver target especially hepatocytes. Among these pathogens, Hepatitis B virus (HBV), hepatitis C virus (HCV) and malaria are the most prevalent liver infectious agents, which combined account for more than 520 million cases of chronic liver disease worldwide (83). Primary hepatocytes represent the gold standard for studying the physiopathology of liver infection since transformed cell lines such as HEPG2 do not

support their life cycle. Thus, HLCs could provide a complementary platform to study mechanisms of infection, life cycle of the virus/parasite, as well as to find novel effective drugs. In this regard, HLCs have proven to be a successful in vitro system for modeling hepatitis virus infection and virus-host interactions. Indeed, it has been shown that hiPSCs acquire the expression of genes involved in hepatitis infection only after passing the definitive endoderm stage, as they differentiated towards HLCs. Notably, these HLCs are not only able to produce the viral RNAs and proteins following infection, but they can support the entire life cycle of the virus (83, 94). Furthermore, it seems that HLCs are capable of producing an appropriate antiviral response including interferon (IFN) production in response to inoculation with HCV (83, 94). Interestingly, HLCs can survive in vitro for up to a week after inoculation with HCV (83). This property offers an opportunity not only to examine the effects of relatively long-term infections on hepatocyte function but also to study in more detail the mechanisms that control permissiveness to viral infection, such as the up-regulation of micro-RNA-122 and suppression of antiviral gene IFN-induced transmembrane protein 1 (84).

Altogether these observations demonstrate that HLCs provide a promising platform to analyze hepatocyte responses to virus infection. However, some shortcomings must be addressed before major conclusions can be made. For example, Sakurai et al. reported that virus titers detected in culture supernatants of HBV-infected HLCs were much lower than those of primary human hepatocytes (PHHs). They attributed this result to the lack of functional maturation of the HLCs derived with current protocols of differentiation (83). Furthermore, it will also be essential to increase the diversity of hiPSCs lines used in these analyses, in order to assess the impact of the genetic background of host cells on the cellular response and efficiency of infection.

Inherited Metabolic Disorders

Almost 70 inherited metabolic disorders (IMDs) affecting the liver have been described. Although the incidence of IMDs is relatively rare, together they affect 1 in 1000 individual and remain a major cause of liver transplantation, particularly in children (10, 75, 77). One of the most common IMDs is α1-antitrypsin deficiency (A1ATD), an autosomal recessive disorder that affects 1 in 2000 individuals of North European descent. It results from a single point mutation in the A1AT gene (the Z allele; Glu342Lys) that causes protein aggregation specifically within hepatocytes. The accumulation of protein polymers induces hepatocytes cell death which ultimately causes cirrhosis (95). While researchers have been trying to model this disease for a long time, efforts involving the use of hiPSCs are relatively recent. Accordingly, a seminal study published in 2010 by Rashid et al., reported the generation of hiPSCs from patients with this pathology. Interestingly, they showed that these hiPSC lines were able to differentiate into HLCs that displayed key features of the cellular pathology, including accumulation of mutant α1-antitrypsin polymers in their endoplasmic reticulum (78). Importantly, in a more recent study, Tafaleng et al. used detailed microscopic and ultramicroscopic analysis to demonstrate that this system model recapitulates not only some of the biochemical features of the disease but also the morphological manifestations observed in patients. These included a delayed degradation and an abnormal accumulation of partially glycosylated A1AT protein in pre-Golgi compartments, the presence of dilated rough endoplasmic reticulum and globular inclusions partially covered with ribosomes. Interestingly, these abnormalities were not observed in HLCs derived from either wild-type donors or patients that only presented with the lung disease (13). These findings reaffirmed the notion that HLCs could be used to predict susceptibility and progression of the disease.

Glycogen storage disease type 1α (GSD1a) and familial hypercholesterolemia (FH) represent two other IMDs which have been modelled using hiPSCs (77, 96). GSD1a regroups

368 autosomal recessive metabolic disorders caused by a deficiency of glucose-6- phosphatase activity,
369 which catalyzes the hydrolysis of glucose-6-phosphate to glucose and phosphate, the final products
370 in gluconeogenesis and glycogenolysis. This pathology affects 1 out 100 000 individuals and is
371 associated with inability to maintain glucose homeostasis resulting in growth retardation,
372 hepatomegaly, lactic acidemia, and hyperlipidemia (97). Interestingly, GSD1a-hiPSCs derived
373 hepatocytes displayed higher levels of intracellular glycogen and lipids, which was concomitant
374 with elevated production of lactic acid, compared with hepatocytes generated from hiPSCs derived
375 from healthy individuals. Of note, these cells were also able to respond transcriptionally to
376 glucagon stimulation, demonstrating that the hepatocytes generated from the GSD1a-derived
377 hiPSC lines display some functionality related to lipids/glycogen metabolism and can response to
378 a key hormone of intermediary metabolism (78).

379 Familial hypercholesterolemia (FH) is an autosomal dominant dyslipidemia caused by
380 mutations in the LDL receptor gene (LDLR) that result in elevated levels of LDL-C in the plasma
381 and premature cardiovascular disease. As hepatocytes are the principal cells that control
382 cholesterol flux in the body, FH patients can be successfully treated with liver transplantation (11).
383 Accordingly, some of the pathological manifestations of FH can be readily recapitulated in vitro
384 by using patient-specific hiPSC-derived HLCs. Indeed, hiPSCs obtained from patients with FH
385 could be differentiated into HLCs that are incapable of incorporating LDL, even though they
386 displayed levels of differentiation and functionality comparable to wild type HLCs (78).
387 Furthermore, in a more recent report, Cayo et al. showed that hiPSCs derived from a patient with
388 cardiovascular disease were also able to produce HLCs. However, in addition to their incapacity
389 to uptake LDL, these HLCs were unable to respond to statin treatment, and displayed an
390 approximately eight-fold increase in the level of secreted apoB-100, compared with HLCs derived

391 from genetically independent control pluripotent stem cell lines (11). Together these observations
392 demonstrated that FH-derived hiPSCs could be used effectively to model diseases affecting lipid
393 uptake and storage in the liver.

394 The next step for the field is to demonstrate that complex liver diseases which are not
395 defined by single genes with Mendelian penetrance can be modeled in vitro using hiPSCs. Of
396 particular interest, hiPSCs derived HLCs could be used to better understand the molecular
397 mechanisms by which genetic variation influences quantitative phenotypic traits related to liver
398 function in humans. Accordingly, a recent report showed that population-based cohorts of hiPSC-
399 derived HLCs could be used to perform genome-wide mapping and validation of functional
400 variants and/or genes involved in metabolic functions related to the liver (8). These results confirm
401 that hiPSCs could indeed be useful to model complex liver diseases. Nonetheless, further studies
402 will be necessary to demonstrate that the results obtained are relevant in vivo and, in this context,
403 animal models remain necessary to provide complementary validations.

404 **Hepatic Organoids for Modeling Developmental Liver Disease**

405 Modeling diseases affecting liver development needs to consider that hepatocytes are not
406 generated in isolation during embryonic life. In this regard, the generation of complex hepatic
407 organoids from hiPSCs could represent a valuable tool to study developmental disorders affecting
408 the liver. Accordingly, a recent report showed the generation of complex HOs generated through
409 a process meant to recapitulate the natural path of liver development, including endoderm, foregut,
410 and hepatoblast differentiation. Importantly, the mRNA and protein expression patterns observed
411 during HOs formation are highly consistent with those patterns observed during liver development
412 in vivo. As an example, on day 3, the majority of the endoderm cells expressed SOX17 and

CXCR4. By day 6, the posterior foregut–like structures express FOXA3, and by day 9, the developing HOs expressed multiple markers of the hepatoblast stage such as TBX3 and AFP. Finally, HOs express the hepatocyte marker CK18, and the formation of luminal structures that resemble bile ducts, which were surrounded by cells expressing the cholangiocyte marker CK7. Overall, these data suggest that HOs are capable of recapitulating liver development. Consequently, HOs displayed many features that are typically observed in the liver in vivo, including the expression of the tight junction marker ZO-1, which is important for the formation of bile canaliculi; and primary cilia, commonly observed in primary cholangiocytes in vivo. Moreover, the HOs display biosynthetic and drug biotransformation properties characteristic of the human liver, with some level of CYP34A-dependent activity. Additionally, HOs contained cells that accumulated glycogen and also were capable of secreting albumin and several types of bile acids into the supernatant. Importantly, HOs displayed some capacity of self-renewal and thus could be expended in vitro (12). Considering all these properties, it was proposed that HOs could be used to study the impact of genetic mutations on human liver development. As a proof of principle, HOs were used to characterize the effect of different mutations in the JAG1 gene on biliary tract development. Mutations in the JAG1 gene cause the majority (~97%) of the cases of Alagille Syndrome (ALGS), an autosomal dominant genetic disorder where the NOTCH signaling pathway is severely impeded, causing defects in the intrahepatic biliary tree (12, 98-103). Consequently, hiPSCs derived from ALGS patients (ALGS-hiPSCs) formed HOs that developed normally through the hepatoblast stage, including the formation of HLCs. However, they did not contain cholangiocytes or bile ductular structures, neither they were capable of regenerating secondary organoids. Furthermore, HOs showed signs of intrahepatic cholestasis, and fibrosis, which could be explained by the reduced levels of RNA expression for several NOTCH signaling

436 components (JAG1, NOTCH2, HEY1) and cholangiocyte markers (CK7, CFTR). These results
437 showed that mutations in the JAG1 gene can influence not only the efficiency of HOs formation
438 but their ability to form duct-like structures containing cholangiocytes, as well as their capability
439 to transport bile and their capacity of regeneration (12).

440 Interestingly, not all mutations in the JAG1 genes were able to affect the formation of
441 normal HOs. Indeed, iPSCs generated from individuals carrying the Gly274Asp JAG1 mutation,
442 which is present in patients with cardiovascular defects but without any liver or bile duct
443 abnormalities, were able to produce HOs with the same morphology and efficiency as control
444 hiPSCs. Together these findings indicated that HOs can recapitulate with fidelity the phenotypes
445 dictated by different types of JAG1 mutations in vivo (12). It is worth mentioning that, so far, there
446 is no strong correlation between the type and location of the JAG1 mutation and the severity of
447 the disease. Accordingly, it has been suggested that additional genomic modifiers may be
448 responsible for the highly variable clinical manifestations that characterizes ALGS (98-103).

449 The preeminent thesis posits that ALGS is caused by haploinsufficiency of JAG1 on the
450 basis that individuals with whole gene deletions can have identical phenotypes to those with
451 intragenic mutations (99-104). However, it has also been reported that at least in vitro, mutant
452 JAG1 proteins could act through a dominant negative mechanism to inhibit Notch signaling (98-
453 103). Hence, it remains to be answered whether ALSG is consequence of haploinsufficiency or a
454 dominant-negative effect of the mutated JAG1 gene. In this regard, Guan et al. also showed that
455 unlike hiPSCs with a heterozygous ALGS mutation (ALGS-hiPSCs), hiPSC lines engineered to
456 carry a heterozygous JAG1 knockout were able to efficiently form intact liver organoids that
457 presented all the features displayed by control HOs. Interestingly, when ALGS-hiPSCs were
458 converted to haploinsufficient knockouts, these new hiPSC lines were able to form functional HOs.

These data strongly suggest that ALGS liver abnormalities are caused predominantly by a dominant-negative effect of the JAG1 gene mutation (12).

Overall, liver organoids seem to reproduce critical events of liver development and thus could be useful to uncover new insights into human developmental disorders such ALGS. This system could overcome some of the limitations of previous in vitro methods. Increasing the complexity of the organoids by incorporating other non-parenchymal cells could also further increase the spectrum of liver disease that can be modeled in vitro.

Nonalcoholic Fatty Liver Disease (NAFLD) and Nonalcoholic Steatohepatitis (NASH)

The first manifestation of nonalcoholic fatty liver disease (NAFLD) is the accumulation of lipids in hepatocytes (105, 106). The persistence of this condition triggers a plethora of alterations including mitochondrial dysfunction, endoplasmic reticulum stress, hepatic insulin resistance, and inflammatory response, which evolves toward nonalcoholic steatohepatitis (NASH) (106, 107). 29% of patients with NASH will progress to cirrhosis and a third of these will develop cancer (108). Consistent with this statistic, NAFLD/ NASH is the second most common cause of liver transplantation (107). However, predicting the evolution of the disease is currently impossible while diagnosis involves invasive methods such as liver biopsy which are not well tolerated by patients (106, 107). Modeling NAFLD/NASH y using HLCs could facilitate the identification and functional validations of biomarkers for prognosis, stratification and drug development. Accordingly, a first attempt to model NAFLD in vitro has been recently reported by Graffmann et al., who showed that HLCs could be used to model intracellular lipid accumulation (108). Furthermore, this study shows that the resulting HLCs display the biochemical alterations associated with steatosis including an upregulation of the lipid droplet coating protein Perilipin 2,

as well as of numerous genes of the peroxisome proliferator-activated receptor pathway (108). Thus, this HCL model can recapitulate some of the metabolic features of NAFLD. Although these results represent a step forward in modelling NAFLD/NASH, further development is required to recapitulate in full the human pathophysiology. Indeed, disease progression is linked with lipotoxicity and not only lipid accumulation. Furthermore, it involves a complex inflammatory response which can only be produced by immune cells such as macrophages. Hence, developing co-culture systems where hepatocytes interact with other non-parenchymal cells will help further improve the utility of HLCs in recapitulating the features of NAFLD/NASH.

CONCLUSIONS

Animal models have provided a vast amount of knowledge concerning diseases and have helped to develop most of the drugs currently available. However, they clearly have limitations for modelling human complex disorders especially when genetic diversity is involved. Consequently, hiPSCs-derived from patients could deliver a novel tool to model human diseases, and their genetic mechanisms. Accordingly, hiPSCs are already used to generate cells of virtually every organ of the body and the resulting cells have been used to model a diversity of diseases. However, only a handful of studies have used hiPSCs-derived disease models to actually uncover new mechanisms of disease or to identify new drugs. The technical requirement for such work, and the lack of standardization in cell culture systems, explain in part this relatively slow progress. Furthermore, data generated from an in vitro model needs to be validated, ideally using clinical information from individual patients. Those comparative studies are time and resource consuming. Finally, a lack of functional maturation may also limit the direct relevance of cells generated from hiPSCs. However, protocols are progressing rapidly and new approaches are constantly being developed to improve

hepatocyte functional maturation levels, differentiation consistency, reproducibility, and scale-up conditions. In parallel, increasingly complex models for human tissues and organs are being engineered. The hope is that tissue organoids containing more than one cell type, embedded into 3D conditions mimicking organ architecture will improve the function of hiPSCs-derived cells. The careful selection of control cell lines and development of hiPSC line cohorts derived from multiple genetic backgrounds will also increase reproducibility and interpretation of the data obtained from hiPSCs. Ultimately, these improvements will not only transform our capacity to study the impact of genetics on disease onset but will also allow the development of truly personalized medicine.

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879 Figure Legends

880 Figure 1. Schematic Representation of the Potential Use of PSCs in Biomedicine. PSCs can be
881 generated either by isolating them from the inner cell mass (ICM) of human embryos or by
882 reprogramming somatic cells through overexpression of a small set of transcription factors. Using
883 cocktails of cytokines and growth factors that mimic the natural paths of development, the
884 derivatives of the three primary germ layers—ectoderm, mesoderm and endoderm— are generated
885 and subsequently differentiated into a diverse number of somatic cells. By choosing the right
886 controls, PSCs can be utilized to study phenotypes associated with disease and to perform drug
887 screening assays.

888 Figure 2. (A) Timeline of HLCs Differentiation and its Relationship with Early Human Embryonic
889 Developments. PSCs are equivalent to the pluripotent cells from the epiblast (Carnegie Stage 5,
890 Day 7-12 post fertilization). Definitive Endoderm (DE) cells are very efficiently generated upon
891 induction of PSCs differentiation using a define medium containing Activin A, BMP4, FGF2, and
892 WNT3A. This process involves a series of morphogenetic changes that resembles DE formation
893 during gastrulation. Specification of Foregut and Hepatoblast cells is favored by the stimulation
894 DE cells with high levels of Activin A and B27. HLCs formation and maturation is stimulated by
895 Oncostatin M and HGF. (B) Overview of the Gut Endoderm formed During Early Mouse
896 Embryonic Development. CS: Carnegie Stage; A (Anterior), P (Posterior), V (Ventral), D (Dorsal),
897 FG (Foregut), MG (Midgut), HG (Hindgut). Days on top panel represent age post fertilization.
898 Days on bottom panel represent days of in vitro differentiation. All embryonic stages are based on
899 data from the Carnegie Collection (https://embryology.med.unsw.edu.au/embryology/index.php/

900 Carnegie_Collection), which collects data from the Human Development Anatomy Center (USA)

and the Carnegie Institute (USA). Also, from New Insights into Early Human Development: Lessons for Stem Cell Derivation and Differentiation (114), and from Developmental Anatomy of the Human Embryo – 3D-Imaging and Analytical Techniques (115).

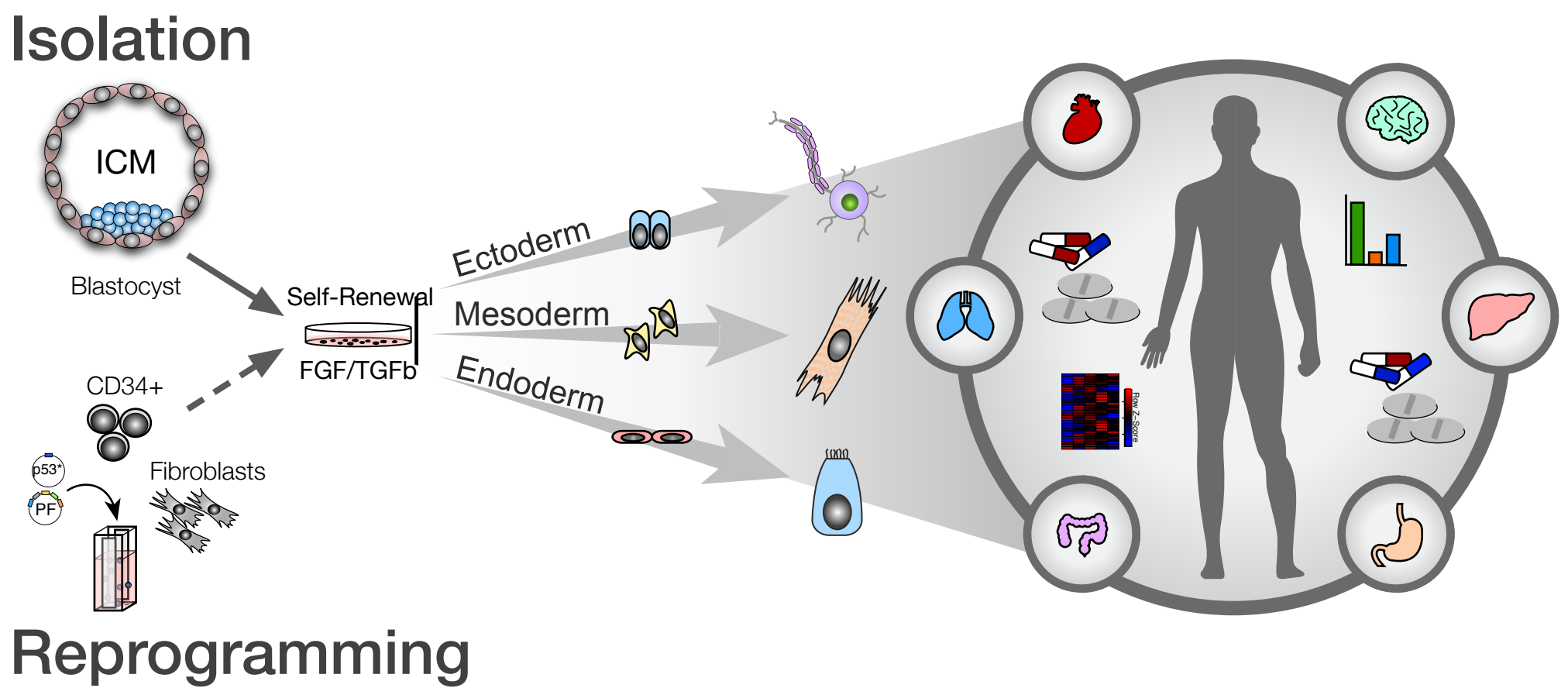
Figure 3. Characterization of hiPSC-derived HLCs. Immunofluorescence depicting the presence and localization of Albumin (green) and HNF4a (86) in 30-day differentiated hepatocyte-like cells from hiPSCs using the protocol described in Hannah et al 2012. Nuclei were counterstained with DAPI (blue).

Figure 4. Schematic representation of the adult liver (8) with the gallbladder, common bile duct and network of intra-hepatic bile ducts (green) [left panel]. Yellow boxes depict examples of diseases affecting either the liver of the bile duct network. The liver consists of several hepatic lobules, which are formed by sheets of hepatocytes (8) surrounded by a network of sinusoids (blue), lined by endothelial cells [middle and left panels]. Oxygenated and nutrient-rich blood flows through the sinusoids from the portal triad (composed by the portal vein, hepatic artery and biliary duct) towards the central vein at the centre of the lobule, allowing the exchange of metabolites between the blood and the hepatocytes. Hepatocytes produce bile which is secreted into bile canaliculi (green) and transported through the bile ducts (lined by cholangiocytes) into the gallbladder. In addition, hepatic stellate cells reside in the space of Disse, between the hepatocytes and the sinusoids; and Kupffer cells, liver's resident macrophages, reside in the liver sinusoids.

Table 1. Functional properties of hepatocyte-like cells (HLCs) derived from hiPSCs.

Table 1: Functional properties of hepatocyte-like cells (HLCs) derived from hiPSCs

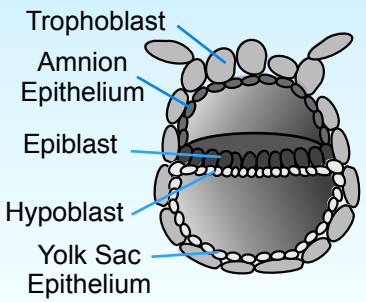
Functional Feature	Adult Hepatocytes	HLCs	Reference
Binucleation	Yes	Yes	78, 81, 83, 84, 86, 89, 91, 100
Albumin expression/secretion	Yes	Yes	70, 78, 81, 83, 84, 86, 87, 88, 89, 90, 91, 100
a1AT expression/secretion	Yes	Yes	78, 81, 83, 86, 87, 88
AFP expression/secretion	No	Yes	70, 78, 81, 83, 84, 86, 87, 88
Urea production	Yes	Yes	83, 100
Glycogen storage	Yes	Yes	78, 83, 86, 87, 88, 91, 100
Indocyanine green uptake	Yes	Yes	81, 100
LDL uptake	Yes	Yes	78, 100
Bile canaliculi formation	Yes	Yes	90, 91
CYP3A4 expression/activity	High	Low	78, 81, 83, 84, 87, 88, 89, 91, 100
CYP3A7 expression/activity	Low	High	70, 81, 91, 100
EPCAM expression	No	Yes	89
CK18 expression	Yes	Yes	70, 78, 83, 89
HNF4a expression	Yes	Yes	70, 84, 85, 88, 89, 90, 91, 100
Susceptible to viral infection	Yes	Yes	85, 86


Isolation
ICM
Blastocyst
Self-Renewal
FGF/TGFb
CD34+
p53*
PF
Fibroblasts
Ectoderm
Mesoderm
Endoderm
Row Z-Score
Reprogramming

A

Embryonic Development

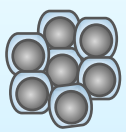
Implantation



CS 5
Day 7-12

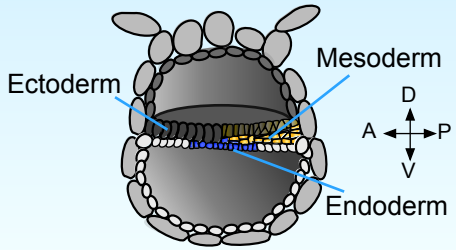
FGF2
TGFb

Day 3



PSCs

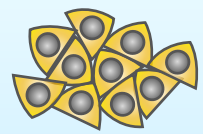
Gastrulation



CS 6-7
Day 13-17

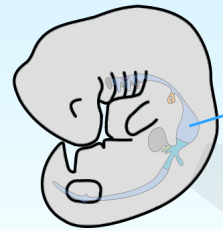
FGF2
ACTIVIN A
WNT3A
BMP4

Day 4-8



Definitive Endoderm

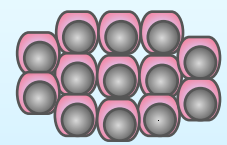
Organogenesis



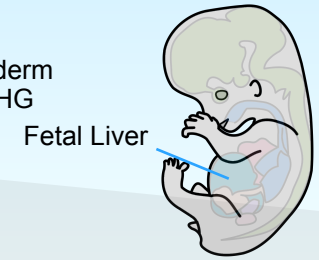
CS 12-15
Day 26-38

ACTIVIN A
B27

Day 9-12



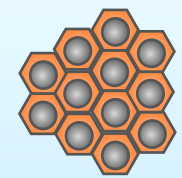
Foregut Endoderm



CS 23
Day 60

ONCOSTATIN M
HGF

Day 13-30



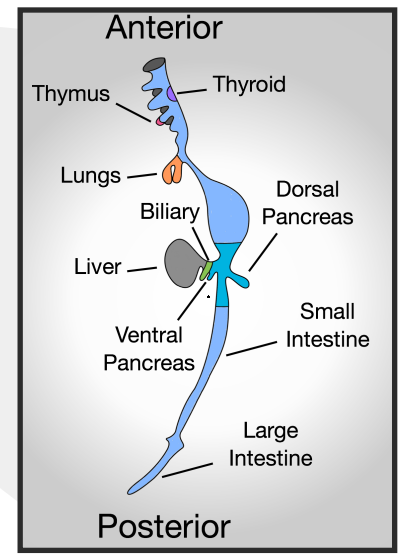
Hepatoblasts

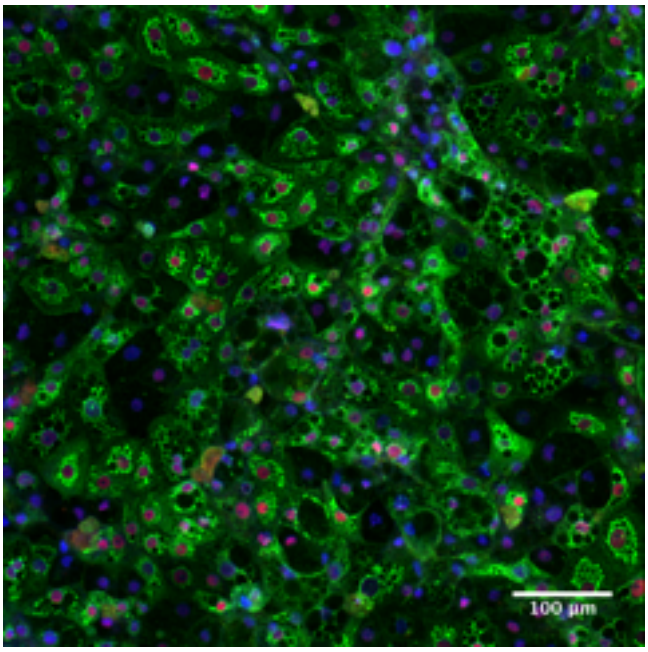
HLCs

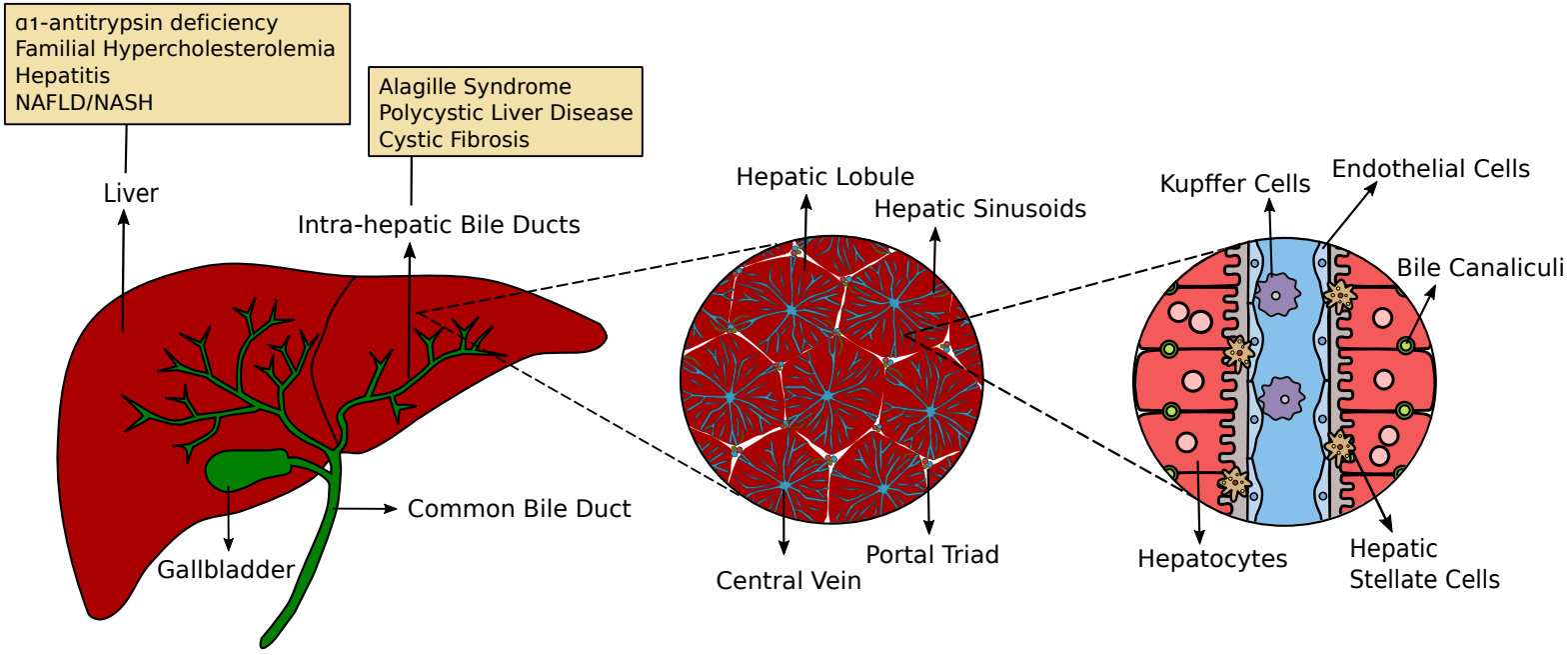
In Vitro Differentiation

B

Derivatives from Gut Endoderm




100 µm


α1-antitrypsin deficiency
Familial Hypercholesterolemia
Hepatitis
NAFLD/NASH
Alagille Syndrome
Polycystic Liver Disease
Cystic Fibrosis
Liver
Intra-hepatic Bile Ducts
Common Bile Duct
Gallbladder
Hepatic Lobule
Hepatic Sinusoids
Central Vein
Portal Triad
Kupffer Cells
Endothelial Cells
Bile Canaliculi
Hepatocytes
Hepatic
Stellate Cells