Aspects of Molecular Medicine 2 (2023) 100025

Contents lists available at ScienceDirect

Aspects of Molecular Medicine

journal homepage: www.journals.elsevier.com/aspects-of-molecular-medicine

Isogenic hiPSC-derived liver-on-chip platforms: A valuable tool for modeling metabolic liver diseases

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ARTICLE INFO

Keywords:
Liver
hiPSC
Organ-on-chip
Inflammation
Fibrosis

ABSTRACT

The liver is a complex organ with vital functions in metabolism, detoxification, and immunity. The anatomy, physiology, and cellular composition of the liver are crucial for comprehending its spatial heterogeneity and regulation of homeostasis. Hepatocytes, liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs), and liver macrophages play pivotal roles in liver function and pathology. Liver diseases such as NAFLD, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), and Liver fibrosis are prevalent and pose significant health challenges. Studying liver development provides insights into liver model evolution, including differentiation protocols for human hepatocyte-like cells (HLCs), hiPSC -derived endothelial cells, stellate cells and macrophages.

Currently, the research landscape of liver tissue models encompasses in vivo and in vitro approaches, including 2D and 3D liver cell culture methods, on-chip systems, and patient-derived hiPSC-based liver disease models. The integration of hiPSCs with micro-physiological systems holds promise for recapitulating liver function and disease in vitro. However, challenges remain in achieving the physiological relevance and scalability of liver models. Advances in the research landscape of liver tissue models are discussed, providing insights into identifying individual patient groups, the current status quo, and prospects of liver research at the interface of developmental biology, tissue engineering, and disease modeling which will allow conclusions to be drawn about the molecular mechanisms of liver diseases and ultimately the targeted use of suitable therapeutics.

1. General overview

1.1. The liver

The liver represents the largest organ of the human body (Davies et al., 2020). It is essential for the metabolism of carbohydrates, proteins, and lipids, as well as for the clearance of toxins and pathogens. The wide range of different biotransformation processes is primarily executed by hepatocytes, the main cell population of the liver. Depending on their localization in the periportal, mid-, and pericentral zones of the hepatic lobules, hepatocytes are exposed to various biophysical and biochemical cues, creating a complex microenvironment that leads to cell fate and functional determination (Trefts et al., 2017; Kietzmann, 2017). Hepatocytes and cholangiocytes (i.e., liver parenchymal cells) are surrounded by a network of non-parenchymal cells (e.g., liver macrophages – the so-called Kupffer cells (KCs) –, liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells (HSCs)) in the hepatic sinusoid which contributes to and regulate metabolic activities and immunological responses (Tricot et al., 2022). As a share of the dynamic balance, cellular interactions between different types of liver cells influence homeostasis and metabolic functions through multiple mechanisms. Due to this, perturbations in the hepatic sinusoid balance influence disease outcomes in the liver and neighboring organs (Gibert-Ramos et al., 2021). In the following section, we describe the anatomy and physiology of the liver.

1.2. Anatomy and physiology of the liver

Anatomically, the liver is located below the diaphragm and occupies most of the upper right quadrant of the abdomen (Abdel-Misih et al., 2010). The superior posterior aspect of the liver contains an open space where the diaphragm and the inferior vena cava are located. The rest of the liver is covered by the visceral peritoneum which meets the diaphragm at the border of the bare area forming the coronary ligament (Vernon et al., 2021).

The liver is divided into two main lobes - a larger right lobe and a smaller left lobe, separated by the falciform ligament (Abdel-Misih et al., 2010). Each functional lobe can be subdivided into its respective sectors

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https://doi.org/10.1016/j.amolm.2023.100025
Received 14 June 2023; Received in revised form 31 August 2023; Accepted 16 October 2023
Available online 17 October 2023

by the hepatic veins that traverse the liver. The liver lobes are microscopically divided into hexagonal shaped hepatic lobes consisting of polygonal hepatocytes that surround the central vein.

Within each interface of the hexagonal lobule is located the portal triad which is bundled in connective tissues and consists of two afferent vessels, the hepatic artery and the hepatic portal vein, as well as the intrahepatic bile duct. The hepatic artery and the liver portal vein carry out the liver blood supply. Here, the hepatic artery delivers oxygenated blood originating from the systemic circulation, while deoxygenated, nutrient-rich blood from the intestine is provided by the hepatic portal vein (Trefts et al., 2017). Moreover, the liver is not vascularized like most other organs of the human body, but by sinusoid capillaries that surround the different hepatic cells (Trefts et al., 2017; Wake et al., 2015). During the movement of blood through the liver, liver cells perform a variety of functions. The primary functions of the liver are defined based on vascular, immunological, metabolic, secretory, and excretory functions (Mitra et al., 2009).

Vascular function: In a healthy person, the liver holds 10%–15% of the total blood volume of the body. It can act as a storehouse of blood for systemic circulation once the peripheral circulation is overloaded. The liver circulation can release blood into the systemic circulation when the body loses blood for any reason (Maestro et al., 2021).

Immunological function: The liver is a highly immune-active organ that mediates local immunoregulatory activity, as well as systemic barrier functions (Cheng et al., 2021). Here, the main contributing cell population is represented by the KCs which perform the immunological function of macrophages and constitute a component of the body's phagocytic system, thereby serving as mediators of inflammation and immune response (Bennett et al., 2021). Furthermore, approximately 50% of the body's circulating lymph is produced by the liver (Tanaka et al., 2016).

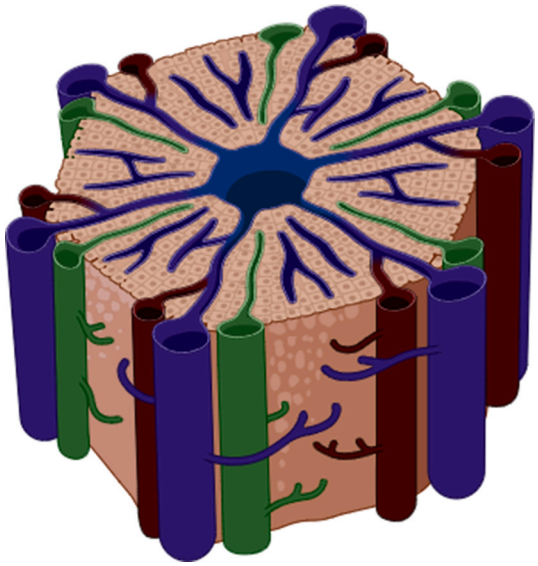

Fig. 1. Architecture of the hepatic sinusoid (created from biorender.com).

Metabolic function: Hepatocytes are the main cell population executing the metabolic function of the liver. They are involved in the metabolization of carbohydrates, proteins, fats, and the biotransformation of endogenous compounds such as steroids, heme, or bilirubin (Schulze et al., 2019). Furthermore, maintaining blood glucose levels while fasting is one of the crucial tasks performed by the liver (Mitra et al., 2009; Alamri, 2018).

Secretory function: The liver produces bile components like water, bile salts, bilirubin, electrolytes, and cholesterol and mediates the subsequent dissemination of those components into the intestinal lumen. Here, the biliary fluid assists in the absorption and digestion of fat-soluble fat-soluble dietary vitamins, as well as the uptake of cholesterol and vitamin K (Hundt et al., 2023).

Excretory function: The detoxification and elimination of drugs and other xenobiotics (Kulsharova et al., 2021; Khoury et al., 2015) represents another vital function of the liver (Trefts et al., 2017). By converting lipophiles into more water-soluble metabolites, these substances are excreted more efficiently via urine (Grant, 1991). Furthermore, bile is excreted through the bile duct as it flows in the opposite direction toward the portal triad located near the hepatic portal vein and hepatic artery. Transport of nutrients and metabolic waste products between blood arteries and liver cells is enabled by the fenestrated endothelial cell layer found in the sinusoid hepatic capillaries (Trefts et al., 2017) (Refer to Fig. 1).

1.3. Spatial heterogeneity and gatekeeping functions of the liver

Numerous organs and tissues exhibit spatial variability along their major axis of blood flow or depth (Kang et al., 2018). With two major blood sources, the liver represents one of those organs. Arterial and venous blood mix within the hepatic sinusoids that were mentioned beforehand (a network of specialized capillary beds in the liver). The blood ultimately drains into the systemic circulation in the center of the hepatic lobules through the liver vein, thereby creating spatial heterogeneity in the liver. This heterogeneity includes cell morphology, gene expression patterns that regulate different functions, and most importantly the modulation of metabolic activities both in health and disease (Kang et al., 2018). Rather than only differences in cellular composition (i.e., ratios of different hepatic cell types), these metabolic activity patterns can be observed between the same cell type (i.e., hepatocytes) at different locations. This variability can be caused by dynamic gradients of modulators, such as nutrients, oxygen, cytokines, and other signaling molecules. A more dynamic pattern of tissue behavior might

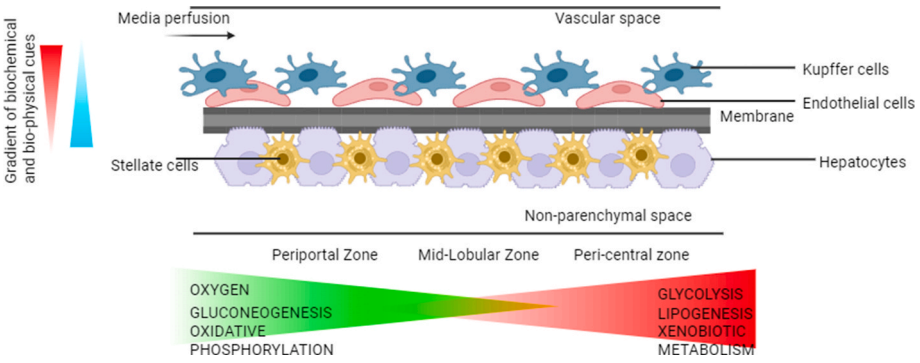

Fig. 2. Different liver cell types and Metabolic pattering in the hepatic sinusoid (left side) design of the liver model composed of non-parenchymal and parenchymal cells cultured in a micro-physiological environment created by biophysical and biochemical gradients. (Blue and Red arrows indicate dynamic cues) Image created in biorender. com). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

be caused leading to the non-uniformly partitioning of cellular functions (Trefts et al., 2017). Furthermore, signaling events during developmental processes can induce stable gene expression and metabolic patterns in liver tissue that help stabilize functional partitioning (Kietzmann, 2017; Cunningham et al., 2021). This phenomenon is known as metabolic zonation and enables the distinction of three different liver zones: the periportal, pericentral, and midlobular zone (Trefts et al., 2017). (Refer to Fig. 2). However, metabolic zones are more discrete than liver zones, which are more flexible with regard to the functional attributes of the cells located in those zones (Trefts et al., 2017). The term zonation refers to the functional heterogeneity (hepatocyte) along the porto-central axis (Kietzmann, 2017), while metabolic zonation describes the spatial compartmentalization of metabolic processes within the functional liver lobules (Martini et al., 2023). Landmark zonated genes are used to distinguish the specific metabolic zones not only spatially but also spatiotemporally, the latter is orchestrated by the circadian clock (Martini et al., 2023). Due to this, periportal, pericentral and midlobular hepatocytes perform different metabolic functions along the porto-central metabolic zonation axis to optimize liver function as shown in Fig. 2 (Martini et al., 2023). Maintaining liver homeostasis while inducing a differentiated phenotype involves an interplay of pathways. These pathways include the Wnt/β catenin, Hedgehod pathway (Hh) and the hypoxia-inducible pathway (HIF). Of significant importance is the Wnt/βeta-catenin signaling as it is a major regulator of hepatic zonation (Wild et al., 2020). Approximately one third of the liver zonation genes represent targets of the Wnt pathway. The most important transmembrane proteins that spatiotemporally modulate the Wnt/βeta-catenin signaling gradient in the liver are the Wnt R-spondin (RSPO) ligands, the repeat-containing repeat-containing G protein-coupled receptors 4–6 (LGR4-6), zinc ring finger 3 (ZNF3) and its homolog ring finger 43 (RNF43) (Sun et al., 2021).

The interdependence of intracellular and extracellular signaling pathways facilitates the construction of a dynamic physical microenvironment that allows cells to communicate with one another and with their surroundings. It has been shown that Wnt/β-catenin signaling and the Hh pathway can be modulated by the HIF system (Kolbe et al., 2019). The non-parenchymal cells such as cholangiocytes and HSCs are primarily found in the portal tract and periportal area, respectively. These cells secrete Hh signals that, in turn, are transported in the peri-venous direction, thereby creating an oxygen gradient. Hh signaling partially suppresses Wnt/βeta-catenin signaling (Martini et al., 2023). Conversely, the low oxygen content in the peri-venous zone activates the HIF system, induces LGR5 expression, and activates β-catenin as well as suppresses the expression of the negative β-catenin regulator adenomatous polyposis coli (APC). RSPOs secreted from the central vein endothelial cells, activate β-catenin via LGR5 in perivenous hepatocytes. To maintain homeostasis, hypoxia activates the expression of Hh components in hepatocytes to feedback inhibit β-catenin.

Protocols facilitating the directed differentiation of induced pluripotent stem cells (iPSCs) to liver-fate reflect this fact with the administration of Wnt/beta-catenin activators Wnt family member 3a (Wnt3a) and Activin A or Glycogen Synthase Kinase 3 (GSK3) inhibitors CHIR9021 have been established (Asumda et al., 2018; Du et al., 2018). Furthermore, provided that cellular gene expression and, hence, cellular phenotype alter in a zone-dependent manner in the context of disease, therapies geared toward "zonal switching" of cell state and phenotype may open a broad range of novel precision medicine-based approaches to treat patients with liver disease.

2. Metabolic liver diseases

In the last 50 years, the research of pathologists and hepatologists has led to increased knowledge about many non-neoplastic liver diseases; among them are NAFLD, NASH, and liver fibrosis which both can be considered as a liver manifestation of the metabolic syndrome (Torbenson et al., 2020; Hashimoto et al., 2013). Furthermore, the prevalence of NAFLD, NASH and associated comorbidities is increasing worldwide, eg, diabetes, obesity and dyslipidemia, necessitating the need for an ideal liver model that can simulate the major aspects of these diseases (Hashimoto et al., 2013). In the following, a short overview of NAFLD, NASH, and liver fibrosis will be provided, and its main drivers will be provided as depicted in Fig. 3.

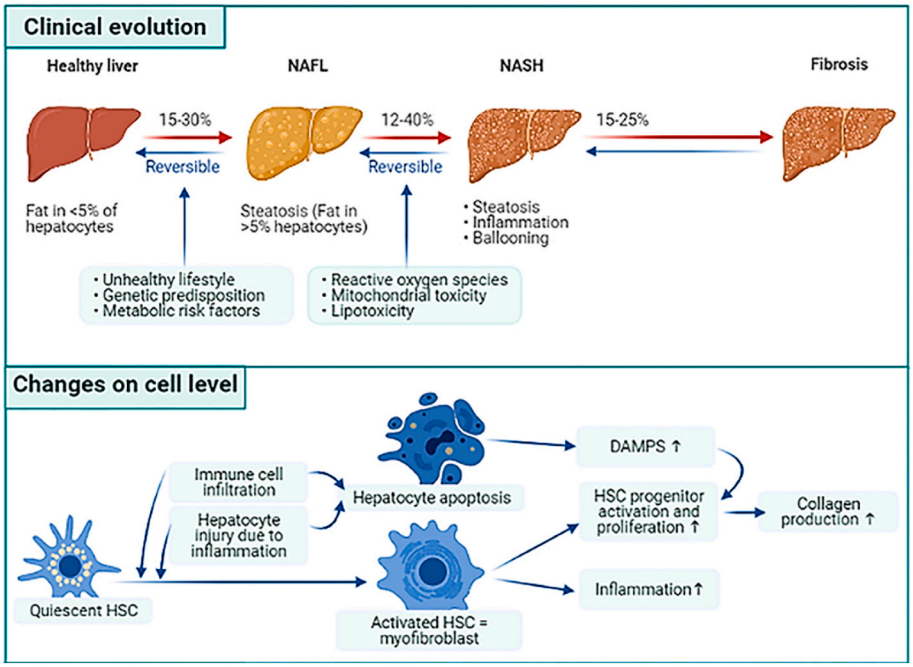

Fig. 3. Overview of the evolution of NAFLD-related fibrogenesis at the clinical and cellular level. (NAFLD manifests clinically as simple steatosis (NAFL). Inflammation is caused by abnormal accumulation of lipid droplets that invade immune cells and secrete cytokines. This is known as nonalcoholic steatohepatitis (NASH), which can lead to hepatic fibrosis. On the cellular level, quiescent cells (HSCs) are activated by immune cell infiltration and hepatocyte injury due to inflammation. The activated HSC transdifferentiates into collagen-producing myofibroblasts, and furthermore the myofibroblasts trigger HSC progenitor proliferation and activation. Figure adapted from Leen Heyens) (Heyens et al., 2021) Free to re-use by copyright CCC license.

2.1. NAFLD

NAFLD represents a spectrum of liver pathologies and can be subdivided into two clinical manifestations, simple steatosis and NASH (Hashimoto et al., 2013; Michelotti et al., 2013). The hallmark of NAFLD is the accumulation of trigylcerides within hepatocytes, that is, steatosis, which is seen in association with the main risk factors, such as obesity and metabolic syndrome (Michelotti et al., 2013). NAFLD can culminate in end-stage liver disease, cancer, and even require liver transplantation (Eslam et al., 2018), once it progresses to NASH, while simple steatosis is primarily a benign and asymptomatic clinical entity that accounts for 80–90% of cases of NAFLD (Hashimoto et al., 2013). A combination of genetic factors is involved in the cause of the disease (Eslam et al., 2018). Four genetic variants in four different genes associated with liver lipid metabolism have been linked to the development and progression of NAFLD (Eslam et al., 2018): 1. The patatin-like phospholipase domain that contains the substitution of protein 3 (PNPLA3) isoleucine for methionine at position 148, I148 M is the most robust and well-replicated genetic factor; 2. the substitution of 2 glutamic acid from the member of the lysine transmembrane 6 superfamily at position 167; 3. Genetic variant of the membrane-bound O-acyltransferase domain containing 7, and 4. variations at the locus of the glucokinase regulator gene (Eslam et al., 2018; Ouchi et al., 2019). These genetic factors increase the probability of fibrosis, NAFLD, and the fibrosis phenotype in patients (Eslam et al., 2018).

Treatment with NAFLD/NASH is of great importance due to its increasing prevalence and progressive potential (Muthiah et al., 2020). To date, the Food and Drug Administration (FDA) has not approved drugs, making current drug therapy options limited (Muthiah et al., 2020). Targeting one of the main causes of NAFLD could represent a possible way to prevent or improve the condition. Here, lifestyle interventions, for example, increased physical activity, caloric restriction, and pharmacological interventions, for example, the use of insulin sensitizers, cholesterol lowering drugs, and nuclear transcription factor modulators, could be applicable measures (Muthiah et al., 2020).

2.2. NASH

NASH is a condition that represents a progressed form of NAFLD developed only by a subset of patients with NAFLD (Michelotti et al., 2013). So far, there have been no reliable tests to differentiate between these two conditions. However, 10–20% of patients with NAFLD are suggested to develop NASH (Hashimoto et al., 2013). Once NAFLD has progressed to NASH, a more serious form of liver damage, the incidence of cirrhosis, liver cancer, and liver disease-related death increases strongly (Michelotti et al., 2013). The phenotype of NASH disease is accompanied by excessive steatosis (compare NAFLD), inflammation, injury, ballooning, and hepatocyte degeneration, indicating activation of the apoptotic pathway 103]. Thus, NASH is considered an individual pathological condition (Hashimoto et al., 2013). Genetic and environmental factors that cause and favor NASH are the same as those of NAFLD (compare 1.6.2). Unlike NAFLD, which manifests itself with the main characteristic of simple steatosis, NASH is integrated into a multisystemic disorder (Muthiah et al., 2020). Additional organs such as the kidney, heart, and vascular system are simultaneously affected and can precede or even promote diabetes, obesity, dyslipidemia, and therefore metabolic syndrome (Hashimoto et al., 2013; Muthiah et al., 2020). Therefore, a vicious cycle of disease development, maintenance, and progression could be caused.

Treatment strategies for NAFLD/NASH are limited, as mentioned. To date, the slowest advancements in therapeutic options have been reported compared to, for example, the field of diagnosis and epidemiological studies (Muthiah et al., 2020). Therefore, the challenge of discovering and evaluating therapeutic options for the treatment of NAFLD/NASH is currently under intense investigation, accompanied by an increasing need for human cell-based liver models.

2.3. Hepatic fibrosis

Hepatic fibrosis is an aberrant wound repair response induced by a variety of chronic liver injuries characterized by excessive deposition of diffuse extracellular matrix (ECM) and abnormal hyperplasia of connective tissue and can progress to liver cirrhosis, liver failure, or liver cancer (Friedman, 2003). Although early liver fibrosis can be reversible, due to the balance of pro- and antifibrosis pathways in short-term liver damage, liver fibrosis will not develop. However, when long-term or chronic liver injury occurs, the membrane of the hepatocytes is destroyed, releasing DAMPs, which are damage-associated molecular patterns that directly induce the activation of quiescent hepatic stellate cells (HSCs) (Liedtke et al., 2022). The imbalance between the pro-fibrosis and antifibrosis pathways (Tacke et al., 2012) destroys the structure of liver tissue and normal physiological function, ultimately leading to liver fibrosis. Furthermore, activated HSCs exhibit enhanced contractility, high levels of alpha smooth muscle actin (αSMA), and release cytokines such as transforming growth factor β 1 (TGFβ-1), platelet-derived growth factor (PDGF) (Tilg et al., 2021) and connective tissue growth factor (CTGF). The specific process of reversing liver fibrosis remains unknown, and an effective treatment for liver fibrosis is required. As a result, it is still a major priority for invitro-disease models in antifibrosis drug research development.

3. Liver development as a guide for the evolution of liver models

3.1. Developmental biology of the liver

During gastrulation, liver development is characterized by reciprocal tissue-tissue interactions between the endoderm and the mesoderm (Zorn, 2008). Until now, key genes and signaling pathways that orchestrate development processes have been identified during different stages of liver development and the generation of functional liver tissue (Zorn, 2008; Gordillo et al., 2015; Zorn et al., 2007; Si-Tayeb et al., 2010). The endoderm, the innermost of the three germ layers, is the origin of the epithelial lining of the digestive and respiratory tract and associated organs such as the lung and liver. On the contrary, the surrounding mesoderm represents the origin of organs such as the kidney and heart, as well as muscle, bones, and the vascular system, while the ectodermal derivatives include the central nervous system and skin (Zorn et al., 2007).

The application of knowledge about liver development stages and signaling pathways has allowed the generation of cells with a liver-like phenotype from embryonic stem cells (ESC) or hiPSC (Refer to Fig. 3) (Asumda et al., 2018; Zorn, 2008; Gordillo et al., 2015). In-vitro models based on hiPSC-derived hepatocyte-like cells and non-parenchyma liver cells have great potential not only to test possible therapeutic measures but also to advance regenerative medicine and genome editing techniques for the correction of disease specific mutation (Asumda et al., 2018; Zorn, 2008). This is further discussed in Section (4.0).

3.2. The rationale for liver differentiation protocols

Researchers have discovered that somatic cells harvested from specific individuals can be reprogrammed into human iPSCs (hiPSCs), and human embryonic stem cells (hESCs), offering a new and potentially limitless source of human cells (Takahashi et al., 2006, 2007). (Refer to Fig. 4). hiPSCs are increasingly used to generate liver models. These models are often based on protocols developed first for monolayer differentiation or forward reprogramming with additional use of ECM and/or suspension culture to achieve 3D growth (Harrison et al., 2021). One reason to differentiate hiPSCs is to emulate the developmental route of the desired cell type. According to developmental research, different types of liver cells gradually commit to one fate or another, starting from a bipotent phenotype (Gordillo et al., 2015). Hence, the development of different progenitor cell lineages is often influenced by the same signaling pathways in a different cellular or temporal context (Gordillo et al., 2015). However, the production of the required cell type can be

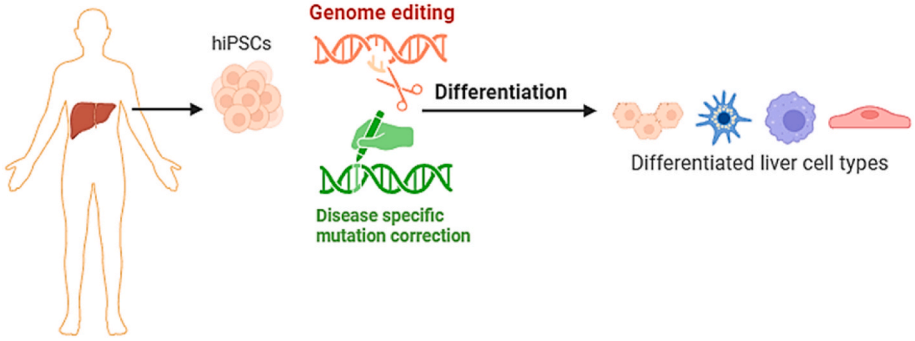

Fig. 4. Generation of hiPSC, their application for disease-specific correction and specification of the germ layer (Figure made from biorender.com).

divided into discrete stages, although it is a continuous process distinguished by distinct expression patterns. Using this information, we can mimic these spatiotemporal stages in vitro, by adding growth factors, which has proven to be an effective process as evidenced by the stepwise differentiation of different types of liver cells (Agarwal et al., 2008). In the following section, the development of the hiPSCs-derived liver cell types will be briefly introduced.

3.3. *hiPSC-derived endothelial cells*

Endothelial cells have been generated from hiPSCs using multiple differentiation procedures. Classically, differentiation protocols for a given cell type deploy sequential growth factor and cytokine cocktails to mimic in vivo differentiation. Only a few studies have reported the derivation of LSEC-like cells from mouse pluripotent stem cells (mPSC) and hiPSC (Koui et al., 2017; Gage et al., 2020). More importantly, relatively little is known regarding the development of LSEC and, hence, the signals required for LSEC differentiation in vitro. LSECs have been hypothesized to be derived from the septum transversum mesenchyme, which is a mass of mesenchymal tissue required for liver specification. (Gouysse et al., 2002). LSECs have also been proposed to originate from various types of cells, including endodermal cells, cells derived from the sinus venosus, cells of large vessels adjacent to the developing liver bud, GATA binding protein 4 (GATA4) positive liver progenitor cells, and hemangioblasts (Gage et al., 2020). However, genetic lineage tracing has recently been shown to identify the endocardial origin of liver vasculature (Zhang et al., 2016, 2021). The production of LSECs derived from hiPSCs will offer a useful approach for investigating the biology of this significant class of liver cells.

3.4. *hiPSC-derived stellate cells*

Hepatic stellate cells (HSCs) play a critical role in the evolution of liver fibrosis by generating extracellular matrices. The inaccessibility of human quiescent HSCs (qHSCs) and a suitable in vitro model that properly reproduces HSC activation has impeded the discovery of medicines to inhibit liver fibrosis (Koui et al., 2021). The embryonic origin of HSCs is a topic of debate, even if their mesodermal origin has gained wide acceptance (Asahina et al., 2011). In contrast to hiPSC-HLC, differentiation protocols, only a few HSC differentiation protocols have been published 127]. The main sources of HSCs now used are primary cells acquired from human liver resections and commercial cell lines (LX-2). However, they have significant drawbacks, such as the difficulty of isolation and expansion and, for cell lines, an overactivated phenotype (Herrmann et al., 2007; Xu et al., 2005; Perea et al., 2015; Sancho-Bru et al., 2005). The main advantage of iHSCs is that they can be expanded extensively, allowing the generation of a large number of progeny for high-throughput in vitro assays, and they can also maintain their quiescence phenotype. Furthermore, the acquisition of HSCs from multiple different patient-specific iPSCs can enable their use in personalized medicine strategies.

3.5. *hiPSC-derived macrophages*

Monocytes and macrophages can be derived from human peripheral blood mononuclear cells (PBMCs) in various biological research fields. Despite the relative ease of the isolation process, it is frequently challenging to obtain large, high-quality cell batches from patient donors outside of specialist research facilities, particularly in the case of rare disease phenotypes. Additionally, there is the issue of donor-to-donor variability and problems usually associated with Human leukocyte antigen matching especially in transplantation studies. (Cao et al., 2020).

Multiple studies have been reported in which hiPSCs are fated to the monocyte/macrophage lineage (Lyadova et al., 2021). Furthermore, resident cells can condition IPSDM to acquire tissue-specific characteristics in vitro. Despite this progress, only one study from Tasnim et al. has shown that hiPSCs can differentiate into cells with characteristics similar to those of KC. hiPSCs can be differentiated into erythroid and myeloid progenitor cells with features that leads to the generation of monocytes and macrophages using a previously adapted protocol. The characterization methods and functional assays described would be applicable to modeling inflammatory diseases in 2D and 3D liver models. (Cao et al., 2020).

3.6. *Research landscape liver tissue models*

Liver tissue models are and have been frequently used to study liver development, regeneration, and diseases, as well as drug toxicity and metabolism (Thompson et al., 2021). As with the knowledge gained, animal liver models lack the ability to obtain human translatable experimental results, in vivo research on the liver organ and, especially, in vitro liver models emerged to perform experiments based on human cells (Thompson et al., 2021; Gupta et al., 2021).

3.6.1. *In vivo research on the liver organ*

The utility of liver cell types is hampered by the overarching shortage of primary material, as high-quality liver tissue is reserved for transplantation purposes whenever possible, leaving less-than-ideal samples for research usage. This is exacerbated by recent developments, such as robotic surgery, that have limited the pool of viable tissue available for further study and transplantation (Messner et al., 2019). Furthermore, in vivo human studies at mechanistic levels are associated with unacceptable health risks, which is why most of our knowledge about liver tissue comes from clinical observation and genome-wide association studies (GWAS). Given the severe global burden of liver-associated diseases, obtaining detailed mechanistic insight into liver pathophysiology is of great importance for prevention studies and the development of therapeutic interventions (Paik et al., 2022).

However, by comparison, animal models allow for more flexibility with respect to experimental interventions and mechanistic investigations. Hence, a great part of our current understanding of the liver organ lead back to the research on animal models. To study liver-associated diseases such as NAFLD and NASH, the concept of "multiple

hits" is widely recognized (Tilg et al., 2021; Peng et al., 2020). To better understand the patho-mechanisms of NAFLD, a variety of rodent models have been developed: Linden et al. developed a mouse model of human PNPLA3^{I148M} knock-in mutation to study NAFLD, the ob/ob mouse as a monogenic model of NAFLD lacking leptin on a protein level (Abe et al., 2019) and the high-fat- diet/high-choline-deficient/carbon tetrachloride HFD/HCD + CCl4 model. The HFD/HCD + CCl4 model, a polygenic model, resembles NAFLD more closely than monogenic models (Tsuchida et al., 2018). However, despite the humanization efforts of rodent models, in many cases, conclusions from animal models cannot be translated into human biology. Especially in terms of metabolism and immunology, differences between species make it difficult to fully mimic human pathophysiology in clinical findings (Reimer et al., 2020).

4. Diagnostic application of gene editing functions in patient-derived liver hiPSCs

An advantage of employing hiPSCs is that the genetic background of the patient can be mirrored in the organoids. Patient-specific iPSC cells derived from patients with liver disease can be used to introduce disease-relevant mutations for use in models that recapitulate a conditions' pathophysiology and progression (Pournasr et al., 2017). The development of genome editing tools e.g. zinc finger nucleases (ZFN), transcription-activator-like effector nucleases (TALENS), and the discovery of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated system (CRISPR-CAS) have allowed the creation of genetically corrected hiPSCs for the study of monogenic diseases (Tilson et al., 2021). hiPSCs and liver organoids can now be genetically modified and studied using gene perturbation techniques such as clustered regularly interspaced short palindromic repeat-CRISPR-associated 9 (CRISPR-Cas9) or CRISPRa/i screening (Dastidar et al., 2018). The most prevalent type of genetic variation among humans is single nucleotide polymorphisms, also referred to as SNIPs. The I148 M mutation in the PNPLA3 gene has been identified as the predominant genetic variable associated with an increased risk of developing NAFLD/NASH in a patient. This single nucleotide polymorphism (SNP) is localized in a diacylglyceride lipase and is known to play a role in liver fat metabolism and triacylglyceride accumulation through regulation of X-box binding protein-1 expression under stress of the endoplasmic reticulum (ER) (Ouchi et al., 2019). Patients who carry the SNP have a 2-fold greater hepatic fat accumulation and are 10-fold more likely to develop from NAFLD to liver cancer (Pournasr et al., 2017). Hepatocyte-like cells generated from human induced pluripotent stem cells (hiPSCs) were utilized by Tilson et al. to investigate the roles of patatin-like phospholipase domain containing 3 (PNPLA3) in lipid build-up and toxicity. They created isogenic hiPSC lines with the I148 M variant or a complete PNPLA3 deletion. The hiPSCs that resulted were subsequently differentiated into hepatocyte-like cells and cultured in the presence of oleic acid (OA) and palmitic acid to assess the intracellular accumulation of lipids and lipotoxicity. The lipid accumulation was exacerbated by the mutant hiPSC phenotypes. They were able to demonstrate that PNPLA3 mutations decrease triglyceride lipase activity, which appears to provide protection against fatty acid-induced lipotoxicity,. However, this model lacked a functional vasculature and the addition of other non/parenchymal liver cells (Tilson et al., 2021). Groger et al. recently revealed that co-culture of insulin-sensitive i-Heps with isogenic iPSC-derived pro-inflammatory macrophages increases glucose production by blocking insulin from suppressing gluconeogenesis and glycogenolysis. They discovered that TNF and IL1 are triggers of insulin resistance mediators in iPSC-Heps, which is indicative of specific effects on insulin signaling and glucose metabolism mediated by NF–B or JNK. Although they could demonstrate that accurate simulation of macrophage-induced hepatocyte inflammation required prolonged release and interaction of several cytokines as given by iPSC-Macrophage co-culture, however the model did not include other non-parenchymal liver cell types such as stellate cells and endothelial cells which are also to contribute to inflammation related processes in metabolic liver diseases (Groeger et al., 2023). Gurevich and colleagues have generated pure end-stage hepatocytes from iPSCs derived from a NASH patient. NASH hiPSC-hepatocytes spontaneously accumulated lipids in the absence of exogenous lipids (Gurevich et al., 2020). The authors reveal the maturation status of the cells and included other non-parenchyma cell types such as endothelial cells and stellate cells, though a functional vasculature was however lacking in this model. Furthermore, hiPSC produced by patients with enzyme deficiencies can be used directly to generate disease-specific organoids, allowing the investigation of inflammatory mechanisms that lead to a steatosis phenotype in these individuals (Ouchi et al., 2019). Proper controls for patient-derived iPSCs are critical during such studies. These control hiPSCs should share a single genetic background with patient-derived hiPSCs, which offers the possibility to eliminate biological noise, resulting from genetic heterogeneity between different hiPSC lines, and thus could serve as the gold standard control for their diseased counterparts (Segeritz et al., 2018). A patient-specific drug testing platform offers the advantage of facilitating drug efficacy and toxicity screening to optimize treatment programs with patient-specific pharmacotherapy (Choi et al., 2013). Another efficient technique for the generation of hiPSC heps was performed by Thomas Rute et al., They postulated that nuclear receptors might be the most promising options to enhance hepatocyte functionality in the differentiation culture system for liver disease modelling (Tomaz et al., 2022). They demonstrated that forward programming could provide a flexible substitute for the directed differentiation approach of in vitro generation of hepatocytes. Their findings indicate that the absence of numerous nuclear receptors is connected with the immature condition of HLCs, which raises the possibility that these elements may be required to promote the functional maturation of hepatocytes (Tomaz et al., 2022). Despite this huge progress, the phenotypic trait of the cell type established through forward programming techniques, and special suitable culture medium are still needed. Although autologous hiPSCs provide a valuable insight in allowing different cell types to be created with the same genetic origins, there are still challenges to be addressed. First, there are only a few iPSC lines currently available for genetic investigations, in which dozens or even hundreds of genetic backgrounds should be examined. The California Institute of Regenerative Medicine (CIRM) stem cell pool and some research groups have genotyped hiPSC from healthy and fatty liver disease patients, and this advantage can be leveraged to generate different liver cell types from healthy and diseased donors (Jamieson et al., 2018). However, the success of such networks will depend on the secure acquisition of long-term funding and clinical know-how, as well as the improvement of data and operational systems that will contribute to therapeutic practice standards and help guide future regenerative medicine initiatives in terms of liver diseases. Secondly, the maturation status of liver cells, especially in monoculture models, might not be fully sufficient to model certain aspects of liver diseases. Furthermore, environmental variables, such as lifestyle effects (such as nutrition, insulin resistance, smoking, and sedentarism), viral infection, and/or comorbidity-driven causes, frequently impact the chance of developing liver-associated diseases. These shortcomings can be solved by the integration of micro-physiological systems also known as microfluidic devices that allows for real time monitoring of biophysical and biochemical cues that would certainly improve the predictive power of in-vitro models.

5. On-chip systems

Currently, on-chip micro-physiological systems represent the best available tools for modelling the liver (Kanabekova et al., 2022). In terms of flexibility and scalability. The integration of a vascular component is of utmost importance regarding a physiologically relevant liver model considering its high metabolic activity. Organ-on-chip (OoC) technology offers the potential to integrate the missing part of more

precisely controlling spatio-temporal, physical, and chemical microenvironments. Furthermore, the metabolism and distribution of drugs are two areas where tissue-to-tissue crosstalk is crucial in physiology and disease. As a result, chambers for various tissue types are increasingly being included in microfluidic devices, which are linked by flowing culture media. Microfluidic, dynamic systems assist in overcoming the constraints of static cultivation models by increasing model complexity. One of the most significant advances is the elimination of waste products and metabolites, as well as the continuous delivery of nutrients, inside the liver.

5.1. OoC technology and state of the art

Microfluidic systems, also known as OoC systems, are made up of perfusable micrometer-sized chambers with porous ECM-coated membranes. This allows replication of tissue-tissue interactions and a more consistent level of tissue and organ functionality (Bhatia et al., 2014). The European Organ-on-Chip Society (EUROoCS) described an OoC as a multipurpose microfluidic device, containing living bioengineered tissue substructures in a controlled microenvironment, recapitulating one or more aspects of organ dynamics, functionality, and patho-physiological response in vivo under real-time monitoring (Mastrangeli et al., 2019). Furthermore, chip platforms can include and combine biophysical and biochemical gradients (e.g., oxygenation levels, pH) and mechanical forces (shear stress, tension and compression, that is, peristaltic movement) (Corral-Nájera et al., 2023). Tissue and organ-level physiology can be fully investigated by replicating minimal functional units rather than full organs, producing results that are more autologous and transferable to the in vivo situation in humans when compared to static in vitro culture systems (Wikswo, 2014). Furthermore, by connecting distinct functional organ units, such on-chip systems enable the inclusion of multiple cell types and even the combination of different functional organ units.

So far, many biomedical studies have relied on cell line-based liver-on-chip models: Although liver cell lines are inexpensive, standardizable and capable of proliferating indefinitely and while they are often animal or tumor derived, they do not resemble the liver organ-specific cell types. Liver primary cells, as an alternative, perform much better in mimicking desired phenotypes and functions on-chip platforms. However, these cells are most times non-proliferative and tough to source in limited cell quantities and by association, limited availability for large-scale experiments (Zeilinger et al., 2016).

Another promising alternative is the use of hiPSCs, which have the advantages of both cell lines (proliferation and standardization) and primary cells (human, morphological, and physiological function), which is a viable asset for OoC technology (Wnorowski et al., 2019). In addition to the cellular features, the mechanical qualities and chemical compositions of the scaffolds utilized on the chip should be similar to the original ECM. Because of their biocompatibility, the majority of polymeric materials employed are organically sourced. Natural hydrogels, while used in the clinic, have poor batch-to-batch consistency and mechanical properties, whereas synthetic polymers generally lack cell-binding site. Therefore, the modification of single-origin materials or the combination of diverse materials is emerging as a new approach to achieve the maximum benefit from liver-on-chip platforms (Sk et al., 2021; Ghahremanzadeh et al., 2021; Chen et al., 2022). The organ-on-chip technology allows for monitoring on multiple levels: (i) online monitoring entails a non-invasive real-time assessment of on-chip conditions obtained through optical interrogation of sensors (Fuchs et al., 2021), (ii) at-line measures are based on assessments of sample effluents extracted from the perfusion medium following interaction with the on-chip tissues (i.e. supernatants), and (iii) off-line measurements, which typically involve intrusive procedures. (Fuchs et al., 2022).

5.2. hiPSCs based micro-physiological systems of the liver

In recent years, biomimetic human LoC models have progressed from simpler 2D cell models to spheroids or organoids to satisfy the growing need to understand the mechanics of complicated liver diseases. Several hiPSC LoC models for co-culturing primary cells and hepatic cell lines have been developed, this technique has also been used in the field of hiPSC-hepatic progeny. Wang et al. differentiated the hepatic lineage in situ using hiPSC-derived embryoid bodies (EBs) in micropillar microfluidic devices. The resultant organoids were used to create HLCs and cholangiocyte-like cells. (Wang et al., 2018). Perfusion improved the expression of mature liver markers (albumin (ALB) and CYP3A4) and the functionality of HLC, including the hepatotoxic reaction to acetaminophen (APAP), in a dose- and time-dependent manner. Similar results were obtained by Leclerc et al., who discovered that the perfusion of immature HLCs on microfluidic biochips induced up-regulation of many pathways involved in cellular rearrangement, stress response, and drug metabolism. The Lee-Montiel group combined liver progeny with hiPSC and cardiac progenitor cells derived from the same hiPSC in a more sophisticated arrangement using a functionally integrated OoC system. As a result, the researchers discovered that ketoconazole inhibited the CYP3A4-induced conversion of cisapride to norcisapride, causing ventricular arrhythmias. (Lee-Montiel et al., 2020). Furthermore, investigators at the LiFEChip Consortium have also developed a liver islet fat platform to mimic obesity and diabetes. To promote insulin resistance in hepatocytes, the authors connected hiPSC-derived hepatocytes (hiPSCHep), adipocytes, and islets with circulating macrophages. This model represents one of the few MPS platforms that uses iHep to develop interorgan communication, thus recapitulating hepatic steatosis (Slaughter et al., 2021). Kostrzewski et al. recently disclosed a NAFLD/NASH MPS system based on PHH with nonparenchymal cells. The NAFLD/NASH phenotype was mimicked by perfusing a medium rich in fatty acids through the system, which was evidenced by intracellular lipid accumulation, the production of inflammatory and profibrotic markers, and a transcriptome profile such as NASH. The NAFLD/NASH phenotype was reversed by the therapeutic candidate Farnesoid X receptor (FXR) agonist obeticholic acid. (Kostrzewski et al., 2020). To model the course of the disease, the researchers also employed mutant PNPLA3 HSCs. Due to this, there are improvements in multicellular 3D hiPSC-Liver-on-chip cultures, such as longer culture durations, improved perfusion strategies and the ability to include patient-specific iHeps, harbouring PNPLA3 SNPs, as well as finding unique traits that are diagnostic or potentially predictive of NAFLD/-NASH in a broader population.

Despite all these improvements in the above models, there is currently no platform that integrates all the different parenchyma and non-parenchyma liver cell types from a single hiPSC donor line in a microfluidic platform. The integration of HLA matched immune cells in future models will provide valuable insight into the study of inflammation-associated liver diseases. Autologous hiPSC MPS devices will offer substitutes for animal research to represent the range of liver steatosis and will more quickly assess medications from non-clinical to clinical testing. This will ultimately pave the way for invitro preclinical LoC models, which could be used to investigate the molecular consequence of host genetic factors, altered cytokine release, liver disease-associated SNIPs, and environmental effects that lead to disordered lipid metabolism. Furthermore, short-chain fatty acids such as (acetate, propionate, and butyrate) and Bile acids (BA) have been strongly linked to the prevention of metabolic liver syndromes and diseases; for instance, dietary supplementation has been shown to protect and reverse liver abnormalities caused by a high-fat diet (Den Besten et al., 2015). However, because of conflicting results, ranging from protective effects to cytotoxicity, the role of microbe-derived products is still hotly debated at the genetic level. It has been suggested that this is exacerbated by the absence of a representative human liver model. BAs created from liver cholesterol, on the other hand, are conjugated to gut bacteria,

which produces structural variants in the microbiota that are linked to plasma triglycerides and can have a direct impact on host physiology. BAs have been identified as the main therapeutic target for metabolic liver diseases (Wang et al., 2021). A liver OoC platform would allow researchers in the future to investigate the GUT-LIVER axis by simulating the fatty liver phenotype using lipid stimuli that influence cell shape and gene expression linked to lipid metabolism.

6. Conclusion

In conclusion, studying liver development and using hiPSC-based liver models provide valuable insights into the evolution of liver models and disease modeling. The integration of hiPSCs with liver microphysiological systems, such as organ-on-chip technology, holds promise for advancing liver research in vitro. However, challenges remain in achieving the physiological relevance and scalability of liver models. Continued advancements in developmental biology, tissue engineering, and disease modeling are needed to improve the fidelity of liver models and enhance our understanding of liver function and pathology. Overall, the research landscape of liver tissue models is rapidly evolving and ongoing efforts in this field hold great potential to advance our understanding of liver biology, disease mechanisms, and therapeutic interventions. In principle, the evaluation metrics for an ideal LoC should include key readouts such as (optical readouts, biomarker analysis, evaluation of molecules secreted into effluent media, recapitulating the liver acinus (the inclusion of parenchyma (Hepatocytes) and Non-parenchyma (Stellate cells, macrophages, and endothelial cells) in a co-culture model and determining its predictive power in terms of disease and PKPD modeling for drug testing). Using this toolbox, a variety of hepatic tissue readouts should be validated: long term (>1 month) viability and functionality on the chip (LoC); Furthermore, functional metabolism of liver enzymes (albumin), uptake and release of retinol and free fatty acids, and the activation of hepatic stellate cell; cytokine secretion profile of immune cells in response to inflammatory stimuli (LoC) must be key caveats that an invitro-liver model should possess. In general, these models highlight that tissue function is not just the sum of contributions from individual cell types but is the result of synergistic interactions occurring in the complex interplay of all different cells in tissue.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

I would love to acknowledge and thank Dennis Torkornoo for his useful insights and suggestions.

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