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Hepatocyte-like cells and liver organoids: the application of iPSCs and their derivants for liver diseases

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Abstract: Liver diseases have become great burden for human health because of the high morbidity and mortality. Orthotopic liver transplantation which has always been considered as the primary treatment for end-stage liver disease is limited in the clinic. The development of cell therapy, especially induced pluripotent stem cells (iPSCs) holds promise for liver diseases. It is reported that the hepatocyte-like cells and liver organoids derived from iPSCs can be applied to establish disease models, test drug hepatotoxicity or directly perform specific functions as grafts. In this article, we will systematically review the two differentiated derivants of iPSCs, show the prospective application of differentiated products, in order to provide experimental and theoretic basis for clinical treatment.

Keywords: Induced pluripotent stem cells; Hepatocyte-like cells; Liver organoids; Liver disease

Abbreviations: iPSCs, induced pluripotent stem cells; MASLD, metabolic dysfunction-associated steatotic liver disease; DILI, drug induced liver injury; 2D, two-dimensional; 3D, three-dimensional; HLCs, hepatocyte-like cells; LOs, liver organoids; MASH, metabolic dysfunction-associated steatohepatitis; FGF2, fibroblast growth factor 2; BMP4, bone morphogenetic protein 4; HGF, hepatocyte growth factor; OSM, oncostatin M; DEX, dexamethasone; AAT, alpha-1 antitrypsin; CYP450, cytochrome P450; CRISPR, clustered, regularly interspaced, short palindromin repeats; ECM, extracellular matrix; NTCP, sodium taurocholate co-transporting polypeptide; ALGS, alagille syndrome; PNPLA3, phospholipase domain-containing protein 3

1. Introduction

Over the past decades, liver diseases have become the major cause of high morbidity and mortality around the world. There are about 350 million people worldwide suffer from liver diseases currently. It is estimated that more than one-fifth of Chinese population experiences liver diseases,

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such as hepatitis B virus infection, metabolic dysfunction-associated steatotic liver disease (MASLD), alcohol-related liver disease, drug induced liver injury (DILI) and liver cancer[1]. Orthotopic liver transplantation is the only curative treatment for end-stage liver diseases[2]. However, the application of liver transplantation is limited because of donor shortage, immunological rejection, and the complexity of operation. In this case, the emergence of cell therapy may weaken these restrictions[3]. There have launched widely preclinical and clinical studies to demonstrate that cell therapy will be a promising treatment for liver diseases.

Cell therapy can provide mounts of cells with regenerative capacity to restore hepatic functions. The cell sources include autologous primary hepatocytes, xenogeneic hepatocytes or types of stem cells. In fact, primary hepatocytes are not only difficult to isolate and expand in vitro, but also strict to cryogenic storage and long-term immunosuppressive therapy[4]. Meanwhile, there are ethical and immune rejection issues which are difficult to solve in xenograft. In terms of stem cells, induced pluripotent stem cells (iPSCs) have the potential of multidirectional differentiation and low immunogenicity, becoming kinds of relatively innovative stem cell source with high application prospect[5].

IPSCs are types of pluripotent stem cells with the potential of multiple differentiation and infinite self-renewal capacity. In 2006, Takahashi first cultured iPSCs by introducing four specific transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) into mouse embryonic cells through retroviral vector[6]. In 2007, iPSCs were first created by reprogramming human somatic cells[7, 8]. This technology won the 2012 Nobel Prize in Physiology or Medicine and then wide association studies on iPSCs has developed worldwide. Almost all cells of somatic tissues can be programmed into iPSCs, including skin tissue, hair, blood even urine[9-12]. As iPSCs are widely derived from individuals, old issues that immune exclusion or ethical crisis can be improved[13].

It was demonstrated that iPSCs can differentiate into neural cells[14, 15], cardiac cells[16], vascular cells[17], functional hepatocytes[18] and other various cellular types (Figure 1).

The multidirectional differentiation ability of iPSCs makes them hopefully applied in neurodegenerative diseases, cardiac diseases, liver diseases and some inherited bleeding disorders[19-22]. Besides, the new emergence of organoids has deepened research in iPSCs fields. Organoids are three-dimensional (3D) structures that replicate high cell-cell and cell-extracellular matrix interactions and simulate the pathophysiology and disease process in vitro. It has been reported that organoids, derived from iPSCs and adult stem cells are studied on disease modeling, drug screening and toxicity testing[23].

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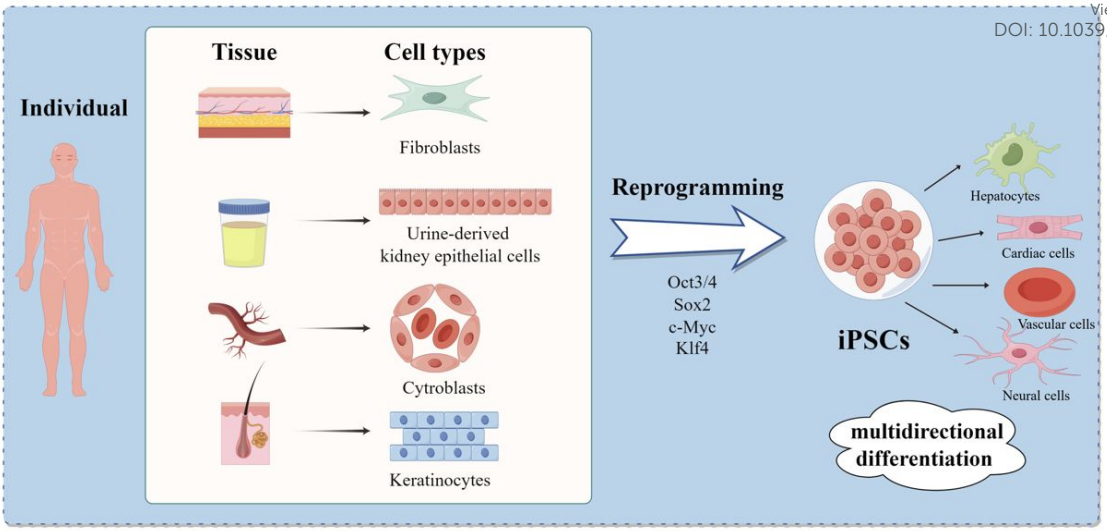

Figure 1. IPSCs can be derived from fibroblasts in human skin tissue, kidney epithelial cells in urine, cytroblasts in blood and keratinocytes in hair, etc. The reprogramming process requires the addition of four classical transcription factors: Oct3/4, Sox2, c-Myc, and Klf4. IPSCs have the ability to differentiate into various tissue cells involving hepatocytes, cardiac cells, vascular cells and neural cells, etc.

In recent years, iPSCs have shown their differentiation ability in terms of liver diseases. The derivants, hepatocyte-like cells (HLCs) and liver organoids (LOs) are prospective tools for the research of hepatitis B, MASLD, metabolic dysfunction-associated steatohepatitis (MASH) and liver tumor. In this review, we will summarize the generation of HLCs and LOs by iPSCs. We will also show their applications on various liver diseases, aiming to provide a trial basis for clinical treatment.

2. Generation of HLCs derived from iPSCs

Liver has numerous cellular types, in which hepatocytes are the most dominant cell type, accounting for more than 80% cells and covering most of the hepatic functions. HLCs are generated from stem cells to function like fetal hepatocytes. Although HLCs are low mature currently, they are conducive to long-term culture and research due to the ability that maintaining the relatively stability of differentiation degree and expanding indefinitely[24].

In 2009, Song et al. and Si-Tayeb's team demonstrated that human iPSCs can be directly induced into HLCs by stepwise differentiation[18, 25]. The initial scheme consisted of four stages: endoderm induction, hepatic specification, hepatoblastic expansion and hepatic maturation. The differentiation protocol stimulated some relevant pathways to imitate key stages in liver development by adding different cytokines or growth factors. Some essential factors included activin A, fibroblast growth factor 2 (FGF2), bone morphogenetic protein 4 (BMP4), hepatocyte growth factor (HGF), oncostatin M (OSM), and the glucocorticoid dexamethasone (DEX), etc[25]. How to induce HLCs with primary hepatocyte phenotype and fuction is the key to culture process along with the progress of research. The common hepatic differentiation protocol of iPSCs lasts about twenty-five days and can be divided into three stages: definitive endoderm differentiation, hepatic progenitor induction, and hepatocyte differentiation[26, 27](Figure 2). Firstly, the definitive endodermal formation is induced by activin A, which can regulate the expression of the iPSC-specific gene NANOG, thereby contributing to the pluripotency maintenance of iPSCs[28]. Sometimes Wnt3a is added at this stage to promote the differentiation of iPSCs towards the endoderm. After about 3 days, exposure to FGF2

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and BMP4 under hypoxic condition to determine differentiation direction, and HGF is added to encourage hepatic progenitor cell proliferation[29]. Then, under normoxic condition, HLCs maturation is promoted in hepatocyte culture medium with OSM and DEX[27]. This generation approach can produce more mature HLCs from human iPSCs effciently, uniformly, and repeatably.

For confirming the differentiation effect of each stage, it's crucial to measure the expression levels of some specific hepatic markers. Similar to primary human hepatocytes, iPSC-induced HLCs successfully express several key markers, such as alpha-1 antitrypsin (AAT), hepatocyte nuclear factor 4 alpha, and alpha fetoprotein, etc[30, 31]. However, HLCs stay in a relatively immature state and retain some characteristics of fetal hepatocytes[26]. There are some defects in functional characteristics of HLCs. A notable issue is that the activity of cytochrome P450 (CYP450) isozyme of HLCs is significantly lower than that of primary human hepatocytes[32]. CYP450 takes part in the process of drug metabolism in liver, its absence is a serious disadvantage when culturing hepatocytes in vitro. Additionally, Laemmle et al. found the lack of aquaporin 9 expression in HLCs. Aquaporin 9 is a membrane channel protein that mediates urea cycle. Its deficiency is the reason why HLCs generally secrete less urea than primary human hepatocytes[33]. Alternatively, low level of α-fetoprotein secretion, alcohol dehydrogenase inactivity are also characteristics of low maturity of HLCs. Meanwhile, the expression of CYP3A and CYP2D6 are also significant matters[34].

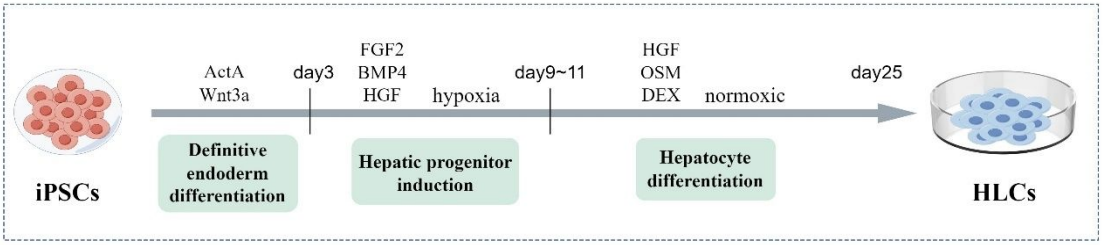

Figure 2. The culture protocol for generation from iPSCs to HLCs takes 25 days which is divided into three stages: definitive endoderm differentiation, hepatic progenitor induction, and hepatocyte differentiation. And the gradual maturation of HLCs is induced by adding different transcription factors at each stage.

Although the production of iPSCs-HLCs needs to enhance maturity, much progress has been made toward the clinic. Human iPSCs provide a powerful platform for disease modeling, personalized medicine and cell-based therapy. Recently, various gene engineering tools, especially clustered, regularly interspaced, short palindromin repeats (CRISPR) have been widely applied[35]. And considerable progress has been realized on rectifying genetic shortcomings. The CRISPR/Cas9 system combined with guided RNA can target a specific genetic site and then effectively repair genetic defects[36]. Taking advantage of this technology, patient-derived iPSCs can be differentiated into particular cell types such as modified HLCs and then directly infused into patients, or introduce specific mutations and establish disease model[37]. But there are still great challenges in clinical translation. In future more clinical trials will be conducted to drive iPSC-HLCs development.

3. Generation of liver organoids derived from iPSCs

Organoid is a 3D structure derived from (pluripotent) stem cells, progenitor and differentiated cells that self-organize through cell-cell ad cell-matrix interactions to recapitulate aspects of the native tissue architecture and function in vitro[38]. As 3D cultivate provides high cell-cell and cell-matrix interaction that involving the dynamic regulation of signaling pathways and paracrine signals,

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the structure of LOs is more similar to the hepatic physiological microenvironment state[39, 40]. Thus, the 3D cultivate can recapitulate complex liver architecture and function better than single two-dimensional (2D) monocultures, which makes LOs promising tools for preclinical applications and regenerative medicine (Table 1). In the past, it has been defined that organoids derive from two types of stem cells: pluripotent stem cells (either embryonic stem cells or iPSCs) and organ-specific adult stem cells[23]. At present, the cell sources of organoids involve pluripotent stem cells, progenitor cells, primary cells, and tumor specimens from patients[41]. Among them, iPSC-HLCs are ideal cell source for LOs due to their features such as patient specificity and unlimited supply[42]. They can be used when studying the occurrence, development and treatment of liver diseases, especially in the establishment of disease models and drug models.

Table1. Comparison between 2D culture and 3D culture.

Comparison	2D cultivation method	3D cultivation method
Derivation of cells	-Cell lines from a single cell	- Pluripotent stem cells
Components	-Only cell lines	-Multiple cell types -Cells - Medium with growth factors -Matrix or scaffold material
Model effect	-Lack the capability of simulating the architectural features of organoids -Low maturity -Have significant inherited, epigenetic, and functional changes	-Replicate high cell-cell and cell-extracellular matrix interactions -Involve the dynamic regulation of signaling pathways and paracrine signals -Have organ structure and genetic stability
Preparation cost	-Low	-High
Expansion	-Easy but not precise	-Long-term and stable
Application characteristics	-Widespread but high failure rate	-In the experimental phase -Bright prospect

Abbreviations: 2D two-dimensional; 3D three-dimensional

The first organoid was generated by researchers using mouse intestinal stem cells to generate 3D intestinal organs[43]. In the following years, the protocol for culturing organoids was successfully applied to other organs including stomach, liver, prostate, pancreas and lungs[44]. LOs were first reported in 2013. Huch's team used Wnt signal to drive long-term expansion of LOs that retain many features of the original epithelial structure[45]. In the same year, Takebe et al. gained vascularized and functional LOs from iPSCs firstly, which could connect to the host blood vessels within 48 hours after transplantation[46]. There have been important research breakthroughs based on iPSCs technology towards LOs in the past decade which bring new benefits to clinic. Herein, we will discribe the approach to LOs generated by iPSCs and show how to apply them to studying liver disease.

The culture process of iPSCs to LOs can be simply summarized as that cells are cultured in the medium with growth factors added, they firstly proliferate and differentiate to a specific mature state in 2D level, then will be collected and embedded in matrix or scaffold material to form a 3D structure and keep continuous growth(Figure 3). LOs can be derived either from the culture of a single cell type, or can be a co-culture system containing multiple cell types. The single type of cell

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culture ensures the proliferation and self-organization of homogenous cells, while co-culture of multiple cells types can better mimic the liver organ structure[47].

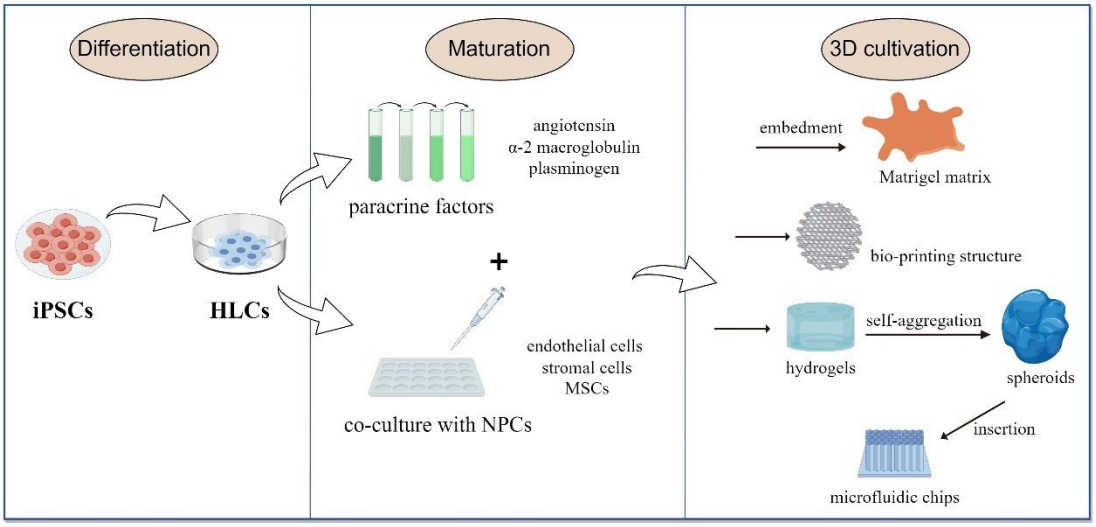

Figure 3. The culture process of iPSCs to LOs. HLCs proliferate and differentiate to a specific mature state in 2D level (either a single cell type or a co-culture system). Then cells are collected and embedded in matrix or scaffold material to form a 3D structure and keep continuous growth.

LOs culture currently mainly adopts a gradually induced differentiation scheme. After being differentiated into HLCs, organoids continue to mature in the medium supplemented with paracrine factors. The paracrine factors that trigger hepatic differentiation involve angiotensin, α-2 macroglobulin and plasminogen, and they jointly simulate the mature signaling environment to transform single cells into organs[48]. Then they are embedded in Matrigel matrix, a gelatinous protein mixture derived from the extracellular matrix (ECM) of the Engelbreth-Holm-Swarm mouse sarcoma that is mainly composed of laminin, collagen IV and entactin[49]. This matrix is extremely bioactive and it supports the structure and proliferation of different organoid types. Currently, most generation relies on Matrigel to provide structural support for the growing organoids and enable them to achieve 3D suspension growth[50]. Hence, the generation of human LOs from iPSCs is realized by adding different growth factors or chemicals at the corresponding stage, and with the structural and functional support provided by Matrigel matrix.

The 3D systems which involving multiple cell types can produce more functional in vivo-like liver models than traditional single cell types. In the liver, non-parenchymal cells are also important part of maintaining fuction. The major trend of organoids development is co-culturing iPSC-HLCs with endothelial cells, stromal cells and mesenchymal stem cells (MSCs), performing 3D cultures using various new biomaterials. It has been reported that available 3D culture models include bio-printed structures, microfluidic chips, or hydrogels[51-53]. Hydrogels mimic ECM properties and are thus ideal scaffolds of in vitro 3D liver models. They can produce organoids with the same efficiency of Matrigel without the possible immunogenic disadvantages[54]. For example, Schoonjans' team utilized a polyethylene glycol hydrogel which complemented with key ECM proteins of native liver such as laminin-111, collagen IV and fibronectin. Finally, they produced LOs with improved hepatic maturation and preserved differentiation capacity[55]. The other group used hydrogels with iPSC-HLCs to self-aggregate as spheroids, then inserted them on microfluidic chips and co-cultured with human umbilical vein endothelial cells and adipose tissue-derived MSCs[56].

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They provided terminal proof that iPSC-HLCs can successfully be cultured in 3D as spheroids and on microfluidic chips, and co-culturing iPSC-HLCs with non-parenchymal cells enhanced their functionality. Besides, some non-parenchymal cells may provide a capability to become supportive lineages, such as pro-fibrotic cells, hepatic stellate cells, and liver resident macrophages, Kupffer cells[57]. Using iPSC-HLCs composed of these cells to derive multi-cellular human liver organoids that overview of steatosis, inflammation, fibrosis and other liver disease phenotypes more completely.

LOs derived from iPSCs show a regenerative property which is similar to human[58]. The expansion of iPSCs in vitro is almost efficient and unlimited. So LOs can achieve long-term expansion in vitro, maintain genetic stability, and provide the possibility for large-scale production to form biobanks. Meanwhile, they can eliminate the lmitation of dependence on primary tissue. Once iPSCs cell lines are established, these cells can be repeated indefinitely to produce HLCs and organoids[59]. The 3D structure can significantly improve LOs' maturation and make them closer to adult liver. For example, their functions in albumin secretion, glycogen storage, drug metabolism and lipid transport have been demonstrated to be well expressed[60]. LOs derived from iPSCs have been applied to the study of oncogenic mutations, based on which the host-pathogen relationships can be studied in vitro. There have been some researches about liver organoids' applications on the mechanism of host-HBV/HCV interactions for primary liver cancer[39, 61]. Altogether, LOs offer a promising tool that can be applied to various liver-related studies. And LO-based disease modeling and drug development will be an important direction for biomedical applications in the field of liver diseases.

4. Application of iPSC-derived HLCs and LOs

HLCs and LOs derived from iPSCs have been widely used in preclinical studies of multiple liver diseases including viral hepatitis, inherited monogenic diseases, non-alcoholic fatty liver disease, drug-induced liver injury, and liver tumor(Figure 4). It is a cutting-edge method to simulate disease pathophysiology in vitro that using CRISPR/Cas9 gene editing technology to introduce or replicate specific mutations in iPSCs cell lines[62]. But on the other hand, gene-editing modeling has obvious limitations as it needs to identify the mutation site of disease. So other techniques are utilized for some diseases with unknown pathogenesis to build disease model, such as simulating the disease environment to induce morbidity. The successful disease simulation in vitro and the establishment of disease models support the possibility of screening therapeutic targets and drugs. Disease models also can be used to the experiment and analysis of human intervention factors and provide a good theoretical basis for clinical strategies, which is of great significance for clinical treatment[63, 64]. Besides, HLCs and liver organoids can be used directly as grafts, especially organoids. They can also be transplanted to implant bio-artificial liver devices, or to form functional units of implantable devices to provide temporary liver function, thereby forming compensations to delay or repair disease progression[65, 66].

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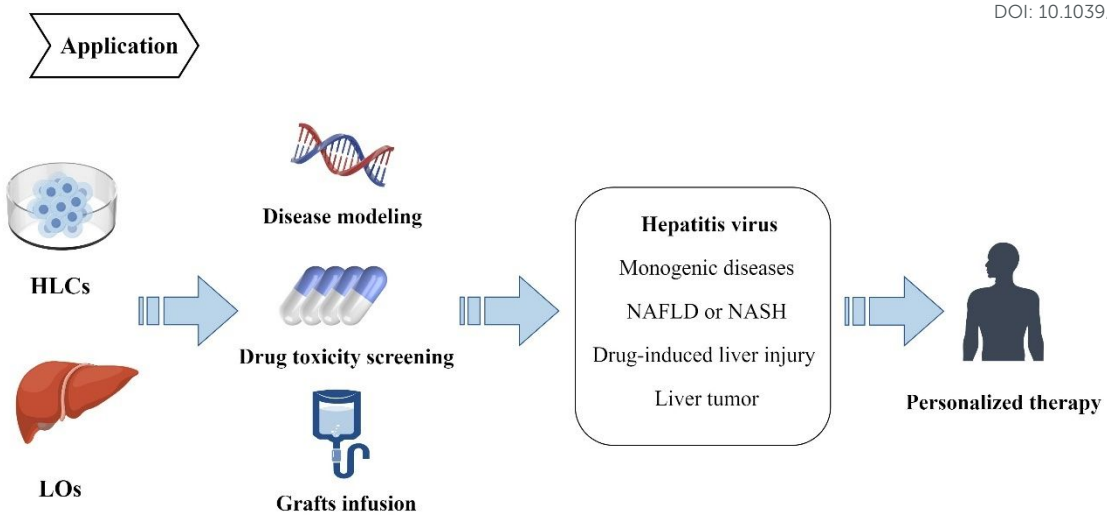

Figure 4. IPSCs can differentiate into HLCs and organoids, which can be applied in the fields of disease modeling, drug toxicity screening and grafts infusion. They have been extensively studied in disease animal models including DILI, HBV or HCV, NAFLD or NASH, providing a basis for the formulation of individualized treatment strategies.

4.1. Hepatitis virus

Hepatitis virus infection is not only a common cause of most liver disease but also the main factor inducing primary liver cancer. HBV and HCV are the most common pathogens, with about 170 million HBV infections and 350 million HCV infections worldwide[67]. In the past, the mechanism of interaction between virus and host was not clear due to the lack of reproducible, amplifiable in vitro modeling systems that could precisely recapitulate the virus life cycle. With the development of iPSCs, Schwartz et al. found that after being infected by HCV, iPSC-HLCs can replicate the whole infection cycle of HCV to establish models in infectious liver disease[68]. Sakurai et al. demonstrated that during the differentiation of iPSCs into HLCs, expression levels of several genes involved in HBV infection were elevated[69]. The sodium taurocholate co-transporting polypeptide (NTCP) is a functional receptor for HBV and overexpress NTCP can improve the efficiency of HBV infection. Li et al. reported the enhanced expression of NTCP and proved that medium containing iPSC-HLCs made cells highly susceptible to HBV infection[70]. As a result, iPSC-HLCs are hopefully to become a platform for studying the host inflammatory response to pathogen infection and then provide a possibility to find new drug targets.

Alongside, LOs with components derived from human iPSCs provide new guidelines for developing models of hepatitis virus in vitro, owing to their prominent susceptibility and capability to precisely recapitulate the virus life cycle[71]. Elisa De Crignis et al. infected liver organoids with HBV, and found that the model successfully expressed HBV RNA and proteins and produced HBV with infectious activity[61]. This organoid platform can be used to screen drugs that inhibit HBV replication and test drug toxicity. In another paper, researcher established a functional organoid by culturing iPSC-derived endodermal, mesenchymal, and endothelial cells in a three-dimensional microwell culture system. This liver organoid is susceptible to HBV and can sustain HBV replication for a prolonged duration, reproducing the viral life cycle and virus-induced liver dysfunction[72]. The above results indicate that iPSC-derived organoids can provide an in vitro infection research system for viral hepatitis.

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4.2. Monogenic diseases

The use of iPSC-HLCs or organoids as a novel model of several monogenic diseases are attracting increasing attention. The most common approach is to introduce known mutations through CRISPR/Cas9 technology to induce differentiation into the desired phenotype.

Alagille syndrome (ALGS) is a multi-organ disorder caused by mutations in the Notch ligand JAG1 gene, which manifests as chronic cholestasis[73]. Yuan et al. exploited a method for differentiating human iPSCs into LOs. During organoids development, JAG1 mutation in ALGS was simulated and the expression of JAG1 mRNA was regulated in vitro model. The features of ALGS liver disease are recapitulated in ALGS organoids[74, 75]. In another report, LOs were succeffully produced to reproduce cystic fibrosis process and as a result, they mimic disease phenotypes such as impaired the activity of transmembrane conduction regulator channel[76, 77]. Moreover, R778L mutation was introduced into iPSCs or embryonic stem cells from patients with Wilson's disease to acquire modified HLCs. The results showed that compared with the wild group, mutational HLCs had more death and higher sensitivity to copper ions, which was closer to the phenotype of Wilson's disease[78]. In 2022, AAT deficiency patient-specific iPSCs were engineered, aiming to recapitulate cellular phenotypes that result from expression of mutant ZAAT alleles, thereby determining the impact of ZAAT alleles on hepatocyte biology[79].

As evidenced by above, iPSCs are research tools in vitro for multiple monogenic liver diseases. The successful phenotype of monogenic disease provides a possibility for the establishment of disease models in vitro. We can try to screen targets and therapeutic drugs based on models, which is of great significance for clinical treatment.

4.3. MASLD or MASH

MASLD is the second most common cause of liver transplantation except for liver tumor. The incidence and severity of MASLD is increasing and there is a risk of progression to end-stage liver disease such as cirrhosis or hepatocellular carcinoma[80]. Additionally, it has been demonstrated that MASLD is associated with type 2 diabetes and even can increase cardiovascular risk[81]. In spite of this, its pathophysiological mechanism is not clear. Research found that the I148M variant of patatin-like phospholipase domain-containing protein 3 (PNPLA3) is associated with MASLD[82]. Tilson's team used CRISPR/Cas9 to modify iPSCs with a knockout of PNPLA3 gene. Then, iPSCs were differentiated into HLCs and treated with either oleic acid or palmitic acid to induce MASLD-like phenotypes. It was found that PNPLA3 loss induced lipid accumulation, predisposition to steatosis, and increased sensitivity to ethanol or methotrexate induced hepatotoxicity[30]. In another study, scientists combined stem cell biology with bioengineering principles, used free fatty acid to induce MASLD's pathological changes on iPSCs organoids. It showed increased lipid accumulation and expression of markers related to lipid metabolism. In addition, the expression of inflammatory and fibrotic markers was also increased[83]. MASH is a severe form of MASLD. The organoids from MASH patients' liver functionally manifested decreased albumin secretion, increased lipid storage and CYP450 metabolism. Transcriptome analysis of liver organoids showed significantly increased expression of markers of liver fibrosis and tumor[84]. The study opened up a new way for MASH. Elbadawy et al. created organoids of various stages of MASH in mice[85]. The pathological effects of the model performed increased expression levels of liver fibrosis markers collagen I and α-SMA, demonstrating that these 3D cultures can reflect the degree of fibrosis in MASH. The above disease models summarize the key pathological features of MASLD or MASH and may provide tools for

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the study of their underlying mechanisms, opening new path for their application in disease exploration and drug discovery.

Intrasplenic transplantation of iPSC-HLCs alleviated excessive accumulation of triglycerides and hepatitis symptoms, reduced the production of inflammatory cytokines and oxides, and reduced the production of apoptotic cells in MASLD mouse models[86]. High throughput analysis based on iPSCs can screen potential therapeutic drug. The technology of chemogenomic library and human iPSCs were used to identify compounds that can significantly reduce intracellular neutral lipid content among 13,000 bioactive compounds, thus promoting development of novel drugs for fatty liver disease[87]. These results suggested that iPSCs may provide an alternative treatment and drug screening pathways for MASLD or MASH-related end-stage liver disease.

Organ crosstalk is a significant matter affecting the development and prognosis of MASLD. It was reported that multiple tissues can influence MASLD development or correlate with MASLD severity, such as adipose tissues, pancreas, muscle, brain, and thyroid[88]. The various or cells tissues co-cultured with iPSC-HLCs can provide different immune mivroenvironment and phenotypic induction, in the future, the organoids models will likely need to combine with these cells or tissues for overcoming multiple organ crosstalk.

4.4. DILI

The drug hepatotoxicity is a major problem in all stages of clinical development. It is the most common cause of post-marketing warnings and withdrawals. So far, establishing a model in vitro which can predict drug hepatotoxicity remains a significant challenge in drug development. With the development of stem cells, iPSC-derived hepatocytes or organoids are gradually became as in vitro tools for DILI detection. These technologies provide predictive models for novel drugs before entering clinical trials and as a potential in vitro diagnostic tool[89].

IPSC-HLCs or organoids can be applied to predict DILI. They are characterized by increased drug-related sensitivity. A study confirmed iPSC-HLCs have increased time-dependent sensitivity to amiodarone, aflatoxin B1, troglitazone, et al.[90]. The results were also beneficial to predict the degree of drug damage. Another study generated liver organoids from a 3D system on a chip, they showed time-dependent and dose-dependent changes in liver toxicity after exposure to acetaminophen[91]. Researchers co-cultured HLCs with 47 known toxic drugs for 6 days and evaluated their toxicity by determining albumin, urea and ATP. The results found that HLCs successfully detected 24 of 37 toxic drugs with a sensitivity of 65%, while 10 non-toxic drugs were tested with 100% accuracy[92]. Shinozawa's team established a high-throughput toxicity screening model based on liver organoids which tested 238 marketed drugs by measuring the transport activity of bile acids[93]. Charles J et al. dispersed LOs derived from three separate iPSC lines in 384-well-formatted plates and exposed to known hepatoxic drugs.The IC_{50} values of compound cytotoxicity are measurable through this high-throughput screening format, showing the potential utility of iPSCs for DILI risk assessment[94]. Wu et al. futher used LOs derived from human iPSCs to model liver fibrogenesis induced by TGFβ or LPS treatment, and established a high-throughput anti-fibrosis drug screening system, which demonstrated that SD208 and Imatinib could significantly suppress fibrogenesis[95].

In conclusion, prediction and screening platforms based on iPSCs have high potential in liver toxicology research, and promoting drug screening applications, and personalized medicine. But the reliability and safety of the test have not been verified. It has not been clear whether the tissue heterogeneity will affect the effect of the drug, and whether the drug toxicity tests passed in the

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mouse model can be safely applied to patients. Therefore, there are still many problems to be explored for drug-induced liver injury from trial to clinic.

4.5. Liver tumor

Tumor heterogeneity accouts for the different drug response among patients, which emphasizes the importance of precision medicine[96]. The personalized strategies related to individual characteristics have crucial clinical relevance. The culture protocols for 3D tumor models have been established successfully in recent years, including the lung[97], pancreas[98], colon[99] and liver[100]. Patient-derived models of tumor provide an operating object in vitro to study the molecular mechanism of tumorigenesis, seek therapeutic targets, test drug effect and formulate personalized strategies. Under the background of primary liver cancer, models based on patient-derived iPSCs or organoids are widely used preclinical models to study precision medicine. They can not only preserve tumor microenvironment but also tumor structure, at the same time, can better recapitulate tumor heterogeneity[101]. Here we show immense progress on establishing liver tumor models (Table 2). Based on tumor models, we can study the molecular mechanism of liver tumor, as well as guide early prevention and treatment.

Table2. Liver tumor models derived from organoids.

Cell derivation	3D cultivation method	Applications	Advantages	Ref
iPSC-EC, iPSC-MC with luciferase-expressing Huh7 cells	Ultra-purified alginate gel	-Induce liver cancer mouse model -Study the mechanism of fibrosis or MASLD TEM in HCC tumor development	-High engraftment rate -Controlled tumor size -Simulation of the original TEM	102
Hepatobiliary tumor tissue of patients	Suspended in Matrigel drops	-Assess the effect of neoantigen-directed therapy -Screen personalized immunotherapy targets	-Recapitulate genetic complexity of original tumors -Replication mutation	103
Hepatocellular carcinoma patients who underwent surgical resection	Seeded in spheroid formation medium	-Evaluate the treatment efficacy of candidate agents	-Maintain the histological features and expression profiles of the derived tumors -Expandable in vitro -High generation efficiency	104
Liver cancer tissues	Mixed 1:2 with Matrigel	-Further pharmaco-proteogenomic analysis in models	-Retention of morphology, multiomics characteristics, and tumor heterogeneity -Recapitulated previous cancer tissue–based molecular subtypes	105
Tumor specimens	Solidification	-Screen sensitive drugs	-Exactly recapitulate the	106

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	of Matrigel	-Evaluate the efficacy of drugs directly targeting tumor cells	histopathological features of the original PLC -Enrich aggressive PLC subpopulations	
Liver tumor from patients who had no history of viral-meditated hepatitis	Cultured in organoids expansion medium	-Biomarker identification -Large-scale drug testing	-Recapitulate the histological architecture and expression profiles of parent tumor -Retain the specific differences between patients as well as between tumor subtypes -Long-term expansion in vitro	[107]

Abbreviations: iPSC-EC iPSC-derived endothelial cells; iPSC-MC iPSC-derived mesenchymal cells; MASLD metabolic dysfunction-associated steatotic liver disease; TEM tumor microenvironment; HCC hepatocellular carcinoma; PLC primary liver cancer

As mentioned above, these tumor organoids have shown the potential to model tumor environment and facilitate basic research of cancers. In the field of personalized drug selection, the tumor organoids have been widely used in hepatocellular carcinoma patients for high-throughput drug screening. Ji's team established a biobank of 65 human liver cancer organoids, screening a series of drugs and compounds that are used in the clinic or are under development like sorafenib or lenvatinib, to achieve high prediction accuracy for drug responses[105]. Furthermore, they also assessed potential drug combination therapies, and predicted that lenvatinib may have better combinatorial effects with temsirolimus. Yuan Zhang et al. also evaluated the in vitro drug response of hepatocellular carcinoma on tumor organoid model[108]. Except that, single-cell RNA sequencing on liver tumor models may be helpful for dissecting drug resistance mechanisms[109]. In the future, research on liver cancer organoids will also be invested in searching for more biomarkers that facilitate early diagnosis and patient stratification[101].

5. Summary and Limitation

After years of unremitting efforts by researchers, iPSCs' derivants have gradually expanded from 2-dimensional space to 3-dimensional space, and from basic research to preclinical study, with wide application and broad prospect in various types of liver diseases.

HLCs or LOs derived from iPSCs provide object for in vitro research that addresses the difficulties of primary cells with sources, amplification, storage. HLCs and cholangiocytes differentiated from iPSCs can be applied to the modeling of molecular mechanisms at the cellular level, toxicity testing of drugs, and cell therapy in regenerative medicine in vitro. Liver organoids have emerged as potential tools in the drug development phase. Currently, the main research topics about organoids are disease modeling and prediction of drug hepatotoxicity to develop individualized treatment strategies[110-112]. In addition, transplantaion of iPSC derivatives as grafts has shown good results in animal models. A study had shown that made iPSC-HLCs into cell patches then attached onto the injury liver surfaces. This method successfully ameliorated lethal acute liver injury induced by the infusion of carbon tetrachloride, suggesting the high potential for cell engraftment of iPSC-HLCs[113]. In recent studies, transplantation protocols for organoids

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demonstrated that LOs have achieved transplantation rates of 80% and survival times of up to 90 days[114].

However, there are some limitations that need to be optimized in the whole iPSCs' application scenarios. First and foremost, the main safety issue regarding iPSC is the risk of tumorigenicity[115]. As all of the four reprogramming factors have been associated with tumor development, especially c-Myc, which is one of the most frequently mutated genes in human cancers[116]. Furthermore, some of the added cytokines also play a role in tumor, such as a dominant negative mutant of p53. Researchers should strictly control the differentiation conditions to aberrant expression of tumor-related mutant genes. Secondly, amplification and culture of iPSCs in vitro inevitably cause genetic alterations, chromosomal abnormalities, gene mutations and other uncontrollable and unpredictable problems. Uncontrolled safety issues in clinical applications pose a problem for cell therapy with iPSC. Thirdly, immune rejection is another critical issue. Early studies have shown that undifferentiated iPSCs were rejected in syngeneic recipients when transplanting iPSC and derivatives as cell therapy[117]. At the same time, this reflects another issue that the differentiation scheme needs for further maturation. HLCs and LOs are composed of cells with different differentiation degrees. The immature cells, on the one hand, will become confounding factors in the culture system and produce unknown effects. On the other hand, disease models cannot fully reproduce disease characteristics which limits their applicability in areas such as drug testing or cell replacement[118, 119]. But in other words, liver is an organ with complex composition and function that it either cannot expect to be understood by analyzing a single type of cell or cannot fully reproduce the pathogenesis of liver-related genetic metabolic diseases by a single cell. The reprogramming of iPSCs is expected to be the main means to promote maturity in the future. In addition, the extracellular matrix components required for LO culture are not clearly defined, which means there are many variables[120]. Fourthly, so far, the vast majority of iPSC-based disease modelling studies performed the comparative evaluation of a few patient-derived iPSC lines. This approach makes it easier to learn about disease-associated variants and phenotypes, while not the actual disease-causing variants. In the future, the biobank needs to be expanded to include enormous disease-related iPSC cell lines to obtain more disease information[121]. Last but not least, there is a lack of large-scale clinical trials to validate the correctness and safety of iPSCs. Interestingly, the number of clinical trials on hepatocytes and LOs is large (Table 3). In the future, the stability and safety of iPSCs should be further clarified, HLCs and liver organoids should be put into clinical trials and develop standardized training model to exert clinical application value.

Table3. Clinical trials using iPSCs, hepatocytes and LOs for liver diseases.

NCT Number	Title	Year	Country	Disease	Status	Results
IPSCs						
NCT03867526	Establishment of Human Cellular Disease Models for Wilson Disease (IPSWILSON)	2018	Pakistan	Wilson disease	Completed	NA
NCT0095369	Patient Specific Induced Pluripotency Stem Cells (PSiPS)	2009	Iran	Hepatic disorders	Completed	NA
Hepatocytes						
NCT04806581	Clinical Study of	2021	China	Liver	Not yet	NA

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	Hepatocyte Transplantation for Liver Cirrhosis			cirrhosis	recruiting	
NCT04496479	Allogenic Hepatocyte Transplantation Into Periduodenal Lymph Nodes	2022	United States	End stage liver disease	Recruiting	NA
NCT05727722	Micro-encapsulated Hepatocyte Intraperitoneal Transplantation in Liver Failure Adults	2023	China	ACLF, CLF	Not yet recruiting	NA
Organoids						
NCT06077591	Prospective Clinical Validation of NGS and PDO Guided Therapy in Patients With Advanced/ Inoperable Solid Tumors (PDO)	2024	China	HCC, Colorectal cancer	Not yet recruiting	NA
NCT05932836	An Organoid-on-chips Technique Based on Biopsy Samples and Its Efficacy in Predicting the Response to HAI in HCC	2023	China	HCC	Recruiting	NA
NCT05183425	Patient-derived Organoids Predicts the Clinical Efficiency of Colorectal Liver Metastasis	2022	China	Colorectal liver metastasis	Recruiting	NA

Abbreviations: iPSC induced pluripotent stem cells; ACLF acute-on-chronic liver failure; CLF chronic liver failure; NGS next generation sequencing; PDO patient-derived tumor organoids; HCC hepatocellular carcinoma; HAI hepatic artery infusion; NA not available

To sum up, hepatocytes and LOs derived from iPSCs represent two dimensions of regeneration methods, which are used into disease modeling, drug screening, personalized medicine and other applications. Although there are still some challenges, iPSCs show their immense capacity and superiority.

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