Narrative review of in vitro experimental models of hepatic fibrogenesis

Review Article

Narrative review of *in vitro* experimental models of hepatic fibrogenesis

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Background and Objective: Hepatic fibrosis is a pathological condition affecting millions of people worldwide that results from an improper tissue repair process, following liver injury or inflammation. Since progressive liver fibrosis can evolve into end-stage liver diseases, it is becoming increasingly important to develop efficient experimental models for evaluating new anti-fibrotic therapies. An important role in the onset and progression of hepatic fibrosis is played by hepatic stellate cells (HSCs), perisinusoidal vitamin A-storing cells that, in the presence of pro-fibrogenic stimuli, acquire a myofibroblast-like phenotype with an increased ability to produce extracellular matrix (ECM) components. In this review, we provide an overview of the traditional two-dimensional (2D) systems and of the innovative bioengineered three-dimensional (3D) models that allow for the screening of novel anti-fibrotic therapies.

Methods: Data presented in this narrative review were retrieved from scientific literature by searching the computerized database PubMed and MEDLINE for original and review papers describing different *in vitro* 2D and 3D culture systems that mimic hepatic fibrosis.

Key Content and Findings: Over the past years, most *in vitro* studies have focused on the mechanisms underlying HSC activation, using liver cells cultured in traditional 2D systems. The development of 3D *in vitro* models allowed studying the complex interactions between HSCs and the surrounding parenchymal and non-parenchymal cells, and between liver cells and ECM, thus improving the mimicking of the situation *in vivo*. Advanced bioengineered 3D models can replace *in vivo* models reducing the ethical concerns and biological issues.

Conclusions: Traditional and innovative *in vitro* cell culture systems represent a valid alternative to *in vivo* animal models in the investigation of the complex mechanisms involved in fibrosis development and in the discovery of new anti-fibrogenic compounds for the treatment of hepatic fibrosis.

Keywords: Liver; fibrosis; hepatic stellate cells (HSCs); 2D and 3D models

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Introduction

Hepatic fibrosis is a pathophysiological process characterized by an excessive accumulation of extracellular matrix (ECM) in the liver. Alterations in the dynamic balance between synthesis and degradation of collagen fibers can be due to different chronic insults, such as toxins, drugs, alcohol abuse, cholestasis, viral or parasitic infections, metabolic and hereditary diseases (1-4). All these different factors may cause liver cell damage, inflammation and contribute to the development of fibrosis, which, if uncontrolled, can eventually lead to cirrhosis, characterized by the distortion of the hepatic architecture and blood flow (5,6). Both hepatic fibrosis and cirrhosis are considered risk factors for the development of hepatocellular carcinoma,

ultimately leading to liver failure. Despite the high incidence worldwide (7), except for liver transplantation, currently no efficient therapies are available against hepatic fibrosis and cirrhosis (8).

Hepatic stellate cells (HSCs) are non-parenchymal cells representing an intracellular storage of vitamin A and are considered key players of liver fibrosis (9). During fibrogenesis, following activation upon liver injury, they undergo morphological changes and acquire a myofibroblast-like phenotype, also increasing their proliferation rate and contractility (10,11). Activated HSCs lose their vitamin A storage and reduce the expression of glial fibrillary acidic protein (GFAP) (12,13), while increasing the expression of alpha smooth muscle actin (α-SMA) (14,15). They also modulate the biosynthesis and release of ECM components, such as collagen types I and III (16), matrix metalloproteinases and their specific tissue inhibitors (17).

The activation of HSCs is considered a key driver of hepatic fibrosis (9). Different pathways and molecular mediators are involved and act collectively or individually. Cytokines, growth factors and the rapid induction of cell surface receptors have been designated as central players in this mechanism. In addition, other events, such as oxidative stress, epigenetic signals and endoplasmic reticulum stress, have been shown to be involved in HSC activation (11).

HSCs respond to the extracellular signals produced by resident and inflammatory cells. Among them, transforming growth factor-beta (TGF-β) is considered one of the most potent fibrogenic cytokines (18,19). TGF-β is stored as an inactive molecule and, once released it binds to specific receptors on the cell membrane, and generally triggers the phosphorylation of SMAD proteins (20). This protein family includes a member, SMAD3, which is able to regulate gene transcription by promoting the expression of pro-fibrotic markers, predominantly α-SMA and collagens, leading to the remodeling of the ECM (20). As shown by Clarke *et al.* (21), the cellular response to TGF-β depends on the concentration of the ligand to which they are exposed and in the proper conditions, it is able to induce HSC activation.

Other important cytokines are platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). The first one is a mitogenic molecule that binds to its specific receptor, the PDGF-β receptor, stimulating cell proliferation and the acquisition of a contractile phenotype (22). On the other hand, VEGF is a potent cytokine able to promote HSC proliferation and fibrogenesis, also required for hepatic tissue repair and fibrosis resolution (23).

During the HSC activation process, an important role is also played by lipid metabolism. In fact, activated HSCs are characterized by the loss of lipid droplets, which are a storage of retinol esters and triacylglycerols (24). Recently, treatment of HSCs with lipopolysaccharide has shown to regulate HSC activation by inducing autophagy and stimulating retinoid hydrolysis, resulting in the decrease of the number of lipid droplets (25). As shown by Hong and coworkers (26), activated HSCs can be also reverted to a quiescent state by culturing them with retinol and oleic acid.

Being anchorage-dependent cells, HSCs regulate intracellular signaling pathways and change their phenotype depending on the stiffness and the bio-mechanical conditions of the surrounding ECM (27). In fibrotic conditions, the ECM stiffness increases and this leads to the switch of HSCs from the quiescent into the activated state (28). *In vitro*, HSC culture on polyacrylamide gels with varying stiffness demonstrated that rat HSCs grown on soft supports (0.4 kPa, corresponding to the stiffness of a normal rat liver) retain their quiescent phenotype, while on stiff supports (12 kPa, comparable to the stiffness a cirrhotic rat liver) transform into activated myofibroblasts (29,30). The increased ECM stiffness correlates with mechanical stretch, which can enhance the expression of TGF-β (31) and induce epithelial-to-mesenchymal transition in HSCs (32). Interestingly, the targeting of two mechano-sensitive nuclear factors, Yes-Associated Protein (33) and Bromodomain-Containing protein 4 (34), is able to induce the reversal of liver fibrosis (35).

The mechanisms underlying hepatic fibrosis are not completely understood: hence, the need for *in vivo* and *in vitro* models that reproduce the characteristics and pathogenesis of human fibrosis. In the last two decades, several *in vivo* research studies on different rodent models have tested the efficacy of various biological molecules and chemical compounds that may possibly attenuate or revert fibrosis (10,36). However, because of animals and humans differ in the genetic background, metabolism and immune response, animal studies are not perfectly predictive of human response to drugs and diseases (36,37). As an alternative, new available technologies may help to develop *in vitro* models of human liver fibrosis that could be more predictive and efficient than animal ones (38). This review presents the most currently used *in vitro* two-dimensional (2D) models and the recent development of three-dimensional (3D) models that help to investigate

Table 1 The search strategy summary

Items	Specification
Date of search	15th October 2021
Databases and other sources searched	MEDLINE and PubMed
Search terms used	Hepatic fibrosis, hepatic stellate cells, 2D and 3D *in vitro* models of hepatic fibrosis
Timeframe	Reviews and papers from 1976 to 2021
Inclusion and exclusion criteria	Inclusion criteria: reviews and experimental articles focused on in vitro models of hepatic fibrosis
	Exclusion criteria: reviews and experimental articles focused on chronic liver disease and liver tumors
Selection process	E.C. selected the papers for the section dedicated to 2D models
	G.C. selected the papers for the section dedicated to 3D models
	E.C. and G.C. worked independently
	E.C., G.C. and S.B. worked together for the introduction and conclusion section
Any additional considerations, if applicable	None

2D, two-dimensional; 3D, three-dimensional.

the mechanisms underlying hepatic fibrosis, and test new therapeutic agents against its development. We present the following article in accordance with the Narrative Review reporting checklist (available at https://dmr.amegroups.com/article/view/10.21037/dmr-21-102/rc).

Methods

The literature research was performed on 15th October 2021 by searching in MEDLINE and PubMed. Only studies in English language were included and the following keywords were used: liver fibrosis, HSCs, 2D and 3D hepatic fibrosis *in vitro* models. The research was focused on 2D and 3D *in vitro* models of hepatic fibrogenesis that mimic the situation *in vivo* and represent a valid alternative to study new anti-fibrotic therapies. In the 2D model section, the attention was concentrated on different liver cell populations, which were further discussed in 3D models. In particular, we focused on HSCs that are the key players of liver fibrosis. Reviews and experimental articles published from 1976 to 2021 were included. *Table 1* resumes the research strategy.

Discussion

2D models of liver fibrosis

Over the past years, most of the *in vitro* studies on liver fibrosis were based on traditional 2D systems of HSCs cultured on flat polystyrene uncoated dishes (39). *In vitro* 2D cultures of HSCs of different sources when stimulated with profibrotic stimuli, as TGF-β, undergo alterations in gene and protein expression (40). However, this approach is not able to fully recapitulate what occurs *in vivo* during the development of hepatic fibrosis, and important differences have been highlighted between *in vitro* cultures and *in vivo* models. The comparison of *in vitro*-activated HSCs with *in vivo*-activated HSCs isolated from fibrosis-induced mice treated with carbon tetrachloride (CCl_4) revealed a different gene expression profile, with an overlap of only 25%, showing that the *in vivo* setting better reflected the interactions between HSCs and the fibrotic liver microenvironment (41).

To obtain relevant and reproducible experiments with 2D *in vitro* models, two important aspects should be considered: the choice of the cell type and the availability of the cells in a quantity sufficient to perform all the studies (42). In the following sections, we will recapitulate the main characteristics of different cell populations employed in liver fibrosis research.

HSC primary cell culture

The first *in vitro* cultures of HSCs were developed in 1984 starting from primary cells isolated from rats through collagenase digestion and subsequent centrifugation (43,44). The classic method of HSC isolation consists in a density

gradient centrifugation, which allows to separate primary HSCs on the basis of their low density, caused by the presence of abundant lipid droplets (45). The floating HSCs can be further enriched by centrifugation or cell sorting. However, the standardization of the isolation procedure is difficult by the occurrence of cell variations originating from the isolation protocols applied by different operators in different laboratories (46) (*Table 2*). When freshly isolated HSCs are seeded on plastic culture dishes, they can be maintained in culture only for a limited number of passages, due to the restricted life span and to the spontaneous differentiation process, which starts independently from the source and the isolation procedure of the primary cells. Moreover, the spontaneous differentiation of HSCs into myofibroblast-like cells in 2D cultures does not reflect the pathophysiological condition occurring during the progression of liver fibrosis. One possible strategy to avoid this spontaneous differentiating process could be to cultivate primary HSCs on Matrigel, which helps to mimic the ECM microenvironment of the liver, while maintaining the cells in a quiescent state (47). Nevertheless, some significant disadvantages should be considered when using primary liver cells. First, the scarce availability of human biological samples and the presence of inter-individual differences limit the large-scale use of these cells (48). Second, the isolation procedures to obtain pure cultures of individual cells from healthy liver tissue are time-consuming and the yield is limited.

Spontaneously immortalized HSC lines

In order to provide a stable source of HSC, efforts have been made to generate immortal cell lines from primary HSCs (*Table 2*). Common approaches to generate HSC immortalized cell lines are based on the exposition of the cell culture to ultraviolet light or, in alternative, on the spontaneous culturing immortalization (46).

One of the first HSC line obtained by spontaneous immortalization is the murine cell line GRX. It derives from hepatic fibrotic granulomas of C3H/HeN mice infected with *Schistosoma mansoni*. Primary cells were isolated by enzymatic digestion and then sub-cultured at low density on plastic supports to purify cell clones. These cells show a myofibroblast-like phenotype and express collagen type I. They are used to evaluate changes in lipid content during the progression of liver fibrosis (49).

Another spontaneously immortalized cell line is PAV-1. It comes from a colony obtained by spontaneous immortalization of primary rat HSCs, which have been isolated from male 8-month-old Wistar rats by pronase-collagenase digestion. They express α-SMA, fibronectin and collagen type I, but not collagen type III. PAV-1 cells are employed to study the role of free fatty acids in alcoholic liver disease (ALD), since ethanol reduces lipid droplets in the cytoplasm, leading to HSC activation (50).

Experimentally immortalized HSC lines

A promising alternative to spontaneous immortalization lies in the experimental manipulation of primary HSCs. A typical procedure consists in the transfection of the cells with a construct that includes a temperature-sensitive mutant of simian virus 40 large T-antigen (SV40T), which is active at 33 ℃, excluding any doubt about possible tumorigenic activities. Another approach is based on the control of gene expression by inducing the ectopic expression of telomerase reverse transcriptase (TERT). These methods allow obtaining cell populations that grow faster than primary HSCs and duplicate between 24 and 72 hours (46) (*Table 2*).

The first human immortalized HSC line was the LI90 line, derived from an epithelioid hemangioendothelioma. This cell line is suitable for characterizing drug targets in HSC activation, but its use is limited by cellular senescence after a certain number of culture passages (51). To overcome this limitation, LI90 line was transfected with a retroviral vector expressing the human *TERT* gene, thus generating a new cell line called TWNT-4. Both LI90 and TWNT-4 express vimentin, α-SMA and collagen types I, III and IV (52).

The hTERT-HSC line was established from primary HSCs derived from surgical specimens of a healthy human liver. To overcome the problem of cellular senescence, these HSCs were transfected with a retrovirus carrying a cytomegalovirus promoter controlling the expression of *hTERT* gene. These cells express typical HSC activation markers, such as GFAP, vimentin, α-SMA, and are suitable for investigating the HSC transition from quiescent to activated phenotype (53).

The most used human immortalized cell line is the Lieming Xu (LX)-2 cell line (54). Together with the LX-1 line, it was isolated from a normal liver enzymatically digested, fractionated through density gradient and then immortalized by transfection with a plasmid expressing the SV40T. The subsequent propagation in low serum conditions of the LX-1 clone (1% fetal bovine serum) led to the final selection of the LX-2 clone. Both these cell lines express the main fibrosis-associated markers, such as α-SMA,

Table 2 Characteristics of cells employed in liver fibrosis research

Name	Source species	Method of isolation	Expressed markers	Investigation purposes	Issues	References
Primary cells						
Primary HSC	Human and murine healthy/diseased tissues	Density gradient centrifugation; cell sorting	HSC activation markers	Cell functionality correlated to the *in vivo* situation	Limited life span; activation after seeding; restricted material; culture heterogeneity	(43-48)
Spontaneously immortalized HSC lines						
GRX	C3H/HeN mice with hepatic fibrotic granulomas	Enzymatic digestion; low-density sub-culturing	Collagen type I	Changes in lipid content during liver fibrosis progression	Genotypic and phenotypic drift; culture selection pressure	(49)
PAV-1	Male 8-month-old Wistar rats	Pronase-collagenase digestion	α-SMA, fibronectin and collagen type I	Role of free fatty acids in HSC activation	Genotypic and phenotypic drift; culture selection pressure	(50)
Experimentally immortalized HSC lines						
TWNT4	Epithelioid hemangioendothelioma	Primary cell transfection with retroviral vector expressing *hTERT* gene	α-SMA, vimentin, collagen type I, III and IV	Drug targets in HSC activation	Genotypic and phenotypic drift; culture selection pressure	(51,52)
hTERT-HSC	Healthy human liver	Primary cell transfection with retroviral vector carrying *hTERT* gene controlled by cytomegalovirus promoter	GFAP, vimentin, α-SMA	HSC transition from a quiescent to an activated state	Genotypic and phenotypic drift; culture selection pressure	(53)
LX-1	Healthy human liver	Enzymatic tissue digestion; density gradient fractioning; transfection with a plasmid carrying SV40T construct	α-SMA, collagen type I and IV, TGF-β receptor I and II, PDGF-β receptor, fibronectin and vimentin	HSC activation process	Genotypic and phenotypic drift; culture selection pressure	(54)
LX-2	Healthy human liver	Cultivation of LX-1 in low serum conditions	α-SMA, collagen type I and IV, TGF-β receptor I and II, PDGF-β receptor, fibronectin and vimentin; high transfection capacity	Molecular pathways involved in HSC activation	Genotypic and phenotypic drift; culture selection pressure	(54)
HSC-T6	Male Sprague-Dawley rats	Primary cell transfection with a vector expressing the SV40T construct	Retinoid receptors; collagen types I, III and IV, vimentin, α-SMA, and TGF-β1	Pathways involved in collagen expression and new therapeutic targets	Genotypic and phenotypic drift; culture selection pressure	(55-57)

Table 2 (*continued*)

Table 2 (*continued*)

Name	Source species	Method of isolation	Expressed markers	Investigation purposes	Issues	References
JS1, JS2, JS3	Wild-type (JS1), Toll-like receptor 4-deficient (JS2) and myeloid differentiation primary response gene 88-deficient (JS3) C57BL/6 mice	Primary cell transfection with a vector carrying the SV40T construct controlled by cytomegalovirus promoter	Lipopolysaccharides; high transfection capacity	Pathways involved in HSC activation	Genotypic and phenotypic drift; culture selection pressure	(58)
Hepatic tumor cell lines as surrogates of hepatocyte activity and metabolism						
HepaRG	Liver tumor affected by hepatitis C	Collagenase and CaCl2 digestion; treatment with hydrocortisone and DMSO	Metabolizing enzymes; hepatobiliary transporters	Drug preclinical testing; co-culture in 3D systems	Genotypic and phenotypic drift; culture selection pressure	(59)
HepG2	Hepatocellular carcinoma	Collagenase digestion; sub-culturing of selected clones	Liver-specific markers	Drug preclinical testing; co-culture in 3D systems	Genotypic and phenotypic drift; culture selection pressure	(60)
Stem cell-derived HSCs						
iPSC-derived HSCs	Any cell type	Generation of iPSCs; cytokine or growth factor-induced differentiation in HSCs	HSC activation markers	Drug preclinical testing; co-culture in 3D systems	Difficulty in recapitulating the phenotype of adult cells	(61)

HSC, hepatic stellate cell; α-SMA, alpha smooth muscle actin; GFAP, glial fibrillary acidic protein; TGF-β, transforming growth factor-beta; PDGF-β, platelet-derived growth factor-beta; LX: Lieming Xu; DMSO, dimethyl sulfoxide; 3D, three-dimensional; iPSC, induced pluripotent stem cell.

collagen types I and IV, TGF-β receptors I and II, PDGF-β receptor, fibronectin and vimentin. Interestingly, only LX-2 cells appear responsive to TGF-β stimulation which in turns, induced the expression of procollagen α I (54). As a cell line that best reproduces *in vivo* HSC activation, LX-2 are suitable for studying the molecular pathways involved in HSC activation.

The HSC-T6 line is one of the most used experimentally manipulated rat cell lines. It is derived from HSCs purified from male Sprague-Dawley rats and transiently transfected with SV40T (55). Since they express retinoid receptors and typical markers of HSC activation, such as collagen types I, III and IV, vimentin, α-SMA, and TGF-β1, they can behave as both quiescent and activated cells. HSC-T6 cells are used to study the pathways involved in collagen expression and to find new anti-fibrotic therapeutic targets (56,57).

The JS1, JS2 and JS3 immortalized murine cell lines come from HSCs isolated from wild-type, Toll-like receptor 4-deficient, and myeloid differentiation primary response gene 88-deficient C57BL/6 mice, respectively (58). They were transfected with SV40T under the control of a cytomegalovirus promoter. Their main experimental application regards the study of the pathways involved in HSC activation.

The advantages of HSC lines are the unlimited supply and lifespan, correlated with the possibility to perform long-lasting experiments, and the possibility of being cultured in standard conditions, common to different laboratories. However, these cell lines are interested by genotypic and phenotypic drifts, originating after extended culture periods. Furthermore, sub-clones might arise due to selection pressures occurring in the laboratory (46).

Hepatic tumor cell lines as surrogates of hepatocyte activity and metabolism

Hepatocytes are involved in the progression of liver fibrosis. Primary hepatocytes (PHs) derived from human samples are preferred because their characteristics reflect

the functionality of the organ *in vivo*, but they can be maintained in culture only for a limited number of passages and in 2D culture, PHs lose their peculiar polarized phenotype due to the disruption of cell-cell and cell-matrix connections (42). Immortalized hepatic tumor cell lines are suitable surrogates of PHs (*Table 2*). These cell lines are easy to handle, phenotypically stable and have an unlimited lifespan. The HepaRG is the most suitable surrogate of human PHs since they express different metabolizing enzymes and hepatobiliary transporters (38). The HepaRG cell line was established starting from a liver tumor affected by hepatitis C. This cell line is bipotent: in fact, it can differentiate into biliary-like or hepatocyte-like cells (59). Primary cells were isolated by digestion with collagenase and $CaCl_2$ and then cultivated onto uncoated dishes. The selected colonies were treated with 5×10^{-5} M hydrocortisone and 2% dimethyl sulfoxide (DMSO) so that only hepatocyte-like cells could survive (59). On the other hand, the HepG2 cell line was established from human liver carcinoma, not contaminated by viral infections. Primary cells were isolated from a well-differentiated hepatocellular carcinoma of a 15-year-old white male, and after *in vitro* sub-culture, the selected clones expressed classical liver-specific markers (60).

Stem cell-derived HSCs

Stem cells are characterized by a marked ability to proliferate and differentiate under proper conditions and recently they become a valuable source of adult hepatocytes. Liver stem/progenitor cells, embryonic stem cells (ESCs), extra-hepatic biliary tree stem cells and induced pluripotent stem cells (iPSCs) can generate HSC-like cells (38) (*Table 2*).

In particular, iPSCs are very promising, since they can be derived from any cell type. These cells differentiate into iPSC-derived HSCs in the presence of cytokines, growth factors and ECM proteins, but they are not able to acquire a phenotype that fully recapitulates the one of adult cells. Unfortunately, the lack of standardized culture conditions does not allow maintaining their differentiated state (10).

A recent study from Coll and colleagues showed the possibility to induce the differentiation of iPSCs into HSCs through incubation with BMP4 and then with retinol and palmitic acid (61). These cells showed HSC-like gene expression profile and functionality *in vivo*, providing the possibility of developing protocols for the generation of HSCs from iPSCs.

3D models of liver fibrosis

Although 2D models of hepatic fibrosis have the advantages of being multiplexing, highly reproducible and easy to handle, they are unable to fully replicate the physiological 3D liver environment. On the other hand, 3D models of hepatic fibrosis allow interactions between cells and among cells and ECM, cellular migration, integrin adhesions, chemotaxis, and traction, and in some cases reproduce gradients of oxygen and soluble growth factors, thus supporting cellular proliferation and differentiation (62) (*Table 3*).

Role of ECM in co-culture systems

HSC monocultures have proved useful in studying the mechanisms of activation of HSCs. However, they do not take into account the interactions between HSCs and hepatocytes or other non-parenchymal cells, such as liver sinusoidal endothelial cells (LSECs) and Kupffer cells, which are critical for the progression of liver fibrosis. Using 2D co-cultures of HSCs in direct contact with other liver cells, it was possible to investigate changes in gene and protein expression triggered by cell-cell interactions. Furthermore, indirect co-cultures based on the use of trans well inserts, which allow separation of HSCs from another liver cell type, have demonstrated the role of paracrine factors in modifying the genetic and functional behavior of HSCs (39).

The use of co-culture systems consisting of HSCs and hepatocytes allows maintaining the HSCs in a quiescent state and keeps the system functional for long periods of time (98,99). By damaging the hepatocytes, which in turn induce HSC activation, it is possible to mimic more closely the *in vivo* mechanism of fibrosis development (38). Usually, co-culture systems are set up using HSC lines in combination with PHs. These HSC-hepatocyte co-culture systems have been improved by seeding cells between two layers of ECM compounds or by culturing in self-assembled 3D systems, both of which help to maintain the 3D architecture of cells (100).

Several studies have pointed out that cellular adhesion pathways and the composition of the ECM may influence cellular behavior (101-103). In particular, HSCs behave differently when grown on plastic or other types of matrices and tend to assume a specific morphology depending on integrin-mediated adhesion to the ECM. When HSCs are cultured on a 2D surface, they adopt

Table 3 3D systems employed in liver fibrosis research

Model	Characteristics	Advantages	Disadvantages	References
Spheroids	Self-assembled 3D system of adult tissue cells using: (A) ultra-low attachment or cell-repellent plates; (B) gravitational aggregation in hanging drop cultures	Cell-cell and cell-ECM interactions; easy to generate; high throughput	Simplified model architecture; lack of perfusion; high heterogeneity in cell composition and size	A: (61,63,64); B: (65,66)
Organoids	Self-assembled 3D system of adult progenitors, stem cells, iPSCs, or ESCs	Self-renewable; imitation of the liver in structure and function	Lack of perfusion; high heterogeneity in cell composition	Cholangiopathies (67-70); ALD (71); NAFLD/NASH (72-75)
Bioprinting	3D model generated using 3D biofabrication technologies, such as inkjet, extrusion, laser-induced forward transfer, and light-assisted	High reproduction of liver phenotype and functionality, by precisely controlling the organization of cells and ECM; possibility to regulate the mechanical properties; possibility to combine different cells and types of biomaterials; high-throughput	Lack of perfusion; biocompatibility problems of the materials; challenges with cell viability and structural integrity; problems with printing speed and resolution; generally high costs	(76-78)
Bioreactors and liver-on-a-chip	Self-assembled 3D culture combined with microfluidic devices that provide fluid flow and shear stress	Possibility to reproduce hepatic sinusoids, with a constant flow of fresh nutrients and oxygen; possibility to reproduce bile canuliculi, with the removal of metabolic waste; possibility to use TEER and ROS sensors for monitoring the development of fibrosis; long-term cultures	Hardly high throughput	(64,79-81)
Precision cut liver slices	3D model generated by cutting fresh livers with an automated vibratome	Intact healthy or fibrotic liver architecture and cellular heterogeneity; accurate cell-cell and cell-ECM interactions; possible association with bioreactors to extend lifespan and functionality; possible association with intestinal slices (inter-organ interaction)	Rapid tissue degradation and loss of function; repair and regenerative response, with consequent HSC activation, triggered by slice preparation	Screening of anti-fibrotic compounds (82-88); HSC activation (89-91); NAFLD (92); NASH (93); ALD (94); Cholangiopathies (95)
Decellularized liver matrix	3D model generated through whole organ decellularization	Intact healthy or fibrotic liver architecture and perfusion; accurate cell-cell and cell-ECM interactions	Reconstitution of the liver with one or two cell types incompletely models cell-cell interactions	(96,97)

3D, three-dimensional; ECM, extracellular matrix; iPSC, induced pluripotent stem cell; ESC, embryonic stem cell; TEER, transepithelial electrical resistance; ROS, reactive oxygen species; HSC, hepatic stellate cell.

a flat and elongated shape that is not characteristic for this cell type. However, when cultured in a 3D microenvironment, HSCs maintain their natural star-shaped morphology (39). Unlike a traditional 2D support, a 3D culture system allows a dynamic exchange of factors between the cells and the surrounding microenvironment. During hepatic fibrogenesis, changes in the protein composition of ECM (mostly collagens, laminins and integrins), lead to the formation of new ECM-cellular networks and the subsequent reassembly of the liver structure (39). One of the most abundant components of the ECM, collagen, is responsible of the increasing liver stiffness during the development of fibrosis and, as well as providing support for communication between cells, it also acts as reservoir for

the secreted cytokines, growth factors, extracellular vesicles and other ECM proteins during liver damage (104).

Self-assembled 3D systems

Self-assembled models arise from the combination of different cell populations that are made to aggregate in a physically constrained environment. The simplest self-assembled system is a spheroid, a 3D cellular structure composed of adult tissue cells (105). The first hepatic spheroids were set up starting from 2D co-cultures of primary rat hepatocytes and HSCs, and employing self-forming aggregation techniques, such as low adhesion culture dishes (98), and poly-DL-lactic acid-coated plates (99,106). Currently, hepatic spheroids are established employing a wide range of new techniques. One example is micromolding, a technique for modeling hydrogels on a microscale: concave micromolds of non-adherent material (e.g., polydimethylsiloxane) has proven efficient to establish spheroids of PHs and HSCs (107), PHs and endothelial cells (108) and tri-culture spheroids of PHs, HSCs and endothelial cells (109). To date, research studies using micromolding-based spheroids have focused on monitoring the hepatocyte phenotype and function in 3D cultures, while HSC activation has not yet been investigated.

Hepatic spheroids were also established using slowly rotating concave 96-well ultra-low attachment or cell-repellent plates (63,64,110). Bell and colleagues (110) developed spheroids of primary human hepatocytes and characterized them by proteomic analysis, showing that the spheroids maintain a stable phenotype comparable to that in the liver for at least 5 weeks. By co-culturing for 8 days these primary human hepatocyte spheroids with non-parenchymal cells (in 2:1 ratio), the presence of HSC, Kupffer cell and biliary cell markers was detected within the spheroids. Similarly, Leite and coworkers (63) set up spheroids of HepaRG hepatocytes and HSCs (in 1:2 ratio), which showed HSC activation upon single and repeated exposure to pro-fibrotic compounds (allyl alcohol and methotrexate). In a recent report by Coll *et al.* (61), iPSC-differentiated functional HSCs remain quiescent when 3D-cultured in the presence of HepaRG hepatocytes, and become activated in response to fibrogenic stimuli. HSC activation in spheroids has been also observed following hepatocyte toxicity induced by acetaminophen (61,63,64), resembling the mechanism of *in vivo* activation of human HSCs. For this reason, this model represents a powerful tool for studying *in vitro* HSC activation in liver fibrosis.

Another method of generating spheroids is through gravitational aggregation in hanging drop cultures. Foty and colleagues (111) have proposed a relatively simple protocol that involves pipetting cells suspension onto the inner side of top lid of a petri dish, and incubating them under physiological conditions, to form spheroids in which the cells are in direct contact with each other and with the ECM components. Recently, this protocol has been successfully applied to generate liver spheroids (112,113) and to develop high-throughput technologies, such as the hanging drop system provided by InSphero AG (Zurich, Switzerland), which allow the generation of human liver 3D microtissues suitable for testing drug-induced hepatotoxicity (114-118) and liver fibrosis (65,66). Using these multicellular 3D culture systems (hepatocytes/Kupffer cells/HSCs), Prestigiacomo and colleagues have shown a fibrotic phenotype upon stimulation with two fibrogenic compounds (methotrexate and thioacetamide), as assessed by gene-expression and protein-deposition of ECM proteins, such as fibronectin and collagens (65). They also compared 3D primary multicellular cultures with 3D monocultures of PHs after exposure to TGF-β1 and observed that the presence of non-parenchymal cells was required in the system to recapitulate fibrosis (66). The same technology was applied (119) to generate a 3D *in vitro* model of non-alcoholic steatohepatitis (NASH). Once the 3D culture systems (hepatocytes/Kupffer cells/HSCs) were exposed to lipotoxic or to inflammatory stimuli, the microtissues developed the pathophysiological features of NASH, including the release of procollagen type I, the increased deposition of ECM components and the regulation of molecular pathways involved in lipid metabolism and inflammation. Treatment of this NASH model with drug candidates modified the gene expression profile of the microtissue and reduced the specific disease parameters. Hence, this model has proved efficient for analyzing the mechanisms involved in NASH pathogenesis and for assessing the efficacy of anti-NASH drug candidates (119).

Recently, organoids are gaining increasingly importance in scientific research, as they can mimic real organs in structure and function. Unlike spheroids, organoids are self-organizing 3D culture systems originating from adult progenitors or ESCs characterized by self-renewal capacity, and which can be induced to differentiate into multiple cell types, thus better replicating the physiological distribution and function of the cells in that specific organ (120). The first liver organoid was established by Huch and colleagues, who isolated and expanded Lgr5-positive biliary cells from a mouse model of hepatic injury (121). In the last decade,

organ-like 3D *in vitro* models to study liver fibrosis have been established using ESCs, hepatoblasts, iPSCs and cells derived from adult tissues (122).

To reproduce the development of fibrosis in congenital disorders of the biliary tract, such as biliary atresia, Chusilp and colleagues (67,68) generated organoids using intrahepatic bile ducts. In this 3D injury model, they tested the acetaminophen-induced fibrotic response in organoids and observed that cholangiocyte apoptosis sustained the fibrotic process by secreting fibrogenic cytokines (67). They also demonstrated that human amniotic fluid stem cells reduced cholangiocyte apoptosis and promoted their proliferation, thus inhibiting the fibrogenic response in the injured organoids through a paracrine effect (68). Another 3D *in vitro* system that aimed to replicate aspects of hepatic fibrosis associated with various congenital cholangiopathies was established by Brovold *et al.* (69), who set up organoids composed of human liver progenitor cells and HSCs (primary activated HSCs or LX-2 cell line). Interestingly, compared to LX-2-containing 3D systems, organoids generated from primary activated HSCs showed larger clusters of biliary like structures, increased ECM-remodeling capacity and higher expression levels of fibrosis-associated genes (69,70).

To investigate liver fibrosis in a 3D *in vitro* model of ALD, human ESC-derived organoids were stably expanded after long-term 3D cultures, and then combined with human fetal liver mesenchymal cells. Upon ethanol treatment, these organoids mimicked typical ALD-associated pathophysiologic features, including oxidative stress generation, inflammation, steatosis, and fibrosis (71). The incorporation of other liver cells (e.g., Kupffer cells) into this 3D model could more accurately reflect the impact of severe fibrosis and steatosis on liver function.

To generate a 3D *in vitro* model of non-alcoholic fatty liver disease (NAFLD), organoids consisting of hepatocytes and HSCs (HepG2 and LX-2, in 24:1 ratio) were exposed to a mixture of fatty acids (palmitic acid and oleic acid) and fibrogenic stimuli. In these particular 3D culture conditions, liver cells were able to increase collagen and intracellular fat levels, thus permitting to investigate the molecular mechanisms underlying fibrosis in NAFLD (72). Interestingly, the inhibition of microRNA (miR)-122 in liver organoids consisting of primary human hepatocytes, Kupffer cells, HSCs and LSECs, led to the development of inflammation, necrosis, steatosis and fibrosis (73). A similar multicellular human hepatic organoid composed of hepatocytes, HSCs, and Kupffer like cells was generated from iPSCs, to study NASH. When treated with a mixture of free fatty acids, these liver organoids reproduce the characteristic features of the pathological progression of steatohepatitis, including steatosis, inflammation, and fibrosis (74). Liver organoids were also established using liver cells isolated from mice with different stages (mild, moderate and severe) of methionine and choline deficient diet-induced NASH and maintained in culture with a combination of Matrigel with various stem cell-stimulating growth factors (75). All of these NAFLD/NASH-mimicking organoids successfully reproduced the characteristics of the liver fibrotic tissue and could be employed to identify potential promising diagnostic markers (genes and non-coding RNAs) of NAFLD/NASH, and to screen for novel compounds for the treatment of liver steatosis.

3D bioprinted liver tissues

Unlike organoids, which reproduce tissue organization using natural developmental processes, 3D biofabrication technologies recreate a more physiological cellular organization, through a precisely controlled process. The 3D tissue microenvironment plays a pivotal role in establishing the typical characteristics of fibrosis development, therefore by precisely positioning cells and ECM within the tissue, and controlling collagen deposition and tissue stiffness, it is possible to improve the reproduction of the disease progression. The aim of 3D biofabricated tissue systems is to reproduce the phenotype and functionality of a particular tissue, with customized geometries, adjustable mechanical properties and high-throughput capabilities (62).

The most commonly used 3D biofabrication technologies are inkjet (droplet-based) (123), extrusion (124), laser-induced forward transfer (125), and light-assisted (126) bioprinting. Each of these techniques has benefits and disadvantages with respect to costs, printing speed and resolution, cell viability, structural quality, and biocompatible materials, and the resulting constructs resemble in varying degrees the mechanical and functional properties of the fibrotic tissue (62,127).

One of the most described extrusion-based bioprinted 3D liver models has been developed by Organovo Holdings (San Diego, CA, USA): this functional model of human liver tissue consists of human PHs co-printed with HSCs and human umbilical vein endothelial cells (HUVECs). The exposure of this 3D liver construct to repeated administration of methotrexate and thioacetamide successfully mimicked progressive fibrogenesis in drug-

induced liver injury, showing increased amount of collagen deposition, cytokine production, and fibrosis-related gene expression profiles (76). Further incorporation of Kupffer cells to this model permitted to examine their impact on the fibrogenic response: following prolonged exposure to TGF-β1 and methotrexate, Kupffer cells shortened the drug-induced injury window, causing a delay in the increase of miR-122, marker of hepatocyte damage (77). Based on these promising results, this 3D bioprinted model could be feasible as a high-throughput model for the screening of anti-fibrotic drugs.

Unfortunately, some drawbacks of this *in vitro* 3D liver model are the proprietary printing technique, and the complex experimental setup, which make it unavailable to most of the research community. Recently, Cuvellier and coworkers set up another extrusion-based bioprinted 3D liver model in which human liver progenitor cells, HSCs and HUVECs were embedded in a matrix of gelatin methacrylate (78). Since collagen deposition was observed only when TGF-β1-activated LX-2 were co-cultured with HepaRG and HUVECs, this functional 3D model could better reproduce hepatic fibrosis than liver cell monocultures. Therefore, bioprinted 3D systems open up new perspectives in studying the molecular mechanisms involved in fibrosis development and help to screen for inhibitors of collagen expression and deposition.

Microfluidic bioreactors and liver-on-a-chip technology

While self-assembled 3D cultures and bioprinting techniques efficiently recreate cell-cell contacts, vascular structures and fluid flow cannot be easily simulated. A possible solution is to combine these 3D culture systems with "organ-on-chip" technology (64), taking advantage of microfluidic devices or microscale reactors that could mimic hepatic sinusoids, by providing a constant flow of fresh nutrients and oxygen, and bile canuliculi, by removing the metabolic waste generated.

The first microfluidic bioreactor that combined self-assembled 3D cultures together with fluid flow and shear stress was established by Griffith and co-workers (128,129). This multi-channel bioreactor consisted in a collagen I-coated silicon 3D scaffold placed on top of a cell-retaining filter, and allowed a continuous flow of nutrients and oxygen through each channel. Spheroids of primary rat hepatocytes seeded into the channels of the bioreactor maintained their viability for up 2 weeks and reorganized to form vessel-like structures (128), as well as bile canaliculi (129).

In an attempt to mimic the perisinusoidal space of Disse and allow paracrine interactions between cells, a bioreactor consisting of two cell culture chambers and a commercial microporous membrane was proposed by Illa and colleagues (130). While activated HSCs were seeded onto the lower chamber of the bioreactor, HUVECs were seeded onto the upper gelatin-coated chamber. Shear stress generated by applying fluid flow onto the HUVECs improved endothelial morphology and reduced the activation status of HSCs, probably due to paracrine factors released by HUVECs. As it helps to keep HSCs quiescent, the hemodynamic flow of this 3D liver system could be exploited in liver fibrosis studies.

Recently, the haemodynamic human liver system set up by HemoShear Therapeutics (Charlottesville, VA, USA) has been used to identify new therapeutic approaches for the treatment of NASH (79). In this bioreactor system, primary human hepatocytes were co-cultured in a transwell system with primary HSCs and macrophages, separated by a synthetic membrane, and perfused with a medium enriched in NASH-associated risk factors, such as glucose, insulin and free fatty acids. Lipotoxic conditions induced a steatotic phenotype in hepatocytes, with increased secretion of TGF-β and expression of ECM-related genes. However, despite the presence of αSMA-positive HSCs, no collagen secretion was detected in the HSC fraction (79).

Another example of dynamic perfused 3D model is the LiverChip platform (CN Bio Innovations, Cambridge, UK), consisting of 12 independent bioreactors for the 3D culture of primary human hepatocytes, HSCs and Kupffer cells in a collagen I-coated scaffold. A pneumatically operated pump allows constant recirculation of culture medium and constant perfusion of the scaffold, to support cell viability and control oxygenation and shear stress on the tissue (80,131,132). Recently, a 3D model of NAFLD has been established using primary human hepatocytes plated in the LiverChip platform (80). Hepatocytes cultured in the presence of free fatty acids showed a steatotic phenotype, with increased secretion of adipokines associated with fibrosis, making this 3D system a valuable tool for testing the efficacy of anti-steatotic compounds.

Notably, Farooqi and coworkers (81) employed sensors of transepithelial electrical resistance (TEER) and reactive oxygen species (ROS) for monitoring the development of fibrosis. They set up a dynamic 3D microfluidic system, in which HepG2 cells and human fibroblasts were co-cultured, and different ECM components (collagen, fibronectin, and poly-l-lysine) were compared to test cell attachment. Treatment with TGF-β1 immediately reduced TEER values

and increased ROS release. After 24 hours of stimulation, especially in the presence of fibronectin as cell adhesion matrix, TEER values increased due to ECM deposition and fibroblasts activation. This liver fibrosis-on-chip model efficiently demonstrated that electrochemical sensors could potentially substitute the conventional end-point assays (e.g., α-SMA and collagen I measurement) for studying fibrosis in 3D microfluidic systems.

3D models preserving liver structure

A 3D *in vitro* model that allows to keep both organ architecture and cellular heterogeneity intact is precision-cut liver slices (PCLS). Liver slices 8 mm in diameter, 200–250 µm thick and containing 70 to 100 lobules, are generated by cutting fresh livers with an automated vibratome, such as the Krumdieck tissue slicer (133). PCLSs can be obtained from different species, such as rodent (82,94,95,134-137), and human liver (83,138,139), and then cultured in flasks, multiwell plates, or bioreactors under gentle rotation.

This 3D system has been extensively employed to test the effect of anti-fibrotic compounds in the liver (82-87) and to explore the molecular mechanisms of liver fibrogenesis in models of NAFLD (92), NASH (93), ALD (94). Recently, hepatic fibrogenesis has been also investigated in PCLSs treated with bile acids to simulate cholestatic liver injury (95). In addition, inter-organ interaction has been also assessed by culturing together liver and intestinal slices (88) in microfluidic devices (140).

The major drawback of this 3D system is that the slice preparation results in 2-cut surfaces, which trigger a repair and regenerative response, with consequent HSC activation within 96 hours (141). In particular, HSC activation has been studied in PCLSs after CCl_4 (89) and acetaminophen (90) exposure, to evaluate the different responses of such slices to these fibrogenic compounds, using gene expression profiling. Another problem is rapid tissue degradation and loss of function of PCLSs, whose lifespan is estimated to be around 48 hours (142). For this reason, PCLSs have been associated with bioreactors to extend their lifespan, functional longevity, and metabolic activity to least 6 days (91).

Another 3D *in vitro* technique that helps to retain liver biochemical and biomechanical features is whole organ decellularization. It consists in flushing the liver with detergents, such as triton X-100 in combination with sodium dodecyl sulphate (143,144), or sodium deoxycholate combined with delipidation with phospholipase A2 and perfusion with nucleases (145). The resulting bioscaffold is a decellularized matrix in which, once the liver cells are seeded, the 3D liver is reconstituted with the preservation of the organ architecture and the intrinsic vascular network. Several protocols have been developed aimed at creating a decellularized matrix suitable for cell cultures from rat (144,146) and human (147,148) livers. Moreover, decellularized livers have been successfully used to test fibrotic compounds (96,97). Serrano and coworkers (96) tested the anti-fibrotic activity of NV556, a novel cyclophilin inhibitor, using LX-2 cells activated by TGF-β1 and seeded on decellularized 3D scaffolds obtained from healthy human livers. NV556 down-regulated transcription levels of collagen I α1 and lysyl oxidase, both markers of activated LX-2, and reduced the production of the ECM collagens. To evaluate the anti-fibrotic effect of sorafenib, Thanapirom and colleagues (97) used decellularized 3D scaffolds obtained from healthy and cirrhotic human livers, and engrafted with TGF-β1-treated HepG2 and LX-2. Treatment with sorafenib inhibited STAT3 phosphorylation, known to be associated with HSC activation, and reduced pro-collagen 1α1 secretion and the expression of TGF-β1-induced fibrogenic markers (collagen 1α1 and lysyl oxidase, fibronectin-1, and IL-6) in HepG2-LX2 co-cultures in both healthy and cirrhotic 3D liver scaffolds.

Conclusions

In the last twenty years, great progress has been made in the development of *in vitro* culture systems to explore the complex mechanisms of liver fibrosis. They have been proposed as an alternative to *in vivo* models, which are very popular in preclinical studies to predict the possible adverse reactions to chemicals in humans. Animal models are preferred to *in vitro* studies for some reasons: the possibility to monitor the biological activity of a candidate drug in relation to the microenvironment, to follow its bio-distribution, pharmacokinetics and pharmacodynamics. Actually, animal models may fail in mimicking the complex human pathogenesis and the final results of pre-clinical testing could be not completely reliable or could be opposite (36). Indeed, animal models are not always truly predictive of the human physiological responses, due to the fact that different species can differ in their metabolic and immune responses (149).

Moreover, ethical concerns lead to consider the replacement of animal models according to the 3R principle: "Reduction, Refinement and Replacement". *In vitro* models

seem like a good alternative because they permit to reduce the use of animal testing in drug safety studies, the timing of the experiments and the costs (38). Furthermore, they allow for testing new therapeutic modalities, such as RNA interference, cell-targeted stimulations or genetic manipulations that can be controlled by the operator.

The employment of 2D culture systems based on HSC monocultures has allowed investigating the activation of HSCs and the signaling pathways involved in this process. However, one of the major drawbacks of traditional 2D cell culture is the lack of the surrounding microenvironment, without which it is not possible to reliably reproduce the pathophysiological mechanisms of fibrogenesis. The development of 3D culture systems permitted a more in-depth study of the intricate cell-cell and cell-ECM interactions involved in the progression of liver fibrosis, also taking into account the importance of tissue vascular perfusion. In particular, the integration of microfluidic devices and bioreactors to 3D culture systems can highly improve their lifespan and functionality. By providing high level of control over cellular and ECM composition, bioengineered and microfluidic 3D models could help to unravel the peculiar characteristics of hepatic fibrogenesis in different liver diseases, such as NAFLD, ALD, and cholangiopathies. Furthermore, by controlling the timing of progression of the damage affecting liver cells, it would be possible to mimic the different stages of hepatic fibrogenesis. Future optimization of the *in vitro* 3D models of fibrogenesis would definitely help to identify new anti-fibrogenic compounds and an efficient therapy to treat hepatic fibrosis.

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Footnote

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