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REVIEW ARTICLE

In Vitro Generation of Luminal Vasculature in Liver Organoids: From Basic Vascular Biology to Vascularized Hepatic Organoids

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Liver organoids have gained much attention in recent years for their potential applications to liver disease modeling and pharmacologic drug screening. Liver organoids produced *in vitro* reflect some aspects of the *in vivo* physiological and pathological conditions of the liver. However, the generation of liver organoids with perfusable luminal vasculature remains a major challenge, hindering precise and effective modeling of liver diseases. Furthermore, vascularization is required for large organoids or assembloids to closely mimic the complexity of tissue architecture without cell death in the core region. A few studies have successfully generated liver organoids with endothelial cell networks, but most of these vascular networks produced luminal structures after being transplanted into tissues of host animals. Therefore, formation of luminal vasculature is an unmet need to overcome the limitation of liver organoids as an *in vitro* model investigating different acute and chronic liver diseases. Here, we provide an overview of the unique features of hepatic vasculature under pathophysiological conditions and summarize the biochemical and biophysical cues that drive vasculogenesis and angiogenesis *in vitro*. We also highlight recent progress in generating vascularized liver organoids *in vitro* and discuss potential strategies that may enable the generation of perfusable luminal vasculature in liver organoids.

Keywords: Blood vessel, Vascularization, Liver, Organoid, Stem cell

Introduction

The liver is a vital organ that plays crucial roles in many physiological processes, including glucose and lipid metabolism, plasma protein secretion, bile acid secretion, blood coagulation, and drug detoxification. The basic structural and functional unit of liver tissue is the liver lobule, which has a diameter of 1.0～1.3 mm (1) and consists of hepatocyte plates, sinusoidal capillaries, and six hexagonally positioned portal triads, each comprising a portal vein, a hepatic artery, and a bile duct. The lobule receives nutrient-rich blood from the portal vein and oxygen-rich blood from the hepatic artery, which flows through the sinusoidal capillaries toward the central vein in the lobule. This complex architecture and vascular system are essential for a wide spectrum of biological processes in the liver. Hepatocytes are responsible for the major functions of the liver However, the interplay between hepatocytes and various non-parenchymal cells (NPCs) such as endothelial cells (ECs), hepatic stellate cells (HSCs), and Kupffer cells (KCs) is crucial in normal and diseased liver as well as in processes of liver development (2, 3). Particularly, specialized liver sinusoidal endothelial cells (LSECs) that line the liver sinusoid are key drivers of physiological and

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pathological processes of the liver (4). The liver vasculature, and its relationships with the surrounding cells and structures, are key factors for accurate *in vitro* modeling of liver development and disease.

Primary hepatocytes cultured under two-dimensional conditions are the traditional *in vitro* model used to investigate liver function and disease. However, primary hepatocytes cannot maintain their functions for long periods in conventional two-dimensional culture systems (5) and cannot be expanded *in vitro*. To overcome these limitations, expandable three-dimensional (3D) liver organoids were derived from leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5)-positive liver stem cells (6). This strategy has been used to generate several types of liver organoids from liver-resident cells, including epithelial cell adhesion molecule (EpCAM)-positive ductal cells (7) and primary hepatocytes (8), and human pluripotent stem cells (hPSCs) (9-11). Liver organoids are emerging as an alternative to primary hepatocytes for drug toxicity screening and cell therapy. However, most organoids lack a complete set of NPCs, which limits their ability to exactly recapitulate the complex liver architecture and pathophysiological events that depend on the interactions between hepatocytes and NPCs.

Recently, multi-lineage liver organoids containing at least one type of NPCs together with hepatocytes have been suggested as an efficient *in vitro* model to mimic the structural complexity and interplays between the different cell types in normal and diseased liver. The liver vasculature has special characteristics that enhance molecular exchange with other blood vessels (12) and are essential for liver development (2) and disease progression (4). Several attempts have been made to produce blood vessels inside liver organoids (13, 14). Particularly, the diffusion limit of oxygen and nutrients is generally considered to be 100～200 μm in tissues or cellular spheroids (15), which hinders the *in vitro* formation or assembly of large organoids. Therefore, the generation of vasculature with perfusable lumens is crucial for the survival of organoids or assembloids that can recapitulate complex liver structures and pathological events *in vitro*. Moreover, the vascularization can improve the tissue resemblance and hepatic functions of liver organoids compared to 2D-cultured hepatocytes or non-vascularized liver organoids (13, 16, 17). This review focuses on the characteristics of liver vasculature under normal and disease conditions and discusses potential mechanisms and strategies that may enable the generation of vasculature in liver organoids. We also highlight recent advances in the generation of vascularized liver organoids.

Characteristics of Hepatic Vasculature

Vasculature in the normal liver

The liver is the main site for numerous physiological processes that are critical to human survival. Hepatocytes, the liver parenchymal cells, play crucial roles in these processes; however, the liver vasculature provides an essential passageway for molecular exchange and contributes to liver regeneration (18). The liver vasculature is an interconnection of three major blood networks in which blood from the portal vein and hepatic artery is mixed and transported through the sinusoidal lumen to the central vein for drainage (19). During liver development, the vitelline vein, posterior cardinal vein, and umbilical vein are incorporated into the liver and give rise to the portal vein, the central vein, and the ductus venosus (20). LSECs have been proposed to originate from various types of cells, including endodermal cells, sinus venosus-derived cells, cells of the large vessels adjacent to the developing liver bud, GATA binding protein 4 (GATA4)-positive hepatic progenitor cells, and hemangioblasts (21, 22). Mature LSEC-specific characteristics are acquired through a three-step process that begins with laminin-rich basement membrane (BM)-encircled cells without fenestrae that express typical EC markers such as CD31 and CD34 (12, 23). Through progressive differentiation, these cells become fenestrated and secrete laminin-poor and tenascin-rich perisinusoidal extracellular matrix (ECM), after which the typical EC markers are gradually down-regulated while LSEC-specific markers such as CD4, CD14, CD32, and intercellular adhesion molecule-1 (ICAM-1) are up-regulated. At birth, the LSECs become zonated in terms of fenestrae size and number, with a general tendency for a decrease in fenestrae size (12, 23). The cellular and molecular signals that govern LSEC differentiation are not completely elucidated. However, vascular endothelial growth factor (VEGF) signals from liver epithelial and mesenchymal cells (24), transforming growth factor beta (TGFβ) repression (25), Wnt signaling (26), and GATA4-mediated transcriptional programs (27) are known to promote LSEC differentiation and proliferation. LSECs are a type of highly permeable EC that lack BM and have fenestrae without diaphragms (Fig. 1A) (22). The high permeability and endocytic capacity of LSECs, along with their spatial localization at the interface of blood and hepatic cells, enable them to interact with other cell types and maintain normal liver physiology by regulating molecular and cellular trafficking, blood flow, immune function, and liver regeneration (22, 28). In particular, the reciprocal interaction between LSECs and pericyte-like HSCs is essential for both normal homeo-

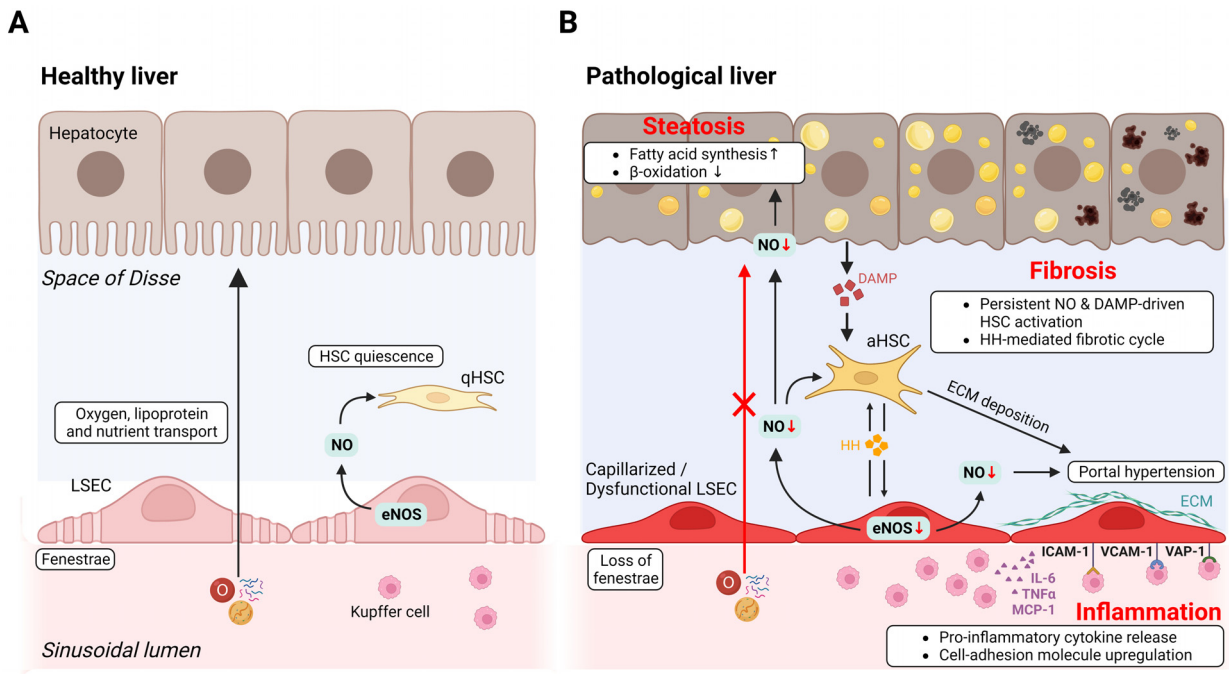

Fig. 1. Interactions between liver sinusoidal endothelial cells and other liver-resident cells in healthy (A) and pathological (B) conditions. LSEC: liver sinusoidal endothelial cell, qHSC: quiescent hepatic stellate cell, aHSC: activated hepatic stellate cell, NO: nitric oxide, eNOS: endothelial nitric oxide synthase, DAMP: damage-associated molecular pattern, ECM: extracellular matrix, HH: hedgehog, ICAM-1: intercellular adhesion molecule-1, VCAM-1: vascular cell adhesion molecule-1, VAP-1: vascular adhesion protein-1, IL-6: interleukin-6, TNF α: tumor necrosis factor alpha, MCP-1: monocyte chemoattractant protein-1.

stasis and disease progression because of the distinct roles of HSCs in quiescent and activated states (4).

Vasculature in the pathological liver

Capillarization is a progressive phenotypic change in the liver sinusoidal epithelium characterized by loss of fenestrae and formation of BM that occurs early in a variety of chronic liver diseases (Fig. 1B) (4). LSEC permeability can be lost as a direct mechanical effect of capillarization, impeding the passage of oxygen, nutrients, and macromolecules across the blood and liver parenchyma. This eventually results in aggravation of steatosis through dysregulation of lipoprotein transport (29) and may promote liver fibrosis through the production of damage-associated molecular pattern (DAMP) molecules driven by cellular hypoxia (30). From a biochemical standpoint, the major dysfunction associated with capillarized LSECs is impairment of endothelial nitric oxide synthase activity, resulting in the increased oxidative breakdown of the vasodilator nitric oxide (NO) (29). This causes intrahepatic vascular resistance and portal hypertension because the decrease in NO levels causes constriction of the sinusoidal vasculature and may also trigger HSC activation, resulting in BM formation around the sinusoid (4). As NO is a key player in the inhibition of fatty acid synthesis, the promotion of β-oxidation, and the attenuation of KC activation, reduced NO bioavailability appears to exacerbate steatosis and inflammation (29). Moreover, LSECs in injured liver tissue produce increased levels of vasoconstrictors that contribute to portal hypertension (31). In the context of liver fibrosis, capillarized LSECs are incapable of suppressing HSC activation, which eventually leads to collagen deposition in the space of Disse (32). In addition, secretion of hedgehog signaling molecules reinforces LSEC capillarization (33), thereby creating a vicious cycle between LSECs and HSCs that promotes fibrotic progression. LSECs also release various angiocrine signals in response to acute or chronic injuries, which regulate liver fibrosis and regeneration (34). In chronic liver inflammation, such as non-alcoholic steatohepatitis, LSECs become activated and acquire pro-inflammatory phenotypes, including progressive overexpression of the adhesion molecules ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), and vascular adhesion protein-1 (VAP-1), which enhance leukocyte infiltration, and production of pro-inflammatory mediators such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNF α), and monocyte chemoattractant protein-1 (MCP-1), which activate KCs and recruit leukocytes (29).

Angiogenesis is a common event in pathological liver tissue, where LSEC capillarization-induced hypoxia and pro-inflammatory mediators upregulate angiogenic gene expression (35). Data from *in vivo* studies of anti-angiogenic therapies suggest that angiogenesis exacerbates inflammation and fibrosis, although further research is required to determine the precise impact of angiogenesis on the progression of chronic liver diseases (22, 29). Nevertheless, dynamic changes of intrahepatic vasculature play an essential role in the onset and progression of liver disease.

Biochemical and Biophysical Cues for Organoid Vascularization

In order to develop strategies to produce vascularized liver organoids *in vitro*, it is important to first understand the mechanism regulating vasculature formation. In this section, we summarize the process of vascularization and highlight the signaling pathways and biological cues that may enable the formation of luminal vasculature inside liver organoids.

Vasculogenesis and angiogenesis

Vasculogenesis and angiogenesis are the processes responsible for the formation of new blood vessels (36). During embryonic development, the first blood vessel is formed through vasculogenesis, which involves a series of events that include the formation of primitive blood islands, blood island fusion, tubular structure remodeling, and the development of the first primitive vascular plexus (37). After the first vascular networks are formed by vasculogenesis, new blood vessels are formed from pre-existing ones through a process known as angiogenesis, which can occur by EC sprouting or by vessel lumen splitting (Fig. 2A) (37). VEGF, the key angiogenic molecule, causes ECs to secrete matrix metalloproteinases (MMPs), which degrade the BM (38), and angiopoietin-2 (ANG2), which de-

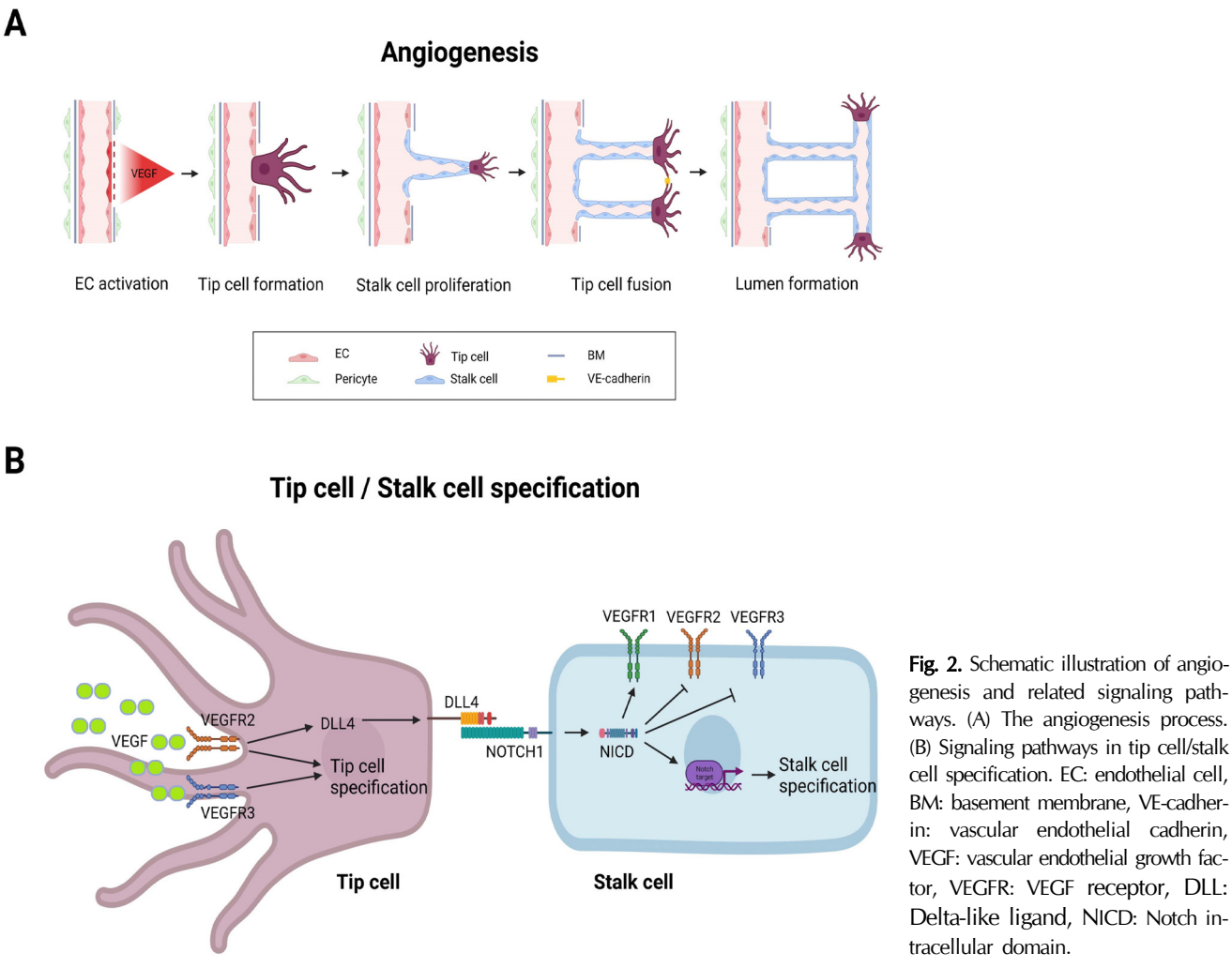

Fig. 2. Schematic illustration of angiogenesis and related signaling pathways. (A) The angiogenesis process. (B) Signaling pathways in tip cell/stalk cell specification. EC: endothelial cell, BM: basement membrane, VE-cadherin: vascular endothelial cadherin, VEGF: vascular endothelial growth factor, VEGFR: VEGF receptor, DLL: Delta-like ligand, NICD: Notch intracellular domain.

taches pericytes from the BM (39). Tip cells and stalk cells are specialized ECs with different roles in blood vessel formation. Tip cells form filopodia in response to VEGF stimulation during angiogenesis and guide new sprouts to migrate from existing vessel walls (40). Following the tip cells, stalk cells proliferate, establish junctions with adjacent cells, and produce BM components, inducing the formation of new vascular lumen (37, 39). When two tip cells from neighboring sprouts make contact, the sprouts fuse and form a new blood vessel for perfusion, which is mediated by vascular endothelial cadherin (VE-cadherin) (39). The resulting neovascular perfusion subsequently promotes vascular maturation and stabilization by recruiting pericytes and depositing BM (39).

Signaling pathways for vascular formation

The VEGF-A and Notch signaling pathways are essential for endowing ECs with the features of tip cells or stalk cells during angiogenesis (Fig. 2B). Tip cells express high levels of VEGF receptor 2 (VEGFR2) and VEGF receptor 3 (VEGFR3) and produce Delta-like Notch ligand (DLL4) in response to VEGF stimulation (40). Subsequent interaction between DLL4 and its receptor Notch1 converts neighboring ECs into stalk cells by down-regulating VEGFR2, VEGFR3, and neurophilin1 (NRP1) and up-regulating VEGF receptor 1 (VEGFR1) (37). Stalk cell activity is suppressed in tip cells by NRP1, which inhibits the activin receptor-like kinase (ALK) signaling pathway.

Recently, the hippo signaling pathway was suggested as a key player in angiogenesis. Yes-associated protein (YAP) activation, which is regulated by VE-cadherin via the phosphoinositide 3-kinase (PI3K)-Akt pathway, increases ANG2 expression in ECs during angiogenesis (41). In addition, the YAP/TAZ- TEA domain transcription factor (TEAD) complex upregulates the expression of amino acid transporters in ECs, which activates the mechanistic target of rapamycin complex 1 (mTORC1) signaling pathway to regulate angiogenic growth (42).

Biochemical and biophysical cues in vascular formation

VEGF and fibroblast growth factors (FGFs) are central factors in the formation of new blood vessels in various tissues (39). VEGF plays a key role in angiogenesis initiation and promotes mitochondrial activities and activation of the Akt3 and mTOR pathways, both of which are involved in vessel formation (43, 44). Basic fibroblast growth factor (bFGF) supports angiogenesis by promoting EC migration and proliferation as well as mural cell wrapping (45, 46). Subsequent maturation of newly formed vessels requires angiopoietin-1 (ANG1), which maintains quiescent ECs and promotes vessel integrity (47). The hepatocyte growth factor also promotes the maturation of newly formed vessels through cytoskeleton remodeling (48) without causing vascular inflammation (49).

Hypoxia-inducible factors (HIFs) are induced by low oxygen tension in many tissues and are suggested as important biochemical mediators of EC sprouting (39). HIF-1–induced neovascularization requires VEGF, as demonstrated by VEGF deletion in TetON-HIF-1 mice (50). ANG2 and sphingosine-1-phosphate (S1P) are also induced by hypoxia (51, 52) and promote vascular growth under hypoxia. Particularly, ANG2 triggers vessel destabilization to enable sprouting (53). In addition, platelet-derived growth factors (PDGFs), TGFs and ephrins all play a role in vessel remodeling and maturation (54).

ECM produced by ECs, pericytes, and smooth muscle cells provides mechanical support for the vascular endothelium and is essential for regulating EC migration and sprouting for blood vessel formation (45). Multiple ECM characteristics, such as density, stiffness, degradability, and key ECM adhesion peptides all need to be considered to precisely mimic blood vessel formation *in vitro* (55). High ECM density reduces the rates of neovascular growth and sprouting (56), whereas high ECM stiffness promotes vessel sprouting and outgrowth of ECs (57). Additionally, ECM stiffness controls VEGF secretion (58) and stimulates tip cell development via YAP signaling (59).

Blood flow is a key physical element that stabilizes vascular assembly and new vessel networks. Because ECs are located inside blood vessels, they are constantly affected by shear stress, the force produced by blood flow (60). Laminar fluid shear stress induces Krüppel-like factor family 2 (KLF2) expression (61) in cultured human vascular ECs and inhibits YAP activity via autophagy and sirtuin 1 (SIRT1) expression in human umbilical vein endothelial cells (HUVECs) (62). These flow-dependent modulations of intracellular signaling and gene expression are crucial for maintaining and remodeling blood vessels.

Vascularized Liver Organoids

Multiple attempts have been made to produce vascularized liver organoids based on the understanding of biochemical and biophysical cues in vasculogenesis and angiogenesis. In this section, we categorize vascularized liver organoids according to the major strategies employed to produce them: self-organization, bioprinting, and microfluidic device-based approaches. We also summarize the characteristics of organoids produced using each strategy (Table 1, Fig. 3).

Table 1. A brief summary of vascularized liver organoids

Generation method	Cell types at the generation stage	Vascularization method	Vessel characteristics	Perfusion	Ref.
Self-organized (condensation on Matrigel bed)	hiPSC-derived hepatic endoderm, HUVECs, MSCs	Spontaneous (mixture of EGM and HCM)	CD31+ EC network (*in vitro*); CD31+ vasculature connected to host vessels (after transplantation); blood and dextran perfusion (after transplantation)	*In vivo*	(13, 14, 66-68)
	Human fetal liver cells, HUVECs, MSCs	Spontaneous (EGM)	HUVEC network (*in vitro* and after transplantation); dextran perfusion (after transplantation)	*In vivo*	(63)
	PHHs, LSECs, MSCs	Spontaneous (mixture of LSEC growth medium and HGM)	CD31+ EC (*in vitro*)	-	(64)
	hiPSC-derived hepatic endoderm/ECs/STM cells or HUVECs, MSCs	Spontaneous (mixture of EGM and HCM)	EC sprouting (*in vitro*); iPSC-EC-derived vasculature connected to host vessels (after transplantation); blood and dextran perfusion (after transplantation)	*In vivo*	(16)
	hiPSC-derived hepatocytes/ECs/MSCs or human aortic ECs, dental-pulp MSCs	Spontaneous (mixture of EGM and HCM)	CD31 and VE-cadherin IF (*in vitro*)	-	(69)
	UCMSC-derived hepatocytes/LSECs/HSCs	Spontaneous	CD31 IF (*in vitro*)	-	(65)
Self-organized (embedding in liver extracellular matrix and cultured in microfluidic device)	Induced hepatocytes/HUVECs	Spontaneous (interstitial flow)	CD31+ EC network (*in vitro*); decreased active caspase 3 in organoids cultured with fluid flow	-	(70)
Self-organized (assembled in ultra-low attachment plate)	3D PHHs, HUVECs	Spontaneous (mixture of EGM and HEM)	None	-	(71)
	hiPSC-derived hepatocytes, primary human HSCs, primary human cholangiocytes, primary human LSECs or hiPSC-ECs	Spontaneous (40 ng/ml VEGF)	CD31+ capillary-like structure	-	(72)
Self-organized (assembled in non-tissue culture treated plate)	Human or mouse liver progenitor cells, mouse primary LSECs or HUVECs	Spontaneous (mixture of WME and EGM, 50 ng/ml VEGF, 50 ng/ml bFGF)	CD31, LYVE-1, CD32b IHC (*in vitro*); LYVE-1 and CD32b IHC (after transplantation)	-	(73)
Self-organized (assembled using microwell film)	hiPSC-hepatoblast/ECs/MSCs	Spontaneous (40 ng/ml bFGF, 100 ng/ml VEGF)	CD31+ EC network (*in vitro*); VE-cadherin, FVIII, and vWF IF (after transplantation); FVIII secretion (*in vitro* and after transplantation)		(74)

Table 1. Continued

Generation method	Cell types at the generation stage	Vascularization method	Vessel characteristics	Perfusion	Ref.
Self-organized (layer-by-layer coating of cells)	PHHs or HepG2 cells, HUVECs, normal human dermal fibroblasts	Spontaneous	CD31+ EC network with lumen (*in vitro*); quantification of vessel length and branching points (*in vitro*); blood perfusion (after transplantation); CD31, vWF, and CD34 IF (after transplantation); aSMA+ pericyte-like cells covering vessel (after transplantation)	*In vivo*	(75)
Self-organized (pulsed overexpression of GATA6 in hPSC)	Genetically modified hiPSCs	Spontaneous	CD31, CD34, CD146+ EC network (*in vitro*); quantification of vessel length and branching points (*in vitro*); single-cell RNA-sequencing (EC markers including PDGFB, NOTCH1, ANGPT2, TIE1, DLL4, KDR, CDH5 and ERG); blood perfusion (after transplantation); CD31+ vasculature connected to host vessels (after transplantation)	*In vivo*	(17, 76)
Bio-printed	PHHs, HUVECs, human lung fibroblasts	Prepatterned (EGM)	CD31+ EC network (*in vitro*)		(77)
	EA.hy926 (human ECs), HepG2/C3A cells	Prepatterned	CD31+ vessel with lumen (*in vitro*)		(78)
	Self-condensed liver organoids derived from PHHs, HUVECs, MSCs	Spontaneous (bFGF, VEGF)	CD31 IF and IHC (*in vitro*), CD31 IHC (after transplantation)		(79)
Microfluidic device (liver sinusoid-like chip)	HepaRG, HUVECs, human HSCs (LX2), human primary macrophages	Prepatterned (EGM, interstitial flow)	VE-cadherin IF (*in vitro*)	*In vitro* (microfluidic chip)	(80)
	PHHs, primary human LSECs or HUMECs, human HSCs (LX2), human KCs (THP-1)	Prepatterned (interstitial flow)	VE-cadherin IF (*in vitro*), SEM (LSEC fenestrae, *in vitro*), fluorescent albumin perfusion and permeability analysis (*in vitro*)	*In vitro* (microfluidic chip)	(81)
Microfluidic device (AngioChip)	Primary rat hepatocytes or hESC-derived hepatocytes,HUVECs, MSCs	Prepatterned (EGM, interstitial flow)	CD31 IF (*in vitro*), FITC perfusion (*in vitro*), immune cell (THP-1 and RAW 267 macrophage) perfusion (*in vitro*)	*In vitro* (microfluidic chip)	(82)
Re-seeding in decellularized liver	Human fetal liver cells, HUVECs	Prepatterned	vWF and eNOS IF (*in vitro*), dextran perfusion (*in vitro*), TEM (vascular lumen, *in vitro*)	*In vitro*	(83)

hiPSC: human induced pluripotent stem cell, HUVEC: human umbilical vein endothelial cell, MSC: mesenchymal stem cell, EC: endothelial cell, STM: septum transversum mesenchyme, UCMSC: umbilical cord-derived mesenchymal stem cell, LSEC: liver sinusoidal endothelial cell, HSC: hepatic stellate cell, IF: immunofluorescence staining, IHC: immunohistochemistry, VE-cadherin: vascular endothelial cadherin, LYVE-1: lymphatic vessel endothelial hyaluronan receptor 1, FVIII: Factor VIII, vWF: von-Willebrand factor, αSMA: alpha-smooth muscle actin, eNOS: endothelial nitric oxide synthase, SEM: scanning electron microscopy, TEM: transmission electron microscopy, EGM: endothelial cell growth medium, HCM: hepatocyte culture medium, HEM: hepatocyte expansion medium, HGM: hepatocyte growth medium, VEGF: vascular endothelial growth factor, WME: Wiliam's E medium, bFGF: basic fibroblast growth factor.

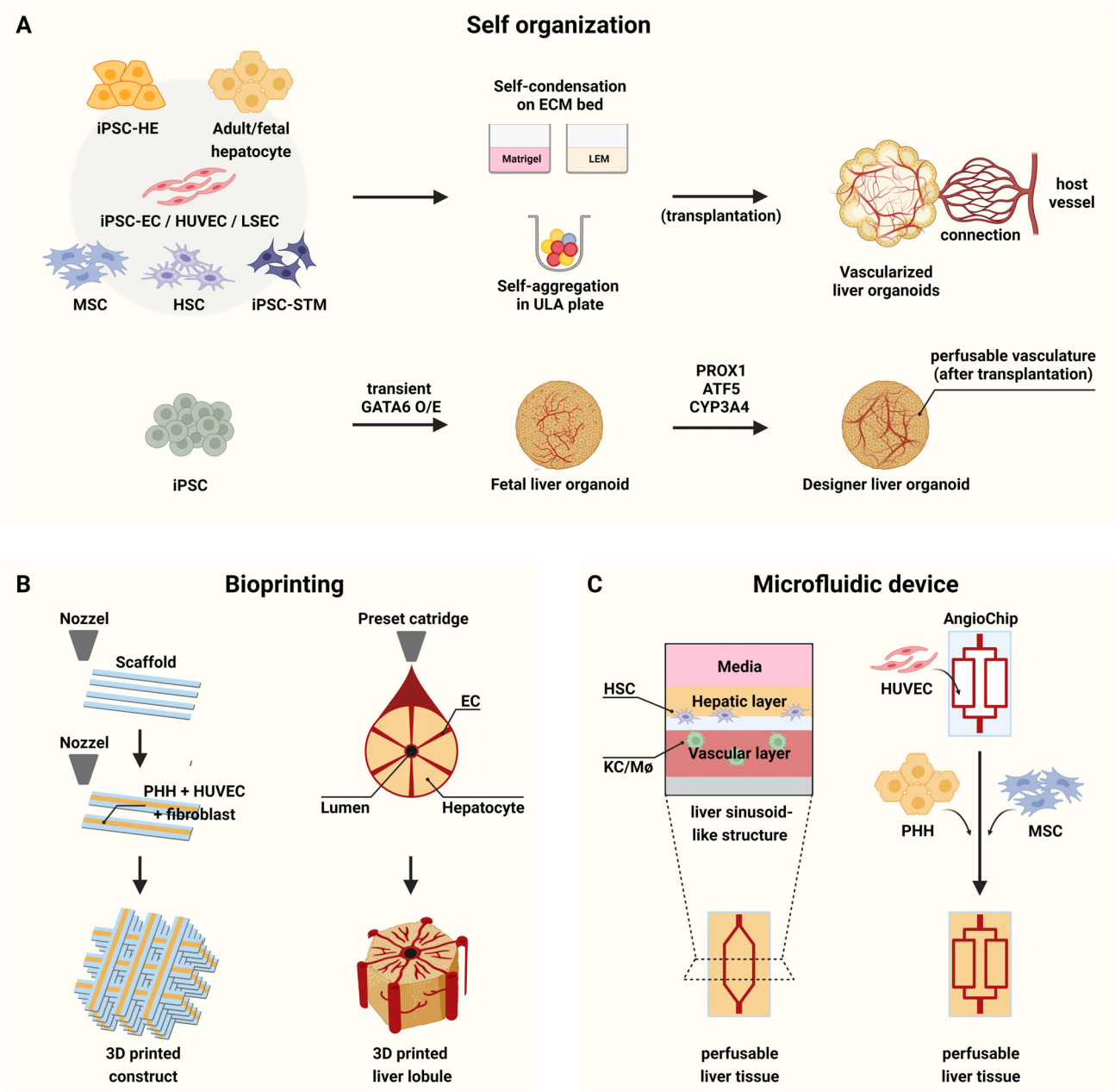

Fig. 3. Current approaches used for generating vascularized liver organoids. (A) Self-organized liver organoids with vasculature by assembling different cell types derived from multiple cell sources. (B) Two different approaches used for printing liver tissue-like architectures from hepatocytes, fibroblast, and ECs. (C) Illustrations representing the strategies for generating microfluidic device-based liver organoids with perfusable vessels. iPSC: induced pluripotent stem cell, iPSC-HE: iPSC-derived hepatic endoderm, iPSC-EC: iPSC-derived endothelial cell, HUVEC: human umbilical vein endothelial cell, LSEC: liver sinusoidal endothelial cell, MSC: mesenchymal stem cell, HSC: hepatic stellate cell, iPSC-STM: iPSC-derived septum transversum mesenchyme, ECM: extracellular matrix, ULA: ultra-low attachment, GATA6: GATA binding transcription factor 6, PROX1: Prospero homeobox 1, ATF5: activating transcription factor 5, CYP3A4: cytochrome P450 3A4, PHH: primary human hepatocyte, 3D: three dimentional, KC: Kupffer cell, MΦ: macrophage.

Self-organization

In 2013, Takebe et al. (13) generated vascularized liver bud-like organoids by culturing and aggregating human induced pluripotent stem cell (hiPSC)-derived hepatic endoderm (HE) cells, HUVECs, and human mesenchymal stem cells (MSCs) on a Matrigel bed. They used a mixture of commercially available culture media to support survival and differentiation of both ECs and hepatocytes in organoids. The

three types of cells self-organized under the MSC-driven force of condensation into 3D aggregates, or liver bud-like organoids, after 48 hours. EC sprouting was also detected in the organoids. The successful generation of organoids were achieved only on the Matrigel bed, but not on agarose, collagen I, or laminin bed, suggesting that ECM is a critical element for self-condensation of the three cell types. FGF and bone morphogenic protein (BMP) signals derived from HUVECs and MSCs were also essential for the organoid condensation, EC network formation, and hepatocyte maturation. After the organoids were transplanted to a mouse cranial window model, they produced extensive networks of human CD31+ vasculature that were connected to mouse CD31+ host vessels in the graft. Dextran perfusion assays showed that the vasculature in the organoids was fully functional after transplantation. The organoid transplants consisting of three different cell types were more similar to the normal liver architecture compared to transplants of HUVEC and MSC aggregates. Because the functional vasculature was generated after transplantation of the organoids, the angiogenic cues derived from host tissues might involve in the vascularization of organoids and are needed to be investigated (13). Based on this strategy, different types of liver organoids were later produced from various cell sources, including fetal liver cells (63), adult primary cells (64, 65), and hPSC-derived cells (14, 16, 66-69). The differentiation of HE, ECs, and septum transversum mesenchyme entirely from hiPSCs, together with the large-scale production of organoids composed of the three cell types, suggested that liver bud-like organoids might be useful for drug testing and cell therapy (16). Incorporating hiPSC-derived ECs and septum transversum mesenchyme (STM), instead of HUVEC and MSCs, increased the hepatic functions of liver bud-like organoids, implying that liver-specific NPCs are important elements for hepatic maturation of the organoids. Moreover, features of organoids with hiPSC-derived EC and STM were more similar to the human liver tissue when compared to the organoids assembled with HUVEC and MSCs, emphasizing that liver-specific NPCs are also crucial for recapitulating the liver tissues (16). The establishment of liver bud-like organoids containing LSECs, instead of HUVECs, was also reported, although further studies are needed to access LSEC-specific features of the ECs in these organoids (64, 65).

Studies have shown that communication among cells of different lineages is essential for liver bud formation from hPSCs and hepatocyte maturation in liver organoids, suggesting that the multi-lineage organoid models might be useful for investigations of cell–cell interactions in liver development (14, 67). Interestingly, ECs in liver bud-like organoids showed increased expression of HIF-1α, a key driving factor of angiogenesis, compared to 2D-cultured ECs (14). *In silico* receptor-ligand pairing suggests that extensive multi-lineage communications are occurred between HE, EC and MSCs in liver bud-like organoids. This study revealed that VEGF signaling is a major factor that promote both the EC network formation and hepatoblast differentiation in the organoids (14). Furthermore, co-culture of HUVECs and MSCs with hiPSC-derived HE induced the expression of multiple proteins which are related to hypoxia, TGFβ signaling, angiogenesis, and hepatic differentiation (67). Therefore, these studies indicate that paracrine signals from different cell types affect the maturation and vascularization of liver bud-like organoids. Multi-lineage liver organoids with vasculature can also be generated by embedding induced hepatocytes, generated by direct reprogramming of fibroblasts, with ECs in decellularized liver ECM (70). This model demonstrated that the combination of a physical cue (media flow) in the culture together with the incorporation of ECs significantly enhances the self-organization, survival, and maturation of liver organoids with complex EC networks. Collectively, the multi-lineage interactions between different cell types, ECM, and interstitial flow, all regulate the maturation and vascularization of self-condensed liver organoids. The self-condensed liver organoids can recapitulate the cell-cell communications that occurs during the liver developmental processes (14, 67), and improve survival of liver-injured mice (13, 16), suggesting the potential use of these organoids as a model system of liver development and a source of cell replacement therapy.

In other approaches, liver organoids were produced by aggregating different cell types on non-tissue-culture-treated or ultra-low-attachment (ULA) multi-well plates (71-74). Liver organoids with EC networks and bile canaliculi were generated by self-aggregation of hiPSC-derived hepatocytes with primary human HSCs, primary human cholangiocytes, primary human LSECs in 96-well ULA plates in the presence of VEGF (72). Other studies used the VEGF and bFGF to induce the EC network formation in organoids by aggregating mouse liver progenitor cells and mouse primary LSECs (73), and iPSC-hepatoblasts, ECs, MSCs (74). All these approaches emphasized the importance of ECs in organoids by showing that ECs 1) enhance the differentiation and maturation of liver progenitor cells (73) and hiPSC-derived hepatocytes in 3D culture (72) and 2) form vessel-like structures that express the LSEC-specific markers lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) and CD32b both *in vitro* and *in vivo* after transplantation (73). Other approaches produced vascularized liver tissues *in vitro* by assembling primary human hepatocytes (PHHs),

normal human dermal fibroblasts, and HUVECs using a fibronectin/gelatin-based layer-by-layer cell coating technique (75). The artificial liver tissues produced by this method developed luminal vasculature that expressed CD31, von-Willebrand factor (vWF) and CD34 and was perfusable after transplantation. Additionally, the vessels were covered with alpha-smooth muscle actin (α-SMA) + pericyte-like cells. Serial coating of each cell type with fibronectin and gelatin before the aggregation increased vessel length and branching points and enhanced VEGF secretion in liver organoids, leading to the formation of complex luminal vasculature *in vitro* without extrinsic angiogenic factors (75).

Liver organoids with blood vessels were also generated from hPSCs by transient overexpression of GATA-binding protein 6 (GATA6) (17, 76). Transient GATA6 overexpression in 2D-cultured hiPSCs spontaneously generated complex liver organoids consisting of hepatocytes, ECs, cholangiocytes, stromal cells, and hematopoietic cells with vascular networks that expressed CD34, CD31 and CD146 *in vitro* (76). Particularly, C-X-C chemokine receptor type 4 (CXCR4) + cells in the organoids expressed key angiogenic factors, VEGF-A and bFGF, suggesting that VEGF-A and bFGF secreted from CXCR4+ cells enabled the vascular network formation in the organoids without additional treatment of exogenous angiogenic factors (76). The hepatic function of the hPSC-derived organoids was improved by clustered regularly interspaced short palindromic repeats (CRISPR)-based transcriptional activation of cytochrome P450 3A4 (CYP3A4) combined with overexpression of Prospero homeobox 1 (PROX1) and activating transcription factor 5 (ATF5) (17). The genetically engineered organoids produced CD31+ and CD34+ vessels covered with Nestin+ pericyte-like cells. The complex vasculature was connected to host vessels and perfusable upon transplantation. The overexpression of CYP3A4, PROX1, and ATF5 increased the expression of key growth factors and receptors involved in angiogenesis and eventually enhanced vascular complexity of the organoids. The CellNet liver gene regulatory network analysis indicated that the organoids generated by overexpressing GATA6, CYP3A4, PROX1 and ATF5 mimic the gene expression profile of human liver tissues better than other liver organoids, including EPCAM+ endoderm-derived organoids (17).

Bioprinting

3D cell printing is a promising technique to recapitulate in culture the complex architecture of liver tissue. There are several approaches to constructing liver-like 3D cellular clusters using bioprinting. Infusion of collagen bioink containing PHHs, HUVECs, and lung fibroblasts into the pre-patterned canals of a hydrogel framework with endothelial growth medium induced the formation of capillary-like networks (77). Furthermore, the heterotypic interaction between the hepatocytes and ECs substantially increased the survival and function of liver cells in the 3D printed construct, emphasizing the importance of vasculature in liver-mimicking microtissues and organoids (77). A different type of 3D bioprinting with a pre-set cartridge designed to resemble the hexagonal structure of liver lobules was used to fabricate hepatic-lobule-like constructs (78). Lobular constructs made by this method had ECs surrounding liver cell clusters as well as the outside of the construct, which eventually became interconnected. The lobular constructs also had enhanced hepatic functions, including drug metabolism and albumin production (78). Large-scale production of 3D liver microtissues or organoids is required for applications in drug screening and cell-based therapy. 3D bioprinting systems equipped with multiple cartridges or needle arrays enable the scalable manufacture of 3D liver-like constructs or organoids that comprise multiple cell types and different biomaterials (13, 79). Although the bioprinting enables the generation of prepatterned vascular and hepatic structures which partially mimic the liver architecture, the potential application of these model for disease modeling and drug screening needed to be further investigated.

Microfluidic devices

Microfluidic systems can be used to simulate many aspects of biological events and are capable of creating biochemical or biophysical microenvironments *in vitro*. Several attempts have been made to use these features to recapitulate the structure and physiology of normal liver vasculature by sequentially seeding different cell types in prepatterned microfluidic devices. A microfluidically perfused biochip that mimics the luminal structure of liver sinusoids was developed by seeding two different mixtures of cells, the first composed of HUVECs and macrophages and the second composed of HepaRG cells and LX2 cells, on each side of a membrane in a microfluidic chip to generate 3D liver organoids (80). In this microfluidic device, perfusion of the medium at an optimal speed increased oxygen supply and improved EC polarization and hepatobiliary functions, probably by inducing flow-mediated shear stress (80). Similarly, a microfluidic chip-based vascularized liver acinus model was developed by sequentially seeding LSECs, THP-1-derived macrophages, LX2 stellate cells, and PHHs with liver ECM (81). In this model, the presence of typical fenestrae structures and the high permeability of the LSEC layer were confirmed by scanning

electron microscopy and fluorescent albumin perfusion. By controlling the flow rates, continuous oxygen zonation was created in the chip, and differential HSC activation was observed across the zone, suggesting the potential application of microfluidic chip-based model for investigating hepatic zone-specific drug metabolism and diseases (81). In a different approach, a prepatterned angiogenesis scaffold (AngioChip) harboring an open-vessel lumen coated with ECs was used to recapitulate the vascular interfaces of the liver (82). Vascularized hepatic tissues engineered using the AngioChip can be produced in a scalable manner and used to monitor the processing of clinically relevant drugs *in vitro* (82). Decellularized liver tissues that preserve ECM and vascular trees can also be used to generate vascularized liver organoids (83). When exogenous ECs and human fetal liver cells were perfused into decellularized liver tissue, the perfused cells were incorporated into their native locations and displayed typical features of each cell type, creating a liver tissue-like construct (83).

Conclusions and Future Perspectives

Strategies to generate vascularized liver organoids have greatly advanced in the last decade. Each method described in this review has advantages and limitations. For instance, self-organized liver organoids recapitulate some of the processes of liver development and vessel formation. However, they do not have a luminal vasculature that is perfusable *in vitro* and cannot accurately mimic the complex architecture of mature liver tissues. Moreover, the unique features of hepatic vasculature, including fenestration, have not been fully analyzed in self-organizing liver organoids. Microfluidic devices provide interstitial flow through channels, thereby enhancing tissue maturation and providing a flow model of the liver sinusoid, but they have limited ability to recapitulate the developmental processes of the liver. Bioprinting can partially mimic hepatic lobular and vascular structures. However, the bioink used in 3D bioprinting needs to be developed further to overcome issues related to tissue specificity, cell viability, shape fidelity, resolution, supportive ability, and biocompatibility.

There are a few reports demonstrating tissue-specific features of blood vessels, including marker expression, characteristics of fenestrae and permeability in liver organoids. However, to our knowledge, there is no report investigating pathobiological changes of the vasculature using liver organoids. As mentioned earlier, LSECs interplay with other liver cells under pathological conditions, losing fenestrae and NO synthesis activity and secrete proinflammatory cytokines and ECM in injured liver. These pathological changes of LSECs are critical for disease progression because they lead to portal hypertension in chronic liver disease and impaired liver function, activating HSCs and KCs. Thus, the investigation of vascular changes in liver organoids is urgently required to precisely model the chronic liver diseases.

Tissue engineering techniques have been evolving to produce 3D liver tissues that more precisely mimic the liver architecture and physiology in the human body. A major limitation of these techniques is that the diffusion of oxygen and nutrients is limited by the thickness of the tissues. Therefore, integrated studies of the vascularization of 3D tissues are urgently needed to generate bigger and more complex artificial liver tissues, organoids, and assembloids that recapitulate *in vivo* conditions. Recently, substantial glomerular networks with perfusable lumens were achieved in hPSC-derived kidney organoids by combining self-organization and microfluidic approaches (84). In another study, brain organoids derived from hPSCs could generate perfusable vasculature with blood-brain barrier characteristics *in vitro* after incorporation of inducible ETS variant 2 (ETV2)-overexpressing hPSCs (85). Other studies vascularized brain organoids by fusing them with blood vessel organoids composed of ECs and perivascular cells and then inducing directed angiogenesis toward the brain organoids (86, 87). The manufacture and engineering of tissue-specific ECM for organoids is another promising strategy to recapitulate tissue-specific features (88).

Vascularized liver organoids provide advanced models for investigations of multi-lineage communication in normal and pathological conditions. The presence of functional luminal vasculature enables even distribution of nutrients, oxygen, drugs, and pathological stimuli to the core of the organoids. Therefore, vascularized liver organoids will allow more accurate screening for drug efficacy and toxicity. In addition, pre-vascularization of organoids *in vitro* may improve the survival and function of grafts after transplantation, contributing to more successful therapeutic outcomes for patients with liver disease.

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Potential Conflict of Interest

The authors have no conflicting financial interest.

References

1. Ho H, Zhang E. Virtual lobule models are the key for multiscale biomechanical and pharmacological modeling for the liver. Front Physiol 2020;11:1061
2. Matsumoto K, Yoshitomi H, Rossant J, Zaret KS. Liver organogenesis promoted by endothelial cells prior to vascular function. Science 2001;294:559-563
3. Si-Tayeb K, Lemaigre FP, Duncan SA. Organogenesis and development of the liver. Dev Cell 2010;18:175-189
4. Iwakiri Y, Shah V, Rockey DC. Vascular pathobiology in chronic liver disease and cirrhosis - current status and future directions. J Hepatol 2014;61:912-924
5. Elaut G, Henkens T, Papeleu P, Snykers S, Vinken M, Vanhaecke T, Rogiers V. Molecular mechanisms underlying the dedifferentiation process of isolated hepatocytes and their cultures. Curr Drug Metab 2006;7:629-660
6. Huch M, Dorrell C, Boj SF, van Es JH, Li VS, van de Wetering M, Sato T, Hamer K, Sasaki N, Finegold MJ, Haft A, Vries RG, Grompe M, Clevers H. In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. Nature 2013;494:247-250
7. Huch M, Gehart H, van Boxtel R, Hamer K, Blokzijl F, Verstegen MM, Ellis E, van Wenum M, Fuchs SA, de Ligt J, van de Wetering M, Sasaki N, Boers SJ, Kemperman H, de Jonge J, Ijzermans JN, Nieuwenhuis EE, Hoekstra R, Strom S, Vries RR, van der Laan LJ, Cuppen E, Clevers H. Long-term culture of genome-stable bipotent stem cells from adult human liver. Cell 2015;160:299-312
8. Hu H, Gehart H, Artegiani B, LÖpez-Iglesias C, Dekkers F, Basak O, van Es J, Chuva de Sousa Lopes SM, Begthel H, Korving J, van den Born M, Zou C, Quirk C, Chiriboga L, Rice CM, Ma S, Rios A, Peters PJ, de Jong YP, Clevers H. Long-term expansion of functional mouse and human hepatocytes as 3D organoids. Cell 2018;175:1591-1606.e19
9. Shinozawa T, Kimura M, Cai Y, Saiki N, Yoneyama Y, Ouchi R, Koike H, Maezawa M, Zhang RR, Dunn A, Ferguson A, Togo S, Lewis K, Thompson WL, Asai A, Takebe T. High-fidelity drug-induced liver injury screen using human pluripotent stem cell-derived organoids. Gastroenterology 2021;160:831-846.e10
10. Kim H, Im I, Jeon JS, Kang EH, Lee HA, Jo S, Kim JW, Woo DH, Choi YJ, Kim HJ, Han JS, Lee BS, Kim JH, Kim SK, Park HJ. Development of human pluripotent stem cell-derived hepatic organoids as an alternative model for drug safety assessment. Biomaterials 2022;286:121575
11. Mun SJ, Ryu JS, Lee MO, Son YS, Oh SJ, Cho HS, Son MY, Kim DS, Kim SJ, Yoo HJ, Lee HJ, Kim J, Jung CR, Chung KS, Son MJ. Generation of expandable human pluripotent stem cell-derived hepatocyte-like liver organoids. J Hepatol 2019;71:970-985
12. Collardeau-Frachon S, Scoazec JY. Vascular development and differentiation during human liver organogenesis. Anat Rec (Hoboken) 2008;291:614-627
13. Takebe T, Sekine K, Enomura M, Koike H, Kimura M, Ogaeri T, Zhang RR, Ueno Y, Zheng YW, Koike N, Aoyama S, Adachi Y, Taniguchi H. Vascularized and functional human liver from an iPSC-derived organ bud transplant. Nature 2013;499:481-484
14. Camp JG, Sekine K, Gerber T, Loeffler-Wirth H, Binder H, Gac M, Kanton S, Kageyama J, Damm G, Seehofer D, Belicova L, Bickle M, Barsacchi R, Okuda R, Yoshizawa E, Kimura M, Ayabe H, Taniguchi H, Takebe T, Treutlein B. Multilineage communication regulates human liver bud development from pluripotency. Nature 2017;546:533-538
15. Jain RK, Au P, Tam J, Duda DG, Fukumura D. Engineering vascularized tissue. Nat Biotechnol 2005;23:821-823
16. Takebe T, Sekine K, Kimura M, Yoshizawa E, Ayano S, Koido M, Funayama S, Nakanishi N, Hisai T, Kobayashi T, Kasai T, Kitada R, Mori A, Ayabe H, Ejiri Y, Amimoto N, Yamazaki Y, Ogawa S, Ishikawa M, Kiyota Y, Sato Y, Nozawa K, Okamoto S, Ueno Y, Taniguchi H. Massive and reproducible production of liver buds entirely from human pluripotent stem cells. Cell Rep 2017;21:2661-2670
17. Velazquez JJ, LeGraw R, Moghadam F, Tan Y, Kilbourne J, Maggiore JC, Hislop J, Liu S, Cats D, Chuva de Sousa Lopes SM, Plaisier C, Cahan P, Kiani S, Ebrahimkhani MR. Gene regulatory network analysis and engineering directs development and vascularization of multilineage human liver organoids. Cell Syst 2021;12:41-55.e11
18. Rafii S, Butler JM, Ding BS. Angiocrine functions of organ-specific endothelial cells. Nature 2016;529:316-325
19. Trefts E, Gannon M, Wasserman DH. The liver. Curr Biol 2017;27:R1147-R1151
20. DeSesso JM. Vascular ontogeny within selected thoracoabdominal organs and the limbs. Reprod Toxicol 2017;70:3-20
21. Zhang X, Tang L, Yi Q. Engineering the vasculature of stem-cell-derived liver organoids. Biomolecules 2021;11:966
22. Poisson J, Lemoinne S, Boulanger C, Durand F, Moreau R, Valla D, Rautou PE. Liver sinusoidal endothelial cells: physiology and role in liver diseases. J Hepatol 2017;66:212-227
23. Ober EA, Lemaigre FP. Development of the liver: insights into organ and tissue morphogenesis. J Hepatol 2018;68:1049-1062
24. DeLeve LD, Wang X, Hu L, McCuskey MK, McCuskey RS. Rat liver sinusoidal endothelial cell phenotype is maintained by paracrine and autocrine regulation. Am J Physiol Gastrointest Liver Physiol 2004;287:G757-G763
25. Yoshida M, Nishikawa Y, Omori Y, Yoshioka T, Tokairin T, McCourt P, Enomoto K. Involvement of signaling of VEGF and TGF-beta in differentiation of sinusoidal endothelial cells during culture of fetal rat liver cells. Cell Tissue Res 2007;329:273-282
26. Klein D, Demory A, Peyre F, Kroll J, Augustin HG, Helfrich W, Kzhyshkowska J, Schledzewski K, Arnold B, Goerdt S. Wnt2 acts as a cell type-specific, autocrine growth factor in rat hepatic sinusoidal endothelial cells cross-

stimulating the VEGF pathway. Hepatology 2008;47:1018-1031
27. Géraud C, Koch PS, Zierow J, Klapproth K, Busch K, Olsavszky V, Leibing T, Demory A, Ulbrich F, Diett M, Singh S, Sticht C, Breitkopf-Heinlein K, Richter K, Karppinen SM, Pihlajaniemi T, Arnold B, Rodewald HR, Augustin HG, Schledzewski K, Goerdt S. GATA4-dependent organ-specific endothelial differentiation controls liver development and embryonic hematopoiesis. J Clin Invest 2017;127:1099-1114
28. DeLeve LD, Maretti-Mira AC. Liver sinusoidal endothelial cell: an update. Semin Liver Dis 2017;37:377-387
29. Hammoutene A, Rautou PE. Role of liver sinusoidal endothelial cells in non-alcoholic fatty liver disease. J Hepatol 2019;70:1278-1291
30. Gracia-Sancho J, Caparrós E, Fernández-Iglesias A, Francés R. Role of liver sinusoidal endothelial cells in liver diseases. Nat Rev Gastroenterol Hepatol 2021;18:411-431
31. Gracia-Sancho J, Laviña B, Rodríguez-Vilarrupla A, García-Calderó H, Bosch J, García-Pagán JC. Enhanced vasoconstrictor prostanoid production by sinusoidal endothelial cells increases portal perfusion pressure in cirrhotic rat livers. J Hepatol 2007;47:220-227
32. Deleve LD, Wang X, Guo Y. Sinusoidal endothelial cells prevent rat stellate cell activation and promote reversion to quiescence. Hepatology 2008;48:920-930
33. Xie G, Choi SS, Syn WK, Michelotti GA, Swiderska M, Karaca G, Chan IS, Chen Y, Diehl AM. Hedgehog signalling regulates liver sinusoidal endothelial cell capillarisation. Gut 2013;62:299-309
34. Ding BS, Cao Z, Lis R, Nolan DJ, Guo P, Simons M, Penfold ME, Shido K, Rabbany SY, Rafii S. Divergent angiocrine signals from vascular niche balance liver regeneration and fibrosis. Nature 2014;505:97-102
35. Coulon S, Heindryckx F, Geerts A, Van Steenkiste C, Colle I, Van Vlierberghe H. Angiogenesis in chronic liver disease and its complications. Liver Int 2011;31:146-162
36. Patan S. Vasculogenesis and angiogenesis. Cancer Treat Res 2004;117:3-32
37. Potente M, Mäkinen T. Vascular heterogeneity and specialization in development and disease. Nat Rev Mol Cell Biol 2017;18:477-494
38. Arroyo AG, Iruela-Arispe ML. Extracellular matrix, inflammation, and the angiogenic response. Cardiovasc Res 2010;86:226-235
39. Potente M, Gerhardt H, Carmeliet P. Basic and therapeutic aspects of angiogenesis. Cell 2011;146:873-887
40. Naito H, Iba T, Takakura N. Mechanisms of new blood-vessel formation and proliferative heterogeneity of endothelial cells. Int Immunol 2020;32:295-305
41. Choi HJ, Zhang H, Park H, Choi KS, Lee HW, Agrawal V, Kim YM, Kwon YG. Yes-associated protein regulates endothelial cell contact-mediated expression of angiopoietin-2. Nat Commun 2015;6:6943
42. Ong YT, Andrade J, Armbruster M, Shi C, Castro M, Costa ASH, Sugino T, Eelen G, Zimmermann B, Wilhelm K, Lim J, Watanabe S, Guenther S, Schneider A, Zanconato F, Kaulich M, Pan D, Braun T, Gerhardt H, Efeyan A, Carmeliet P, Piccolo S, Grosso AR, Potente M. A YAP/TAZ-TEAD signalling module links endothelial nutrient acquisition to angiogenic growth. Nat Metab 2022;4:672-682
43. Wright GL, Maroulakou IG, Eldridge J, Liby TL, Sridharan V, Tsichlis PN, Muise-Helmericks RC. VEGF stimulation of mitochondrial biogenesis: requirement of AKT3 kinase. FASEB J 2008;22:3264-3275
44. Guo D, Wang Q, Li C, Wang Y, Chen X. VEGF stimulated the angiogenesis by promoting the mitochondrial functions. Oncotarget 2017;8:77020-77027
45. Lamalice L, Le Boeuf F, Huot J. Endothelial cell migration during angiogenesis. Circ Res 2007;100:782-794
46. Serbo JV, Gerecht S. Vascular tissue engineering: biodegradable scaffold platforms to promote angiogenesis. Stem Cell Res Ther 2013;4:8
47. Saharinen P, Eklund L, Miettinen J, Wirkkala R, Anisimov A, Winderlich M, Nottebaum A, Vestweber D, Deutsch U, Koh GY, Olsen BR, Alitalo K. Angiopoietins assemble distinct Tie2 signalling complexes in endothelial cell-cell and cell-matrix contacts. Nat Cell Biol 2008;10:527-537
48. Sulpice E, Ding S, Muscatelli-Groux B, Bergé M, Han ZC, Plouet J, Tobelem G, Merkulova-Rainon T. Cross-talk between the VEGF-A and HGF signalling pathways in endothelial cells. Biol Cell 2009;101:525-539
49. Kaga T, Kawano H, Sakaguchi M, Nakazawa T, Taniyama Y, Morishita R. Hepatocyte growth factor stimulated angiogenesis without inflammation: differential actions between hepatocyte growth factor, vascular endothelial growth factor and basic fibroblast growth factor. Vascul Pharmacol 2012;57:3-9
50. Oladipupo S, Hu S, Kovalski J, Yao J, Santeford A, Sohn RE, Shohet R, Maslov K, Wang LV, Arbeit JM. VEGF is essential for hypoxia-inducible factor-mediated neovascularization but dispensable for endothelial sprouting. Proc Natl Acad Sci U S A 2011;108:13264-13269
51. Brindle NP, Saharinen P, Alitalo K. Signaling and functions of angiopoietin-1 in vascular protection. Circ Res 2006;98:1014-1023
52. Williams PA, Stilhano RS, To VP, Tran L, Wong K, Silva EA. Hypoxia augments outgrowth endothelial cell (OEC) sprouting and directed migration in response to sphingosine-1-phosphate (S1P). PLoS One 2015;10:e0123437
53. Qin D, Trenkwalder T, Lee S, Chillo O, Deindl E, Kupatt C, Hinkel R. Early vessel destabilization mediated by Angiopoietin-2 and subsequent vessel maturation via Angiopoietin-1 induce functional neovasculature after ischemia. PLoS One 2013;8:e61831
54. Takuwa Y, Du W, Qi X, Okamoto Y, Takuwa N, Yoshioka K. Roles of sphingosine-1-phosphate signaling in angiogenesis. World J Biol Chem 2010;1:298-306
55. Crosby CO, Zoldan J. Mimicking the physical cues of the ECM in angiogenic biomaterials. Regen Biomater 2019;6:61-73
56. Edgar LT, Underwood CJ, Guilkey JE, Hoying JB, Weiss

JA. Extracellular matrix density regulates the rate of neovessel growth and branching in sprouting angiogenesis. PLoS One 2014;9:e85178
57. Mason BN, Starchenko A, Williams RM, Bonassar LJ, Reinhart-King CA. Tuning three-dimensional collagen matrix stiffness independently of collagen concentration modulates endothelial cell behavior. Acta Biomater 2013;9: 4635-4644
58. Bao M, Chen Y, Liu JT, Bao H, Wang WB, Qi YX, Lv F. Extracellular matrix stiffness controls $VEGF_{165}$ secretion and neuroblastoma angiogenesis via the YAP/RUNX2/SRSF1 axis. Angiogenesis 2022;25:71-86
59. Guo Y, Mei F, Huang Y, Ma S, Wei Y, Zhang X, Xu M, He Y, Heng BC, Chen L, Deng X. Matrix stiffness modulates tip cell formation through the p-PXN-Rac1-YAP signaling axis. Bioact Mater 2021;7:364-376
60. Paszkowiak JJ, Dardik A. Arterial wall shear stress: observations from the bench to the bedside. Vasc Endovascular Surg 2003;37:47-57
61. Dekker RJ, van Soest S, Fontijn RD, Salamanca S, de Groot PG, VanBavel E, Pannekoek H, Horrevoets AJ. Prolonged fluid shear stress induces a distinct set of endothelial cell genes, most specifically lung Krüppel-like factor (KLF2). Blood 2002;100:1689-1698
62. Yuan P, Hu Q, He X, Long Y, Song X, Wu F, He Y, Zhou X. Laminar flow inhibits the Hippo/YAP pathway via autophagy and SIRT1-mediated deacetylation against atherosclerosis. Cell Death Dis 2020;11:141
63. Takebe T, Koike N, Sekine K, Fujiwara R, Amiya T, Zheng YW, Taniguchi H. Engineering of human hepatic tissue with functional vascular networks. Organogenesis 2014;10: 260-267
64. Ramachandran SD, Schirmer K, Münst B, Heinz S, Ghafoory S, Wölfl S, Simon-Keller K, Marx A, Øie CI, Ebert MP, Walles H, Braspenning J, Breitkopf-Heinlein K. In vitro generation of functional liver organoid-like structures using adult human cells. PLoS One 2015;10:e0139345
65. Li J, Xing F, Chen F, He L, So KF, Liu Y, Xiao J. Functional 3D human liver bud assembled from MSC-derived multiple liver cell lineages. Cell Transplant 2019;28:510-521 Erratum in: Cell Transplant 2022;31: 9636897221126859
66. Takebe T, Enomura M, Yoshizawa E, Kimura M, Koike H, Ueno Y, Matsuzaki T, Yamazaki T, Toyohara T, Osafune K, Nakauchi H, Yoshikawa HY, Taniguchi H. Vascularized and complex organ buds from diverse tissues via mesenchymal cell-driven condensation. Cell Stem Cell 2015;16: 556-565
67. Asai A, Aihara E, Watson C, Mourya R, Mizuochi T, Shivakumar P, Phelan K, Mayhew C, Helmrath M, Takebe T, Wells J, Bezerra JA. Paracrine signals regulate human liver organoid maturation from induced pluripotent stem cells. Development 2017;144:1056-1064
68. Ayabe H, Anada T, Kamoya T, Sato T, Kimura M, Yoshizawa E, Kikuchi S, Ueno Y, Sekine K, Camp JG, Treutlein B, Ferguson A, Suzuki O, Takebe T, Taniguchi H. Optimal hypoxia regulates human iPSC-derived liver bud differentiation through intercellular TGFB signaling. Stem Cell Reports 2018;11:306-316
69. Goulart E, de Caires-Junior LC, Telles-Silva KA, Araujo BHS, Kobayashi GS, Musso CM, Assoni AF, Oliveira D, Caldini E, Gerstenhaber JA, Raia S, Lelkes PI, Zatz M. Adult and iPS-derived non-parenchymal cells regulate liver organoid development through differential modulation of Wnt and TGF-β. Stem Cell Res Ther 2019;10:258
70. Jin Y, Kim J, Lee JS, Min S, Kim S, Ahn DH, Kim YG, Cho SW. Vascularized liver organoids generated using induced hepatic tissue and dynamic liver-specific microenvironment as a drug testing platform. Adv Funct Mater 2018; 28:1801954
71. Peng WC, Logan CY, Fish M, Anbarchian T, Aguisanda F, Álvarez-Varela A, Wu P, Jin Y, Zhu J, Li B, Grompe M, Wang B, Nusse R. Inflammatory cytokine TNFα promotes the long-term expansion of primary hepatocytes in 3D culture. Cell 2018;175:1607-1619.e15
72. Ardalani H, Sengupta S, Harms V, Vickerman V, Thomson JA, Murphy WL. 3-D culture and endothelial cells improve maturity of human pluripotent stem cell-derived hepatocytes. Acta Biomater 2019;95:371-381
73. Yap KK, Gerrand YW, Dingle AM, Yeoh GC, Morrison WA, Mitchell GM. Liver sinusoidal endothelial cells promote the differentiation and survival of mouse vascularised hepatobiliary organoids. Biomaterials 2020;251:120091
74. Son JS, Park CY, Lee G, Park JY, Kim HJ, Kim G, Chi KY, Woo DH, Han C, Kim SK, Park HJ, Kim DW, Kim JH. Therapeutic correction of hemophilia A using 2D endothelial cells and multicellular 3D organoids derived from CRISPR/Cas9-engineered patient iPSCs. Biomaterials 2022;283:121429
75. Sasaki K, Akagi T, Asaoka T, Eguchi H, Fukuda Y, Iwagami Y, Yamada D, Noda T, Wada H, Gotoh K, Kawamoto K, Doki Y, Mori M, Akashi M. Construction of three-dimensional vascularized functional human liver tissue using a layer-by-layer cell coating technique. Biomaterials 2017;133:263-274
76. Guye P, Ebrahimkhani MR, Kipniss N, Velazquez JJ, Schoenfeld E, Kiani S, Griffith LG, Weiss R. Genetically engineering self-organization of human pluripotent stem cells into a liver bud-like tissue using Gata6. Nat Commun 2016;7:10243
77. Lee JW, Choi YJ, Yong WJ, Pati F, Shim JH, Kang KS, Kang IH, Park J, Cho DW. Development of a 3D cell printed construct considering angiogenesis for liver tissue engineering. Biofabrication 2016;8:015007
78. Kang D, Hong G, An S, Jang I, Yun WS, Shim JH, Jin S. Bioprinting of multiscaled hepatic lobules within a highly vascularized construct. Small 2020;16:e1905505
79. Yanagi Y, Nakayama K, Taguchi T, Enosawa S, Tamura T, Yoshimaru K, Matsuura T, Hayashida M, Kohashi K, Oda Y, Yamaza T, Kobayashi E. In vivo and ex vivo methods of growing a liver bud through tissue connection. Sci Rep 2017;7:14085

80. Rennert K, Steinborn S, Gröger M, Ungerböck B, Jank AM, Ehgartner J, Nietzsche S, Dinger J, Kiehntopf M, Funke H, Peters FT, Lupp A, Gärtner C, Mayr T, Bauer M, Huber O, Mosig AS. A microfluidically perfused three dimensional human liver model. Biomaterials 2015;71:119-131
81. Li X, George SM, Vernetti L, Gough AH, Taylor DL. A glass-based, continuously zonated and vascularized human liver acinus microphysiological system (vLAMPS) designed for experimental modeling of diseases and ADME/TOX. Lab Chip 2018;18:2614-2631
82. Zhang B, Montgomery M, Chamberlain MD, Ogawa S, Korolj A, Pahnke A, Wells LA, Massé S, Kim J, Reis L, Momen A, Nunes SS, Wheeler AR, Nanthakumar K, Keller G, Sefton MV, Radisic M. Biodegradable scaffold with built-in vasculature for organ-on-a-chip engineering and direct surgical anastomosis. Nat Mater 2016;15:669-678
83. Baptista PM, Siddiqui MM, Lozier G, Rodriguez SR, Atala A, Soker S. The use of whole organ decellularization for the generation of a vascularized liver organoid. Hepatology 2011;53:604-617
84. Homan KA, Gupta N, Kroll KT, Kolesky DB, Skylar-Scott M, Miyoshi T, Mau D, Valerius MT, Ferrante T, Bonventre JV, Lewis JA, Morizane R. Flow-enhanced vascularization and maturation of kidney organoids in vitro. Nat Methods 2019;16:255-262
85. Cakir B, Xiang Y, Tanaka Y, Kural MH, Parent M, Kang YJ, Chapeton K, Patterson B, Yuan Y, He CS, Raredon MSB, Dengelegi J, Kim KY, Sun P, Zhong M, Lee S, Patra P, Hyder F, Niklason LE, Lee SH, Yoon YS, Park IH. Engineering of human brain organoids with a functional vascular-like system. Nat Methods 2019;16:1169-1175
86. Sun XY, Ju XC, Li Y, Zeng PM, Wu J, Zhou YY, Shen LB, Dong J, Chen YJ, Luo ZG. Generation of vascularized brain organoids to study neurovascular interactions. Elife 2022;11:e76707
87. Kook MG, Lee SE, Shin N, Kong D, Kim DH, Kim MS, Kang HK, Choi SW, Kang KS. Generation of cortical brain organoid with vascularization by assembling with vascular spheroid. Int J Stem Cells 2022;15:85-94
88. Heo JH, Kang D, Seo SJ, Jin Y. Engineering the extracellular matrix for organoid culture. Int J Stem Cells 2022;15:60-69