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Review article

Emerging biotechnologies for engineering liver organoids

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ABSTRACT

The engineering construction of the liver has attracted enormous attention. Organoids, as emerging miniature three-dimensional cultivation units, hold significant potential in the biomimetic simulation of liver structure and function. Despite notable successes, organoids still face limitations such as high variability and low maturity. To overcome these challenges, engineering strategies have been established to maintain organoid stability and enhance their efficacy, laying the groundwork for the development of advanced liver organoids. The present review comprehensively summarizes the construction of engineered liver organoids and their prospective applications in biomedicine. Initially, we briefly present the latest research progress on matrix materials that maintain the three-dimensional morphology of organoids. Next, we discuss the manipulative role of engineering technologies in organoid assembly. Additionally, we outline the impact of gene-level regulation on organoid growth and development. Further, we introduce the applications of liver organoids in disease modeling, drug screening and regenerative medicine. Lastly, we overview the current obstacles and forward-looking perspectives on the future of engineered liver organoids. We anticipate that ongoing innovations in engineered liver organoids will lead to significant advancements in medical applications.

1. Introduction

Liver organoids have advanced beyond traditional two-dimensional (2D) cell cultures and animal models, emerging as promising tools for disease modeling, drug screening and tissue engineering [1,2]. Unlike the disordered and simple growth seen in three-dimensional (3D) hepatocyte clusters, liver organoids form well-organized, polarized 3D structures capable of self-renewal and differentiation [3–5]. Liver organoids can mimic essential physiological aspects of actual livers, such as cellular diversity, spatial organization, and the microenvironment, while performing critical liver functions like protein synthesis and drug metabolism [6,7]. Typically, researchers cultivate organoids using primary hepatocytes from tissues, adult stem cells, or induced pluripotent stem cells (iPSCs) within an animal-sourced scaffold known as Matrigel [8–11]. However, these organoid models are constrained by significant variability due to manual processes, the absence of standardized 3D scaffolds, and the difficulty in achieving full maturity and complexity within the constraints of research settings [5,12–14].

Engineered liver organoids are designed to address challenges related to materials, technologies and cellular components seen in traditional models. Key to their success are the precise morphogen gradients and the development of tissue-like scaffolds that simulate the in vivo cellular microenvironment, essential for preserving cell health and functionality [15]. Hydrogels, both natural and synthetic hydrogels, along with decellularized scaffolds, are favored for their availability, biocompatibility and mechanical properties [16,17]. Innovative engineering technologies, including droplet microfluidics, acoustic fluidics, 3D bioprinting and organoids-on-a-chip technologies, offer significant improvements in organoid assembly efficiency, featuring enhanced control, consistency, and scalability compared to conventional approaches [18–21]. In contrast, the future of organoids is inherently tied to the differentiation processes of stem cells, making genetic engineering a powerful approach for fundamental modifications to stem cells [22]. This enables the tailored development of organoids with specific characteristics and functions. A profound comprehension of stem cell dynamics, coupled with the expanding use of genetic engineering, paves

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the way for creating more complex and functional liver organoids.

Herein, we summarized the most recent advancements in liver organoids, focusing on their engineering fabrication and biomedical application (Fig. 1). Initially, we outlined the diverse matrix materials utilized in liver organoids, including natural hydrogels, synthetic hydrogels, and decellularized scaffolds. Subsequently, we discussed the various engineering technologies employed, such as droplet microfluidics, acoustic fluidics, 3D bioprinting and organoids-on-a-chip systems. Next, we delved into the applications of genetic engineering in editing liver organoids, particularly viral transduction and the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9 (CRISPR/Cas9) system. Furthermore, we illuminated liver organoids as a novel frontier in the field of disease modeling, drug screening, and regenerative medicine. Lastly, we addressed the present obstacles encountered by liver organoids and provided insights into their promising role in liver disease treatment and clinical drug development.

2. Matrix materials for engineering liver organoids

The extracellular matrix (ECM) is an intricate network of proteins and polysaccharides that surround cells within tissues [23,24]. Apart from providing essential 3D support to cells, the ECM significantly influences key cellular processes such as cell adhesion, migration, proliferation, differentiation, and the attainment of specific functional states [25–28]. In the context of organoid formation, ECM mimics play a pivotal role by supplying bioactive factors, structural 3D support, and morphological cues to the enclosed cellular clusters [29,30]. The development of liver organoid culture relies on identifying a dependable matrix material that accurately reproduces the in vivo environment, thus aiding in the creation of stable and physiologically relevant models. Hydrogels are increasingly popular as preferred cell-guided scaffolds, paving the way for enhancing the engineering of liver organoids.

2.1. Natural hydrogels

Natural hydrogels are a type of polymers sourced from animals or plants. They can establish a microenvironment resembling the organ being studied, supporting cell growth and differentiation within the organoid [31]. The most frequently utilized 3D matrix for growing organoids is Matrigel, a basement membrane matrix-based hydrogel derived from Engelbreth-Holm-Swarm mouse sarcoma chondrosarcoma murine cells [32,33]. While Matrigel is highly biologically active and aids the proliferation of various organ types, its tumor origin restricts its application in clinical therapy [34–37]. Additionally, it is costly, lacks a precise definition, and exhibits inherent heterogeneity [38,39].

Another category of polymers, known as defined hydrogels, have a clear and controlled composition, including animal-derived polymers like collagen I, laminin, gelatin and fibrin, as well as plant-derived polymers like chitosan, alginate and agarose [31,40–42]. Despite their natural occurrence, these materials can now be artificially synthesized or modified to adjust their chemical and physical properties to meet application needs. For instance, recombinant collagen produced through genetic engineering technology is characterized by its single composition, batch consistency, and enhanced hydrophilicity [43]. Additionally, the deacetylation degree of chitosan can be altered to adjust its solubility and mechanical properties [44]. These polymers offer easily controllable physicochemical properties, enhancing reproducibility in experiments compared to Matrigel [45]. Furthermore, they are plentiful and demonstrate strong biocompatibility. Nevertheless, they do face

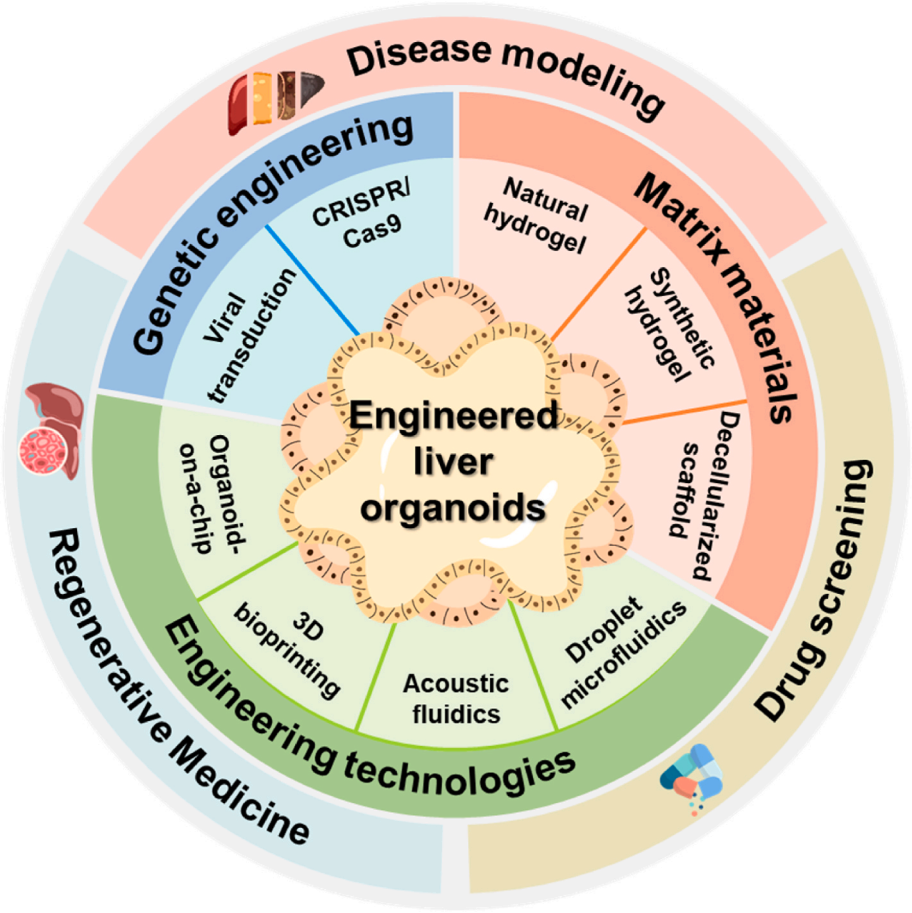

Fig. 1. Schematic of the emerging biotechnologies for engineering liver organoids and their applications in biomedicine.

constraints such as poor stability, inferior mechanical properties, and rapid degradation [46]. Considering these challenges, modified natural hydrogels present more ideal properties and may be modified to respond to external stimuli like temperature, light, and ions. For example, modification of gelatin with methacryloyl groups to form GelMA endows it with tunable mechanical properties and degradation rates, while also enhancing its biocompatibility, and conferring photopolymerization capabilities [47]. This multifunctional, customizable modified natural hydrogel is more suitable for cell culture.

2.2. Synthetic hydrogels

Synthetic hydrogels encompass a group of chemically synthesized polymers, such as polyethylene glycol (PEG) and polyisocyanopeptides (PIC) [47]. These materials are stable and can be easily tailored in physical properties, chemical composition and overall structure, but they may lack biochemical properties [48–50]. To address this limitation, synthetic hydrogels are often improved through various modifications, including functionalizations, integrin commodification, and protein alterations [51]. These enhancements aim to create organoid culture systems with superior mechanical and biochemical attributes tailored for specific research applications [52]. By combining the stability and customizability of synthetic hydrogels with targeted modifications, researchers can develop advanced organoid models that better mimic the complexities of biological systems.

Researchers successfully generated iPSC-derived liver organoids in a PEG scaffold coated with collagen I [53]. Sorrentino et al. found that functionalizing PEG hydrogels with fibronectin, laminin or Arg-Gly-Asp (RGD) leads to varying degrees of organogenesis (Fig. 2a) [54]. Among them, organoids induced by PEG-RGD exhibit physical forms and transcriptomic patterns comparable to those of Matrigel. Similarly, researchers demonstrated that PIC polymers functionalized with RGD successfully promote cell growth [55]. Ye et al. discovered that PIC-RGD was insufficient to stimulate liver organoid proliferation for a possible reason of the lower RGD concentration, but a novel hydrogel combining PIC and laminin effectively induced liver organoid formation (Fig. 2b) [56]. Human recombinant laminin-111 (hr Laminin-111) possesses equivalent proliferative and differentiative support capabilities for liver organoids as the laminin-entactin complex (LEC), and it avoids the tumorigenicity associated with LEC, making it more promising for clinical applications. These studies reveal that synthetic hydrogels are comparable to or even superior to Matrigel. By adjusting the stiffness of the synthetic network, synthetic hydrogels can better match the physiological level of the liver and provide long-term mechanical support compared to Matrigel [57]. This property highlights the significant promise of synthetic hydrogels for cultivating human liver organoids and their suitability in clinical realms like cellular therapy and tissue engineering.

2.3. Decellularized scaffolds

Decellularized scaffolds are a type of ECM obtained by eliminating cells from tissues through physical, chemical, or enzymatic methods [58–60]. Therefore, they are also classified under the domain of native hydrogels. Unlike single-component ECMs and synthetic hydrogels, decellularized scaffolds preserve the complete 3D structure, biochemical composition, and biological activity of the tissue, offering the most authentic cues for cell attachment, proliferation, and differentiation [61–63]. Seeding target cells onto whole decellularized scaffolds is known as recellularization. Whole liver decellularized scaffolds offer the potential to implant various liver cells, including cholangiocytes and hepatocytes, to reproduce the cellular distribution and cellular activity of the natural liver [64]. Chen et al. successfully obtained rat liver whole decellularized scaffolds and recellularized them with highly active primary cholangiocytes (Fig. 3a) [65]. The primary cholangiocytes are evenly dispersed within the lumen, gradually forming a seamless biliary tree network while maintaining robust cellular viability. These functional ductal organoids express specific markers of cholangiocytes and

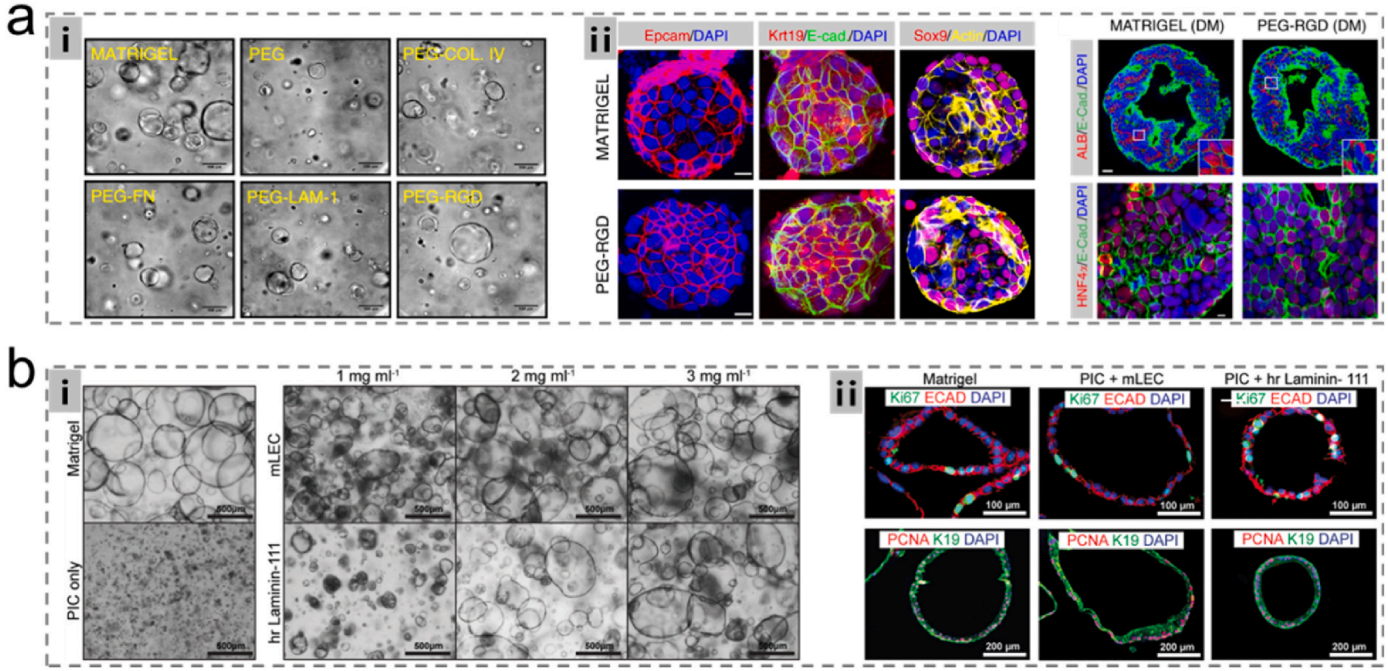

Fig. 2. Comparison of synthetic hydrogels and Matrigel for liver organoid culture. **(a)** (i) Optical images of liver organoids at day 3 grown in Matrigel, PEG, and PEG supplemented with different ECM components; (ii) Immunofluorescence analysis of liver organoids grown in Matrigel and PEG-RGD, including stem/ductal markers such as Lgr5, Epcam, Krt19 and Sox9, and hepatocyte markers such as ALB and HNF4α [54]. Scale bars: 25 μm. Copyright 2020, Springer Nature. **(b)** (i) Optical images of liver organoids at day 7 embedded in Matrigel, PIC, PIC + mLEC and PIC + hr Lmminin-111; (ii) Immunofluorescent analysis of liver organoids embedded in Matrigel, PIC + mLEC and PIC + hr Laminin-111 confirmed an epithelial progenitor phenotype and highly proliferative potential [56]. Copyright 2020, John Wiley and Sons.

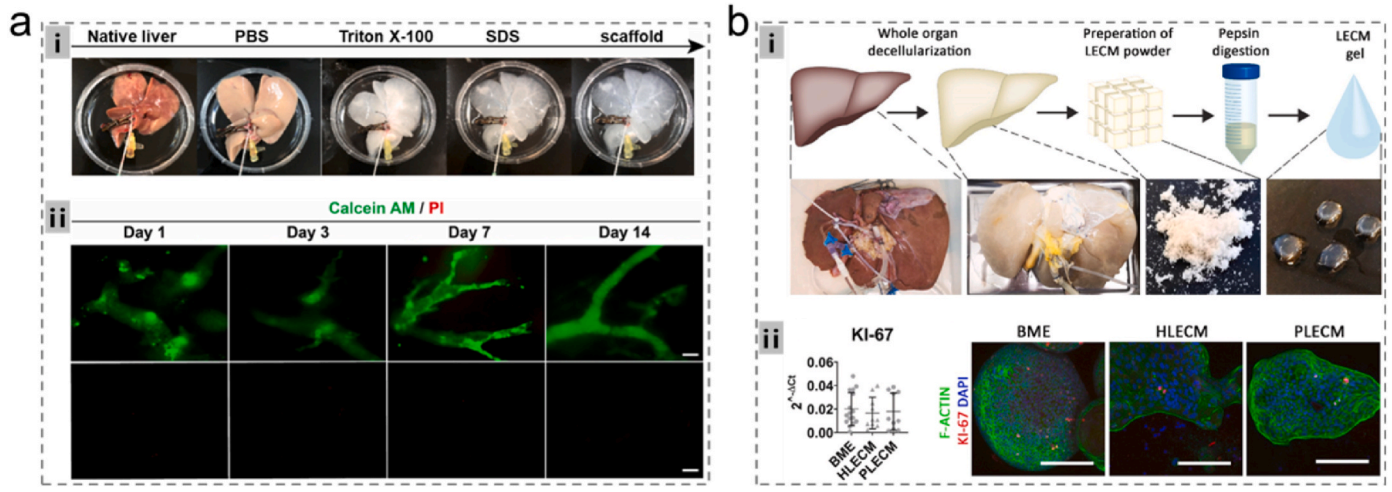

Fig. 3. Liver decellularized scaffold for liver organoid culture. **(a)** (i) Decellularization process of a natural liver; (ii) Cell viability staining of functional duct-like organoids [65]. Scale bars: 100 μm. Copyright 2023, Elsevier. **(b)** (i) A schematic diagram of the preparation of liver extracellular matrix (LECM) gel; (ii) Gene expression and immunofluorescence of the proliferation marker Ki-67 do not show significant differences between HLECM or PLECM and BME controls [66]. Scale bars: 100 μm. Copyright 2022, Elsevier.

exhibit well-defined tight junctions and polarity. The regenerative biliary tree exhibits functions more closely resembling the natural biliary tree. This approach demonstrates the potential of decellularized scaffolds for creating complex organoid models that closely mimic the native tissue environment.

Decellularized matrices can be utilized not only as whole organ scaffolds but also treated to various forms, such as thermal hydrogels [67]. Decellularized liver tissue undergoes enzymatic treatment to produce a solution that forms a LECM gel when heated to 37 °C (Fig. 3b) [66,68]. While the resulting hydrogel may not preserve the structural details of the original tissue, it retains essential biochemical properties that influence cell behavior. Saheli et al. demonstrated that liver organoids, formed through the spontaneous arrangement of human hepatocarcinoma cells co-cultured with other cells within a sheep LECM gel, exhibited enhanced gene expression and functional activity compared to traditional culture methods [69]. Another research group compared the biological differences between human and porcine LECM and confirmed their potential to replace mouse-derived basement membrane extracts (BME) for organoid culture (Fig. 3b) [66]. Factors like pH, light, and magnetism can also trigger the sol-gel transition of hydrogels [15]. Leveraging these findings, decellularized liver gelation can be utilized as a material for droplet microfluidics, 3D printing, and other applications to facilitate the integration of cellular components more easily [70,71]. However, similar to Matrigel, batch-to-batch variability and immunogenicity of the ECM-based hydrogels pose challenges in precisely regulating cells [72]. Researchers continue to explore ways to overcome these limitations and optimize the use of decellularized hydrogels in tissue engineering and regenerative medicine.

3. Engineering technologies for engineering liver organoids

Traditional methods for generating organoids include culturing organoids in Matrigel domes, on non-adherent round bottom plates, in stirred suspension bioreactors or hanging drops (Fig. 4a) [73,74]. These conventional methods are all non-engineered and characterized by labor-intensive and multi-step manual processes, often resulting in the generated organoids exhibiting variability and heterogeneity [75]. To overcome these challenges, a variety of sophisticated engineered approaches, leveraging advanced biomedical engineering technology and state-of-the-art equipment, have been devised to streamline mass

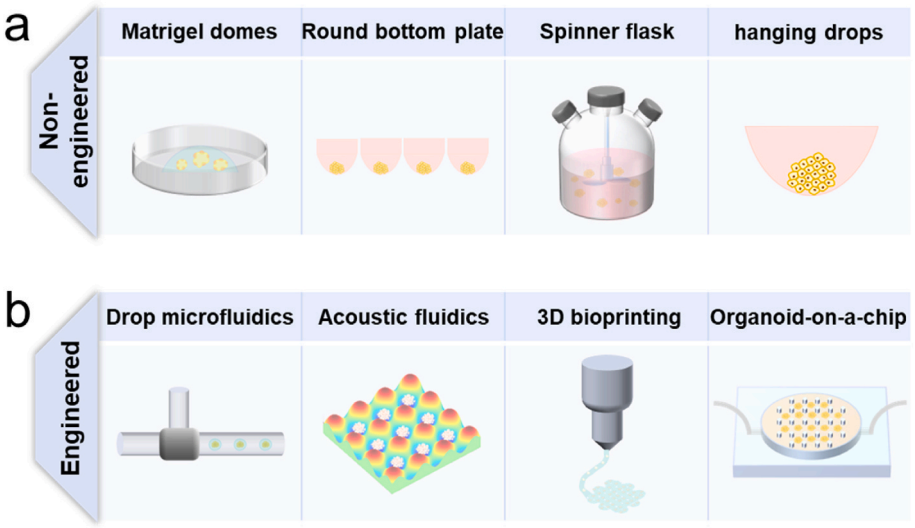

Fig. 4. Strategies for organoid fabrication. **(a)** Non-engineered strategies and **(b)** engineered strategies for organoid fabrication.

production and ensure the consistency of organoids (Fig. 4b). Here, we underscore the pivotal role of droplet microfluidics, acoustic fluidics, 3D bioprinting and organoids-on-a-chip in the assembly of liver organoids. These cutting-edge methodologies provide groundbreaking solutions, enabling the development of more standardized and reproducible organoid models. They pave the way for significant advancements in hepatic research and hold promise for future breakthroughs in drug development, disease modeling, and the realm of personalized medicine.

3.1. Droplet microfluidics

Droplet microfluidics have been developed to create uniform emulsion droplets, microparticles, and microcapsules with intricate structures or compartments [76–79]. It has been shown to be an efficient way of producing large-scale cell spheroids simply with a one-step injection [80–82]. Hydrogel capsules are considered excellent platforms for cultivating 3D cellular spheroids such as organoids, thanks to their precise shapes and large surface area [83]. The process involves dispersing a continuous cell suspension into droplets enclosed in the outer phase by the shear force of the outer phase liquid [84]. The dimensions of the cell spheroids can be precisely regulated by modifying the inner diameter of the channel and the flow rate of the fluid [85,86]. Therefore, droplet microfluidics enables the continuous production of consistent cell microcapsules with controllable sizes.

Engineered liver organoids based on microcapsules have been developed. Hepatic cells differentiated from hiPSCs are arranged into liver organoids with homogeneous dimensions by being encapsulated in composite hydrogel capsules (Fig. 5a) [14]. The size can be adjusted by varying the flow rates and modifying the composition of the hydrogel precursors within core and shell flow. Abbasalizadeh et al. encapsulate hepatic progenitors, mesenchymal stromal cells and endothelial cells from various hiPSC lines in a peptide-based hydrogel to produce cell-loaded microcapsules [87]. Microcapsules undergo self-degradation of their shell, releasing the cell nucleus and eventually developing into liver organoids with interconnected biliary networks and vascular structures (Fig. 5b) [87]. The developed microcapsule system provides a robust and controllable 3D scaffold for organoid creation in a scalable and reproducible manner, helping to address some of the challenges associated with traditional labor-intensive methods.

3.2. Acoustic fluidics

Compared to creating cell microcapsules individually through droplet microfluidics, acoustic fluidics technology has the capability to generate multiple high-density cell aggregations simultaneously within the range of the sound field [88,89]. Acoustic fluidics utilizes piezoelectric transducers to generate sound flow that facilitates cell movement and aggregation [90,91]. When the sound wave ceases, the aggregated cells will gradually disperse. While it is possible for cells to form clusters over time through the gradual secretion of adhesion proteins, this process can be time-consuming, particularly depending on the cell type, leading to decreased productivity [92]. To tackle this issue, Rasouli and colleagues used type I collagen as a bioadhesive to rapidly form cell-collagen aggregates in a matter of seconds [93]. They employed boundary-driven acoustic streaming to create a vortex, where collagen prompts swift adhesion between cells as they are trapped and compressed by the vortex, maintaining compact cell aggregations even after the sound wave subsides (Fig. 6a) [93].

Wu et al. developed a device equipped with four piezoelectric

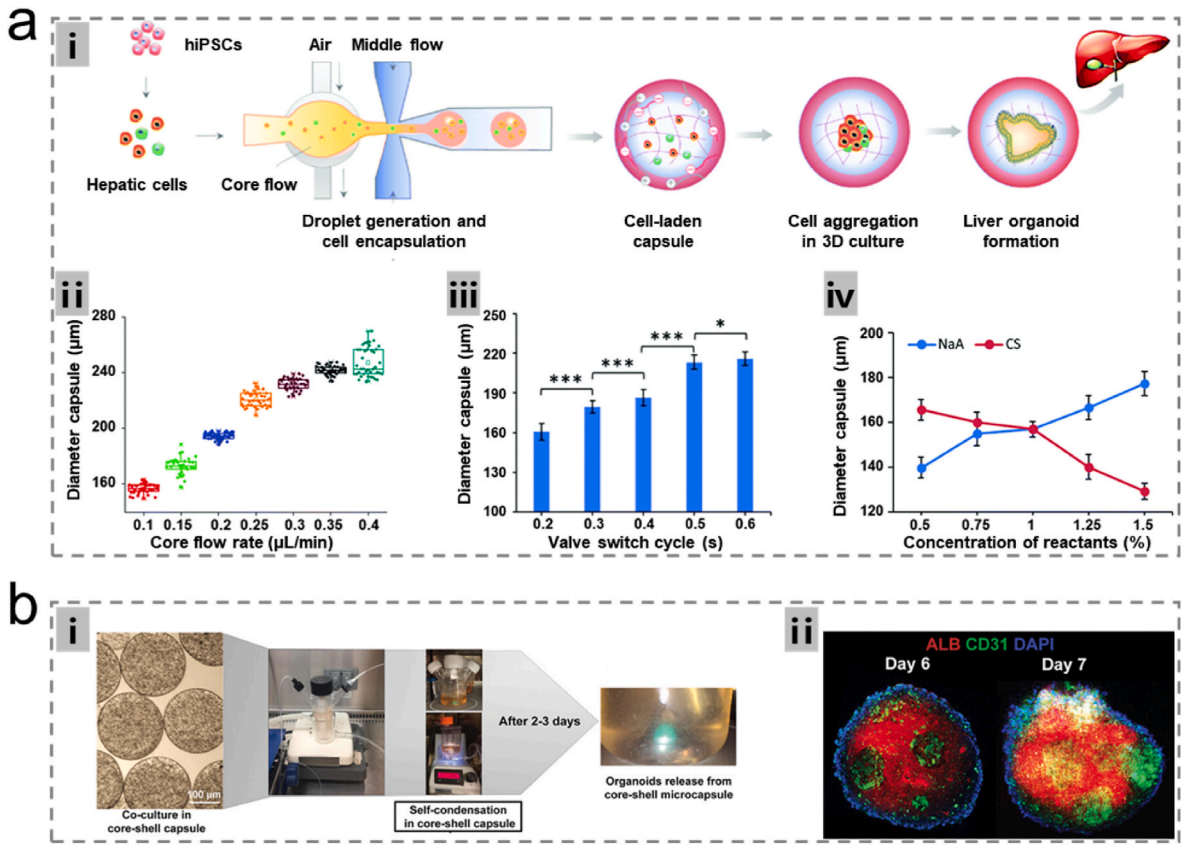

Fig. 5. Droplet microfluidics for liver organoids fabrication. **(a)** (i) Flow chart of engineering human liver organoids from hiPSCs with the defined capsules; Diameter profiles of capsules obtained under different core flow rates (ii), valve switch cycles (iii) and NaA/CS concentration (iv) [14]. Copyright 2020, Royal Society of Chemistry. **(b)** (i) Process of co-culture in microcapsules and dynamic suspension culture after release; (ii) immunostaining of organoids after culture 6 and 7 days for ALB^{+} and $CD31^{+}$ cells [87]. Copyright 2023, John Wiley and Sons.

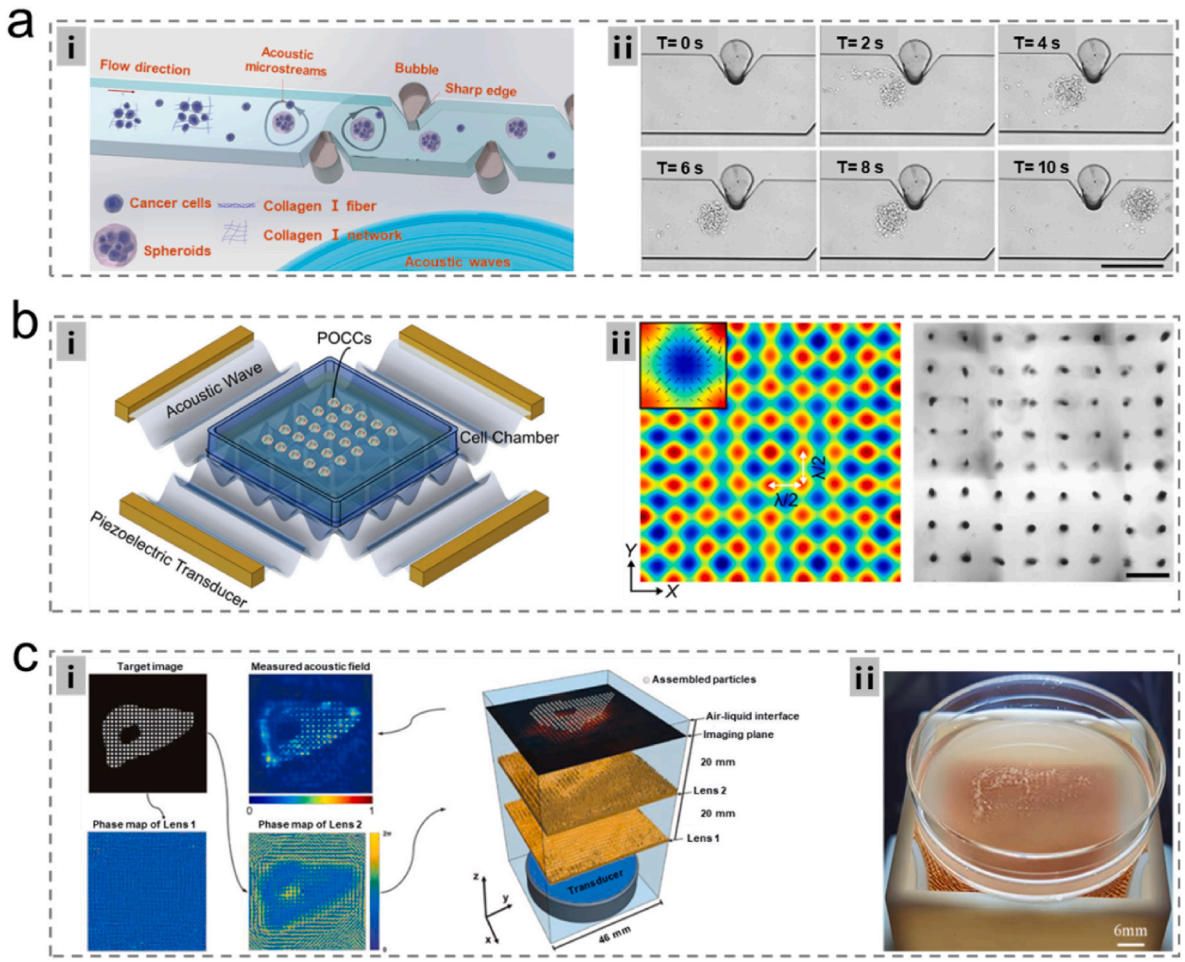

Fig. 6. Acoustic fluidics for cell aggregations fabrication. **(a)** (i) Schematic of cell spheroid by collagen assembly based on boundary-driven acoustic streaming; (ii) Acoustic spheroid formation process in the device over time [93]. Scale bar: 500 μm. Copyright 2021, John Wiley and Sons. **(b)** (i) Schematic of an acoustic cell assembly device; (ii) Simulated sound pressure distributions (left) and actual acoustically assembled cell cluster distributions (right) [94]. Scale bar: 1 mm. Copyright 2023, Springer Nature. **(c)** (i) Workflow for constructing the acoustical holographic lattice with a designed pattern; (ii) Cells undergo 45s ultrasound treatment to form a clustered pattern within a liver-like shape [95]. Copyright 2024, Elsevier.

transducers positioned around a cell pool (Fig. 6b) [94]. By activating the radio frequency signal, a consistent pressure node is established within the cell pool, resulting in the formation of a grid of cell clusters. The cell array was then secured in place by the solidified matrix glue, simplifying subsequent culture procedures. This method has been successfully used to create organoids in breast tumors. Compared to traditional organoids, those formed through acoustic fluidic aggregation exhibit larger volumes and higher uniformity [94]. Researchers from the Chinese Academy of Sciences have utilized holographic acoustic tweezers to act on primary hepatocytes, generating numerous self-assembled cell spheroids with significantly enhanced liver functions compared to traditional 2D and 3D cultures (Fig. 6c) [95]. This research has confirmed the feasibility of assembling liver organoids using acoustic fluidics. Although there are currently limited reports, this is a field that merits in-depth investigation.

3.3. 3D bioprinting

Over the past few decades, 3D bioprinting has made significant strides in creating tissue replicas, offering substantial hope in creating biomimetic liver tissues with precise microstructures in laboratory settings. This cutting-edge technology has also paved new avenues for the construction of liver organoids. Bioprinting provides the ability to precisely control the deposition of cells in 3D space at a speed and scale that can effectively replace the highly laborious and variable manual operations. As demonstrated by Shrestha, microarray printing technology is utilized to evenly deposit droplets of a mixture of foregut cells and Matrigel onto pillar plates in 1 min for the mass production of liver organoids (Fig. 7a) [96]. These bioprinted organoid arrays show lower variability and enhanced functionality due to the efficient delivery of nutrients and oxygen at a microscale. Consequently, bioprinting addresses limitations in variability, throughput, and scalability.

3D bioprinting is employed not only for the precise and efficient distribution of cells but also for the design of intricate shapes that support the development of liver organoids. Bouwmeester et al. have demonstrated the use of extrusion-based bioprinting to fabricate liver constructs with pores measuring 200–400 μm, which ensures efficient molecular diffusion (Fig. 7b) [97]. In addition to stem cells and organoid fragments, the intact organoids for printing are also feasible. Organoids are cellular aggregates that are large in volume and fragile in structure, which poses stringent requirements for printing methods. Non-extrusion-based methods are necessary to avoid clogging issues and structural damage when bioprinting organoids [99]. Bernal et al. have utilized volumetric bioprinting to densely embed intact liver organoids within a 3D lattice in just 20 s, then cultured under perfusion conditions (Fig. 7c) [98]. This 3D model maintains robust cellular activity and function, serving as a biofactory for the study of liver metabolic function. The design flexibility of bioprinting increases the overall complexity and physiological relevance of liver organoids and also enhances the diversity of applications for organoid platforms [100].

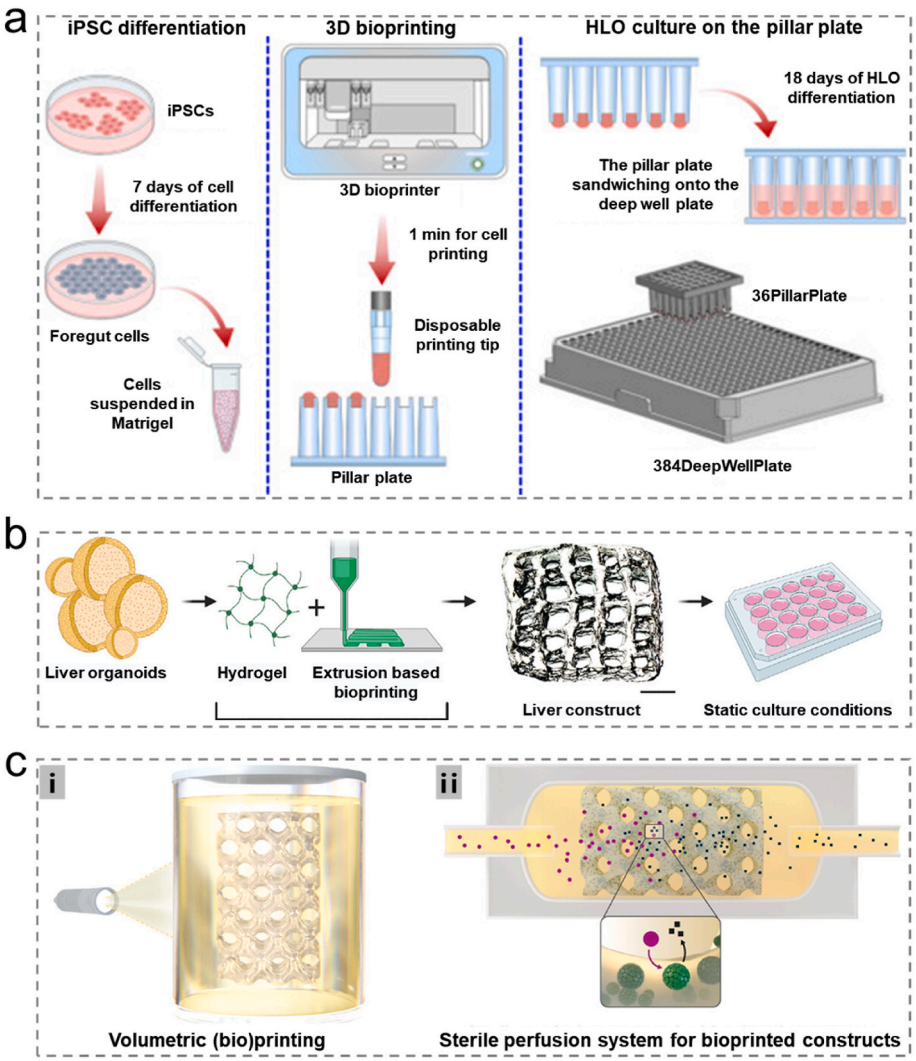

Fig. 7. 3D bioprinting liver organoids. **(a)** Flow diagram of a high-throughput printing of liver organoid array on pillar plate [96]. Copyright 2024, Royal Society of Chemistry. **(b)** Schematic overview of the experimental procedure for bioprinting liver constructs using extrusion-based bioprinting [97]. Copyright 2021, John Wiley and Sons. **(c)** (i) Schematic representation of the volumetric printing process and (ii) illustration of liver organoid-laden printed biofactory cultured under dynamic perfusion [98]. Copyright 2022, John Wiley and Sons.

Bioprinting of organoids still needs to address a series of issues, such as improving printing resolution, reducing cell damage, and developing new types of bioinks. In the future, complex organoids generated through bioprinting will be better applied in the field of biomedicine.

3.4. *Organoids-on-a-chip*

Microfluidic chips have been widely accepted and are extensively used for the successful cultivation of organoids. These microfluidic devices feature hollow channels of varying sizes and shapes, lined with living cells and tissues that are nurtured under conditions of dynamic fluid circulation [101,102]. Leveraging micro-nano manufacturing technology enables microfluidic chips to closely match the size of organoids, and the incorporation of micro-pumps and micro-valves can enhance automation and industrialization processes [103,104]. The distinct advantage of microfluidic technology lies in its dynamic regulation of the culture medium, thereby facilitating molecular diffusion and exerting shear stress, both known to foster cell differentiation [105,106]. Additionally, microfluidic chips can achieve specific stimuli needed for particular research purposes, such as chemical gradients and mechanical forces [51,107,108]. Consequently, utilizing organoid models on microfluidic chips can yield more reliable outcomes in disease modeling and drug response studies.

Wang and colleagues fabricated a 3D perusable micropillar chip system by soft lithography techniques (Fig. 8a) [109]. The microcolumn chip allows for the controllable formation of embryoid bodies, in-situ liver differentiation, and extended 3D cultivation for producing liver organoids directly on the chip. The chip platform ensures the perfusion culture of liver organoids, and the expression of important markers in organoids grown under these conditions is significantly stronger than in static culture. This improvement is attributed to the continuous nutrient provided by the dynamic flow, coupled with the shear stress it exerts. Furthermore, the liver organoids displayed hepatotoxic response to acetaminophen that varied according to the dosage and duration of exposure, showcasing the system's suitability for drug testing and trials.

The integration of liver organoids with other complex organoids-on-a-chip components, such as heart organoids and islet organoids, holds great potential for studying inter-organ interactions. Drug-induced side effects on liver and heart are a significant reason for drug failures in clinical trials. Qin's team has developed a multi-organoids-on-chip system by combining liver and heart organoids in a 3D setup to assess the impact of antidepressants on heart post-metabolism by the liver (Fig. 8b) [110]. This bioengineered organoid chip system comprised four layers, with the upper chamber dedicated to liver organoid culture for drug

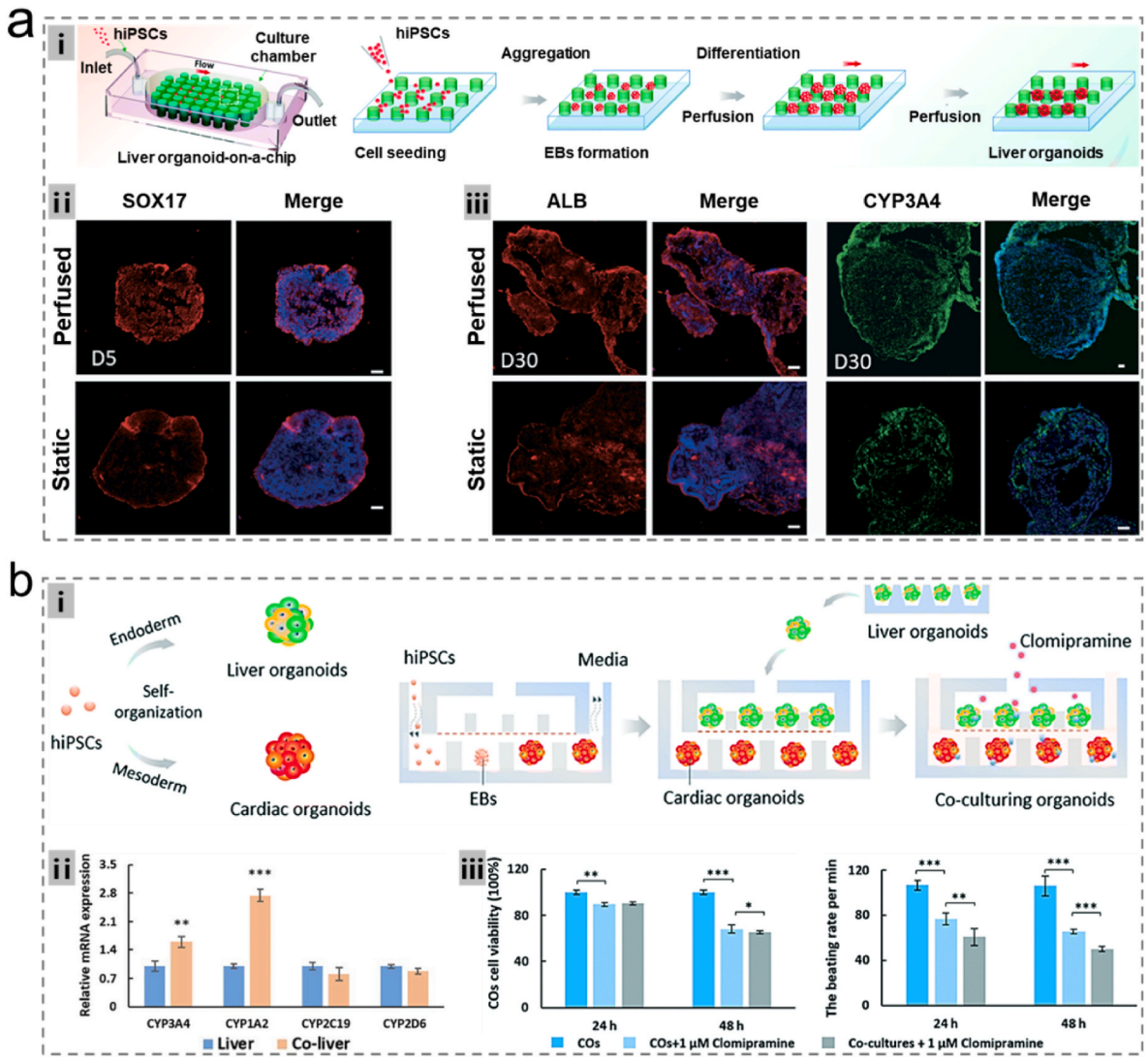

Fig. 8. Liver organoids-on-a-chip. **(a)** (i) Conceptual illustration of liver organoids-on-a-chip; Immunohistochemical staining for (ii) endodermal marker (SOX17) and (iii) hepatocyte markers (ALB and CYP3A4) in liver organoids under perfused or static culture conditions [109]. Scale bars: 50 μm. Copyright 2018, Royal Society of Chemistry. **(b)** (i) Conceptual diagram of liver-heart organoids-on-chip for drug assessment of clomipramine; (ii) The mRNA expression of liver-specific metabolic enzymes (CYP3A4, CYP1A2, CYP2C19 and CYP2D6) in liver organoids with (co-liver) or without (liver) cardiac tissues coculture on chip; (iii) Cell viability and beating rate of the cardiac organoids were evaluated with clomipramine (1 μM) treatment for 24 h and 48 h in the presence and absence of liver organoids [110]. Copyright 2021, Royal Society of Chemistry.

metabolism and the lower micropillar array for heart organoid differentiation and toxicity assessment. Under co-culture conditions, the expression levels of liver-specific metabolic enzymes are significantly increased. Researchers could investigate the effects of clomipramine on heart organoids according to cardiac viability and function with or without hepatic metabolism. This approach provides valuable insights into drug metabolism and responses across multiple organs in vitro, offering a new method for evaluating drug toxicity in a physiologically relevant manner.

Researchers have created a microfluidic dual-organoid platform to replicate the complex glucose regulation process between the liver and islet in a living organism [111]. The liver organoid culture chamber and the islet organoid culture chamber are connected through a microchannel network to realize dynamic interaction. Additionally, Skardal's group has developed a 3-organoid platform and a 6-organoid platform incorporating liver organoids [112,113]. These examples demonstrate that multi-organoid systems exhibit superior physiological functions compared to single organoids. Therefore, multi-organoids-on-a-chip configurations represent an enhanced physiologically appropriate in vitro model for exploring complex biological processes and drug responses.

4. Genetic engineering for engineering liver organoids

Enhancing the external conditions, whether through matrix design or technological advancements, is crucial for achieving effective, efficient and reliable preparation of liver organoids. While these elements have a substantial impact on manipulating the building blocks of organoids, the cells themselves present a promising avenue for further improvement [114]. By programming the intrinsic properties of cells, we can significantly enhance organoid robustness and customize them for specific applications. Genetic engineering has emerged as a powerful tool to guide liver organoid morphogenesis at the genetic level [22]. Techniques such as viral transduction and CRISPR/Cas9 system have become prominent tools in the realm of customizing liver organoids, allowing researchers to precisely manipulate the genetic makeup of cells to achieve desired outcomes [115]. These approaches offer a valuable opportunity to advance the field of liver organoid research and open up

new possibilities for tailored applications and studies.

4.1. Viral transduction

Viral vectors, such as lentivirus, adenovirus, and adeno-associated virus (AAVs), are valuable tools for facilitating stable gene transduction and expression in various applications, including the modification of liver organoids [116–118]. These vectors can integrate into host genomes, allowing for the overexpression or silencing of specific genes and the regulation of protein expression within cells. For example, lentivirus transduction was used to deliver under-expressed transcription factors into fetal liver organoids, to investigate the impact of their overexpression on a range of hepatic and endothelial markers [119]. Lentiviral vectors transfer the COMMD1 gene into COMMD1-deficient organoids, creating engineered liver organoids with restored function for autologous transplantation therapy [120]. Besides, AAV vectors can manipulate target genes, investigating the mechanism of cholangiocyte differentiation into hepatocytes in liver organoids, which contributes to the development of liver regenerative medicine [121]. Viral transduction, known for its safe and efficient gene addition, has been innovatively harnessed for delivering the CRISPR-Cas system [122,123]. This advancement allows viral vectors to serve as carriers for the CRISPR-Cas complex, expanding their utility beyond simple gene transfer to include precise gene editing [124].

The transduction efficiency of viral vectors in hepatocytes is influenced by various factors, including serotype, dose, and the host's immune status. For instance, AAV8 and AAV2 have shown high efficiency in transducing hepatocytes both in vitro and in vivo, making them ideal for delivering therapeutic genes to the liver [125–127]. While AAVs are effective, they have a limited packaging capacity, which restricts the size of the genes that can be delivered. Additionally, there is the potential for the risk of off-target effects, which can lead to unintended genetic alterations. To mitigate these challenges, researchers are exploring a variety of optimization strategies [128]. These strategies include the development of new AAV serotypes, modification of existing AAV capsids to improve tissue specificity and transduction efficiency, and the development of AAV vector systems that can evade host neutralizing antibodies [129–131].

4.2. CRISPR/Cas9

CRISPR/Cas9, inspired by a bacterial defense mechanism against viral infections, has revolutionized the field of gene editing and emerged as a powerful third-generation technology, surpassing earlier methods like zinc-finger nucleases and transcription activator-like effector nucleases [132–134]. This technology has become popular throughout molecular biology owing to its simple design, superior efficiency and effectiveness. CRISPR/Cas9 editing process involves a single guide RNA directing the Cas9 protein to a targeted site, causing a double-stranded break that leads to subsequent insertions, deletions, and mutations [135]. Its potential for organoid design is emerging because it works well in the stem cell field [114,136,137]. This technology offers a new avenue for engineering organoids, and there have been notable studies applying CRISPR/Cas9 to liver organoids, showcasing its promise in advancing organoid research and customization. The precision and versatility of CRISPR/Cas9 make it a valuable asset for creating genetically modified organoid models tailored for specific applications and research goals.

Traditional methods of inducing liver organoids often result in immature phenotypes, necessitating alternative strategies for achieving maturation [138]. While implanting organoids into animal hosts can foster maturation, this approach faces challenges including time-consuming, resource-intensive, and limited control over tissue growth dynamics [139–141]. Fortunately, the revolutionary CRISPR/-Cas9 technology has presented a promising avenue for targeting key transcription factors and guiding fetal liver organoid morphogenesis toward a mature phenotype. One crucial aspect of liver organoid maturation is the attainment of CYP3A4 levels comparable to those found in adult livers [142]. CYP3A4 is a crucial hepatic enzyme involved in the metabolism of both endogenous compounds and a wide range of pharmaceuticals [143,144]. Researchers have explored designer liver organoids (DesLO), which involve the introduction of PROX1, ATF5 and CYP3A4 to fetal liver organoids (FeLO) (Fig. 9a) [119]. By stimulating CYP3A4 activity through CRISPR-mediated transcriptional activation, researchers were able to significantly upregulate CYP3A4 expression, with levels increasing approximately 400-fold (Fig. 9b) [119]. Moreover, a synergistic effect was observed when CYP3A4 was delivered alongside PROX1 and ATF5 circuits, resulting in a remarkable upregulation of CYP3A4 expression by nearly 20,000 times to match the levels found in adult liver tissue. The engineered DesLO demonstrated mature functionality and vascular supply, highlighting the significant impact of gene regulation in directing organ maturation (Fig. 9c and d) [119]. This innovative approach holds promise for unraveling intricate pathways and functionalities within liver organoids, offering a glimpse into the potential applications of tailored genetic interventions in hepatic research.

CRISPR/Cas9 can manipulate genetic mutations in healthy human organoids, as well as correct genetic issues in patient-derived organoids (Fig. 10). By transitioning from healthy to mutant organoids, researchers can simulate disease phenotypes, offering crucial understanding into disease mechanisms and possible therapeutic approaches. This approach has already been employed to study liver diseases [145,146]. For example, researchers have designed CRISPR-engineer APOB−/− and MTTP−/− organoids to model nonalcoholic fatty liver disease (NAFLD) [147]. The platform helps in studying steatosis etiology and identifying putative drug targets. Conversely, mutant-to-health organoids could be an ideal source for autologous transplantation to repair tissue damage caused by mutant diseases, representing a promising direction for CRISPR/Cas9 as a gene therapy tool. For instance, in Wilson's disease, researchers used CRISPR/Cas9 to correct ATP7B mutation in iPSCs derived from patients for the transplantation treatment of diseased mice [148]. A recent study employed CRISPR/Cas9 to correct the genetic defect in primary hepatocytes derived from patients with tyrosinemia, successfully treating the condition in afflicted mice following transplantation [149]. The results highlight CRISPR/Cas9's transformative role in gene therapy and clinical transplantation. This pioneering method holds substantial promise for propelling the field of personalized medicine and for crafting therapies tailored to genetic conditions.

5. Applications of engineered liver organoids

Throughout recent decades, both monolayer cell cultures and animal models have significantly contributed to the progress of liver research and the development of treatments. However, each method has its limitations. While monolayer cell cultures are relatively cost-effective and easy to handle, lack the ability to replicate the intricate cellular microenvironment that liver cells experience in vivo [150–152]. On the other hand, animal models, despite their utility, exhibit biological disparities with humans and pose ethical debates [153–155]. Engineered liver organoids have emerged to address these shortcomings by serving as model systems that better mimic liver development and regeneration processes. Filling the void left by conventional cell cultures and animal models, liver organoids offer the potential for disease modeling, regenerative medicine, toxicology testing, and personalized drug development (Table 1).

5.1. Disease modeling

Liver diseases, including non-alcoholic fatty liver disease, viral hepatitis, cirrhosis, and liver cancer, pose a significant global health challenge [156–159]. Currently, researchers have developed a variety of organoid models that effectively mimic the pathological hallmarks of

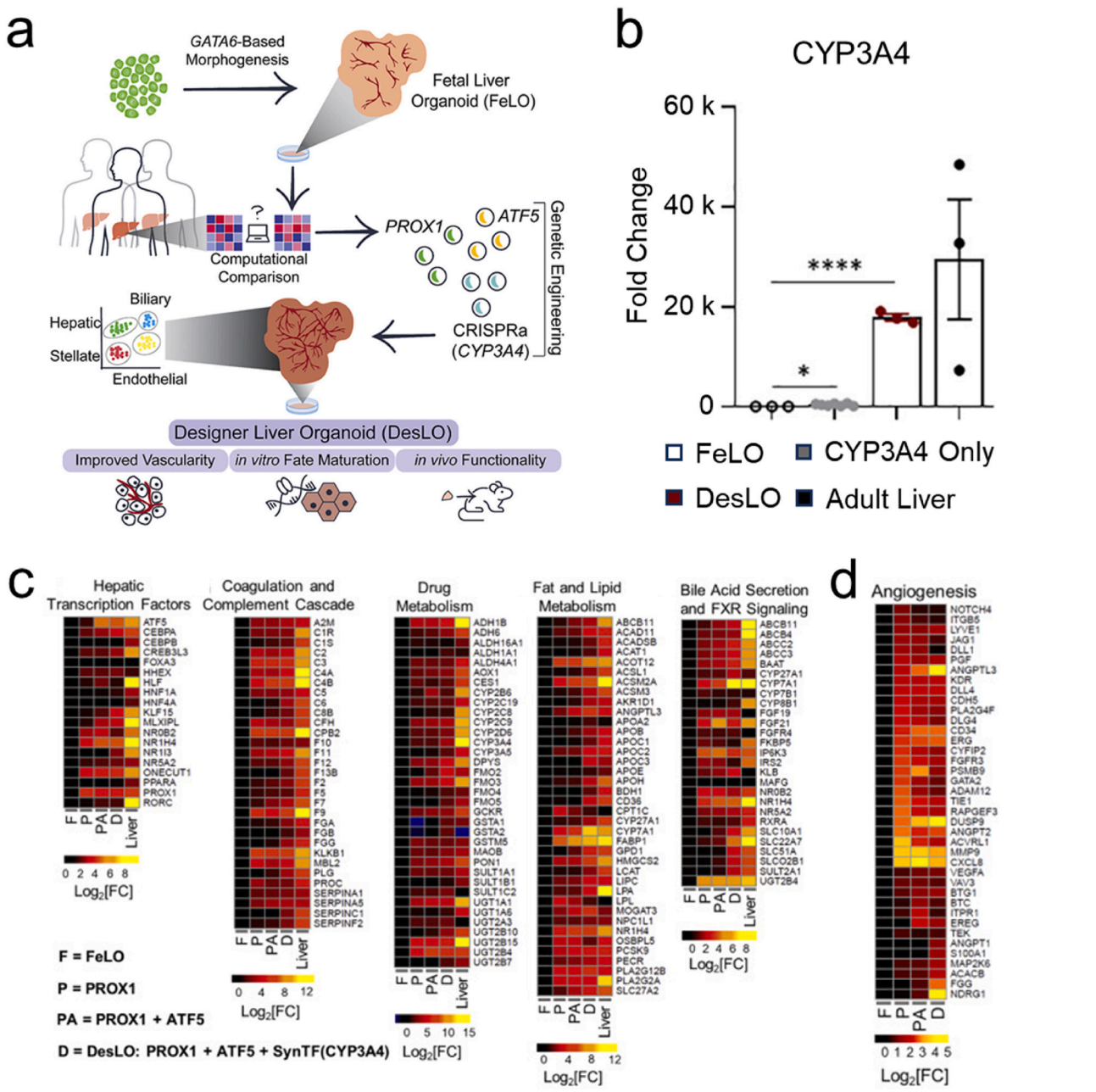

Fig. 9. CRISPR/Cas 9-regulated DesLO. **(a)** Overexpression of ATF5, PROX1 transcription factors, and CRISPR-mediated activation of CYP3A4 to advance the maturity and vascularity of human iPSC-derived fetal liver organoids in vitro [119]. **(b)** qPCR data for significant CYP3A4 upregulation with SynTF(CYP3A4) and synergistic effect of PROX1 and ATF5 co-expression on CYP3A4 activation [119]. **(c)** Heatmaps showing enrichment in pathways relative to FeLO for hepatic transcription factors, complement and coagulation cascade, drug metabolism, cholesterol and lipid metabolism, glucose metabolism, and bile acid secretion and FXR signaling by increasing the components transduced at day 5 from FeLO to DesLO in day 17 tissues [119]. **(d)** Heatmap showing the expression relative to FeLO of angiogenesis-related genes in FeLO (F), PROX1 (P), PROX1+ATF5 (PA), and DesLO (D) transduced at day 5 (except FeLO) in day 17 tissues [119]. Copyright 2021, Elsevier.

these liver conditions, serving as crucial tools for disease research [160–162]. Organoids-on-a-chip platforms are capable of not only providing physiological microenvironments but also creating specific mutagenic environments to induce liver cell pathologies for the study of acquired liver diseases. Wang et al. characterized the pathological features of NAFLD by subjecting liver organoids-on-a-chip to long-term dynamic exposure to free fatty acids [163]. Gene engineering is commonly used for reprogramming iPSCs or healthy donor cells to generate liver organoids that can mimic the genetic makeup of patients with specific monogenic diseases. CRISPR-mediated BAP1 mutation in healthy donor-derived liver organoids leads to phenotypic changes, which are reversed upon lentiviral vector-mediated restoration of BAP1 expression [145]. It revealed the important role of BAP1 in the development of cholangiocarcinoma. Similarly, researchers have transformed normal liver organoids into hepatocellular carcinomas models by lentiviral transduction of the oncogene c-Myc [164]. By combining the Cas9 gene editing system with the piggyBac transposon system, researchers have engineered human liver organoids to express disease-causing mutations found in autosomal recessive polycystic kidney disease (ARPKD). Studying the pathological mechanisms of liver fibrosis and evaluating the efficacy of potential anti-fibrotic therapies have been made possible by this disease model [165]. Therefore, liver organoids as disease

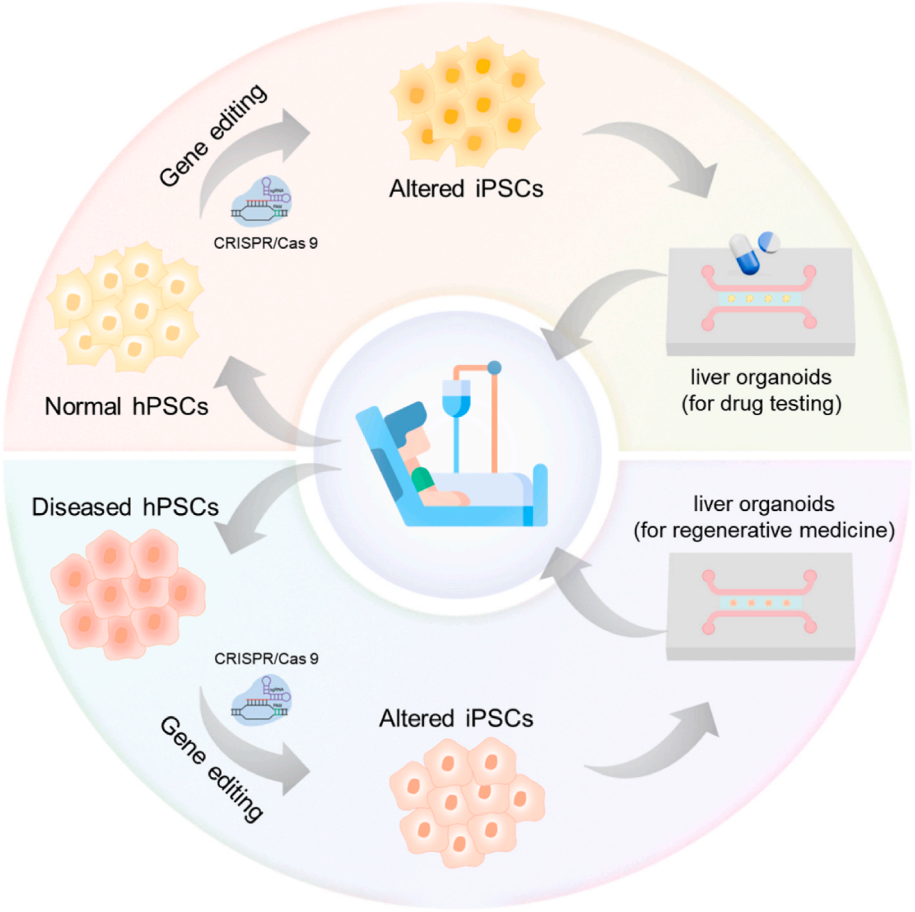

Fig. 10. The applications of CRISPR/Cas9 in engineering liver organoids.

models not only expand our knowledge of liver diseases but also serve as valuable tools for assessing drug effectiveness.

Liver organoids derived from patient-specific primary cells or iPSCs retain the genetic background of diseases [166–168]. Correcting suspected pathogenic genes through gene editing and then assessing whether this leads to a restoration of normal function or a recovery from the pathological state provides a rational approach to studying disease mechanisms. In a notable example, researchers have successfully reverted a disease-causing mutation in iPSCs derived from patients with Alagille syndrome (ALGS) [169]. The generated engineered liver organoids formed an increased number of bile ducts with well-organized structures compared to the mutated organoids. In an additional case, the introduction of ASS1 cDNA into citrullinemia type 1 (CTLN1) patient-derived iPSCs via lentiviral transduction has yielded liver organoids that overexpress ASS1, effectively reversing ammonia accumulation [170]. These applications also demonstrate the potential of gene therapy in the treatment of liver diseases.

5.2. Drug screening

Toxicity assessment of drugs is a critical component in the development of new pharmaceuticals and their clinical application. Due to the liver's critical involvement in drug metabolism, the response of liver cells is essential in drug toxicity screening [171]. For assessing the risk of drug-induced liver injury, liver organoids have emerged as promising predictive models, providing a valuable approach for screening novel drugs before clinical trials [172]. Jiang et al. employed droplet microfluidics to generate uniform organoid precursors for prediction of anticancer drug toxicity [173]. Zhou et al. leveraged the high-throughput microfluidics and 3D printing to rapidly generate reproducible liver organoids for multi-timepoint drug administration and large-scale drug screening, exploring the temporal rhythmicity of hepatotoxicity induced by oxaliplatin and providing guidance for optimal drug administration timing [174]. Exploring the time-dependent toxicity of drugs to achieve optimal efficacy or minimal toxicity will become a significant aspect of future drug development, and droplet microfluidics is poised to substantially increase the efficiency of this screening process.

Given that the human body functions as a multi-organ system, understanding how drugs affect other organs after being metabolized by the liver is crucial in drug development [175]. Multi-organoid chips allow the exploration of prodrug metabolism and mutual toxicity among different organs. For example, a three-organ platform consisting of liver, cardiac, and lung organoids was used to investigate the hepatic conversion of the prodrug capecitabine into 5-fluorouracil and to monitor the subsequent toxicity effects on cardiac and lung organoids [176]. Similarly, a multi-organ platform incorporating liver, heart, lung, endothelial, brain, and testes organoids was employed to study neurotoxicity induced by liver metabolites of the alkylating prodrug ifosfamide [176]. These studies show the significance of multi-organoid systems in assessing the systemic effects of drug metabolism and toxicity beyond the liver.

5.3. Regenerative medicine

Liver diseases can compromise liver functions, and while the liver possesses notable regenerative capabilities, severe cases may necessitate organ transplantation. However, the limited availability of healthy donor tissues poses a significant challenge [177–179]. Liver organoids, with their potential for expansion and differentiation, offer an ideal alternative source for transplantation [140]. Due to the potential

Table 1
Applications of engineered liver organoids.

Engineered technologies	Specific classification	Application	Year	Reference
Hydrogel	Liver decellularized scaffolds	Regenerative medicine (liver failure)	2022	[187]
	Sodium alginate and gelatin	Drug screening (cabazitaxel, oxaliplatin and sorafenib)	2022	[188]
	Hyaluronic acid and collagens	Disease modeling (genetically high levels of plasma Lp(a)) and drug screening (mevastatin)	2024	[41]
3D bioprinting	GelMA	Drug screening (acetaminophen)	2021	[97]
	Sodium alginate and gelatin	Regenerative medicine (liver failure)	2021	[185]
	Matrigel	Drug screening (sorafenib and tamoxifen)	2024	[96]
Droplet microfluidics	Matrigel	Drug screening (paclitaxel, homoharringtonine, epirubicin and daunorubicin)	2020	[173]
	Alginate	Regenerative medicine (liver failure)	2024	[42]
	Matrigel	Drug screening (oxaliplatin)	2024	[174]
Organoids-on-a-chip	Liver-cardiac-lung organoids-on-chip	Drug screening (acetaminophen, epinephrine, propranolol and bleomycin)	2017	[112]
	Liver organoids-on-a-chip	Drug screening (acetaminophen)	2018	[109]
	Multi-organoids-on-chip	Drug screening (bromfenac, tienilic acid, troglitazone, astemizole, cisapride, mibefradil, pergolide, rofecoxib, terodiline, valdecoxib, aspirin, ibuprofen, ascorbic acid, loratadine, and quercetin)	2020	[113]
	Liver-heart organoids-on-chip	Drug screening (clomipramine)	2021	[110]
	Liver-islet organoids-on-chip	Disease modeling (Type 2 diabetes)	2022	[111]
	Liver organoids-on-a-chip	Disease modeling (NAFLD)	2023	[163]
Genetic engineering	CRISPR/Cas9 + lentiviral vector	Disease modeling (Cholangiocarcinoma)	2019	[145]
	Lentiviral vector	Disease modeling (Hepatocellular carcinomas)	2019	[164]
	Lentiviral vector	Disease modeling (CTLN1)	2019	[170]
	CRISPR/Cas9	Disease modeling (ARPKD)	2021	[165]
	CRISPR-Cas9	Disease modeling (ALGS)	2021	[169]
	CRISPR/Cas9	Disease modeling (Wilson's disease)	2022	[148]
	CRISPR/Cas9	Disease modeling (NAFLD)	2023	[147]

biosafety, there are currently no clinical application cases of liver organoids, preclinical experiments have shown encouraging results, demonstrating their therapeutic potential. A pioneering report from Huch and colleagues has shown the feasibility of liver organoid transplantation. Transplantation of Lgr5+ stem cell-derived mouse liver organoids into Fah−/− mutant mice led to a significant increase in survival [180,181]. Further research has shown that human-derived organoids can be converted into functional hepatocytes readily following transplantation into damaged mouse liver [182].

Considering the potential tumorigenic and immune risks of Matrigel, organoids derived from natural or synthetic hydrogels are the preferred choice for liver transplantation. Studies have shown that encapsulating cells in hydrogels can provide immune protection for the cells [183]. Song et al. encapsulated liver organoids in alginate capsules and transplanted them into immunocompetent mice, which avoided direct immune rejection and also reduced safety issues due to PSC-derived teratomas [184]. Researchers have reported the use of a mixture of sodium alginate and gelatin as a bioink to print liver organoids, which were transplanted and significantly extended the survival of mice with liver failure [185]. Recellularized liver decellularized scaffolds are considered a perfect source of transplant donors. Since decellularized scaffolds preserve the vascular architecture, they can facilitate the establishment of vascular anastomosis between donor and recipient after transplantation. An essential problem requiring resolution is the vascular reconstruction of the decellularized scaffolds to prevent potential thrombosis and obstruction of blood supply post-transplantation [186]. The efficiency of reendothelialization can be improved by modifying the scaffolds with heparin or coating with antibodies to endothelial cell surface proteins [186]. Researchers have successfully recellularized engineered livers with PHHs and endothelial cells, surviving and functioning for up to 28 days post-transplantation [187]. These studies offer convincing evidence in favor of the potential of liver organoid transplantation in the treatment of end-stage liver failure. Despite still being in the preliminary stages of clinical trials, the promising outcomes indicate a transformative impact on liver disease treatment.

6. Conclusion and perspective

Liver organoids exhibit substantial promise in liver disease modeling, clinical drug testing, and regenerative medicine. However, the challenge lies in developing liver organoids that faithfully replicate the intricate tissue architecture, cellular diversity, and crucial functions of the native liver. The variability in the composition of liver organoids due to undefined growth conditions underscores the need for bioengineering solutions to enhance their fidelity. Bioengineering approaches offer promising strategies to address these challenges. Engineered hydrogels can be employed to provide microenvironments that closely mimic the native liver niche, thereby improving the functionality and viability of liver organoids. Innovations such as droplet microfluidics and acoustic fluidics have revolutionized organoid production from manual to mechanized processes, enhancing both quality and yield. Additionally, 3D bioprinting technologies enable the construction of liver microstructures in vitro by integrating hydrogels and cells, facilitating the development of more sophisticated organoid models. Microfluidic systems, such as organoids-on-a-chip, provide a means to subject organoids to liquid shear forces, deliver nutrients, and simulate multi-organ interactions by connecting multiple organoids. These advanced systems enhance the physiological relevance of organoids and offer a more comprehensive model for studying complex biological processes [189]. Furthermore, genetic engineering techniques can be employed to enhance the functional capabilities of organoids, further improving their utility in disease modeling and drug testing. By integrating advanced bioengineering strategies, researchers are advancing the development of liver organoids with enhanced homogeneity and functionality, paving the way for more accurate disease modeling, personalized medicine applications, and drug discovery studies.

The realm of liver organoid technology has undoubtedly witnessed

significant advancements; however, to seamlessly integrate these organoids into clinical practice, several formidable challenges necessitate resolution. Firstly, the establishment of liver organoids from iPSCs presents both opportunities and challenges. While iPSCs can differentiate into various cellular lineages, there are concerns about genetic and epigenetic anomalies that might occur in the course of reprogramming and differentiation [190]. Ensuring the genetic stability and consistency of these organoids is crucial for their use in clinical applications. Secondly, significant research on liver organoid transplantation is still limited to Matrigel-based approaches. For liver organoids to be considered appropriate for tissue engineering and clinical use, they must meet stringent safety criteria, including being free of animal components, no risk of tumorigenicity, and scalability to meet clinical demands. Thirdly, mono-cellular liver organoids primarily consist of hepatocyte-like cells and lack supportive cells such as hepatic stellate cells and Kupffer cells, which are essential for a fully functional liver model. The development of multi-cellular liver organoids is a promising direction for research. However, the spatial distribution and proportion of various cell types within these organoids must be finely tuned to reflect the in vivo liver environment.

Solving the aforementioned problems requires ongoing trials and refinements. Traditionally, the search for optimal strategies has relied on literature data and ongoing in vitro experiments, which tend to be both costly and time-consuming, with a propensity for limited success. Artificial intelligence (AI) stands to play a transformative role in addressing the challenges associated with liver organoid technology. Upon training on large datasets, AI systems demonstrate exceptional capabilities in learning and analysis. For instance, AI algorithms have been utilized to analyze the levels of differentiation and morphological changes in iPSC cultures, establishing reliable frameworks for cell validation and further developing automated screening systems [191]. Moreover, AI can capture correlations between hydrogel parameters and performance from vast datasets, effectively integrating with the needs of organoid growth to become a powerful tool for streamlined hydrogel design and validation [192,193]. In addition, AI is capable of efficiently processing large volumes of organoid data, including microscopic images, gene expression profiles, and proteomics data, to extract valuable information and predict the growth and differentiation patterns of organoids. The integration of AI at these critical points can significantly reduce the substantial workload and inaccuracies inherent in manual screening and analysis. However, the exploration of AI in the organoid field is still in its infancy. A crucial task at present is to construct reliable databases and refine AI models. Looking ahead, there is optimism for the establishment of an AI-driven automated system for organoids, encompassing automated cultivation, monitoring, and analysis, which would markedly enhance the efficiency and precision of experimental procedures. The vast potential of AI is expected to elevate the development of engineered liver organoids to unprecedented levels.

In summary, while the technology for generating liver organoids has come a long way, there is still much work to be done to overcome the existing challenges and to fully realize their potential in clinical applications. We are confident that the growing demand for sophisticated liver organoids will be a driving force for advancement in this field. To overcome these challenges and further advance liver organoid development, a collaborative approach among biologists, clinicians and professionals in other fields is crucial. By encouraging interdisciplinary cooperation and harnessing our combined knowledge, we can hasten the improvement of liver organoids. Through such collaborative efforts and innovative thinking, we can strive to develop superior liver organoid models. These models have the potential to be truly transformative, revolutionizing the landscape of liver research and disease treatment.

CRediT authorship contribution statement

Junqi Zhao: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation. **Yue Zhi:** Writing – review & editing, Validation, Investigation. **Haozhen Ren:** Writing – review & editing, Visualization, Methodology, Investigation. **Jinglin Wang:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Yuanjin Zhao:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

Yuanjin Zhao is an editorial board member for Bioactive Materials and was not involved in the editorial review or the decision to publish this review. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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