

Microporous membrane-based liver tissue engineering for the reconstruction of three-dimensional functional liver tissues in vitro

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Abbreviations: 2D, two-dimensional; 3D, three-dimensional; AQP, aquaporin; BAL, bioartificial livers; BC, bile canaliculi; BEC, biliary epithelial cells; BSEP, bile salt export pump; bFGF, basic fibroblast growth factor; CFTR, cystic fibrosis transmembrane regulator; CO, carbon monoxide; DMSO, dimethylsulfoxide; ECM, extracellular matrix; FDA, The Food and Drug Administration; HNF-4, hepatocyte nuclear factor 4; HSC, hepatic stellate cells; MRP, multidrug-resistance associated protein; NO, nitric oxide; NPC, non-parenchymal cells; PC, polycarbonate; PEG, poly(ethylene glycol); PET, polyethylene terephthalate; PLGA, poly(*D,L*-lactide-co-glycolide); PLLA, poly-*L*-lactic acid; SEC, sinusoidal endothelial cell; TAT, tyrosine aminotransferase; TO, tryptophan-2, 3-dioxygenase

To meet the increasing demand for liver tissue engineering, various three-dimensional (3D) liver cell culture techniques have been developed. Nevertheless, conventional liver cell culture techniques involving the suspending cells in extracellular matrix (ECM) components and the seeding of cells into 3D biodegradable scaffolds have an intrinsic shortcoming, low cell-scaffold ratios. We have developed a microporous membrane-based liver cell culture technique. Cell behaviors and tissue organization can be controlled by membrane geometry, and cell-dense thick tissues can be reconstructed by layering cells cultured on biodegradable microporous membranes. Applications extend from liver parenchymal cell monoculture to multi-cell type cultures for the reconstruction of 3D functional liver tissue. This review focuses on the expanding role for microporous membranes in liver tissue engineering, primarily from our research.

Current Status of Therapies for Liver Diseases

The liver is the largest internal organ in the body, accounting for 2% of the weight of an adult (~1.5 kg),¹ and responsible for more than 500 functions, such as the metabolism of sugars, proteins/amino acids, and lipids, detoxification of exogenous chemicals, production of bile acids, and the storage of various other essential chemicals, such as vitamins and iron.² Thus, its failure has potentially fatal consequences. Indeed, loss of liver function causes 40,000 deaths/year³ and is the twelfth most frequent cause of

death in the US.⁴ To date, orthotopic liver transplantation is the only clinically accepted therapy for patients with end-stage liver disease or acute liver failure. Although about 10,000 patients are added to the waiting list annually, fewer than 7,000 undergo transplantations. It is estimated that the high prevalence of hepatitis C (~3%) will increase demand significantly over the next decade.⁵ Additionally, orthotopic liver transplantation requires life-long immunosuppression. Thus, there is a continuing need for alternative therapies to the liver transplantation.

One such alternative is hepatocyte-based extracorporeal bioartificial livers (BALs). The liver is unique in its capacity to regenerate from even massive injuries, able to restore its original mass even if less than 20% of the cells remain undamaged.^{6–8} Thus, BALs could provide temporary support for patients who receive a partial hepatectomy due to acute liver failure or are awaiting orthotopic liver transplantation. Demetriou et al. have developed a BAL loaded with microcarrier-attached porcine hepatocytes and tested it in clinical trials.⁹ However, clinical success has yet to be achieved despite research extending over more than two decades.¹⁰

Another possible alternative to liver transplantation is hepatocyte transplantation. Because the procedure is less invasive than orthotopic liver transplantation and can be performed repeatedly, it could also be used in patients who are severely ill and unable to tolerate organ transplantation. Some studies have demonstrated its efficacy, experimentally and clinically.¹¹ However, poor engraftment of transplanted hepatocytes remains a major barrier to the successful expansion of hepatocyte transplantation therapy.^{12,13}

Tissue engineering is an attractive approach to improvement of cell engraftment. Enhancing cell-cell contact and providing nonimmunogenic matrices before transplantation has been shown to improve cell engraftment in animal models.¹⁴ Furthermore, tissue-engineered products, such as skin substitutes and cartilage replacement, have already helped thousands of patients^{15,16} and

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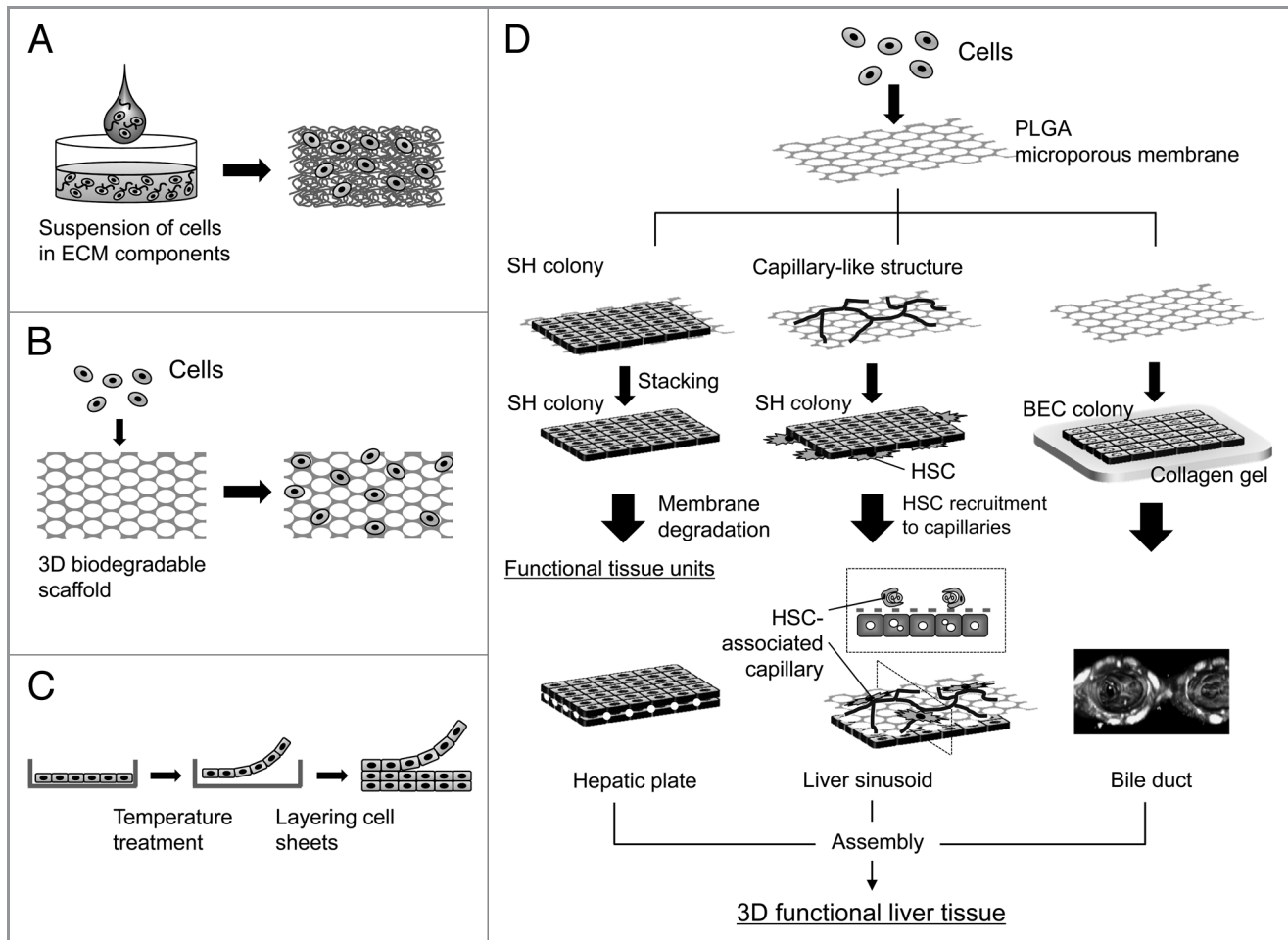


Figure 1. Liver tissue engineering approaches. (A) Cells are suspended in extracellular matrix (ECM) components and then poured into a mold to let the suspensions being polymerized. (B) Cells are seeded onto three-dimensional (3D) porous biodegradable scaffolds. (C) Intact cell sheets are layered to construct cell-dense 3D tissues. (D) Cells are cultured on thin and highly porous poly(*D,L*-lactide-co-glycolide) (PLGA) microporous membranes to fabricate smaller functional tissue units. The tissue units can be then assembled into functional 3D liver tissues by stacking.

other artificial tissues, such as bladder, cornea, bronchial tubes and blood vessels, are in clinical trials.^{15,16} Thus, liver tissue engineering is considered a potentially valuable new therapeutic modality for liver disease.

Liver Tissue Engineering for Reconstructing 3D Liver Tissues

In a common approach to liver tissue engineering, parenchymal cells alone and/or a mixed population of parenchymal and non-parenchymal cells (NPCs) are combined with various forms of three-dimensional (3D) scaffolds and appropriate signaling molecules, such as cytokines or growth factors, that facilitate cell growth, organization, and differentiation. These processes can be classified into two categories. The first involves suspending hepatocytes in extracellular matrix (ECM) components (Fig. 1A). Hepatocytes grown on collagen-coated polystyrene beads in roller bottle cultures with NPCs were allowed to form cell clusters and were implanted in Matrigel, self-organizing into hepatic plate-like architectures three-dimensionally.¹⁷ Fetal liver progenitor cells

were co-cultured in 3D fibrin gel with endothelial cells, resulting in the formation of vascular structures by the endothelial cells and increased proliferation and function of liver progenitor cells.¹⁸ Hepatocytes were transplanted into the subcutaneous space, where new vascular network formation was induced in advance by transplanting a polyethylene terephthalate mesh device coated with poly(vinylalcohol) that allowed for the gradual release of basic fibroblast growth factor (bFGF), resulting in persistent survival for up to 120 d.¹⁹ Photo-polymerization of a hepatocyte-suspended poly(ethylene glycol) (PEG)-based hydrogel has been developed for the reconstruction of 3D liver architectures.²⁰ Hepatocytes suspended in a pre-polymer solution were photo-immobilized locally within a 3D cell-hydrogel network, thus forming a functional 3D liver construct with complex internal architectures.

Three-dimensional scaffolds composed of biodegradable materials can provide platforms for hepatocyte attachment (Fig. 1B). Fetal liver cells seeded in poly-*L*-lactic acid (PLLA) 3D macroporous scaffolds formed small clusters and showed higher levels of hepatic function, comparable with those of adult

hepatocytes.²¹ Similarly, colonies of small hepatocytes (SHs), hepatic progenitor cells, placed on a collagen sponge with NPCs proliferated and expanded to form a hepatic organoid with highly differentiated functions.²² Hepatocytes seeded on PLLA and/or poly(*D,L*-lactide-*co*-glycolide) (PLGA) sponges were engrafted when they were implanted at a site associated with abundant vascular networks with appropriate surgical stimulation.^{23,24}

Both approaches for liver tissue reconstruction thus seems efficacious, since cell behavior can be controlled using materials with various structural and functional properties. However, these earlier studies using ECM or scaffold-based designs to engineer tissues face a major drawback, poor cell density. In native liver tissue, cell density is significantly higher, compared with other tissues, such as bone and cartilage. Accordingly, hepatocytes within native liver tightly interconnect to form layered structures, termed hepatic plates. Additionally, there is only a slight gap between hepatocytes and liver sinusoids, liver-specific microvessels, facilitating rapid exchange of macromolecules between plasma and hepatocytes. Thus, cell-sparse constructs engineered with those scaffolds often do not closely resemble the native liver architecture.

In contrast to earlier studies using ECM or biodegradable materials, scaffold-less cell-sheet engineering has been proposed for construction of 3D cell-dense liver tissue (Fig. 1C). For example, culture dishes, the surfaces of which were modified with a temperature-responsive polymer, have been used. Using such temperature-responsive culture surfaces, hepatocytes can be harvested as intact sheets and cell-dense thick tissues can be constructed by layering these cell sheets.^{25,26} However, a highly complex fabrication process is needed to covalently graft the temperature-responsive polymer onto dish surfaces²⁷ and it also takes more than 30 min to harvest a cell sheet.²⁸ Magnetite cationic liposomes have been also used to label cells and to form multilayered sheet architectures. A magnetic field is then used to accumulate the magnetically-labeled cells onto ultralow attachment culture surfaces and form multilayered sheets.²⁹ Cells can be harvested readily as intact cell sheets by pipetting. However, when this method was applied to hepatocytes, the sheets were not sufficiently strong for recovery.³⁰ Furthermore, because cells have to be harvested as an intact sheet in the two methods above, it is difficult to construct the complex 3D liver architectures that are made from smaller tissue units. In the liver, hepatocytes form hepatic plates while sinusoidal endothelial cells (SECs) form sinusoids. Additionally, biliary epithelial cells (BECs) form bile ducts, tube structures that carry bile secreted by hepatocytes. The liver is generated by repeating these functional tissue units. Thus, it is difficult to reconstruct the complex architecture of the liver in vitro using only cell-sheet engineering-based approaches.

Microporous Membrane-Based Liver Tissue Engineering

To overcome the drawbacks of the culture techniques discussed above, we developed a novel approach for constructing complex 3D liver tissues. In our approach, two-dimensional (2D) tissues, such as hepatic plate-like tissues,³¹ microvascular networks and intrahepatic

bile ducts³² constructed on biodegradable microporous membranes are stacked and allowed to form cell-dense 3D tissues by degradation of the membranes in vitro or in vivo after their implantation (Fig. 1D).

Our approach, stacking two-dimensional (2D) tissues cultured on biodegradable microporous membranes to create complex 3D liver tissues, has advantages over scaffold-less cell-sheet engineering. First, PLGA was used to fabricate microporous membranes. PLGA is a biodegradable material that has been approved by the US Food and Drug Administration (FDA) for use in drug delivery, diagnostics, and other applications in clinical and basic science research. PLGA has already been used in tissue engineering as a 3D cell scaffold in various foams,³³ fibers³⁴ and sponges.³⁵

To fabricate microporous membranes, PLGA, dissolved in dioxane with various levels of moisture content, was spin-coated on a polyethylene sheet and then dried to generate micropores by the dioxane-water phase separation.³⁶ These membranes can be readily peeled off from the sheets with tweezers and cut into the desired shapes, which is relatively easy, compared with other approaches based on cell-sheet engineering. Second, damage to cells can be minimized because the cells are harvested with the membranes with no direct manipulation, such as enzymatic, thermal, or electric treatments.³⁷ Third, although cells in scaffold-less cell sheet engineering have to be layered as intact contiguous sheets to construct 3D tissues, 2D tissues with various structures containing not only sheets but also network structures and duct structures can be stacked using our technique, enabling stepwise construction of complex 3D liver tissues from smaller 2D tissue units. Importantly, a previous study demonstrated that kidney-like tissues could be reconstructed from a combination of epithelial tissues and mesenchymal tissues,³⁸ suggesting that tissue-by-tissue assembly can be used to reconstruct complex tissues from cultured cells in vitro. We also have demonstrated that the stepwise assembly of hepatic plate-like tissues and microvascular networks is necessary for reconstruction of liver sinusoidal structures in vitro.³⁹

Using the microporous membrane-based approach we can thus overcome the problem associated with scaffold-less cell-sheet engineering approaches, the applicability of which to liver tissue engineering is limited.

Functional Liver Tissue Engineering with Microporous Membranes

To fabricate functional 3D liver tissues in vitro, our group developed a bottom-up approach that assembles smaller functional 2D tissue units using microporous membranes. This approach mimics much of the native liver architecture, in which functional 2D tissue units such as the hepatic plates, the sinusoids and bile ducts are the repeating structures. In this chapter, we review the progress in functional 2D tissue unit reconstruction using microporous membranes.

Hepatic plates. We first explored the efficacy of the microporous membrane-based tissue assembling approach in reconstructing hepatic plates using polycarbonate (PC) microporous membranes.⁴⁰

Pairs of membranes were prepared and rat SHs were separately cultured on each. After the SH colonies had developed, one membrane was inverted on top of the other to form an SH bilayer. In the stacked-up structures, the SHs of the upper and lower layers adhered to one another, and that bile canaliculi (BC) formed between them, resulting in the formation of native hepatic plate-like tissues. The stacked-up structures were maintained for more than a month. The cells within the tissues exhibited mRNA transcription of hepatic-differentiation markers such as albumin, multidrug-resistance associated protein (MRP), hepatocyte nuclear factor 4 (HNF-4), tyrosine aminotransferase (TAT), tryptophan-2, 3-dioxygenase (TO) and maintained a relatively high level of albumin secretion for more than a month. Thus, hepatic plate-like tissues with highly differentiated functions, including functional BC, can be reconstructed by stacking layers of SHs. However, the membranes remained in the reconstructed structures permanently, because the PC membrane was not biodegradable, suggesting that stacking more than two layers of SHs would be problematic.

Thus, we next explored the possibility of using PLGA microporous membranes in the stacking culture method for the construction of hepatic plate-like structures to overcome the problems described above.³⁶ SHs were cultured on a membrane to allow them to proliferate and form colonies and then stacked on top of those in the dish so that the membrane was sandwiched between two SH layers. More than two layers can be stacked if the membranes disappear by biodegradation after stacking of the cell layers. The membranes degraded gradually and disappeared almost completely by 14 d after stacking, resulting in the reorganization of the cells into hepatic plate-like tissues. As in the case when two SH layers were stacked to attach to one another,⁴⁰ the cells in the constructed structures formed BC. We confirmed that these hepatic plate-like structures could be maintained at least more than 2 weeks after stacking of the cell layers. In addition, the cells exhibited a relatively high level of mRNA transcription of hepatic-differentiation markers such as albumin, MRP2, bile salt export pump (BSEP), TAT and TO. This upregulation of hepatic differentiated function was also confirmed by continuous secretion of albumin and urea into the culture medium for at least 20 d.

Liver sinusoids. Unlike the microvasculature in other tissue beds, liver sinusoids have highly specialized structures, where hepatocytes and sinusoidal lining cells, including SECs and hepatic stellate cells (HSCs), intimately associate with each other.⁴¹ Based on these anatomical characteristics, the concept of a hepatocyte-HSC-EC complex that functions as a unit for transduction between the bloodstream and the hepatic parenchyma has been proposed.^{41,42} Recent studies support the concept that HSCs serve as a bridge that mediates bidirectional metabolic interactions between sinusoids and hepatocytes, using prostanoids and/or gaseous mediators, such as nitric oxide (NO) and carbon monoxide (CO), as signaling molecules.^{42,43} Thus, reconstruction of the liver sinusoidal architecture is essential in constructing functional liver tissues *in vitro*.

We first established a SH-HSC-EC tri-culture system in which these cells form an *in vivo*-like physiological complex.⁴⁴ SHs and HSCs were first isolated from adult rat livers and cultured on

polyethylene terephthalate (PET) microporous membranes. The SHs formed single-layered colonies on the membranes while HSCs resided in the micropores. Then, ECs were inoculated on the opposite side of the membranes, resulting in a formation of the HSC-mediated structures. To obtain these structures, spatial control of HSC behavior by changing the pore size was important, suggesting that the membranes can be used as not only carriers but also modulator of cellular morphogenesis. Furthermore, HSCs were confirmed to mediate the SH-EC communication, in terms of EC morphogenesis. These results indicated that the SH-HSC-EC physiological complex could be achieved in the reconstruction of HSC-mediated structures.

Using the above tri-culture system, the effect of direct contacts between HSCs and ECs on EC capillary formation was then determined.³⁹ The HSC-EC contacts are increasingly recognized for their roles in EC capillary morphogenesis.^{45,46} However, the hypothetical role of HSC-EC contacts in morphogenesis remained unclear in the tri-culture. HSC-EC contacts were shown to inhibit EC capillary morphogenesis, suggesting that the HSC-EC contacts may be an important factor in EC capillary formation. Additionally, ECs responded to the induction of capillary morphogenesis before the formation of HSC-EC contacts, suggesting that both spatially and temporally, HSC behavior is a key engineering strategy for the reconstruction of sinusoidal tissue *in vitro*.

Finally, we demonstrated reconstruction of HSC-incorporated sinusoidal structures.⁴⁷ In the sinusoids, HSCs surrounded the outer surface of EC capillary structures. To generate these structures, the heterotypic cell-cell interactions across the membranes need to be improved. Thus, PLGA microporous membranes with higher porosity and reduced thickness were used. When the pore size and porosity of the membranes were optimized, HSCs migrated toward the EC capillary structures by passing through the membrane's pores and then surrounded them, resulting in the reorganization of sinusoidal-like structures. These structures were maintained more than 20 d. The HSC-incorporated sinusoidal-like tissues retained higher levels of albumin secretion and hepatocyte-differentiated markers such as MRP2, BSEP, TAT and TO compared with SH-HSC organoids.

Bile ducts. One problem remaining in the constructed hepatic plate-like tissues mentioned above is the accumulation of bile, which is known to be toxic to hepatocytes.⁴⁸ To reconstruct hepatic tissues with a bile drainage system, formation of bile ducts during culture is important. We demonstrated formation of bile ductular networks when rat BECs were cultured between two layers of collagen gel, with stimulation by dimethylsulfoxide (DMSO) in the culture medium.³² These bile ductular networks were found to possess apical domain markers such as Cl⁻/HCO₃⁻ anion exchanger 2 and cystic fibrosis transmembrane regulator (CFTR), and well developed microvilli on their luminal surfaces and also expressed apical [aquaporin (AQP) 1, MRP2 and CFTR] and basal (AQP4 and MRP3) domain markers of BECs. Furthermore, the cells in the bile ductular networks responded to secretin stimulation and transported metabolized fluorescein from the basal side to the luminal space, demonstrating that the reconstructed LBDs were functionally and morphologically

similar to the bile ducts in vivo. However, the thick collagen gel layers prevented co-culturing of bile ductular networks with hepatic plate-like structures in close proximity for the formation of hepatic tissues with a bile drainage system. To overcome this, we have explored the efficacy of the PLGA microporous membranes as alternative cell scaffolds to collagen gel (unpublished data). Bile ductular networks can be co-cultured with hepatic plate-like structures in close proximity if the membranes are biodegraded after formation of the networks. We preliminarily confirmed formation of bile ductular networks when BEC colonies cultured on collagen gel were overlaid with microporous membranes, and their morphologies could be controlled by changing the pore-size of the membranes, again suggesting that the membranes can be used as not only carriers but also modulators of cellular morphogenesis. Furthermore, the ductular networks could be maintained for more than 90 d even after the membranes were degraded.

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Conclusions

We have described a novel liver tissue engineering approach using microporous membranes. Although the approach has been used only in construction of 2D tissue units, we are currently working on assembling these 2D tissue units into functional 3D liver tissues in vitro. We believe that effective application of microporous membrane-based liver tissue engineering will provide new possibilities in the field of liver regenerative medicine.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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