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Extracellular Microenvironmental Control for Organoid Assembly

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Organoids, which are multicellular clusters with similar physiological functions to living organs, have gained increasing attention in bioengineering. As organoids become more advanced, methods to form complex structures continue to develop. There is evidence that the extracellular microenvironment can regulate organoid quality. The extracellular microenvironment consists of soluble bioactive molecules, extracellular matrix, and biofluid flow. However, few efforts have been made to discuss the microenvironment optimal to engineer specific organoids. Therefore, this review article examines the extent to which engineered extracellular microenvironments regulate organoid quality. First, we summarize the natural tissue and organ's unique chemical and mechanical properties, guiding researchers to design an extracellular microenvironment used for organoid engineering. Then, we summarize how the microenvironments contribute to the formation and growth of the brain, lung, intestine, liver, retinal, and kidney organoids. The approaches to forming and evaluating the resulting organoids are also discussed in detail.

Keywords: microfabrication, biomaterials, organoid, hydrogel

Impact Statement

Organoids, which are multicellular clusters with similar physiological function to living organs, have been gaining increasing attention in bioengineering. As organoids become more advanced, methods to form complex structures continue to develop. This review article focuses on recent efforts to engineer the extracellular microenvironment in organoid research. We summarized the natural organ's microenvironment, which informs researchers of key factors that can influence organoid formation. Then, we summarize how these microenvironmental controls significantly contribute to the formation and growth of the corresponding brain, lung, intestine, liver, retinal, and kidney organoids. The approaches to forming and evaluating the resulting organoids are discussed in detail, including extracellular matrix choice and properties, culture methods, and the evaluation of the morphology and functionality through imaging and biochemical analysis.

Introduction

ENGINEERED CELL CLUSTERS with similar cellular organization and physiological function to tissues and organs of living organisms, also known as “organoids,” have

been gaining more attention in biomaterial and bioengineering research. Organoids create a platform to better understand and regulate the biomolecular and cellular activities responsible for development, homeostasis, and regeneration. In the past, tissue explants excised from

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human or animal models, called “*ex vivo* tissue,” were cultured in a controlled extracellular environment for both fundamental and applied bioscience studies. However, these model systems have drawbacks due to imaging limitations, source availability, rapid phenotype loss, and profound differences between species.¹ Cell clusters have also emerged as a promising alternative to *ex vivo* tissues; however, these simple cell aggregates called “spheroids” do not reproduce the delicate ultrastructure and function of their corresponding tissues or organs.

In contrast to *ex vivo* tissue or spheroids, organoids are generated from pluripotent and multipotent stem cells, progenitor cells, and precursor cells for self-assembly. These cells differentiate to lineage-committed tissue cells and reproduce the developmental niche during the differentiation process. These cell clusters can be molded into various forms such as spheres,^{2,3} hollow tubes,^{4,5} and sheets,^{6,7} all of which can further integrate to form partial or entire organs.

In the early years of organoid engineering, the focus was to control the self-organization of cells to reproduce the ultrastructure of target organs. Recently, the new focus is to establish the physiological functionality of engineered organoids. It is common to induce aggregation between cells in a suspended state. However, this cell culture method disregards the important factor of cell–matrix interactions modulated by extracellular microenvironments. According to developmental biology studies, these microenvironmental factors significantly affect the structure and physiological function of the resulting organs.⁸ In addition, the cell–matrix interactions regulate the cell–cell interactions and cellular response to soluble factors.⁹

Accordingly, efforts are increasingly made to design advanced culture platforms of organoid that recapitulate the multifaceted extracellular microenvironment in a bottom-up approach. In this regard, individual or aggregated cells are cultured on a two-dimensional (2D) substrate or in a three-dimensional (3D) matrix that reproduces the *in vivo* microenvironment. Target extracellular matrices (ECMs) are provisional matrices resulting from injuries, connective tissues of an embryo, or mature tissues. Consequently, cells cultured in a proper platform yield functional organoids, including the optic cup,¹⁰ brain,¹¹ intestine,^{12,13} liver,¹⁴ heart,³ and kidney.¹⁵

The extracellular microenvironment is engineered by orchestrating an ECM along with soluble factors. Common ECM materials used to develop organoids include Matrigel and collagen. Their popularity stems from their ease of access, implementation in 2D and 3D cell culture platforms, historic reliability for cell culture applications, biocompatibility with cells and biochemicals, and tunability of the elastic modulus. Because they are natural ECM components or can easily be modified with natural proteins, they can be tailored to a variety of different organoid environments. For example, Matrigel can be found in retinal, liver, intestine, lung, and kidney organoid experiments despite an elastic modulus range from 1.8 to 20 kPa. These ECM materials are coated on various microfluidic platforms or substrates made with polydimethylsiloxane (PDMS) to recapitulate *in vivo* extracellular environments. PDMS is a silicone elastomer that is thermally stable, optically transparent, and biocompatible, thus widely used for cell and organoid culture.¹⁶ This review article will explain the applications of these common biomaterials, specific to organoid culture.

This review article outlines extracellular microenvironment variables which factor into the choice of extracellular microenvironments used for organoid formation and growth. In particular, we focus on the extent to which extracellular chemical and mechanical environments modulate cellular organization and subsequent morphology and physiological function of organoids. The role of extracellular microenvironments will be explained with the formation of the brain, lung, intestine, liver, eye, kidney, and cardiovascular organoids. Overall, this review article will significantly serve to advance the development of biological tools useful to improving human health.

Controlling Extracellular Microenvironment for Brain Organoid Assembly

Increasing efforts have been made to advance brain organoids by recapitulating the physiological function of brain. For example, neural developmental events which usually take months to mature in *in vivo* models can be studied within weeks using brain organoids.¹⁷ The bioelectrical activity of brain organoids should shift from random bursts of spontaneous activity like in developing embryos to coordinated synchronized activity. Since a brain consists of multiple neuronal cells, including neurons, glia, and oligodendrocytes, pluripotent or multipotent stem cells are frequently the starting point for a bottom-up approach to form brain organoids. To date, most studies are focused on creating morphologically similar tissue to the specific regions of the brain, like the cortex or hippocampus, or attempt to recreate a brain disease, like microcephaly.^{18–20}

The brain is one of the softest tissues in the body with an elastic modulus ranging between 1.8 and 2.3 kPa according to measurement using magnetic resonance elastography.²¹ The brain has an intricate structure relating to different functions in behavior and movement. The brain also has a unique blood–brain barrier (BBB) that allows for specific molecules to pass the vascular wall. This barrier keeps the brain free from contaminants circulating in the blood stream and hinders the ability of drugs to reach the internal brain tissue for disease treatment. As such, much of the research on brain organoids is divided into three directions: (1) growth of morphologically correct tissue to understand function and synaptic activity, (2) recapitulation of the BBB, and (3) development of the strategies to treat neurodegenerative diseases and acute/traumatic brain injury. This section will discuss how the extracellular microenvironment controlled with biomaterials contributes to these efforts.

Engineering of brain organoids

Due to the brain’s structural complexity and the heterogeneity of the cells involved with making neural networks, many researchers use pluripotent or multipotent stem cells to create brain organoids along with biomaterials designed to mimic the multiple chemical and physical environments of the brain. For example, Lindborg *et al.* cultured cerebral organoids from pluripotent stem cells in a hyaluronan and chitosan hydrogel.¹⁸ Hyaluronan was chosen due to its recognition as an integral part of the ECM in developing brains. The hydrogels were made by first lyophilizing the chitosan base into leaflets, mixing with dry hyaluronan, and finally hydrating the powders with a hydration fluid (Cell-

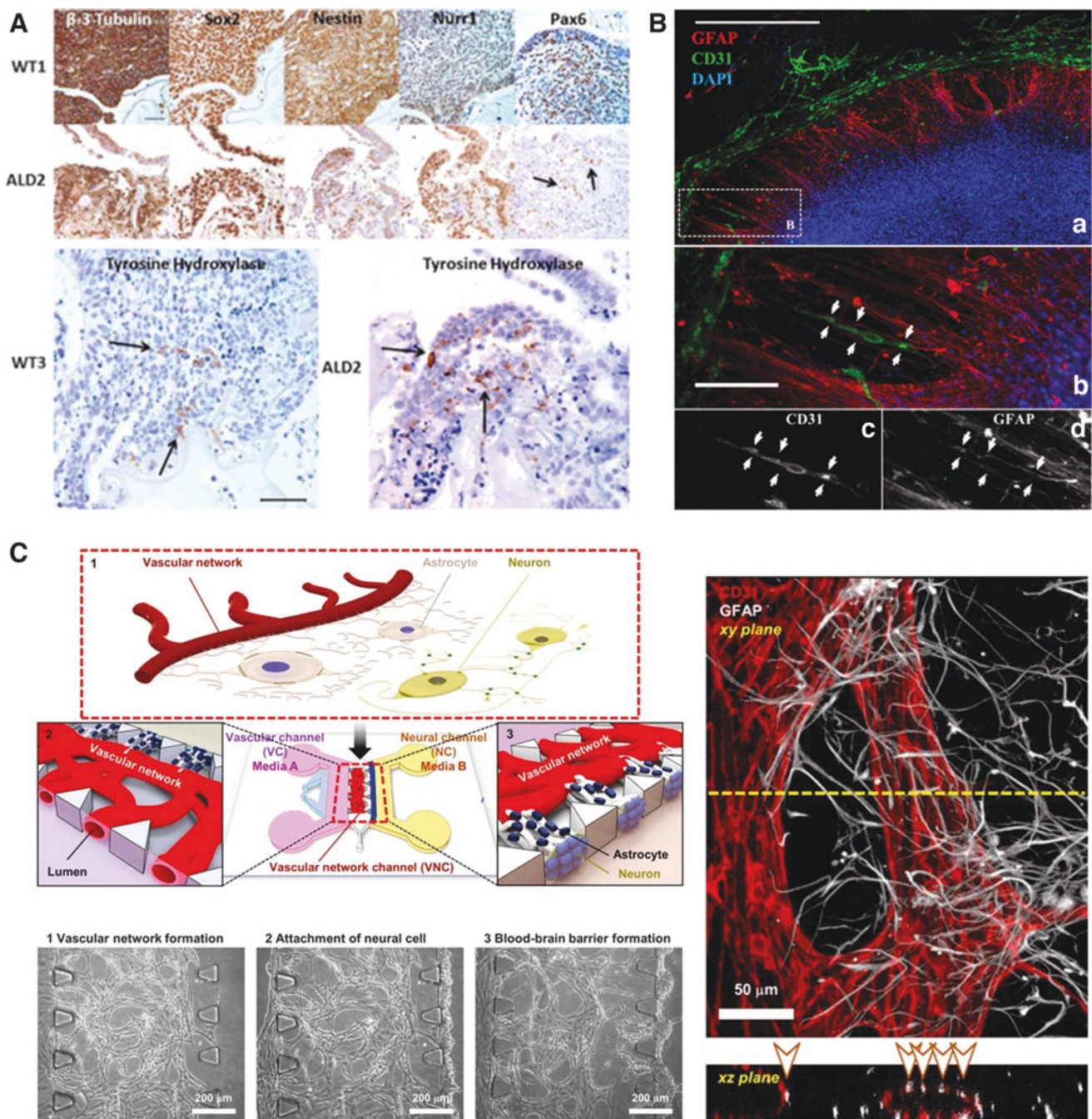


FIG. 1. Cerebral organoid models. (A) Immunohistochemical staining of cerebral organoids derived from control (WT1, WT3) or an adrenoleukodystrophy patient's (ALD2) iPSCs. Reproduced with permission.¹⁸ Copyright 2016, Wiley. (B) Vascular networks of brain organoids cultured in polyethylene glycol-norbornene hydrogels modified with cRGDs. (a) neural construct after 21 days. Endothelial cells (CD31) are stained in *green*, glial cells (GFAP) are stained in *red*, and cell nuclei (DAPI) are stained in *blue*. (b) Zoom of the boxed region shown in A to illustrate association and alignment for a capillary tubule and radially oriented glial cells (arrows). The cells in B are shown as single channel grayscale images for (c) CD31 and (d) GFAP. Reproduced with permission.¹⁹ Copyright 2015, National Academy of Sciences. (C) Scheme of the microfluidic platform for a neurovascular unit, including the BBB and phase contrast and immunofluorescent images of the vascular network formed in the microfluidic chip. Endothelial cells (CD31) are stained in *red*, and glial cells (GFAP) are stained in *white*. Reproduced with permission.²³ Copyright 2017, Springer Nature. BBB, blood-brain barrier; cRGD, cyclic RGD peptide; DAPI, 4',6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; iPSCs, induced pluripotent stem cells. Color images are available online.

Mate3D). The modulus of the hydrogels was measured to be between 9 and 13 kPa, slightly stiffer than the normal brain tissue. From human induced pluripotent stem cells (iPSCs) incorporated into the hyaluronan and chitosan hydrogel, brain organoids were formed with distinct regions of fore-brain, midbrain, and hindbrain as confirmed with protein and gene expression (Fig. 1A). Human iPSCs used herein were derived from six patients: three normal control subjects and three from patients with cerebral adrenoleukodystrophy, a genetic brain disorder causing breakdown of the myelin sheath. The resulting organoids are being used to develop an *in vitro* model of adrenoleukodystrophy.

Schwartz *et al.* used human embryonic stem cell-derived progenitor cells, mesenchymal stem cells, and microglia/macrophage precursors to create a brain organoid used to test for neural toxicity.¹⁹ They modified collagen with cysteines to crosslink 8-arm poly(ethylene glycol) (PEG)-norbornene. Cyclic arginylglycylaspartic acid (RGD) peptides that act as cell adhesion epitopes were then added as a 2 mM solution. The various cell types were seeded in order of appearance during *in vivo* brain development: radial glia was formed first with GABAergic and glutamergic neurons layering outward. Vascular cells were introduced to the organoids on day 9, a relatively early time point in their neural construct's development, which allowed for dispersive capillary formation throughout all neuronal layers (Fig. 1B). Control organoids, which lacked vascular cells, had detectable amounts of vascular endothelial growth factor (VEGF); however, this amount was not necessary to form interconnected vasculature. They characterized the construct's ability to accurately predict toxicity by running a gene expression profile using 31 control compounds such as food additives or pharmaceuticals with no neurotoxicity and 39 toxin compounds.¹⁹

Recapitulation of the BBB formation

The brain is also characterized by the BBB, one of the most restrictive barriers of the body. The barrier consists of the capillary basement membrane separated from the neurons by astrocytes and endothelial cells which create tight junctions.²⁰ This structure reduces the fenestrations that are present in other capillaries around the body. Interestingly, various transporters such as amino acids, glucose, and waste export allow certain molecules and immune cells to cross the BBB, facilitated by the astrocytes and the central nervous system. Chemical cues from the body, like histamine, travel to the astrocytic end-feet, which surround the endothelial cells of the blood vessel, allowing for homeostasis of capillary permeability in the brain.²²

Bang *et al.* recreated this tight physical barrier in a microfluidic chip.²³ By sequentially adding human umbilical vein endothelial cells and then rat cortical neurons into channels of the PDMS microfluidic chip (Fig. 1C), endothelial cells, neurons, and astrocytes orchestrate to form a BBB. Astrocytes' end-feet are in contact with capillaries like the native BBB. A fibrinogen was injected into the channel with human umbilical vein endothelial cells and thrombin to activate formation of a provisional fibrin matrix appropriate to support vasculogenesis.

The neurons were also in direct contact with the astrocytes which formed the relevant protective mechanical environment found in the brain. The resulting BBB exhibited comparable

permeability, which was evaluated with molecular diffusivity. For instance, diffusion coefficients of fluorescein isothiocyanate-dextran molecules *in vivo* (i.e., 0.45×10^{-6} cm/s for 20 kDa molecules and 0.36×10^{-6} cm/s for 70 kDa molecules) are similar to that of *in vivo* models (0.24×10^{-6} cm/s for 20 kDa molecules and 0.15×10^{-6} cm/s for 70 kDa molecules).

Extracellular Microenvironment Control for Lung Organoid Assembly

The lungs are responsible for transmitting oxygen (O_2) from the air into the blood at the alveolar-capillary interface.²⁴ In addition to supporting this phase boundary, the lungs undergo dynamic and constant motion with each breath.²⁵ During inhalation, the intrapleural pressure decreases, causing the alveoli to expand and fill with external air. Therefore, from a design perspective, lung organoids should exhibit elasticity and mechanical resilience through repeated expansion. By doing so, organoids should be able to undergo nonuniform radial strain that causes around a 50% change in surface area and Poisson ratios of 0.41–0.45 without fracture.^{26–28} The pleural membrane should also withstand high tensions, ranging from 10^3 to 10^4 dyne/cm in dogs, which is similar to that of humans.²⁹ This tension increases with the membrane surface area.

In native tissue, these mechanical properties are attained by an epithelial cell layer at the alveoli surface that allows for rapid gas diffusion, surrounded by an elastic basement membrane. The consistent mechanical stretching of the epithelial cell layer is important for the overall function of the lung, playing a role in phospholipid production, organ growth, cellular viability, and signaling.^{27,30–32} Since the epithelium and underlying tissue in the lungs are constantly undergoing dynamic changes, the overall elastic and shear moduli of this region vary.

Recreating the lung's mechanical microenvironment

Since the lungs are mechanically dynamic, lung organoid cultures should be designed to be equally dynamic to support and simulate the complex physical environment in which the cells reside. Researchers often accomplish this goal by culturing cells on devices that can mechanically oscillate to simulate the strains caused by respiration.²⁷ Similarly, researchers have simulated the alveolar-capillary interface by engineering multichanneled microfluidic chips.^{33,34} For instance, a study by Huh *et al.* formed two chambers within a PDMS mold separated by a porous polyester membrane to simulate this interface (Fig. 2A). Small airway epithelial cells were cultured on this membrane with microchannels of 300 μ m diameter to mimic the gaps caused by conducting airways and bronchioles. The cell culture media was flown past the cells with the physiologically relevant speed of 1.5 mm/s.

Interestingly, these devices can reproduce the secretory activity of native cells.³⁵ The epithelial cells at the interface differentiated in response to the air/liquid interface. This platform was used to experimentally test the injuring effects of lung collapse and subsequent reopening by propagating a liquid plug through the microchannels.³⁴ A follow-up study coated the PDMS membrane with fibronectin or collagen, cultured the epithelial cells with human pulmonary microvascular

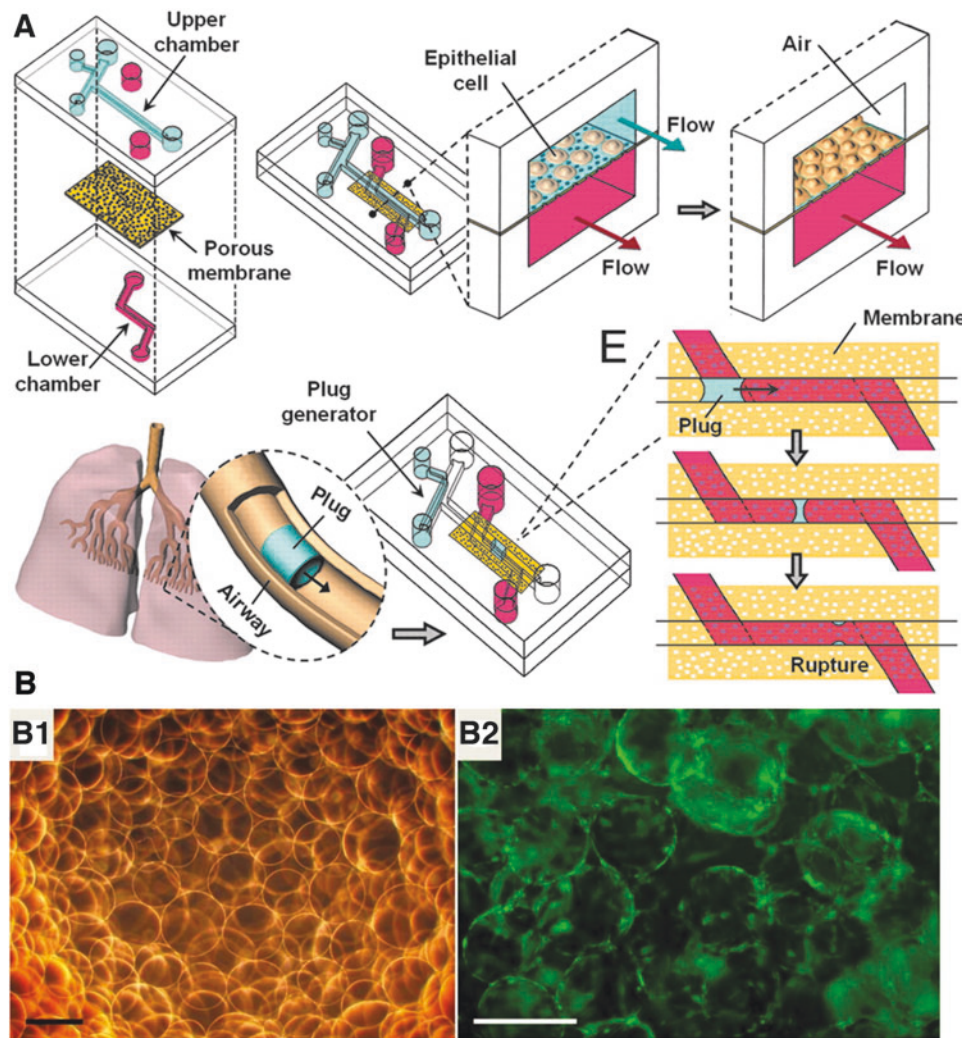


FIG. 2. Lung organoid models. (A) Compartmentalized microfluidic airway systems for lung organoid culture. Reproduced with permission.³³ Copyright 2007, National Academy of Sciences. (B) (B1) Bright-field image of alginate beads and (B2) fluorescence image of human lung fibroblast spheres after 24 h in the bioreactor. Fibroblasts are stained in green. Reproduced with permission.³⁷ Copyright 2016, The American Physiological Society. Color images are available online.

endothelial cells, and applied 10% cyclic strains to mimic the native tissue. This cell culture platform increased lung surfactant production, viability, and various biofunctional markers compared to the control without any applied cyclic strain. For example, intercellular cell adhesion molecule-1 expression was increased by around three times, and reactive O_2 species production was increased by around four times relative to the amount produced without strain.³⁶

Small molecule determinants of morphological development

Rather than mimicking the mechanical environment of the lung, some researchers have instead simulated the fluctuations in O_2 content to simulate bronchopulmonary dysplasia.³⁷ Human fetal lung fibroblasts were cultured on alginate beads that were coated with collagen. The cell-gel constructs were suspended in Tris-buffered dopamine hydrochloride using a rotating bioreactor for 96 h, then matured in an incubator that oscillated its O_2 gas content between 10% and 70% every 24 h (Fig. 2B). This gas content aimed to replicate the gas content in diseased patients and resulted in upregulation in biomarkers such as α -smooth muscle actin and the Notch pathway compared to cells cultured in normoxic conditions ($\sim 20\%$). Ultimately, the human fetal lung fibroblasts, which were cultured

on the collagen-coated alginate beads in hypoxic conditions, formed lung organoids which had similar structural changes as what was seen in infants with bronchopulmonary dysplasia. Thus, the biomaterial substrate accurately mimicked the disease-state environment.

Success has also been found by engrafting mouse fetal pulmonary cells below the renal capsule by Mondrinos *et al.*³⁸ These cells were cultured in a hydrogel made from 1.2 mg/mL collagen type I mixed with tenascin-C, an ECM glycoprotein. The hydrogels improved the circulation of nutrients to the organoids and engraftment, resulting in vascularization into the gels by endothelial cells. Differentiation and organoid formation were further promoted by media supplemented with fibroblast growth factors (FGFs) and modulated by concentrations of tenascin-C, VEGF, and sonic hedgehog compared to serum-free control medium.

Overall, lung organoid culture presents interesting mechanical challenges to sufficiently mimic the native environment. Lung tissue undergoes constant changes in transpulmonary pressure, which imposes cyclic changes in the elastic modulus experienced by the cells. To address these cyclic changes in elastic modulus, lung cells deposit an elastic ECM so that they can withstand repeated mechanical deformations. As such, researchers have developed technologies, such as oscillating bioreactors, to recapture the mechanically dynamic

environment of native lungs during culture. Cell-adherent scaffolds like collagen or Matrigel were used to support the growth and guide the organization of mature lung organoids. However, since the lungs are a dynamic organ, scaffolds should be rigid and flexible enough to sustain large deformations without fracture. Future studies in engineering functional lung organoids will likely combine rigid scaffolds with oscillating bioreactors.

Extracellular Microenvironment Control for Intestine Organoid Assembly

The intestinal epithelium consists of a monolayer of epithelial cells folded to form villus and crypt domains.³⁹ The microstructure of the intestine has an important role in providing increased surface area for nutrient absorption. Besides, epithelial cells exhibit active self-renewal in which cells are replaced every 4–5 days.⁴⁰ Thus, the majority of intestinal organoid engineering studies have focused on controlling the pathways that promote differentiation and self-renewal of the intestinal stem cells. In the epithelium, Lgr5 intestinal stem cells are populated at the intestinal crypts where the differentiation and self-renewal are controlled by the bone morphogenic protein (BMP) and Wnt pathways.^{41–43} Thus, studies are focused on regulating BMP and Wnt pathways in the intestinal cells. Due to the importance of controlling the genetic pathways, studies have introduced various methods to genetically stimulate the cells.^{12,13,44,45} These engineered intestinal organoids hold valuable therapeutic potential for scrutinizing the intestinal *in vitro* and screening drugs for intestinal diseases.

Macroscale manipulation of biomaterial shape to facilitate the intestinal organoid formation

In addition to these methods, researchers used a simpler robust method by manipulating the material where the intestinal organoids were formed. Due to the geometric characteristic of the intestine, which consists of a villus and crypt region, certain topographical features can support intestinal organoid formation. Several studies demonstrated that epithelial cells assemble into a villi-like morphology geometry on pillar arrays made of collagen or collagen-polyethylene glycol diacrylate (collagen-PEGDA), which act as cell adhesion substrate.^{46–48} For instance, Sung *et al.* fabricated a PDMS mold to engineer a collagen and PEG-based substrate with a villi-like topography⁴⁶ (Fig. 3A). Human colon carcinoma cells (Caco-2) cultured on both the control collagen-only substrate, as well as the collagen-PEGDA substrate, formed a villi epithelium. Caco-2 cells were chosen for their widespread use in *in vitro* drug absorption studies. Wang *et al.* also reported that a collagen substrate with micro-sized pillars supports intestinal organoid formation compared to control conditions of culturing crypts in wells without Matrigel or in Matrigel overlaying the microwells.⁴⁹ By having both ridges and grooves on the substrate, the engineered organoids showed both crypts and villi.

A concave microwell also produced a 3D colonic epithelium.⁴⁹ A PDMS microwell filled with Matrigel assembled the intestinal organoid. In the well, the crypts isolated from mice successfully formed an epithelial tissue that resembles the proliferative and nonproliferative zones of the intestine *in vivo*. These results suggest that controlled topographical

features of the substrate allow for the formation of an organoid morphologically similar to the intestine *in vivo*.

Intestinal villi and crypts are exposed to fluid flow, which influences the rate at which absorption of drugs or nutrients enter the body. Many animal models cannot accurately predict the uptake of molecules in the human gut because of differences in metabolism and transport phenomena. Thus, there is a need for an *in vitro* human intestinal model. For example, microfluidic systems are increasingly used to reproduce fluid flow of the natural interstitial fluid.^{50–52} Kasendra *et al.* used a PDMS microfluidic chip with two vacuum chambers on either side of a stacked cell culture channel⁵² (Fig. 3B). The chip was fabricated using soft lithography of the PDMS portion, and the porous membrane that separates the gut epithelium channel from a culture media channel was fabricated using a silicon wafer with a micropillar array.⁵³ Interestingly, duodenal organoids resulting within this microfluidic chip resembled the human duodenum more closely than static 2D studies of Caco-2 cells on Transwell plates. The Transwell plates were coated with the same mixture of collagen and Matrigel as for the chip device.

Focus on biomaterial chemical composition

The chemical composition of a substrate is another factor to support intestinal organoid formation. Conventional methods of engineering intestinal organoids focused on using Matrigel.^{44–47} Recently, various biological materials such as alginate, collagen, and PEG are being used to grow intestinal organoids *in vitro* because of their cell compatibility and workability in forming complex substrate structures. In particular, type I collagen gel, the most abundant matrix protein in the intestine, supports long-term maintenance of the intestinal organoids.^{54–56} In addition, embedding the proliferating intestinal organoids in a type I collagen gel allowed the cells to form hollow tube structures.⁵⁷ Interestingly, while the organoids formed in the Matrigel only case formed single budding cysts, the intestinal stem cells embedded in the collagen gel formed a continuous tube, similar to the natural intestine (Fig. 3C).

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The intestine is a dynamic organ that undergoes acute pulsatile motion. Future studies may utilize oscillatory tensile force bioreactors, such as those used in lung organoid culture,

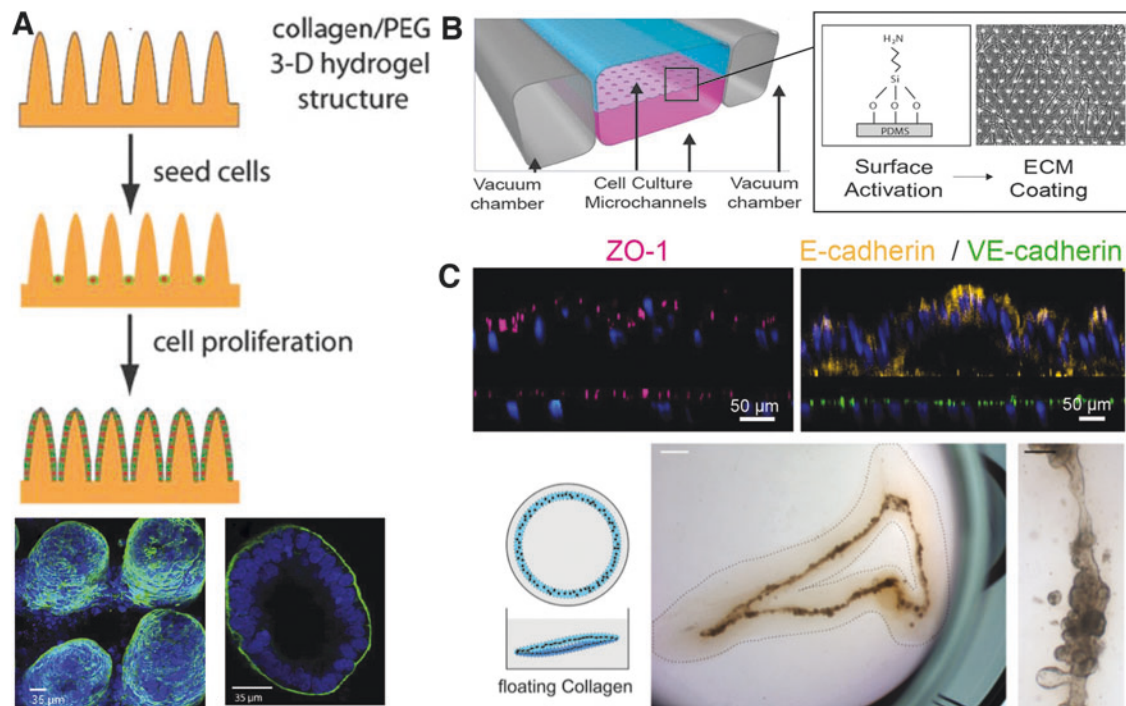


FIG. 3. Intestinal organoid models. (A) Schematic image of 3D hydrogel for intestinal organoid with villi-like topography and confocal images of Caco-2 cells on 3D hydrogel. Actin filaments are stained in green, and nuclei are stained in blue. Reproduced with permission.⁴⁶ Copyright 2011, Royal Society of Chemistry (B) The scheme of the intestine-on-a-chip platform cross-sectioned and confocal immunofluorescence images showing vertical cross-sections of the intestine chip. Tight junction protein (ZO-1) is stained in pink, E-cadherin is stained in yellow, VE-cadherin is stained in green, and cell nuclei are stained in blue. Reproduced with permission.⁵² Copyright 2018, Springer Nature. (C) Intestinal organoids form in floating collagen hydrogel rings. The scale bar represents 1 mm in the middle brightfield image and 100 μm on the right brightfield image. Reproduced with permission.⁵⁷ Copyright 2017, Company of Biologists. 3D, three-dimensional. Color images are available online.

to elicit more *in vivo*-like behavior. Alternatively, these dynamic conditions could be reproduced by controlling fluid flow in a microfluidic chip used to engineer the intestinal organoid.

Manipulation of Extracellular Microenvironments for Liver Organoid Assembly

Primary in filtration and aiding in digestion, the liver is one of the organs which provides nutrition to the body by aiding in the breakdown of food. The liver also protects the body by filtering and destroying harmful materials. The capacity and efficiency for liver function stem from the organization of tissue at the cellular level. Efforts have been made to engineer liver organoids with physiological functionality by understanding the mechanical properties and permeability of normal and diseased livers. For instance, elastic moduli vary from <6 kPa in a healthy liver, to 8–12.5 kPa in early fibrotic liver disease, and >20 kPa in high liver cirrhosis.⁵⁸ In addition, liver disease elevates the blood pressure gradient to higher than 10 mm Hg, leading to a rapid decrease in the production of proteins and filtration, eventually resulting in death from internal bleeding, liver failure, or hepatocarcinoma.⁵⁸

Mixed biomaterial matrices to tune mechanical and chemical properties

Since the liver organoids were grown from primary or stem cells cultured in Matrigel, there has been a push toward growing these miniature organs in various environments

mimicking normal and diseased liver tissue using synthetic and natural ECMs with controlled matrix stiffness and bioactive molecules.^{14,59} For instance, the HepG2 liver cancer cells grown in collagen gels tailored to have an elastic modulus of 4–7 kPa using PEG disuccinimidyl ester cross-linker suppressed malignancy. In particular, cells retained hepatocyte-like detoxification activity and formed the lobule-like lumen in the gel (Fig. 4A). In contrast, the same cells cultured in a softer collagen gel with an elastic modulus of 0.7 kPa showed minimal detoxification activity.

In addition, Klaas *et al.* demonstrated that cells in a diseased or tumorigenic liver could restore activities to form a liver organoid in matrices with proper chemical composition.⁶⁰ In this study, cells isolated from mild and severe fibrotic livers were cultured on dishes coated with fibronectin, type I collagen, or type IV collagen. Cells cultured on the type IV collagen exhibited enhanced proliferation compared to cells cultured on the fibronectin or type I collagen, where a large fraction of cells lost hepatic phenotypes. In addition, decellularized matrix produced by bone marrow-derived mesenchymal stem cells could serve to make HepG2 liver cancer cells form hepatic clusters retaining detoxification activities (Fig. 4B).⁶¹

Using hepatic stromal cells

Separately, efforts have been made to recapitulate complex liver tissue in which hepatocytes and supporting

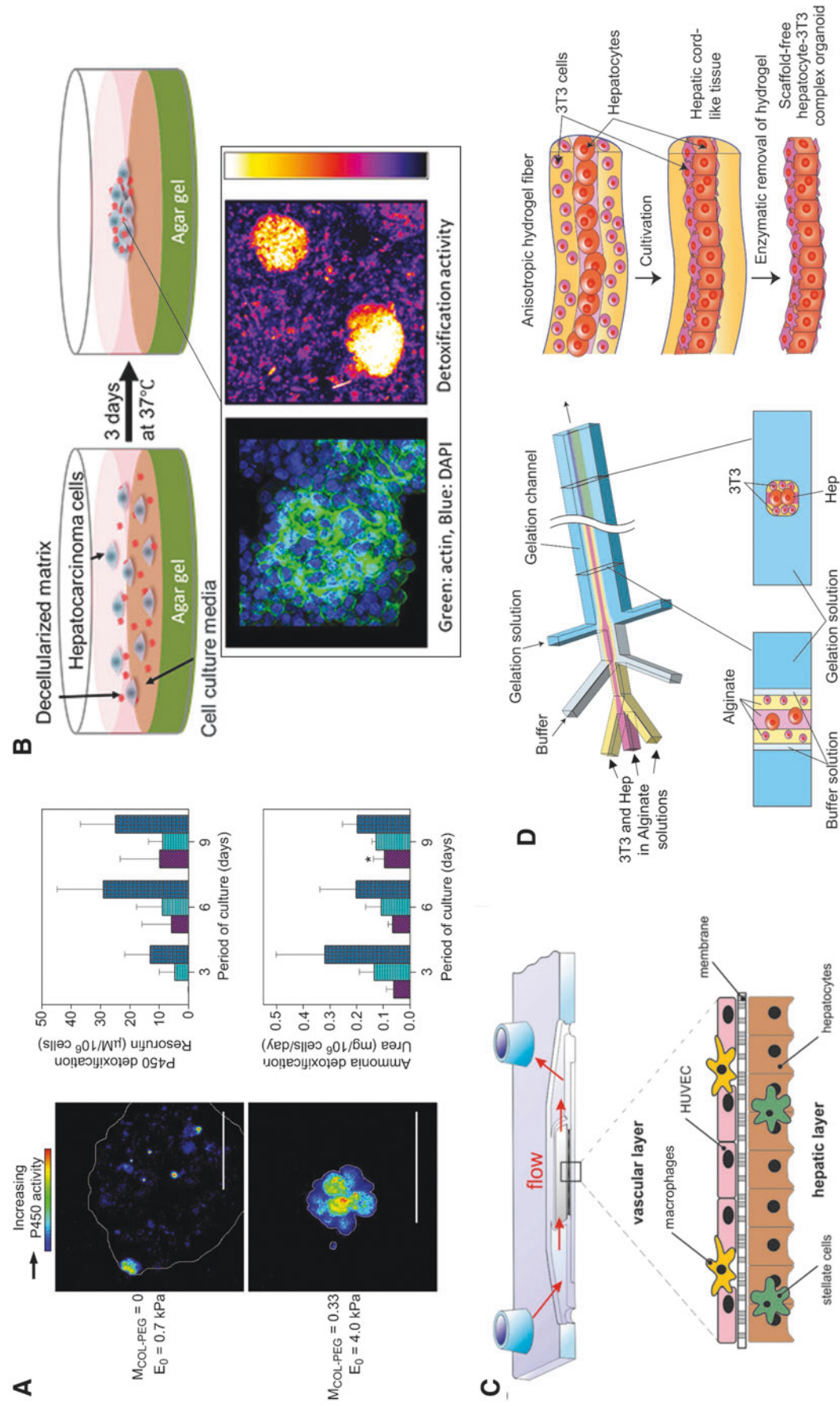


FIG. 4. Liver organoid models. (A) The effect of hydrogel modulus on detoxification activities of 3D hepatocarcinoma cell spheroids, analyzed with cytochrome P450 activity. Reproduced with permission.⁵⁹ Copyright 2011, Elsevier. (B) The schematic image of formation process of a hepatic cluster with decellularized matrix and immunohistochemical image and detoxification assay of hepatic cluster. Actin filaments are stained in *green*, and cell nuclei are stained in *blue*. Reproduced with permission.⁶¹ Copyright 2018 American Chemical Society. (C) The cross-sectional scheme of the biochip liver model. Reproduced with permission.⁶³ Copyright 2015, Elsevier. (D) Schematic illustrations of the microfluidic system for hydrogel microfiber incorporated hepatocytes and 3T3 cells and formation process of hepatocyte-3T3 complex micro-organoids in the microfiber. Reproduced with permission.⁶⁴ Copyright 2012, Elsevier. Color images are available online.

endothelial cells such as fibroblasts, stellate cells, and Kupffer cells are organized to form a liver sinusoid-like structure using a microfluidic system.⁶² For example, Rennett *et al.* seeded liver cells and endothelial cells on opposite sides of the cyclic olefin copolymers-TOPAS[®] biochip.⁶³ Then, this microfluidic system reproduced the layering of the cells around a liver sinusoid structure (Fig. 4C). Because nonparenchymal cells support the function of the liver, cells were introduced into the media flowing through the chip. By doing so, cells formed the supportive stroma. Overall, the microfluidic system increased retention of *CYP3A4* expression, commonly lost through prolonged cultures of liver cells, and exhibited a significantly higher synthesis of albumin and urea than static hepatocyte cultures. To create large liver organoids, liver organoids were also vascularized by hollow endothelial lumen. For instance, certain approaches used biodegradable scaffolds made from alginate and cocultured with vascular endothelial cells and fibroblasts (Fig. 4D).^{64–66}

The future of liver organoids points toward creating larger tissues that could be implantable into humans to replace excised tissues from cancer or disease. While the introduced studies were mostly successful in forming vasculature in the organoid, there are still limitations in recapitulating the full function of the natural liver. These limitations can be solved by culturing vascularized liver organoids in microenvironments that better model the liver. For example, Sasaki *et al.* used a layer-by-layer approach coupled with ECM molecules.⁶⁷ They coated the human liver cells and dermal fibroblasts with fibronectin and gelatin and then added human umbilical vein endothelial cells to create a densely vascularized tissue. The tissues were then implanted into the subcutaneous space of immunocompromised mice. After 21 days, the newly formed vasculature from the liver organoid was engrafted to the host vasculature, making the organoid active to produce human serum albumin.

Manipulation of Extracellular Microenvironments for Retinal Organoid Assembly

The eye is one of the most complex and compact organs. It originates from the optic vesicle connected to a bilayer optic cup with a neural retinal cell layer enclosed by a retinal pigment epithelium. The front of the eye consists of dynamic, light-focusing structures, including the lens enclosed by the ciliary body and iris with the cornea surrounding the entire structure.⁶⁸ While solutions such as glasses and laser eye surgery can correct improper focusing of light onto the retinal cells in the back of the eye, few solutions exist for repairing function of the optic cup itself where retinal cells capture light and transmit signals through the optic nerve.⁶⁹ Therefore, as a first step toward total eye reformation, researchers have developed culture methods to form optic cup organoids *in vitro*.

Mimicking retinal developmental stages

Interestingly, retinal cells possess a substantial ability to self-aggregate and organize *in vitro*, leading researchers to mimic developmental processes.^{70,71} For example, Sasai and coworkers have developed culture methods with embryonic stem cells that guide the stem cells' differentiation into distinct neural retina and retinal pigment epithelium layers

without the need for external force.^{10,72} These processes use low adhesion 96-well plates for the initial cell differentiation and aggregation. The cultures were then embedded in 2% v/v Matrigel and grown in serum-free growth factor-reduced media charged with 40% O₂/5% carbon dioxide. Although both mouse and human cells form organoids with this technique, the resulting organoids present differences in size, relative amounts of rods and cones, and speed of growth.⁷³

Moreover, during organogenesis, the initially spherical cell cluster undergoes extensive reorganization, eventually forming a spatially organized multilayered cup without any external forces. To underlie the mechanism, the Sasai and Eiraku laboratories combined an *in vitro* and an *in silico* approach to model the 3D mechanical forces that deform the overall structure at a single cell layer.⁷⁴ They found that the deformation is driven primarily by cellular proliferation at precise locations, causing a mechanical instability and buckling of the hollow cell sphere. Understanding how stem cells mechanically form organoids was further explored by Lowe *et al.* from the perspective of the cytoskeleton and cellular adhesions.⁷⁵ They cultured human embryonic stem cells suspended within a Matrigel construct. The construct was then broken apart to form floating fragments. Around 5 days, the cells formed cysts and gradually expressed retinal progenitor eye-field markers such as *SIX3*, *RAX*, and *PAX6*. Upon further growth of the cysts on tissue culture multiwell plates, the intrinsic programming for the stem cells served to form retinal organoids by modulating actomyosin forces through Rho kinase activation.^{76,77}

Völknner *et al.* have established a protocol for retinal organoid formation that exhibits high organoid yields and cell diversity. This method does not rely on the evagination of optic vesicle structures. Instead, the researcher manually cut the developing organoid into three equal pieces.⁷⁸ By trisectioning the organoids in an unbiased manner, each will potentially contain eye field-determined neuroepithelium, thus allowing for the growth of three organoids from each starting aggregate. According to analysis of *PAX6* reporter expression, which is a major regulator of neurogenesis, the organoids recaptured a similar expression level as native mouse retina.

Alternative Matrigel extracellular microenvironment to facilitate nutrient transport

While Matrigel is the most widely used material to culture retinal organoids, researchers also have cultured organoids through a combination of poly(2-hydroxyethyl methacrylate) coated plates followed by culture in a rotating culture chamber at progressively faster rotation speeds.⁷⁹ The rotation speed is optimized such that the organoids are kept in a stationary suspension, also known as simulated microgravity. In this rotating vessel environment, the organoids had improved proliferation and differentiation into ganglion and S-cone photoreceptors compared to organoids cultured in static vessels. Other researchers have also found that culturing cells in similar stirred or shaken bioreactors improves the yield of photoreceptor cells.^{80,81} The better cell survival is due to an enhanced nutrient, O₂ transport, and waste removal in dynamics cultures, allowing for larger cell organoids to form by combating the usually poor transport within cell clusters.

Despite its relative complexity of design and function, retinal organoids exhibit a substantial ability to reorganize

TABLE 1. THE FUNCTIONALITY OF ORGANOID DEPENDING ON EXTRACELLULAR ENVIRONMENT

<i>Organoid</i>	<i>Type of cells</i>	<i>Extracellular matrix</i>	<i>External device</i>	<i>Functionality</i>	<i>Reference</i>
Brain	Human induced pluripotent stem cells	Hyaluronan/chitosan	3D culture within hydrogel	Distinct regions of forebrain, midbrain, and hindbrain	17
	Human embryonic stem cells	Collagen	3D culture within hydrogel	Formation of 3D neural constructs with interconnected vascular network	18
	Astrocyte and Human umbilical vein endothelial cells	Fibrin	Microfluidics	Increasing of BBB permeability	22
Lung	Rat pulmonary epithelial cells	Fibronectin/collagen	Microfluidics	Increasing of lung surfactant production, viability, and biofunctional markers	33
	Human fetal lung fibroblasts	Alginate/collagen	3D microbeads	Mimicking similar structure with bronchopulmonary dysplasia	36
	Mouse fetal pulmonary cells	Collagen	3D culture within hydrogel	Increasing of differentiation and organoid formation	37
Intestine	Human colon carcinoma cells	Collagen	Microstructure	Morphologically similar structure with <i>in vivo</i>	45
	Mouse crypt	Matrigel	Microstructure	Morphologically similar structure with <i>in vivo</i>	48
	Human primary crypt	Collagen/Matrigel	Microfluidics	Resembled the human duodenum	55
Liver	Human hepatocellular carcinoma cells	Collagen	3D culture within hydrogel	Retaining hepatocyte-like detoxification activity and lobule-like lumen formation	58
	Human hepatocellular carcinoma cells	Decellularized matrix	3D culture on nonadherent substrate	Retaining detoxification activities	60
	Mouse embryonic fibroblasts/Rat hepatocytes	Alginate	Microfluidics	Increasing albumin secretion, urea synthesis, and hepatocyte-specific gene	63
Retinal	Human embryonic stem cells	Matrigel	3D culture within hydrogel	Increasing retinal progenitor eye-field markers	74
	Mouse embryonic stem cells	Matrigel	Rotating culture chamber	Improving proliferation and differentiation into ganglion and S-cone photoreceptors	78
Kidney	Human embryonic stem cells	Matrigel	3D culture within hydrogel	Formation of 3D tubular structure similar to <i>in vivo</i>	87
	Human proximal tubule epithelial cells	Gelatin/fibrin	3D bioprinting	Enhancement of albumin uptake level and natural proximal tubule morphology	90

3D, three-dimensional; BBB, blood–brain barrier.

and form eye-like structures semiautonomously. Most optic cups and retinal organoids can be formed on Matrigel and rotating bioreactors. The organoids present cell signaling pathways crucial for cell reorganization. However, while organoids can be formed to mimic some of the components of the eye, researchers are yet to assemble a single organoid using the diverse cell types found at all layers of the eye. Future work in this field will require a detailed knowledge of the biological mechanisms behind the cellular organization, as well as proper mechanical supports and localized differentiation of different cell populations. Biomaterials would be well suited to this end by providing supports of physiological relevance while allowing transport of cell signaling molecules to induce organoid formation.

Manipulation of Extracellular Microenvironments for Kidney Organoid Assembly

In the body, the kidney filters wastes, controls blood pressure, regulates fluid, and balances the pH. Nephrons, the kidney's functional units, are responsible for serving these roles.⁸² The kidney undergoes complex developmental stages that require reciprocal interaction between endoderm and mesoderm.⁸³ Therefore, researchers have focused on both recapitulating the environment necessary for the endoderm and mesoderm to interact and induce the formation of functional nephrons. In this regard, kidney organoids hold potential applications in modeling renal diseases and drug screenings, as well as testing the toxicity of chemicals.⁸⁴

Similar to other organoid engineering strategies, methods to supply biological molecules such as basic FGF, retinoic acid, and BMP are commonly used to control the differentiation lineage of renal organoids from human embryonic stem cells to ureteric bud progenitor cells.^{15,85,86} By providing growth factors at different time points, the researchers were able to develop kidney organoids similar to the developmental process. The predominantly used material to support kidney organoid growth is Matrigel.^{87–90} The Matrigel served to increase the number of adhered cells; however, Matrigel forms variable organoids. Because of its tumorigenic origins, the relative concentrations of dissolved growth factors and extracellular proteins result in high batch-to-batch variability. Thus, efforts are required to identify or synthesize an ECM proper for kidney organoid formation.

A new 3D bioprinting method that directly prints proximal tubule cells into a gelatin-fibrinogen matrix was used to generate a kidney organoid.⁹¹ Previous studies focused on recapitulating the renal proximal tubules which are responsible for reabsorption of fluid that is filtered through glomerulus. A fugitive ink, Pluronic® F127, was printed in a tubule shape inside of the gelatin–fibrin gel. The gelatin and fibrin mixture allowed for a controllable gelation time. This structure was connected to a peristaltic pump and covered again with gelatin–fibrin mixture and the fugitive ink. Finally, the two fugitive ink layers were removed by cooling the entire gel to the lower critical solution temperature for the Pluronic which produced an open channel. The epithelial cells grown in these proximal tubules showed enhanced albumin uptake levels and exhibited morphologies similar to the natural proximal tubules. Therefore, this study showed an effective way to mimic the complex intracellular architecture using 3D printed biomaterials.

Conclusion

As organoid development becomes more prevalent in research, differing approaches have developed to improve controllability of the dimensionality, differentiation, and assembly of cell clusters (Table 1). However, the goal of highly functional and morphologically similar counterparts to real-life organs is challenging for conventional cell culture techniques. Researchers have invested tremendous efforts in synthesizing microenvironments that can regulate organoid formation and physiological functions using ECM-mimicking biomaterials mixed or tethered with bioactive molecules. To reproduce the microstructure of tissues, continuous gel matrices, fibrous networks, 3D printed structures, or microfluidic chips are used to advance the quality of organoids. Alternatively, decellularized matrices attained from native tissue or stem cell spheroids are other options used for organoid engineering.

However, the results are diversified depending on the cells' emergent behavior mitigated by cell–cell interactions, neovascularization, and other extrinsic factors such as external physical forces. In addition, the extracellular environment is another factor that influences a development and homeostasis of organoids because it changes with stages of development and progression of disease. By reproducing transitory changes of extracellular microenvironment using stimulus-responsive biomaterials, it may be possible to gain insight into how mechanical and chemical

cues affect the growth and functionality of organoid cultures over time. In addition, ECMs must be defined better to recapitulate the structure and function of target organs. Furthermore, a high-level microscopic and spectroscopic analysis could also be used to improve the quality and controllability of organoids. These methods would provide an in-depth understanding of the extracellular microenvironment at the molecular and cellular levels.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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