Cellular Self-assembly and Biomaterials-based Organoid Models of Development and Diseases

Shivem B. Shah¹ and Ankur Singh^{1,2*}

¹Meinig School of Biomedical Engineering, Cornell University, Ithaca, NY 14853, USA

²Sibley School of Mechanical and Aerospace Engineering, Cornell University, Ithaca, NY 14853, USA

*Prof. A Singh

Sibley School of Mechanical and Aerospace Engineering,

College of Engineering,

Cornell University,

Ithaca, NY 14853, USA

Email: as2833@cornell.edu

ABSTRACT

Organogenesis and morphogenesis have informed our understanding of physiology, pathophysiology, and avenues to create new curative and regenerative therapies. Thus far, this understanding has been hindered by the lack of a physiologically relevant yet accessible model that affords biological control. Recently, three-dimensional *ex vivo* cellular cultures created through cellular self-assembly under natural extracellular matrix cues or through biomaterial-based directed assembly have been shown to physically resemble and recapture some functionality of target organs. These “organoids” have garnered momentum for their applications in modeling human development and disease, drug screening, and future therapy design or even organ replacement. This review first discusses the self-organizing organoids as materials with emergent properties and their advantages and limitations. We subsequently describe biomaterials-based strategies used to afford more control of the organoid’s microenvironment and ensuing cellular composition and organization. In this review, we also offer our perspective on how multifunctional biomaterials with precise spatial and temporal control could ultimately bridge the gap between *in vitro* organoid platforms and their *in vivo* counterparts.

1. Introduction

Organogenesis and morphogenesis involve the formation of complex tissue and organs from single cells. These intricate processes include orchestrated yet complex and dynamic chemical and physical signals that influence the self-assembly of non-prepatterned cells into the target tissue or organ. Biologists and bioengineers have studied both processes to better understand the mechanisms of development, organ physiology, and pathophysiology to inform curative and regenerative therapies [1-3]. In the past, models of organogenesis have included regular and genetically engineered *in vivo* systems, but these models suffer from limited accessibility, complexity, and often restricted multidimensional and multiscale biological control. Also, several conditional knockouts are embryonically lethal, further imposing limitations on understanding cell development [4, 5]. On the other hand, conventional two-dimensional (2-D) *in vitro* models are often an oversimplification and are unrepresentative of the three-dimensional (3-D), multi-cellular natural milieu of typical target organs. Recently, organoids have shown potential for bridging the high-throughput, cost-effective nature of *in vitro* systems with the physiological relevance of *in vivo* models, as indicated in [Figure 1A](#) and described in [Table 1](#) [6-16].

Organoids, as defined here, are *ex vivo* three-dimensional (3-D) cellular structures that either self-organize or are directed to assemble under specific organogenesis cues; these structures must also physically resemble, either fully or partially, the architecture, cellular organization, and composition of an *in vivo* organ while recapturing partial or complete functions of the organ. Organoids can be engineered based on secreted soluble signals, non-modular extracellular matrix signals, and modular extracellular matrix, and a comparison across throughputness, tenability, stochasticity, patterning, and scalability is presented in [Figure 1B](#). A major focus of organoids research has been with pluripotent stem cells (PSCs), which, when grown in a 3-D microenvironment, self-organize and acquire physiologically relevant cellular patterning to develop into several endoderm- and ectoderm-derived tissues, often mimicking their *in vivo* counterparts. Several elegant reviews have highlighted PSC-derived organoids from a development and cellular assembly perspective [12, 14, 17, 18], including 3D aggregates, or embryoid bodies [17]. The focus of this review is to highlight the materials-based approaches that mammalian cells, including PSCs and others, adopt for self-assembly and controlled development of complex tissues, such as brain, gut, and immune system. In this review, we first discuss the organoids that result from self-assembly of cells under secreted and/or exogenous growth factors or natural extracellular matrix (ECM) instructed cues (e.g. [Matrigel](#)), and review the emergence of these organoids as materials with novel and important properties. We next emphasize the role of semi-synthetic and synthetic biomaterials in controlling assembly of organoids. We extensively discuss both approaches for three organoid models – intestine, brain, and immune – in the context of modeling development and disease. [These models are](#)

emphasized because of their complexity and upcoming efforts in biomaterials organoids. We finally provide perspective on how new biomaterials with spatial and temporal control of bio-ligand signaling could guide tissue dynamics, morphogenesis, vascularization, and organogenesis, *ex vivo*.

2. A Materials Approach to Self-assembly of Cells

The flow of information between cells and their neighboring microenvironment is often considered bidirectional leading to a diverse set of biological phenomenon, one of which is the assembly of tissue structures and organs. Pioneering work of Mina Bissell and colleagues has established an understanding of how the mechanical and chemical composition of the extracellular matrix regulates cell behavior and function in malignant cells [19-22]. Likewise, organoid models have used animal derived-ECM networks, such as Matrigel [23], collagen [24], and other basement membrane matrices (e.g. GelTrex [25]), to influence cellular self-assembly and tissue formation. Within Matrigel platforms, myriad organoids have been developed and elucidated key functional and pathological mechanisms [26-28]. Although Matrigel ultimately enhances the self-assembling capacity of PSCs, likely because of the complex distribution of nutrients and protein gradients, this strength can also serve as the platform's major weakness due to the ensuing complexity [23, 29], compositional variability [30-32], and lack of control of individual parameters, such as ECM ligands (**Figure 1**). Within these platforms, cells often assemble into heterogeneous organoids in terms of viability, size and shape [33], and suffer from relatively random spatial positioning of tissue regions. Importantly, the cocktail of growth factors and signaling cascades in Matrigel work simultaneously, which may inhibit complementary pathways, making it difficult to tune and control the signal transduction in cells undergoing organogenesis. Finally, animal-derived hydrogel materials present a major roadblock in clinical application of organoids because of the possibility of pathogen transfer and immunogenicity. Yet, Matrigel remains the most popular material of choice for organogenesis.

An ability to sculpt the biophysical and biochemical microenvironment with control over signaling milieu may help increase the faithfulness of stem cell- and progenitor-derived organoids to real organs, and open up the possibility to guide morphogenesis and organogenesis in culture. To meet this need, the uses of biomaterial-based synthetic or recombinant ECM analogs have recently gained momentum to provide a more precise and better control of the organoid's microenvironment [34-37]. As summarized in Table 1, organoids have already captured several key organ functions and elucidated differences in developmental and dysfunctional mechanisms as well as interactions with therapies in comparison to conventional 2D cell culture models. Of these, organoids using both 3D cellular self-assembly and biomaterial-controlled microenvironment approaches have been established for the intestine, secondary lymphoid organ, and brain and therefore we discuss them in greater detail than other organoid models. A

summary of different types of biomaterials used to engineer these three organ systems and their effect on organoids is provided in **Table 2**. In the following sections, we describe the components and function of each these three organs, followed by the cellular self-assembly and biomaterial based organoid methods used to study development and dysfunction. This development of ex vivo organoid platforms opens up many exciting translational opportunities in basic science, drug discovery, deep sequencing efforts for personalized medicine, and will likely be critical for the emerging cell and tissue bio-manufacturing sector (**Figure 1C**). The organoids can facilitate de novo growth of isotype-matched human cell types in a tissue- and patient-specific manner and enhance treatment of diseases through rational discovery, testing, and translation of new class of therapeutics (**Figure 1C**).

2.1 Gut Organoids: The Intestines

Primarily known for its role in digestion, nutrient absorption, and waste removal, the intestine regulates metabolism and feeding behavior via endocrine hormones. It also serves as a protective barrier by restricting the extensive population of microbes to the gut lumen [38, 39]. These physiological functions are supported by the intestines' complex structure and architecture. Structurally, the intestinal tract averages about 8 meters and can be divided into the small and large intestines [40]. Along the proximal-to-distal axis, the small intestine is subdivided into the duodenum, jejunum, and ileum, followed by the large intestine's sections – the cecum and colon [41]. Each section of the intestines contains an inner lumen surrounded by a multilayered intestinal wall, including tissue from all three germ layers. The mesoderm-derived vasculature, lymphatic, and immune systems alongside the ectoderm-derived nervous system are integrated within the layers and are necessary for complex intestinal functions, including regulation of food transported by peristaltic contractions. However, specialized structures within the mesoderm-derived epithelial layer encircling the inner lumen along with this layer's overall structure account for a large portion of absorption, barrier protection, and endocrine signaling [42]. In the small intestine, epithelial absorptive enterocytes and the secretory goblet cells, Paneth cells, and enteroendocrine cells carry out epithelial function (**Figure 2A**). Goblet and Paneth cells provide barrier protection by respectively secreting mucin and antimicrobial factors, and enteroendocrine cells regulate metabolism through production and secretion of gastrointestinal hormones. Similarly, the colon contains absorptive colonocytes and secretory goblet cells. These epithelial cells are arranged into folds to increase the epithelium's surface area and absorptive capacity. The epithelium is also highly regenerative, renewing every 4-5 days, driven by the recently discovered Lgr5⁺ crypt-base columnar intestinal stem cells (ISCs) residing at the base of the epithelial folds in the crypts of Lieberkühn [38, 43], as seen in **Figure 2A**. Upon discovery of the ISCs, organoid research has focused on recreating functional intestinal epithelium for its widespread application in understanding intestinal development and dysfunction as well

as drug absorption and toxicity. While genetic mouse models are immensely useful, engineering suitable *ex vivo* culture platforms that recapitulate gut provide a unique opportunity to easily tune various development factors for the study of gastric epithelial homeostasis and associated cancer.

Table 1: Summary of emulated target organ physiology and pathophysiology, with cell source indicated in parenthesis

Organ	Function	Dysfunction
Brain	Functional hippocampal neurons [44], cerebellum [45], and forebrain [46, 47] generation (hESCs) Cerebral cortex, choroid plexus, retina, and meninge formation (hIPSCs)[10]	Microcephaly (hIPSCs) [10] Interactions with Zika virus (hIPSCs & hESCs) [13, 48] Idiopathic Autism spectrum disorder (hIPSCs) [46]
Esophagus	Self-renewal and stress response (mouse esophageal epithelial cells) [49]	Barrett's esophagus (human Barrett's esophageal cells) [50]
Eye	Optic-vesicle & Optic-cup morphogenesis (mESCs & hESCs) [51] [52] Retinogenesis (mESCs & hESCs) [53, 54] Retinal Repair and engraftment (mESCs) [55]	Loss of Photoreceptor segments: blindness, retinitis pigmentosa, etc (mESCs) [56]
Inner ear	Otic Placode Induction (mESCs) Functional inner-ear hair cells (mESCs) [57, 58]	
Intestines	Continuous budding (mouse intestinal crypts, Lgr5+ mouse stem cells) [26, 50] Absorptive and secretory function (hIPSCs) [28] Prenatal Development (fetal mouse intestine cells) [59, 60] Nutrient absorption (mouse intestinal crypts) [61] Interaction with immune cells (mouse intestinal crypts) [62] Engraftment (Lgr5+ stem cells, fetal mouse intestine cells, and hIPSCs)[59, 63, 64]	Interactions with bacterial pathogens (mouse intestinal crypts, hIPSCs) [65-67] Intestinal fibrosis (hIPSCs)[68] APC-deficient adenomas (human sample) [50] Colorectal cancer (human Lgr5+ stem cells, human colorectal cancer cells) [69-71] Cystic fibrosis (mouse and human intestinal crypts) [15]
Kidney	Nephrotoxicity screening (mouse kidney cells) [72] Nephrogenesis, nutrient endocytosis, response to toxins (hIPSCs) [7, 72]	
Lingual	Regeneration & engraftment (lingual epithelial cells) [73]	Lingual carcinoma (lingual epithelial cells) [73]
Liver	Functional hepatocyte expansion (Lgr5+ mouse stem cells) [74, 75] Implantation and subsequent vascularization (hIPSCs) [76, 77] Duct formation with functional cholangiocytes (hIPSCs) [78, 79]	α 1-antitrypsin deficiency (human biopsy) [75] Alagille syndrome patient (human biopsy)[75] Polycystic liver disease [79] Cystic fibrosis-associated cholangiopathy [79]
Lung	Proximal and alveolar airway-like structure generation (hESCs & hIPSCs) [80] Engraftment (mouse fetal pulmonary cells)	

	[81]	
Pancreas	Expansion and formation of endocrine cells[82, 83]	Pancreatic cancer (murine and human pancreatic tissue) [84]
Pituitary Gland	Functional adenohypophysis and engraftment (mESCs) [85]	
Prostate	Reconstituted prostate glands (murine and prostate epithelial cells) [27]	Prostate cancer (human prostate cancer biopsy) [86]
Salivary Glands	Self-renewal and transplantation (mouse salivary gland cells) [87]	Hyposalivation (mouse salivary gland cells) [87]
Lymph Node	Germinal center formation (B cells) [88]	Non-Hodgkin B and T-cell lymphoma, including Follicular Lymphoma and DLBCL (explant[89] [37]
Stomach	Regeneration (Lgr5+, mESCs) Functional parietal cells (mESCs) Pepsinogen and gastric acid secretion (mESCs) [90-93]	Menetrier disease [93] H. pylori infection (mouse and human gastric epithelial cells, hESCs) [9, 94-97] Diffuse gastric cancer (mouse glandular stomach epithelial cells) [98]
Taste Buds	Functional taste receptor cell generation (Lgr5+ cells) [99, 100]	
Thymus	Implantation and in vivo thymopoiesis, humoral response, and tolerance (thymic tissue) [101]	

Table 2: Summary of different types of biomaterials and their effect on organoids

Organ	Design Rationale	Known 3D Biomaterial Solution	Effect of Biomaterial Type
Intestine	Controlled biochemical and mechanical cues	Recombinant engineered extracellular matrix (eECM) [35]	Displays intestinal contraction
	Controlled spatial organization	PEG hydrogels with a mechanically stable backbone (sPEG) and hydrolytically degradable polymer (dPEG) [34]	Elucidates the role of the mechanical regulation of ISCs using designer matrices that offer modulation of stiffness, bio-adhesivity, and bio-degradability of the niches
Brain	Controlled tissue organization and chemical and mechanical regulation	Hyaluron and Chitosan [102] PEG-hydrogels [103]	Enables key cellular interactions between the different cells present in neural development through spatial organization
Immune System	Rapid induction of phenotype	Gelatin-Silicate Nanoparticle Hydrogel [88] [104] PEG-MAL Hydrogels [37] [36]	Enables rapid generation of germinal center-like B cells in a user controlled tunable manner and provides adhesive survival signals absent in 2D cultures.
	Enhanced survival Controlled biochemical and mechanical cues Patient specific signaling		Enables microenvironment specific and patient specific functionalization of key signaling ligands (e.g. integrin binding ligands) to study the

			effect of microenvironment type and density
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2.1.1 Cellular Spheroids & Organoids

Healthy Intestinal Organoids

Building upon the identification of the Lgr5⁺ ISCs in intestinal crypts, Hans Clevers and colleagues created intestinal epithelium organoids based on ISC self-assembly [26]. Prior to isolating and culturing mouse ISCs, they first established long-term in vitro culture conditions of mouse crypts that preserved basic crypt physiology by focusing on the known intestinal growth requirements and key endogenous stem cell niche components - Wnt agonist R-spondin 1, epidermal growth factor (EGF), and the BMP inhibitor Noggin (**Figure 2B**). Also, an important structural and pro-survival component present in the niche was laminin-rich Matrigel, which mimicked the composition of native crypt bases. The cellular self-assembly process resulted in whole crypt organoids that resembled the crypt-villus architecture of intestinal epithelium, and major differentiated cell types were present. Organoids were able to undergo continuous budding events, similar to crypt fission, and remained functional in culture for over 8 months [26]. The resulting ISC structures resembled full crypt organoids and native intestinal epithelium with terminally differentiated cells present in the [physiologically relevant](#) locations; ISC hierarchy was additionally maintained for long-term culture⁷. Based on their mouse small intestinal cultural system, the Clevers group engineered similar mouse colon, human small intestine, and human colon whole organoid systems. For mice colon organoids, culture conditions included recombinant human Wnt3a, along with nicotinamide, Alk inhibitor and p38 inhibitor for human small intestine and colon organoids [50].

The engineered intestinal platform provided a simplified but elegant organoid system to study the development and biology of the crypt-villus epithelium. Still, mesenchymal and non-epithelial cells were not present that may further aid in cell-cell communication and secreting building blocks necessary for organogenesis. Other research groups have focused on creating intestinal organoids utilizing pluripotent and more readily available cell sources, such as human embryonic and induced pluripotent stem cells [105-111]. Specifically, Spence et al. differentiated human pluripotent stem cells (hPSCs) into intestinal tissue by mimicking the key steps of intestinal development – endoderm formation, posterior patterning to create the midgut and hindgut, and intestinal morphogenesis [28]. Mature organoids reflected native intestinal epithelium architecture and cellular composition with intact absorptive and secretory function [28]. Organoids also contained a mesenchymal layer that developed in a stepwise manner similar to embryonic development. By imitating embryonic development, this platform provides an avenue to study human development. As an initial example, NEUROG3, known to have a role in mice enteroendocrine

development, was confirmed to also affect human development [28]. There is also a particular interest to better understand the differences between fetal and adult intestinal stem cells to find new therapeutic avenues in intestinal cancer and wound repair. To further study these differences, intestinal organoids with ISCs from several stages in prenatal development have been developed [59, 60]. Early prenatal cells assembled into fetal spheroids, which lacked folds and expressed the markers Lgr4 and Cnx43 as opposed to Lgr5. It remains unclear whether or not the cells were differentiating to an extent to secrete factors critical for structural assembly of these 3D tissues. Fetal spheroids did not require exogenous Wnt ligands and had varied responses to Wnt signaling. Further mechanistic studies using these organoids may yield an understanding of the reduced Wnt signaling and its possible relevance to migration of cells, self-assembly of 3D structures, cancer biology, or intestinal repair.

To test the true differentiation potential of the stem cells in question and for translational applications, organoids created from adult mouse ISCs, fetal mouse ISCs, and hiPSCs have been tested for functional *in vivo* engraftment. With adult mouse ISCs, transplantation into sites of acute colitis had varied success and required optimization, as freshly isolated donor cells were much less successful than expanded stem cells [63]. Fetal transplants had similar results, but transplants were already purified for stem cells [59]. They are also easier to obtain in comparison to human intestinal biopsies, but potential ethical issues and immunosuppression make it less attractive cell source than hiPSCs. Intestinal organoids from hiPSCs have also been shown to include a supporting mesenchymal layer, which is needed for successful engraftment in models exposing host mesenchyme following injury. With this approach, Watson et al. [64] created hPSC intestinal organoids that successfully engrafted and responded to systemic *in vivo* signals. After 6 weeks, in comparison to *in vitro* counterparts, transplanted organoids were more mature and differentiated, resembling native architecture and cell composition. Transplants were also supported by mouse vasculature and lymphatic ingrowth. Functionally, *in vivo* organoids were capable of peptide uptake, demonstrating digestive function. Following ileocecal resection in mice with transplants, organoids responded to systemic signals, such as humoral factors, highlighting their use as an *in vivo* intestine model to study broad physiological responses.

Intestinal models have also advanced as *in vitro* platforms to study drug absorption, endocrine signaling, and complex interactions. Recent studies have highlighted nutrient absorption, drug diffusion, and endocrine signaling within the organoid as well as its interactions with the immune system [61, 62]. Nutrient absorption and endocrine signaling are of particular interest in metabolic disorders, such as obesity and type 2 diabetes. The physiological relevance, ease to create knock out models, sensing ability, and capacity for live-cell intracellular imaging makes the organoid platform more broadly applicable to drug bioavailability and sensory science studies.

Disease Models of Intestine

As intestinal epithelial organoids became more established, they were also used to study common intestinal pathological states, including interactions with an enteric pathogen, fibrosis, cancer, and genetic disorders. Simple intestinal organoids models were first used to study the interactions of bacterial pathogens with the epithelium without confounding factors, such as commensal microbiota. *Salmonella*, for its burden on global health as well as its ability to invade the epithelium, was a common test bacterial pathogen. In adult mouse crypt-derived organoids, *Salmonella* led to morphologic changes, disrupted tight junctions, activation of inflammatory pathways through NF- κ B, and decreased expression of stem cell markers Lgr5 and Bmi1 [65]. A microinjection model was also developed to study the impact of Paneth cell α -defensin antimicrobial peptides on *Salmonella* growth [66]. Forbester et al. transitioned the microinjection model to hiPSC organoids for a greater physiological relevance in terms of genetic specificity, human-restricted pathogens, and architecture, and she similarly found that the bacteria invaded the epithelium and led to cytokine expression changes. Bacteria invasion was also decreased by a mutation in the bacteria's SPI-1 invasion system [67]. By transitioning to hiPSCs, this study also opened the possibility to study host genetic and structural variation in relation to bacterial pathogenesis.

Complex intestinal epithelium organoids with mesenchymal cells were used to study pathologies with cell-based effectors as in the case of fibrosis caused by myofibroblasts. Previously described to have a mesenchymal layer, hiPSC intestinal organoids were also shown to have a population of myofibroblasts that had a dose-dependent pro-fibrotic response to the fibrogenic cytokine TGF- β . This fibrotic response was also reduced by spiro lactone, a cardiac anti-fibrosis drug [68]. As no pharmacologic therapies exist for intestinal fibrosis with research delayed by the simplification of 2D models and irrelevance of animal models, this research alludes to an exciting opportunity to create new therapeutics.

The optimization of culture of developing intestinal organoids saw a concurrent effort in creating diseased organoids, including cancer organoids, for their implications in personalized medicine. Clevers and colleagues have established long-term in vitro culture conditions for adenomatous polyposis coli (APC)-deficient adenomas and human colon cancer [50]. APC-deficient adenomas formed simple cystic structures. Since APC loss leads to constitutively active Wnt signaling, R-spondin 1 was not needed in culture. Noggin was dispensable, but it led to the loss of Lgr5 expression. Since Axin2 expression remained, expansion of APC-deficient adenomas continued. Similarly, colon cancer organoids did not require R-spondin or Noggin. In most cases, EGF was also dispensable, but some organoids rapidly decelerated in proliferation. These findings indicate that in the absence of cues, cells organize but do not truly form properly representative structures. Unlike APC-deficient adenomas, colon cancer organoids formed irregular compact structures [50]. The structural variation and various cultural conditions pointed

to the biological differences that could be studied and induced to create cancer organoids. To transform normal epithelium, multiple mutations are needed to drive the epithelium into an invasive and metastatic state. For colorectal cancer (CRC), the adenoma-carcinoma sequence model states that APC gene mutations would induce adenoma, or premalignant tumor of epithelial origin, followed by subsequent mutations in KRAS, SMAD4, and P53 for the induction of metastasis [112]. By modulating cellular proliferation, differentiation, and apoptosis, these mutations offer a growth advantage to the mutated epithelium and drive carcinogenesis. By using the genome-editing tool CRISPR/Cas9 on human ISC organoids to induce these mutations, throughputness of the organoids can be improved as organoids no longer required niche factors, *in vitro*, for growth and formed tumors upon transplantation into mice. Quadruple-mutant colorectal cancer organoids also tolerated the P53-stabilizing drug nutlin-3, displayed chromosomal instability, and aneuploidy, furthering their semblance to colorectal cancer [69, 70]. This study suggested that 'driver' pathway mutations are sufficient to maintain stem cells in the hostile tumor microenvironment, but not the invasive behavior. Still, intra-variability present within colon cancer organoids alluded to the inherent heterogeneity of cancer and the need for more personalized therapy. To meet this need, tumor organoid cultures from 20 colorectal patients were used to create a “living biobank” that was genetically analyzed to ensure representation of most CRC molecular subtypes [71]. Organoids were then applied as high-throughput drug assays and detected unique drug-gene associations, supporting their future use in personalized patient treatment.

Patient-based organoids and genome engineering has also been applied to the study of genetic diseases, such as cystic fibrosis. Mutations in the cystic fibrosis transmembrane conductor receptor, commonly a deletion of phenylalanine at position 508 in exon 11, lead to cystic fibrosis. Adult intestinal stem cells from two cystic fibrosis patients were used to create organoids, which mirrored the pathological phenotype. Upon transfecting the organoids with sgRNAs to target the mutation, corrected organoids were created, which displayed wild-type phenotype. The restored phenotype along with the engraftment potential of organoids demonstrates the possible use of organoids for stem cell and gene therapy [15].

2.1.2 Biomaterials-based Intestinal Organoids

To address the limitations of existing organoid systems, the use of biomaterials has gained momentum to precisely control the organoid's microenvironment in terms of size, architecture, and both mechanical and biochemical cues. We believe that a more modular and specific approach could ultimately bridge the gap between *in vitro* organoids and their *in vivo* counterparts, as well as could lead to development of whole organ systems. As previously described, most cellular spheroid and organoid work have largely focused on the addition of exogenous signaling molecules, as highlighted in the models of

modulating intestinal organoid development, biology, and dysfunction. The characteristics of the microenvironment, particularly biochemical and mechanical cues, have not been explored, and the limitations and variability of Matrigel have been largely overlooked. Imprecise control of the microenvironment has precluded organoid models from exhibiting more complex intestinal mechanical functions, including contraction needed for peristalsis.

Recently, DiMarco et al. engineered mice intestinal epithelium organoids in collagen I and modulated matrix density, culture geometry, and oxygen availability to promote intestinal contraction. Oxygen availability was first modulated based on culture configurations by comparing the conventional submerged culture configuration against air-liquid interface cultures. The air-liquid interface culture includes organoids cultured on a suspended transwell insert. This exposes cultured organoids to open air conditions and only allows nutrient diffusion to occur in a bottom up fashion. This configuration increased the likelihood of contraction to ~10% of all samples. Prior to this finding, previously described cellular spheroids and organoids did not display intestinal contraction. However, this contraction only occurred in a narrow range of collagen densities, based on collagen stiffness, adhesivity, and architecture. For contraction, the matrix must be compliant enough to elastically deform and recover with an architecture that provides sites for strong cell adhesion and void space for cellular migration. The spatial geometry of the microenvironment also proved to be important as myofibroblast networks at the interface of the collagen matrix and culture wall served as anchorage points and efficient force propagators. Therefore, in larger cultures, the contraction rate was lower due to the smaller relative ratio of interfacial surface area to culture volume. The matrix density and spatial geometry of the microenvironment proved to be the main factors dictating organoid functionality [113].

However, the matrix density, including the architecture and biochemical cues, could not be decoupled from the mechanical stiffness in collagen I. DiMarco has followed up the study and combined intestinal organoids with a new biomaterial for the precise control of the microenvironment's biomechanical and biochemical cues. The biomaterial is a recombinant engineered extracellular matrix (eECM) with an elastin-like structural backbone and extensions of cell-adhesive, fibronectin-derived RGD amino acid sequences. This structure enabled independent tuning of matrix stiffness and adhesivity to understand the individual effect of matrix biomechanical and biochemical forces on intestinal organoids. Compliant matrices (180 Pa) resulted in greater organoid formation efficiency than stiff matrices (1220 Pa). Increasing RGD concentration up to 3.2 mM resulted in similar organoid yields to naturally derived collagen I. It is also important to note that high matrix metalloproteinase activity was present in stiff gels, and inhibition of this activity severely reduced organoid formation (~71.5% to ~40.0%) [35]. Further optimization of the microenvironment to allow for dynamic mechanical forces and

cellular remodeling may lead to further intestinal organoid functionality.

Importantly, in a recent study, Lutolf and colleagues engineered a “designer matrix” that is mechanically dynamic, fully defined culture system for ISC expansion, differentiation, and organoid formation[34] (Figure 2C, D). This important study, for the first time, elucidated the role of the mechanical regulation of ISCs by considering the effects of matrix stiffness and integrin ligand density, an avenue that remained unexplored because of the lack of mechanical control of Matrigel. The study used RGD ligands (that generally bind to integrin $\alpha 5 \beta 1$ and $\alpha v \beta 3$ [36]), and noted that the ISC expansion was optimal within matrices of intermediate stiffness (1.3 kPa), but not in soft matrices (300 Pa). They found that ISC expansion in stiff matrices required integrin-based adhesion and cytoskeletal tension, which they believed was facilitated by YAP – an effector of the Hippo signaling pathway and mechano-sensitive transcriptional regulator. However, colonies within a stiff matrix are susceptible to compression over time, thereby inactivating YAP and hindering growth and morphogenesis. Therefore, they sought to create chemically-defined and mechanically dynamic matrix that was initially stiff for YAP activation and ISC expansion and then would soften to impede compressive forces. Specifically, they created PEG hydrogels with a mechanically stable backbone (sPEG) and hydrolytically degradable polymer (dPEG) functionalized with RGD and coated with laminin-111 (**Figure 2E**). By varying the ratio of sPEG to dPEG, they controlled the softening profile of the gel (Figure 2E) and were able to observe extensive organoid formation using both mouse and human cells (**Figure 2F**)[34]. This study’s precise control of the microenvironment helps researchers understand the necessary cues in development, differentiation, and for specific functions, and it grants intestinal organoids scalability and reproducibility for large-scale uses.

2.2 Cerebral Organoids: The Brain

The brain serves as the command center, parsing inputs and dictating output behavior and movement. The finer mechanistic aspects behind this system that define our humanity are just beginning to be understood. Simple functions that in vitro models could replicate would be the electrical and chemical basis for known signaling, as well as the overall structure of the brain.

The brain structure forms from three sections: forebrain, midbrain, and hindbrain. The forebrain develops into the cerebrum, which accounts for most of the brain’s weight. The cerebrum’s outer layer, the cerebral cortex, has been found to be the region associated with complex thought. The midbrain becomes the brainstem, and the hindbrain forms portions of the brainstem and the cerebellum. The brainstem primarily controls information transmission between the brain and the rest of the body, as well as the regulation of key physiological functions including cardiac and respiratory function. Finally, the cerebellum is associated with motor control [114]. The entire structure of the brain is broadly composed

of two main types of cells – neurons and glial cells. Neurons transmit electro-chemical signals, and glial cells provide support functions, such as providing nutrition, protecting neurons against pathogens, and forming myelin [114]. Brain based models have sought to replicate the intricate brain structure and composition to better understand the mechanisms behind each function. In the following sections, we describe the cellular and biomaterial-based approaches to create brain organoids for the study of brain development and dysfunction.

2.2.1 Cellular Spheroids & Organoids

Healthy Developmental Models of Neural System

The self-organizing capacity of stem cells was adapted early on to form cortical neuroepithelium [115]. Mouse embryonic stem cells (ESC) were differentiated into neural lineages using serum-free suspension culture. In suspension-free culture, embryoid bodies differentiate autonomously without extrinsic induction. To create suspension free-culture, ESC were disassociated and then cultured on low-cell adhesion, or U-bottomed, 96 well plates to allow quick self-assembly into uniformly sized embryoid bodies. The quickness in restoring aggregation improved differentiation reproducibility and selectivity into specific lineages. After three days, the embryoid bodies expressed early neurodermal markers and then formed continuous, polarized neuroepithelial structures by day 5. This same apical-basal arrangement has not been previously seen in other dissociation-rearrangement cultures, including neurospheres. Thus, simple sorting mechanisms via selective cell adhesion between cortical components cannot fully account for apical-basal arrangement. Eiraku et al. suggests that the continuous presence and function of polarized mitotic neuroepithelia is needed for this organization, which is dependent on Notch signaling [115].

This process of serum-free floating culture of embryoid body-like aggregates with quick self-assembly was then applied to more clinically translatable cell sources, including hiPSCs, to study and better understand human neural diseases [116]. Through this approach, specialized organoids for specific brain regions, such as the forebrain [46, 47], hippocampus [44], and cerebellum [45], were also created with the region's functional outputs. Lancaster and colleagues expanded on Eiraku's initial embryoid-body work to create the first whole-brain organoid [10]. In these studies, the use of patterning growth factors was bypassed in favor of creating the proper environment and growth conditions for the stem cells to intrinsically self-assemble into a brain organoid. Building upon the serum-free suspension technique to similarly generate neuroectoderm, Lancaster et al. maintained the resulting neuroectodermal tissues in 3D culture. To develop further tissue growth based on microenvironmental cues, tissues were embedded into Matrigel and then transferred to a spinning bioreactor to facilitate nutrient absorption. After 8 to 10 days in culture, neural identity appeared, and defined brain regions formed from days 20 to 30. Organoids grew

to 4 mm in size and have been cultured past 10 months in the spinning bioreactor [10]. However, due to a lack of vasculature, organoids reached a limit in size and developed a necrotic core.

Morphologically, the cerebral organoids had regions that resembled the cerebral cortex, choroid plexus, retina, and meninges. Immunohistochemistry in serial sections revealed regions corresponding to the forebrain, dorsal cortex, prefrontal cortex, hippocampus, ventral forebrain, and choroid plexus. Many of these sections were immature and were not in the spatially relative location. Still, cerebral organoids did display discrete, interdependent regions. The cerebral cortex was also shown to include progenitor populations that properly organized and produced mature cortical neuron subtypes. These organoids also mirrored human cortical development based on progenitor zone organization and copious outer radial glial stem cells [10]. [Similarly, Kadoshima et al. also engineered cerebral organoids that demonstrated spontaneous development of intracortical polarity, curving morphology, and complex zone separations, similar to normal human brain development \[47\].](#) In summary, this cerebral organoid proves to be a strong initial step in better understanding and modeling human brain development.

Disease Organoid Models of Neural System

By modeling brain development, brain developmental disorders and interactions with pathological agents can be studied. Lancaster et al. expanded upon her cerebral organoids to model microcephaly – a neural developmental disorder that reduces brain size and can lead to delayed development, intellectual disability, and life-threatening physiologic conditions [10]. hiPSC-derived microcephaly organoids displayed smaller neuroepithelial tissue, a large degree of neuronal outgrowth, and smaller neural tissues with few progenitor regions. To further explore the cause of hypoplasia in microcephaly, organoids were examined earlier, and microcephaly organoids had only occasional neuroepithelial regions, with decreased radial glials, and increased neurons when compared when controls. The increased neuron number suggested premature neural differentiation. Radial glial spindle orientation, which should be precisely horizontal for symmetric expansion of neural stem cells, was often oblique or vertical in patient-specific organoids. Overall, this finding suggested that loss of *CDK5RAP2* gene led to premature neural differentiation and improper progenitor maintenance, further elucidating a possible genetic cause of microcephaly [10].

Microcephaly has also been recently linked to infection with the Zika virus during pregnancy. Recently, Zika virus was shown to infect human cortical neural progenitor cells in vitro, leading to increased cell death and dysregulated transcription [117]. Results from another group have shown that this trend of decreased viability and growth occurs in infected brain organoids. Thus, brain organoids may be used to study the mechanistic interaction of Zika virus with neural progenitor cells in the proper physiological context [48]. A recent study has already linked the Zika virus to increased activation of

Toll-like-Receptor 3 (TLR3), a mediator of the innate immune response, which led to a dysregulation of neurogenesis-related genes [13]. Advance in brain organoid research can further elucidate other dysregulated mechanisms in disease, and it holds promise to better understand and possibly develop therapies for neural diseases and disorders, such as Parkinson's, Alzheimer's, epilepsy, and traumatic brain injuries. For example, using Matrigel, Choi et al [118] have reported a three-dimensional human neural cell culture model of Alzheimer's disease.

2.2.2 Biomaterials-based Cerebral Organoids

The primary limitation of cellular cerebral organoids is the lack of control over biological and mechanical signals that are crucial to tissue organization. Although it has been noted in past that patterning growth factors were not used in favor of using more intrinsic cues, there should be some compensation for the lack of surrounding tissue and body axis for spatial geometries resembling native tissue [10, 119]. The inherent variability of Matrigel could result in random spatial organizations and hinder reproducibility. Organoids also lacked additional cell types, such as microglial cells that constitute a majority of the human brain, and prevented the study of cellular interactions for development and dysfunction. Lastly, organoids were also limited in size and often faced necrotic cores due to nutrient diffusion and an absence of vasculature.

Similar to intestinal organoids, in this case, the microenvironment can be adapted to present precise biochemical cues for scalability and possible spatial control. Recently, Lindborg et al described a cerebral organoids (cOrgs) engineered from human PSCs by using a chemically defined hydrogel material and chemically defined culture medium [102]. These organoids were engineered using a hydrogel composed of Sodium hyaluronan (HA-Na) and Chitosan (CT) protonated with formic acid (CTNH₃⁺). HA has been widely recognized as an important niche component in neural tissue engineering. The organoids showed formation of neural rosette and neural tube-like structures and demonstrated early corticogenesis. These cerebral organoids demonstrated protein and gene expression representative of forebrain, midbrain, and hindbrain development and showed responses to glutamate and depolarization in many cells, consistent with neural behavior. These cerebral organoids were used to study adrenoleukodystrophy (ALD), which is a progressive and degenerative neurologic condition that affects the nervous system, adrenal cortex, and testis, resulting from mutations in the ABCD1 gene [102]. Importantly, this study emphasized the need for cell-matrix interactions and mechanical properties of hydrogel for efficient formation of cerebral organoids. Mechanical stiffness analysis using an oscillating plate rheometer methodology indicated that the Young's modulus for the HA-CT hydrogels were between 9 – 13 kPa. This stiffness is markedly higher than native brain (0.5–1 kPa) and previous in vitro reports. Work from Schaffer and colleagues show that soft hydrogels (100-500 Pa) promoted neuronal lineage

while stiff hydrogels (1-10 kPa) promoted glial differentiation [120, 121]. Similarly, soft ECMs with a stiffness similar to that of the neural tissue (100-700 Pa) promoted the generation of early neural ectoderm from hPSCs, while this effect was less pronounced for hard ECMs (7.5 kPa) [122]. Therefore, the biomechanical elasticity of ECMs is a potent regulator for neural lineage commitment of hPSCs and adult stem cell progenitors [123]. Fu and colleagues demonstrated that soft, micropatterned substrates significantly increased the yield of functional motor neurons (spinal cord cells that innervate muscles and initiate movement) differentiated from cultured hPSCs when compared with those cultured in the same differentiation conditions but on standard culture dishes [124]. Likewise, 3D ECM has been extensively explored for neural differentiation from progenitor sources and PSCs [125-127]. Chitin-alginate 3D microfibrillar scaffolds have been shown to support efficient neuronal differentiation (positive expression of SOX1 and PAX6) and maturation under chemically defined culture conditions [125]. Although such constructs are better defined steps toward large-scale production of stem cell derivatives, they have not yet shown the potential to form brain organoids as reported using Matrigel system [10].

In another relevant neural tissue engineering work, Schwartz et al. created 3D neural constructs by combining PEG-hydrogels with human embryonic stem cell-derived neural progenitor cells, mesenchymal stem cells, and microglia/macrophage precursor cells in serum-free culture [103]. The aim was to create a predictive platform for neural toxicity that captured the key cellular interactions between the different cells present in neural development. To create these constructs, PEG hydrogels were simply modified to include matrix metalloproteinase-degradable peptide cross links and CRGDS peptides to respectively facilitate proteolytic remodeling and cell adhesion. To mimic the formation of the neural tube and subsequent recruitment of blood vessels and microglia, neural progenitors were initially seeded on PEG hydrogels, followed by vascular cell and microglia cells seeding nine and thirteen days later, respectively. After the first seeding, neural progenitors formed multiple layers with radially organized glial cells and neural cells, including GABAergic and glutamergic neurons. The second seeding resulted in extensive capillary networks that associated with the glial cells throughout all neuronal layers. The final seeding resulted in dispersed microglia-like cells (Iba1+) were found throughout the construct, associating with endothelial tubules. Changes in the gene expression profile accurately classified 9 out of 10 compounds as toxic or nontoxic, supporting its use in predicting neural toxicity. However, the extent to which the gene expression profile is actually indicative of normal human neural development and function is not known [103]. Therefore, although this biomaterial-based neural construct cannot be fully classified as an organoid, its complexity regarding cell type, scalability, and ability to be patterned is encouraging for the advancement of current cortical organoids.

Several other polymer system and synthetic platforms have also been developed to enable

successful neural differentiation. These platforms have focused on three criteria: matrix adhesivity, mechanical properties, and dimensionality. In particular, a major thrust of matrix adhesivity research has been towards engineering substrates that present ligands that bind integrin $\alpha 6 \beta 1$, which is central to neural progenitor differentiation. Presence of laminin and laminin-rich Matrigel substrates favor neuronal generation and neurite outgrowth, as compared to substrates that present fibronectin, poly-D-lysine, and collagen I [128]. LaPlaca and colleagues showed that mice receiving neural stem cells within the laminin-based scaffold performed significantly better than untreated mice on a spatial learning task, supporting the notion that functional recovery correlates positively with donor cell survival as directed by a biomaterials niche [129]. Laminin, collagen IV, and heparan sulfate have also been shown to regulate the balance of neuronal and glial cell differentiation [130]. Even in the absence of external substrates, endogenous ECMs derived from multicellular cell aggregates, such as embryoid bodies, that have a high content of fibronectin, laminin, collagen IV and vitronectin can accelerate neural differentiation, suggesting that cells themselves secrete building blocks that enable robust neural differentiation and lineage commitment [131].

While a major focus of cerebral organoids and neural tissue engineering has been on healthy tissue regeneration, there is a significant clinical need for biomimetic, tissue engineered brain tumor platforms. Very few studies have taken initiative to engineer spatio-temporally relevant platforms that are able to recapitulate regulatory elements of the complex tumor microenvironment [132-134]. In a recent study, Harley and colleagues engineered a spatially graded hydrogel platform as a 3-D tumor model of glioblastoma (GBM, **Figure 3**) [132], the most common form of brain tumor in adults. GBM tumors are highly heterogeneous and display significant spatial and temporal gradations of cell-cell signaling, ECM protein and growth factors, as well as mechanical and structural properties. Unlike brain, which is mostly rich in glycosaminoglycan hyaluronic acid (HA), GBM tumors display fibers of key ECM proteins such as laminin, fibronectin, and collagen [132]. Although Matrigel cultures have been reported, they suffer from previously mentioned concerns and do not allow in-depth control over gelation kinetics or the final network structure. As a result, Matrigel is not suitable for generating engineered systems with spatial-gradation. Importantly, Matrigel's complexity, as highlighted earlier, makes it difficult to deconstruct secretome data from growing tumors [132]. To overcome these limitations, Harley and colleagues integrated microfluidic-mixing technologies with functionalized gelatin and HA hydrogels to generate a series of hydrogels containing controlled spatial gradients. The engineered platform is the first to report rapid generation of overlapping patterns of GBM tumor cell, hypoxia, ECM, and biomolecule signals within a 3-D hydrogel niche, and is amenable to parallel cellular-biochemical analyses [132]. Engineering such platforms and integrating them with cerebral organoids may permit in situ analysis of brain cell malformations, tumorigenesis, tumor-immune cell interactions, and enable understanding tumor cell

heterogeneity at the molecular and genomic level.

2.3 Immune Organoids: Thymus and Lymph Nodes

In the immune system, lymphocytes first form and mature in primary lymphoid organs, such as the thymus [135] and bone marrow [136, 137]. Following lymphocyte maturation, many lymphocytes constantly move throughout secondary lymphoid tissues, where crucial interactions that underlie homeostatic regulation, peripheral tolerance, and the effective development of adaptive immune responses take place. These secondary lymphoid organs are not only important for the initiation of adaptive immune responses, but they are also increasingly recognized as sites for the regulation of peripheral tolerance to control autoreactive T cells that escape deletion in the thymus. It is critically important to understand the factors that influence the recruitment, interaction, and functioning of immune cells as more studies are focused on developing minimal functional units that recapitulate tissue- and organ-level functions. Given the importance of immune cells and organs in the body, creation of *ex vivo* immune organoids for culturing primary immune cells and allowing them to interact could afford researchers an inside look into the dynamic events that take place in lymphoid and non-lymphoid tissues.

Recent years have seen an increasing interest to create *ex vivo* thymus and lymph nodes organoids [37, 88, 101]. The thymus is composed of two identical lobes with a central medulla, peripheral cortex, and outer capsule. This entire structure is composed primarily of epithelial cells of the medulla and cortex, dendritic cells, and thymocytes, or T-cell progenitors. This structure supports the thymus's primary function of thymopoiesis, or the creation of mature T lymphocytes. These mature T lymphocytes along with other lymphocytes then migrate to the secondary lymph nodes.

Lymph nodes function as the body's filtering system. Positioned throughout the lymphatic system to filter lymph, lymph nodes limit the spread of pathogens and facilitate the induction of the adaptive immune response [138-142]. In addition to the hallmark B, T, and dendritic cells, the lymphoid microenvironment consists of a spatially patterned extracellular matrix, follicular dendritic cells and follicular reticular cells to create the appropriate stromal matrix to allow immune cells to migrate and achieve their function. For the adaptive immune response, the lymph node can be seen as three zones – an outer antigen-sampling zone, the B-cell activation zone (B-cell follicle), and the T-cell activation zone (cortex) [138, 139, 143-146]. These three zones result from dynamic migration processes and can effectively describe how the adaptive immune response is initiated. In the outer sampling zone, antigen-presenting cells, such as dendritic cells (DCs), bind to antigens that will then be presented to the lymphocytes, or B-cells and T-cells. Since the number of DCs and the frequency of antigen-specific T cells are low, T-cells migrate at rapid speeds to survey antigen-bound DCs [143-146]. Upon recognition of an antigen, T-cells are then activated and fulfill their effector functions in cellular-mediated immunity

and in B-cell activation. B-cells can either be activated by antigen presentation from DCs or follicular helper T cells. In response to activation, B-cells then form germinal centers to differentiate into antibody-secreting cells for the induction of humoral immunity. During the germinal center differentiation process, B cells undergo three main processes: affinity maturation, isotype switching, and terminal differentiation into antibody-secreting cells [143-146].

Engineering immune organoids can enable better understanding of the mechanisms behind tolerance, germinal center development, and lymphoid cell dysfunction as well as to create antibody-based therapeutics for the treatment of various pathological states. We subsequently describe the cellular and biomaterial based approaches used to create thymus and lymph nodes for the study of tolerance, germinal centers, and production of antibodies.

2.3.1 Cellular Spheroids & Organoids

Healthy models of Lymphoid Organoids

There is particular interest in creating thymus organoids to establish tolerance to prevent rejection in patients who receive organ transplants and allografts. Fan et al. was able to create the main example of a thymus organoid by decellularizing a mouse thymus with subsequent seeding of thymic epithelial cells and lymphocyte progenitors from the bone marrow [101]. When transplanted into athymic nude mice, these grafts enabled homing of lymphoid progenitors, thymopoiesis, antigen-specific humoral responses upon immunization, and tolerance to skin grafts [101]. Similar to Matrigel and other naturally occurring extracellular matrices, decellularization approaches enhance the self-organizing capacity of these progenitor cells; however, this strength comes at the sacrifice of control, precise composition, and batch-to-batch reproducibility. In this respect, biomaterials could be explored to supplement current decellularization approaches to afford precise control of the organoid's microenvironment.

Likewise, secondary lymph nodes are also garnering interest to better understand germinal center physiology and pathophysiology and to create antibody-based therapies. As primary lymphatic organs are the sites of lymphocyte formation and maturation, lymphocyte deriving stem cells are not normally present in secondary lymphoid organs, such as lymph nodes. Thus, the use of stem cell self-assembly has not been broadly applied to the creation of immune organoids and germinal centers. As a post-developmental process, germinal centers formation is thought to be highly coordinated based on not only cellular-cellular interactions but also precise cellular-microenvironmental interactions. Germinal center B cells are naturally prone to apoptosis unless rescued by anti-apoptotic signals [143, 147, 148]. This limitation has prevented successful culture of GC B cells *ex vivo*. As a result, attempts to successfully create *ex vivo* organoids using matrigel have never been reported or even differentiation using genetically

engineered cells with specific activation signals have limitations. For example, an improvement to the 2D culture was reported by Nojima et al. [149], who described the generation of the 40LB stromal cells that presented CD40L and BAFF to naïve B cells. Although this study reported the ability to produce activated B cells from naïve cells, the 2-D system, in our hand and others, is highly dependent on the source of animal-based serum to induce B cell maturation. Not only does this impose a significant challenge to reproducibility and rigor, it presents a roadblock to long-term humanization of the technology. The 2-D co-culture approach by Nojima et al required the cells to be transplanted back into mouse models for memory B cell generation. In sum, no 2D *in vitro* system has demonstrated the ability to modulate the extent of germinal center reaction or has successfully generated long lived, high-affinity, activated B cells.

Diseased models of Lymphoid Organoids

Another successful application of biomaterials-based lymphoid organoids have been reported in the case of lymphoid transformation, called lymphomas, which are the lymphoproliferative cancer of B and T cells. In the case of follicular lymphoma, cellular spheroids have been developed. Follicular lymphoma (FL) is a slow-growing non-Hodgkin B cell lymphoma in which the body develops abnormal B-cells in lymph nodes. Prior to spheroid creation, most *in vitro* studies included cell suspensions that did not account for the three-dimensional architecture and its ensuing cell-cell and cell-matrix interactions. Thus, Gravelle et al. used the hanging drop method to create aggregates of follicular lymphoma cells [89]. Gene expression studies revealed that over 7000 genes were differentially expressed when cultured in 3D, with the most differentially expressed genes functionally involved in the response to hypoxia, NF- κ B signaling, negative regulation of apoptosis, and regulation of the cell cycle. The gene expression profile relating to hypoxia closely resembled to ones observed in FL biopsies, suggesting that 3D culture made them more similar to native FL. In comparison to suspension cells, aggregates also displayed an activated NF- κ B pathway and far more frequent quiescent status. Unlike suspension cells, FL aggregates were also found to resistant to doxorubicin and bendamustine, two drugs conventionally used in FL treatment, and they also were less sensitive to purified natural kills [89]. These findings emphasized that the spatial organization and the change in cell-cell and cell-environment interactions that resulted from three-dimensional culture dramatically changes the biology in FL lymphoma. However this work did not take into account the extracellular matrix cues of the lymphoid tissues and tumor heterogeneity.

2.3.2 Biomaterials-based Organoids

The simple introduction of 3D culture alluded to the importance of controlled cell-cell and cell-ECM interactions in lymph node development and function. Specifically, our lab has focused on implementing biomaterials to control the mechanics, architecture, and biochemical cues present in lymph

nodes to study and modulate germinal center development and dysfunction. Prior to our work, 3D scaffolds have been used in the context of secondary lymph nodes to form synthetic lymph nodes *in vivo* or to study immune cell migration [150-152]. For instance, to form synthetic lymph nodes, Suematsu and Watanabe et al. first encapsulated stromal cells expressing Lymphotoxin (LT)- β receptor, LT- α , and vascular cell adhesion molecule (VCAM-1) in collagen sponge scaffolds. They then implanted these scaffolds to exploit the host microenvironment to form organized secondary-lymph node structures with follicular dendritic cell networks, T cell clusters, B cell clusters, and GCs [153]. To study immune cell migration, Stachiowiak et al. formed lymphoid-mimicking hydrogel structures composed of a macroporous polyethylene glycol (PEG) infused with collagen and encapsulated T cells and dendritic cells. In this scaffold, T cell migration proved to be dependent on connecting pore size and could be further induced with motility inducing cues, such as CCL21 [154]. Although very valuable, these studies did not demonstrate GC formation and differentiating B cell survival *ex vivo*; thus, our lab seeks to use biomaterials to not only enable GC formation *ex vivo* but also to modulate GC dynamics and dysfunction.

In terms of germinal center-like development, we engineered immune organoids composing of RGD-presenting gelatin that was further reinforced by silicate nanoparticles [88, 104]. These organoids encapsulated naïve murine B cells and 40LB cells, genetically engineered stromal cells capable of activating B cells through CD40L and B cell activating factor expression (**Figure 4A-C**). Exogenous IL-4 was also added for B-cell activation. We first mechanically optimized our material to resemble the native lymphoid microenvironment. Silicate nanoparticle addition resulted in uniform microarchitecture and porosity similar to native tissue. We modulated the biochemical cues based on the presence of RGD – a cellular motif recognized by several integrin mechanoreceptors and plays a role in cellular adhesion in B cells. By comparing our organoid to 2D cultures, we observed a ~100-fold increase in germinal center-like B cells in biomaterial-based organoids within 4 days, in a tunable manner, as compared to modest 10-20 fold with standard 2D cultures. These organoids further induced robust antibody class switching, a hallmark of B cell differentiation and the proliferation was dependent on integrin-ECM interactions [88]. Thus, regulation of the environment's microarchitecture, biochemical composition, and mechanical forces along with key cell-cell interactions enabled us to control the magnitude and rate of the germinal center reaction. Our results stress that the microenvironment's microarchitecture, mechanics, and biochemical signaling as well as controlled cellular signaling is crucial to understand for germinal center development and antibody creation.

We built upon this finding by controlling biochemical forces, particularly integrin-specific ligands, to better understand the effect of microenvironmental cues on the GC reaction [36]. Rather than the previous gelatin model, organoids were now composed of 4-arm polyethyleneglycol (PEG-MAL) for

its ability to present a precisely tuned microenvironment through the simple yet specific conjugation of thiolated bio-adhesive peptides at defined densities (**Figure 4D**). Enzymatically degradable di-thiolated peptide crosslinkers were also incorporated to allow for cellular remodeling and migration. To study the GC reaction, organoids encapsulated murine B cells and 40LB cells, as in the case of the previously described gelatin organoids. We engineered organoids specific for integrins $\alpha_v\beta_3$ and $\alpha_4\beta_1$, as the expression level of the former changes distinctly throughout the GC while that of the latter remains constant [36]. Integrin $\alpha_4\beta_1$ -specific organoids induced a more robust early-germinal center phenotype in a ligand concentration dependent manner in comparison to $\alpha_v\beta_3$ -specific organoids. This enhanced induction can be explained by the fact that the B cell receptor (IgM) co-localized and clustered with integrin β_3 in $\alpha_4\beta_1$ -specific organoids. It is also further supported by the fact that simultaneous presentation of $\alpha_v\beta_3$ and $\alpha_4\beta_1$ resulted in phenotypes similar to that of $\alpha_v\beta_3$ -specific organoids, suggesting a regulatory role for $\alpha_v\beta_3$ in the B cell niche. Overall, the modularity of this system further allowed us to mechanistically understand B cells and the GC reaction.

We built upon this finding by controlling biochemical forces, particularly integrin-specific ligands, to better understand the effect of microenvironmental cues on malignant B-cell and T-cell Non-Hodgkin lymphomas (NHL) [37, 155]. To model lymphomas, organoids encapsulated follicular dendritic cells as a supporting stromal cell subtype along with either malignant B cell or T cell lymphoma cell lines. Through this modular platform, we showcased that B and T cell lymphomas have different integrin-signaling requirements that affect their survival. In comparison to 2D cultures, the organoid models displayed increased proliferation and clustering as well as higher drug resistance to common therapeutics and even an epigenetic histone deacetylase inhibitor, Panobinostat. Mechanistically, in Diffuse Large B Cell Lymphoma cell lines, integrin-specific organoids upregulated B cell receptor expression, possibly explaining their enhanced survival. The change in drug resistance and gene expression highlights how our integrin-specific organoid model seems to better model the lymphoid neoplasm-like heterogeneous microenvironment and alludes to the model's potential in drug screening and translating novel therapies.

3. *Perspective*

Overall, informally regarded as in vitro “miniature organs”, organoids have garnered interest to better understand disease, to model development, and to evaluate the efficacy of new drugs and therapeutics. The sheer complexity of current Matrigel platforms lead to an incomplete understanding of self-assembly mechanisms facilitated by specific microenvironment cues in addition to an inability to manipulate the microenvironment to facilitate greater mechanistic understanding. Alongside with a lack of engineering control, the inherently inconsistent composition and batch-to-batch variability of Matrigel has resulted in issues with reproducibility and a lack of consistent cellular organization, tissue types, functionality, and

maturity in developmental organoids. Within disease states, these same drawbacks have resulted in issues with genetic drift and precluded studies of later disease states that are dependent upon the presence of additional cell types or a dynamic biophysical microenvironment. Genetic drift should be considered in cases where niche factors drive outgrowths of particular tumor sub-types instead of capturing total tumor heterogeneity. In both developmental and disease models, issues with reproducibility have been slightly mitigated due to studies increasing the throughput of organoid system [156], but a well-defined biochemical and biophysical microenvironment is needed to overcome the additional issues. Therefore, organoids have incorporated biomaterials to control the microenvironment's size, architecture, mechanical stiffness, and biochemical cues. These biomaterial-based microenvironments and microsystems present a more modular, controlled approach and have shown enhanced organ functionality, cellular composition, and overall mimicry of their *in vivo* counterparts.

Through the incorporation of biomaterials to control organoid's microenvironment in terms of size, architecture, and both mechanical and biochemical cues, individual organoids are more reproducible, scalable, and better suited for translation. In particular, microsystems have been incorporated to control organoid geometry and size, including in the case of answering questions regarding branching morphogenesis in the mammary gland and lung [22, 156-158]. Synthetic matrices, such as the eECM and PEG-based systems (i.e. designer matrix, immune organoids and lymphoma organoids), also have been used to control the physical, chemical, and cellular composition of the organoid. The design of these organ-specific synthetic matrices can be further modulated based on the results of multifactorial approaches, such as artificial niche microarrays, that probe the effect of individual cellular, mechanical, and ECM-based microenvironmental signals on stem cell fate in a single-cell, high throughput fashion [159]. This reductionist mentality would also facilitate the design of more efficient therapies better suited for rapid translation.

We believe that further incorporation of biomaterials can continue to enhance organoid function through specific spatiotemporal control of microenvironmental cues [160]. The complex interplay of multiple parameters, including gradients of agonists and antagonists, mechanical stiffness, adhesive intercellular interactions, and cytoskeletal and extracellular matrix (ECM) remodeling regulate these processes. Specifically, for cell-cell interactions, these factors direct cell motility and location and are necessary for angiogenesis, innervation, and immune responses. Similarly, biophysical gradients are needed in disease models as they mirror the progression of many diseases, including the stiffening observed in breast cancer tissue. Lastly, in stem cell differentiation, these factors individually and collectively vary as a function of spatial locale and temporal stages of development [160], and drive the formation of cellular structures *in vivo*.

To imitate *in vivo* spatiotemporal dynamics, materials engineered to elicit targeted cellular assembly and responses must be similarly dynamic in their spatiotemporal presentation of bio-ligands and material stiffness [161-167]. Mechanical gradients can be created with photolabile and photoactivatable crosslinkers to corresponding soften or harden specific matrix locales. Dynamic chemical gradients can also be generated through flow-based approaches, such as microfluidic devices, to guide migration, differentiation and cellular organization necessary for advanced organ function and pathologies, as previously accomplished for human liver and intestinal epithelium platforms [168-170]. For further spatiotemporal control, biomaterials can be used to sequestered soluble signals in exact locations. For instance, the biomaterial heparin has been engineered into stem cell culture platforms, in order to spatially control stem cell proliferation and differentiation via sequestration of growth factors such as FGFs and BMPs [171-177]. For additional temporal control, the aforementioned techniques could also include photosensitive caging of specific biomolecules or adhesive sites to respectively facilitate further physical and chemical signaling for vascularization, cell-cell interactions, and advanced disease morphology [166].

In summary, organoids have already influenced our understanding of organ function and dysfunction as well as our approach to creating more effective and even personalized therapeutic approaches. With the incorporation of biomaterials, organoids have started to provide a more mechanistic understanding of necessary microenvironmental cues for organogenesis. We believe that the addition of controlled spatiotemporal signaling will bridge the gap between *ex vivo* organoids and their *in vivo* counterparts and facilitate the advent of efficient, specific therapies.

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Figure Legends

Figure 1: Organoids: Types, formulation strategies, and application. A.) Visualization of physical structure and cellular composition of major organoid platforms. B) Organoid formulations based on secreted soluble signals, non-modular extracellular matrix signals, and modular extracellular matrix are listed on the left, followed by visualization of organoid formulation and the expected organoid structure. On the right, relative advantages and disadvantages of each strategy are summarized with respect to ease of throughput, scalability, tunability, and patterning as well as the resulting stochasticity. Relative increases are indicated by increases in the width of the triangle below each parameter. C) Pyramids show predicted timeline of application of organoids across basic research, drug discovery, and cell-based therapy or regenerative medicine. Bottom pyramids demonstrate expected progression or utility of organoids in drug discovery and translation.

Figure 2: Recent Biomaterials Design Considerations for Intestinal Organoids. **A)** A schematic illustrating the small intestine where LGR5+ crypt base columnar stem cells are intercalated with Paneth cells at the crypt base and enterocytes, tuft cells, goblet cells and enteroendocrine cells on the villi. **B)** A schematic illustrating the Matrigel-based 3-D self-renewing epithelial organoids system that supports the growth of self-renewing, near-native intestinal epithelia in the absence of stromal niche to form discrete crypts with intercalated stem cells and Paneth cells at physiologically relevant positions. **C)** Lutolf and colleagues [178]. reported a biomaterials-based intestinal organoid with design rationale that define different stages of organoid formation within well-defined matrices. **D)** A schematic illustrating hybrid sPEG–dPEG-based mechanically static and dynamic hydrogels used to engineer designer intestinal organoids. **E)** Mechanical characterization of designer hydrogels as a function of dPEG. **F)** Organoids formed in artificial matrices harbored differentiated intestinal cells. Scale bars, 50 μm . Adopted with permission from [178, 179].

Figure 3: A Biomaterials approach to engineer heterogeneous brain-tumor microenvironment. **A)** Harley and colleagues [132] reported a microfluidic-biomaterials-based hybrid platform that mimicked the GBM tumor heterogeneity by considering overlapping patterns of tumor-inspired matrix signals. **B)** The microfluidic mixing device consisted of a microfluidic diffusive mixer with staggered herringbone features (inset) and a connected external mold. Gradients in biomaterial crosslinking density were generated using opposing solutions of functionalized gelatin with dissimilar degree of methacrylate functionalization and hyaluronic acid. Adopted with permission from [132].

Figure 4: Recent Biomaterials Design Considerations for Secondary Lymphoid-like Immune Organoids. **A)** Rational design of biomaterials for immune organoids requires understanding the key extracellular matrix signals involved in immune cell survival and activation. Shown here is the surface protein expression level of integrin, a pro-survival bioadhesive receptor, in immunized animals. Sheep red blood cell (SRBC) immunized C57BL6 mice developed GC over 8 days, and B220+GL7+Fas+ germinal center B cells indicate differential expression of integrins α_4 , α_v , β_1 , and β_3 as compared to naive B cells. **B)** Visual representation of *in vivo* cellular interactions present in B cell follicles of secondary lymph nodes (top) as well as the cellular interactions present in the immune organoid (bottom). In lymphoid tissue, follicular T helper cells (T_{FH} cells) activate B cells through antigen presentation and co-stimulation with CD40 ligand (CD40L). Follicular dendritic cells further support germinal center formation and activated B cell survival through B cell activating factor (BAFF) secretion. In immune organoids, these interactions are emulated using a genetically engineered stromal cell line (40LB) that simultaneously presents T_{FH} specific CD40L and secretes BAFF. **C)** Singh and colleagues [36, 88] engineered healthy immune organoids using integrin $\alpha_v\beta_3$ -binding gelatin that allow *ex vivo* germinal center formation (schematic is shown on the left). Silicate nanoparticles (SiNP) reinforce gelatin and enables ionic crosslinking to form stable hydrogel at 37°C. On right, *ex vivo* germinal centers are displayed through immunofluorescent images of GC B cells and 40LB cells inside organoid. **D)** Schematic of underlying Michael-addition chemistry to form designer PEG-MAL hydrogels that are conjugated with thiolated peptide of interest and encapsulating cells. Confocal images represent IgM BCR expression, clustering, and co-localization with integrin β_3 in 3D immune organoids in a niche-specific manner. Integrin $\alpha_v\beta_3$ -specific organoids (RGD) result in diffused surface expression of IgM BCR that co-localize with integrin, whereas integrin $\alpha_4\beta_1$ -specific organoids (REDV) induce microclustering of IgM BCR. Adopted with permission from [36, 88].

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Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

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Figure 4

Figure 4