REVIEW

Bioengineering approaches to guide stem cell-based organogenesis

Nikolche Gjorevski*, Adrian Ranga and Matthias P. Lutolf*

ABSTRACT

During organogenesis, various molecular and physical signals are orchestrated in space and time to sculpt multiple cell types into functional tissues and organs. The complex and dynamic nature of the process has hindered studies aimed at delineating morphogenetic mechanisms *in vivo*, particularly in mammals. Recent demonstrations of stem cell-driven tissue assembly in culture offer a powerful new tool for modeling and dissecting organogenesis. However, despite the highly organotypic nature of stem cell-derived tissues, substantial differences set them apart from their *in vivo* counterparts, probably owing to the altered microenvironment in which they reside and the lack of mesenchymal influences. Advances in the biomaterials and microtechnology fields have, for example, afforded a high degree of spatiotemporal control over the cellular microenvironment, making it possible to interrogate the effects of individual microenvironmental components in a modular fashion and rapidly identify organ-specific synthetic culture models. Hence, bioengineering approaches promise to bridge the gap between stem cell-driven tissue formation in culture and morphogenesis *in vivo*, offering mechanistic insight into organogenesis and unveiling powerful new models for drug discovery, as well as strategies for tissue regeneration in the clinic. We draw on several examples of stem cell-derived organoids to illustrate how bioengineering can contribute to tissue formation *ex vivo*. We also discuss the challenges that lie ahead and potential ways to overcome them.

KEY WORDS: 3D culture, Bioengineering, Biomaterials, Organoid, Stem cell

Introduction

Morphogenesis and organogenesis, the processes that transform a spherical blastula into a system of fully formed tissues and organs, have piqued human interest for more than a millennium (Aristotle and Peck, 1943), and have been under intense investigation for more than a century (Spemann, 1938; Thompson, 1917). Classic embryology and, more recently, molecular approaches have been highly successful in using invertebrate animal models to elucidate the mechanisms whereby biological form is generated. The advent of sophisticated *in vivo* tools, including conditional knockouts, chimeric labeling and deep tissue imaging, has even provided a glimpse into the organogenetic machinery of mammals. However, owing to the highly multifactorial and dynamic nature of mammalian organogenesis, a comprehensive understanding of the process has remained out of reach. Furthermore, although knowledge obtained from animal models adequately describes a large part of organogenesis in humans, numerous aspects of human development and disease are unique to our species (Hansen et al., 2010; Rangarajan et al., 2004). Organotypic tissue culture can help overcome the limitations of animal models, and offer a closer look into how human tissues and organs develop.

Far from being a new idea, three-dimensional (3D) organotypic culture has facilitated the study of many developmental and neoplastic processes over the past two decades, and has helped to define the indispensable role of the microenvironment in their regulation. In traditional 3D tissue culture, organ-specific immortalized cells or parenchymal fragments are embedded in extracellular matrix (ECM) gels, such as collagen and Matrigel (Debnath and Brugge, 2005; Nelson and Bissell, 2005). Exposing the cells to defined culture conditions triggers various morphogenetic, physiological or pathological processes specific to their tissues of origin, which can then be readily observed and studied. However, the limited number of cell types (usually one or two) included in these models constrains the histological complexity of the tissues, as well as their developmental repertoire. A major advance in organotypic 3D culture was the recent introduction of stem cell-derived organoids (Sasai et al., 2012). These models rely upon the potential of initially pluripotent or multipotent stem cells to yield multiple differentiated cell types, which then self-organize into 3D tissues with unprecedented morphogenetic and histological realism. Although stem cell-derived organoids recapitulate a much wider range of cellular and developmental phenomena compared with traditional 3D culture, differences from the native organs still exist, suggesting that microenvironmental components (chemical, physical or cellular) are either lacking or presented incorrectly in space and time.

Tissue engineers have also attempted to reconstitute tissue formation *ex vivo*, albeit for reasons and objectives historically different from those of cell and developmental biologists. At its conception, the field of tissue engineering sought to build tissues and organs for use in the clinic, i.e. for replacement of the heart, kidney, liver and other organs, which, owing to donor shortage, are limited in supply. Although the generation of fully functional and transplantable organs using engineering strategies has proven difficult to accomplish, the interests of the field have expanded over the years to encompass more fundamental concepts. Notably, bioengineers have set their sights on deconstructing the complexity of the *in vivo* microenvironment and recapitulating it in culture in a modular and highly controlled manner. Bioengineering in general, and biomaterials-based approaches in particular, have been successfully used in elucidating numerous phenomena in basic cell biology (Chen et al., 1997; Engler et al., 2006; Gilbert et al., 2010; McBeath et al., 2004). Recently, the bioengineering techniques that enable tight control over the biochemical and biophysical environment of cells have been translated to the 3D realm, thus expanding the toolkit for tackling inherently 3D phenomena, including morphogenesis and organogenesis. We believe that by combining advances in the biomaterials field with advances in organotypic tissue culture, i.e. stem cell-derived organoids, it will be possible to increase the *in vivo* faithfulness of these models, and to answer questions that have evaded classic approaches.

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In this Review, we will first introduce the recently demonstrated cases of self-organizing stem cells, and discuss similarities and differences with respect to organ formation *in vivo*, and why these differences may exist. Next, we will cover major bioengineering advances that may be complementary to stem cell-based 3D organoids in studying tissue development. Finally, we will discuss the necessity for establishing and assessing tissue function in culture, and how approaches using 'organs-on-a-chip' (see Glossary, Box 1) can contribute to that end.

Self-organizing stem cells

The behavior of stem cells *in vivo* is crucially influenced by their interactions with the various biochemical and biophysical niche components, which underscores the importance of the microenvironment in regulating stem cell fate. Researchers have attempted to rebuild aspects of the niche *in vitro*, with the goal of both studying the extrinsic signals that govern it, and enhancing the maintenance and expansion of stem cells that are otherwise difficult to culture. The role of the microenvironment in regulating stem cell fate is well-illustrated by the case of embryoid bodies (EBs), aggregates of pluripotent stem cells cultured in suspension, which have been used to model early development and patterning of the embryo (Itskovitz-Eldor et al., 2000). Importantly, bioengineering-based approaches applied to EBs have proven very useful in addressing the role of microenvironmental parameters that are difficult to study with traditional culture approaches (Bratt-Leal et al., 2009; Woodford and Zandstra, 2012). For example, such platforms have been instrumental in demonstrating that aggregate size and shape control stem cell fate

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Box 1. Glossary

Bioconjugation. Linking of biomolecules via a covalent bond.

Hydrodynamic flow focusing. Sequential reduction of the width of a liquid stream flowing over a surface, such that certain regions of the surface are exposed to the liquid for longer periods of time. In the context of gradient formation, the liquid contains molecules that bind to the underlying surface with a specific affinity. Prolonged exposure of sequentially narrower regions of the surface to the flowing liquid results in local accumulation of the molecule.

Light-mediated patterning. The concept of shining light onto a restricted region within a light-sensitive material, with the purpose of inducing local mechanical, structural or chemical changes.

Organs-on-a-chip. Bioengineering devices that aim to replicate the key physiological functions of real organs in a miniaturized, simplified and well-controlled environment.

Photoinitiator. A chemical compound that breaks down into smaller, highly reactive chemical groups when exposed to light. In the polymer field, photoinitiators and their decomposition products are used to trigger polymerization processes.

Photolabile chemical groups. A class of photo-sensitive small molecules that undergo degradation in response to light.

Photolithography-based microfabrication. Creating nanometer- or micrometer-scale features within photo-sensitive materials through controlled exposure to light.

Photo-sensitive moiety. A chemical group that undergoes a certain change in response to light.

Poly(ethylene glycol) (PEG). A polymer of ethylene oxide, available in a range of molecular weights and structures. Upon chemical or enzymatic crosslinking, PEG macromers can form solid transparent hydrogels with large water content, which are suitable for cell culture. PEG hydrogels are used in multiple industrial, commercial and medical contexts.

Soft lithography. A technique to fabricate or transfer nanometer- or micrometer-scale features using 'soft' elastomeric stamps or molds.

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within EBs (Bauwens et al., 2008; Hwang et al., 2009; Karp et al., 2007) and implicating the ECM as a potent modulator of EB differentiation (Battista et al., 2005; Li et al., 2013). The recently introduced stem cell-derived organoids have demonstrated that, aside from modeling early embryogenesis, stem cells can be used to mimic aspects of rodent and human organogenesis. A common feature of the stem cell-derived organoids described above is the use of Matrigel in their generation, whether in solution (the optic cup) or as a solid scaffold (the mini-gut and the mini-brain). The crucial requirement for Matrigel in these protocols, along with mounting evidence for the role of the ECM in the morphogenesis of multiple organs, including the salivary (Larsen et al., 2006; Sakai et al., 2003) and the mammary (Brownfield et al., 2013; Gjorevski and Nelson, 2011; Muschler and Streuli, 2010) glands, suggests that influences from the microenvironment may be complementary to self-organization in driving organoid formation. We will outline the formation of retinal, intestinal and cerebral organoids in culture, highlighting differences from their native counterparts that may potentially be attributed to an absence of the complex microenvironmental cues encountered *in vivo*.

The optic cup

The development of the eye has intrigued scientists, including pioneer embryologists Spemann and Lewis, for more than a century (Lewis, 1907; Spemann, 1938). Despite its prominent place in the history of embryology and developmental biology, several aspects of eye formation, and notably the role of adjacent epithelial and mesenchymal tissues, remained unclear (Sasai, 2013). Recently, Sasai and colleagues recapitulated aspects of eye development *in vitro*, showing that mouse pluripotent embryonic stem cells (ESCs) can self-organize into a bilayered optic cup-like structure when cultured in 3D (Eiraku et al., 2011). In their culture system, aggregates of mouse ESCs in suspension were first cultured in medium containing Matrigel and minimal growth factors. Within six days, regions of the spherical neuroepithelium lumenized and started to express the retinal marker Rx (Rax – Mouse Genome Informatics) while evaginating to form spherical buds. The distal portion of these buds subsequently underwent invagination, finally giving rise to a two-layered structure that approximates the architecture of the optic cup *in vivo*. Beyond the morphological similarities, at the molecular level these inner and outer layers expressed neural retina (NR) and retinal pigment epithelial (RPE) markers, respectively, indicating that cell differentiation followed that of the developing retina *in vivo*.

Perhaps the most important implication of the stem cell-derived optic cup is the self-sufficiency of the retinal epithelium in executing morphogenesis. Sasai and colleagues demonstrated that retinogenesis in culture occurs independently of surrounding tissues, including the lens ectoderm and the periocular mesenchyme, thus weighing in on a long-standing debate in the field (Eiraku et al., 2012). Although the self-driven nature of optic cup development in culture is evident, the *in vivo* environment of the developing eye is substantially more complex: multiple neighboring tissues influence the chemical environment by secreting numerous soluble signals, including members of the wingless pathway (Wnts), bone morphogenetic proteins (BMPs), fibroblast growth factor (FGF) and sonic hedgehog (Shh) (Adler and Canto-Soler, 2007; Esteve and Bovolenta, 2006; Fuhrmann, 2008; Muller et al., 2007). Whereas some of these signals may be permissive or inductive, others may be suppressive, and, as such, may hinder the tendencies towards autonomous retinogenesis seen in culture. In addition, surrounding tissues may play physical roles, albeit passive ones, by presenting volumetric constraints. Indeed, several recent studies present evidence suggesting roles for the lens, the periocular

mesenchyme and the surface ectoderm in eye development (Smith et al., 2009). To evaluate the potential roles of neighboring tissues, while retaining the optical and physical accessibility of 3D culture, one would ideally mimic their chemical and physical attributes, without actually incorporating their cellular aspects.

The small intestine

The identification of Lgr5 (leucine-rich G protein-coupled receptor 5) as a bona fide marker of intestinal stem cells (ISCs) by Clevers and colleagues (Barker et al., 2007) was a major turning point in the stem cell and intestinal biology fields. Importantly, Lgr5$^+$ cells, which were shown to be long-lived and multipotent in vivo (Barker et al., 2007), can divide and give rise to multicellular organoids, often referred to as 'mini-guts', when cultured in 3D Matrigel, in the presence of epidermal growth factor (EGF), R-spondin and noggin (Sato et al., 2009). These ever-expanding organoids comprise multiple crypt-like domains, which protrude radially from a central lumenized structure, and harbor Lgr5-expressing cells. Adjacent to the Lgr5$^+$ cells, organized in the characteristic checkerboard pattern observed in vivo, are the Paneth cells, as well as the absorptive enterocytes, entero-endocrine cells and mucus-producing goblet cells that line the central lumen. As such, 'mini-guts' re-enact multiple aspects of intestinal development and homeostasis, including perpetual cycles of stem cell self-renewal and differentiation into multiple functional cell types, as well as the correct spatial arrangement and self-organization of these cells into a lumenized and polarized epithelial structure.

An important morphological difference that sets intestinal organoids apart from the native organ is the clear absence of villous structures. This difference may also be attributed to the lack of cellular or extracellular components, which are normally present in vivo. For example, Matrigel, which is rich in laminin-111 and collagen IV, may fail to provide additional proteins required for full morphogenesis of the intestinal system. Laminin-511, in particular, is enriched in the villus basement membrane, and has been implicated in the proper formation of the villi in vivo: conditional deletion of the laminin α5 chain results in fusion of the villi and adoption of a colonic mucosal architecture (Mahoney et al., 2008). A recent study also proposed a role for non-epithelial cell types, which are absent in the organoid culture, in the formation of the villi (Shyer et al., 2013). Specifically, the sheath of smooth muscle cells enveloping the intestine was found to provide circumferential restraint to the growing intestinal mucosa, thus generating compressive forces that result in the epithelial buckling and folding that precedes villus formation. Controlled modifications of the existing culture model to reflect these complexities may help reconcile in vitro and in vivo observations.

The human brain

The human cerebral cortex is evolutionarily the most complex tissue in the animal kingdom, and no animal model can fully recapitulate its unique features (Lui et al., 2011). To obtain information about human cerebral development, function and disease, we have historically resorted to observing brain shape and activity by bioimaging or by analyzing postmortem brain samples (Bae and Walsh, 2013). As such, the human brain makes the strongest case for creating culture models of human tissues and organs. Recently, Knoblich and colleagues took that leap and established a 'cerebral organoid' culture, which faithfully models multiple histological and developmental features of the human brain (Lancaster et al., 2013). In brief, EBs generated by aggregation of human ESCs or induced pluripotent stem cells (iPSCs) were embedded in Matrigel, and further cultured in a spinning bioreactor for improved oxygen and nutrient transport. Within 8 to 10 days, neuronal differentiation is

observed, and in 20 to 30 days the aggregates grow into large compartmentalized 3D structures containing brain region-specific cellular layers. The wide range of brain regions that are represented in these organoids, albeit to different degrees of maturity, includes the forebrain, midbrain, hindbrain, meninges, choroid plexus, hippocampus and retina. Notably, the outer subventricular zone and the inner fiber layer are also observed in the mini-brains, whereas they are completely absent in mice (Shitamukai et al., 2011). These zones contain neural stem/progenitor cells and are credited with the large neuronal output and brain size seen in humans (Fietz and Huttner, 2011).

Although stem cell-derived cerebral organoids are a major leap in modeling and understanding neural development and disease, recreating the full developmental program of the human brain (to arrive at an adult-like tissue in culture) remains a distant goal. Poor nutrient availability, owing to lack of vascularization, leads to massive cell death in the interior of the organoids and limits overall growth to several millimeters. The zones within the organoids that correspond to different brain regions at present lack the spatial organization and shape of the native organ. Also evident is the absence of late-appearing cells, such as astrocytes and oligodendrocytes, which constitute the majority of cells in the adult brain. Improving nutrient and metabolite transport by introducing vasculature or by alternate means may help sustain growth long enough to allow recapitulation (either self-driven or assisted) of both morphological and cellular aspects that are likely to be stage dependent.

From organoid to organ: instructive biomaterials may bridge the gap

Designing synthetic matrices for 3D organoid culture

Much emphasis has been placed on the self-organizing nature of stem cell-derived organoids, whereas the surrounding extracellular environment has been largely regarded as passive 3D scenery in which a pre-scripted cellular program can play out. However, these mini-organs are currently imperfect copies, which would suggest that their native counterparts rely upon both the self-organizing potential of the stem cells as well as complementary influences from the microenvironment to establish final architecture and function. Furthermore, the ECM used in stem cell-derived organoid cultures is in most cases a 'one-size-fits-all' type of matrix: physicochemical properties cannot be controlled to accommodate tissue-specific needs, nor can the course of the morphogenetic processes be manipulated. Thus, the time is ripe to move stem-cell derived organoids into designer ECMs, with compositions that can be tailored to reflect organ-to-organ microenvironmental variations. One could imagine taking advantage of such control to direct stem cell fate decisions within organoids during their formation, thus steering the overall development of the organoid at will.

Over the past several years, we and others have demonstrated that incorporating essential signals of native ECMs into synthetic polymer matrices renders these otherwise bioinert environments permissive to biological processes (e.g. Lutolf and Hubbell, 2005; Nguyen and West, 2002; Saha et al., 2007; Tibbitt and Anseth, 2012) (Table 1). For example, cross-linking poly(ethylene glycol) (PEG) (see Glossary, Box 1) with cell-signaling components and oligopeptides that are susceptible to proteolytic degradation endows the resulting 3D scaffold with biofunctionality, making it permissive to cell migration, proliferation and tissue morphogenesis and regeneration in vivo (Lutolf and Hubbell, 2003; Lutolf et al., 2003b). Aside from chemical or enzymatic cross-linking, self-assembly of peptides that are both hydrophilic and lipophilic in aqueous media has been used to form an intriguing class of synthetic biomaterials (Luo and Zhang, 2012;

Table 1. (Semi-)synthetic hydrogel matrices for 3D cell culture

Class of materials	Description	Key advantages	Key limitations	References
Natural biomolecule-polymer 'hybrids'	Comprise natural proteins and polysaccharides (e.g. hyaluronic acid, fibrinogen, albumin, heparin) crosslinked with a synthetic polymer or smaller crosslinker	Combined benefit provided by biomolecules (e.g. adhesiveness, sequestration of soluble growth factors) and polymer backbone (control over structure and mechanical properties)	High molecular weight proteins and polysaccharides can introduce complexity and variability	(Shu et al., 2004; Almany and Seliktar, 2005; Marklein and Burdick, 2010)
Self-assembling hydrogels	Nanostructured hydrogels formed by weak interactions between small building blocks, such as amphiphilic peptides	Mimics fibrillar nature and complex physical behavior of native ECM gels. Can present adhesive ligands at a high concentration.	Provides a more narrow range of mechanical properties and types of incorporated molecular signals	(Silva et al., 2004; Gelain et al., 2006; Dankers et al., 2005; Jayawarna et al., 2006)
'Blank-slate' polymer hydrogels	Formed by crosslinking of synthetic polymers [e.g. poly(ethylene glycol), polyacrylamide, poly(hydroxyethyl methacrylate), poly(N-isopropylacrylamide-co-acrylic acid)]	Modular, versatile and fully chemically defined. Mechanical properties can be tightly controlled across a wide range. Biofunctionalities (e.g. proteolytic degradability, adhesiveness, soluble proteins and cell-cell interaction proteins) are readily incorporated. Spatiotemporal control of mechanics and tethered signals by photopatterning.	Uniform structure and simple mechanical behavior do not approximate the porous, fibrillar structure of the natural ECM and its complex mechanics	(Mann et al., 2001; Lutolf et al., 2003a; Kim et al., 2005; Peyton et al., 2006; Kloxin et al., 2009; Phelps et al., 2012; Tsurkan et al., 2013; Wylie et al., 2011)

Zhang et al., 1993). Adding adhesive or proteolytically cleavable sites found in natural matrices endows these materials with additional biofunctionality, allowing cells to interact with and remodel them (Kisiday et al., 2002; Silva et al., 2004). Indeed, self-assembling peptide systems have been shown to enhance EB formation and neuronal differentiation (Li et al., 2012, 2013; Silva et al., 2004).

In addition to mimicking the various functionalities of native ECMs, these approaches can be used to deconstruct the physiological complexity of native environments and define their key instructive features. The biochemical and biophysical properties of these synthetic ECM analogs can be modulated across a wide range of variables and nearly independently of each other, which offers a powerful platform to probe systematically the influence of individual or combinations of ECM signals on basic cellular and morphogenetic processes (Fig. 1). To expand the number of different variables that can be analyzed using these combinatorial microenvironments, they can be interfaced with existing high-throughput approaches in bioengineering and cell biology (Ranga and Lutolf, 2012).

High-throughput approaches to define the key extrinsic regulators of organogenesis

Defining the microenvironmental components that regulate organogenesis, as well as their modes of action, requires rigorous multivariate, systems-level analyses. Owing to their poorly defined, highly complex and variable composition (Hughes et al., 2010; Vukicevic et al., 1992), Matrigel-rich extracellular environments are unsuitable for performing such analyses. The synthetic matrices introduced above, by contrast, offer a physically and chemically defined 'blank canvas', which can then be decorated with microenvironmental signals in a modular and controlled fashion, and used to define their effects on stem cell-derived organoid formation.

To this end, motivated by an interest in breaking down the combinatorial complexity of stem cell niches and cancer microenvironments, the bioengineering community has borrowed technologies from the DNA microarray fabrication field to create 'cellular microarrays'. Here, various microenvironmental signals, including ECM proteins, cell-cell interaction proteins and soluble cues, are tethered individually or in defined combinations at discrete spots, thus generating arrays of 'artificial niches' (Ranga and Lutolf, 2012) (Fig. 1). The response of stem cells to the various members of the combinatorial library is then recorded in a high-throughput manner, allowing researchers to draw links between particular signals or groups thereof and specific cell behaviors (Flaim et al., 2005; LaBarge et al., 2009; Soen et al., 2006). To move the cells away from the stiff glass slides and tissue culture plastic to more physiologically appropriate substrata, high-throughput screening approaches have been used to identify synthetic polymers for the expansion of embryonic or induced pluripotent stem cells (Anderson et al., 2004; Brafman et al., 2010). Our group investigated the influence of mechanics, cell-cell interactions and cell-ECM interactions in regulating stem cell behavior by using an indirect spotting technique to generate protein arrays at the surface of PEG hydrogels with controllable mechanical properties (Gobaa et al., 2011) (Fig. 1). The systems-level analysis afforded by this platform unveiled a synergistic interaction between biophysical and biochemical factors in the regulation of mesenchymal and neural stem cell fate. Whereas these two-dimensional cellular microarrays have been highly informative, they are not suitable for dissecting the extrinsic regulators of morphogenesis and organogenesis, which are inherently 3D processes. Nonetheless, the underlying technologies can be merged with the 3D artificial ECMs discussed above to create arrays of 3D combinatorial microenvironments, which can then be used to both introduce and study the complex external signals that may currently be lacking in stem-cell derived organoid culture.

The potential benefits of subjecting stem cell-driven organoid formation to a multi-parameter analysis within a synthetic environment are multifold. First, a comprehensive and dynamic screen would identify the precise components through which the microenvironment, including the ECM, confers its influence on organoid formation, and link particular cues to stage-specific cellular behaviors. The systems-level aspect would be especially useful in uncovering synergistic or antagonistic interactions between individual components, which may not be unraveled by classic approaches. Second, combining the
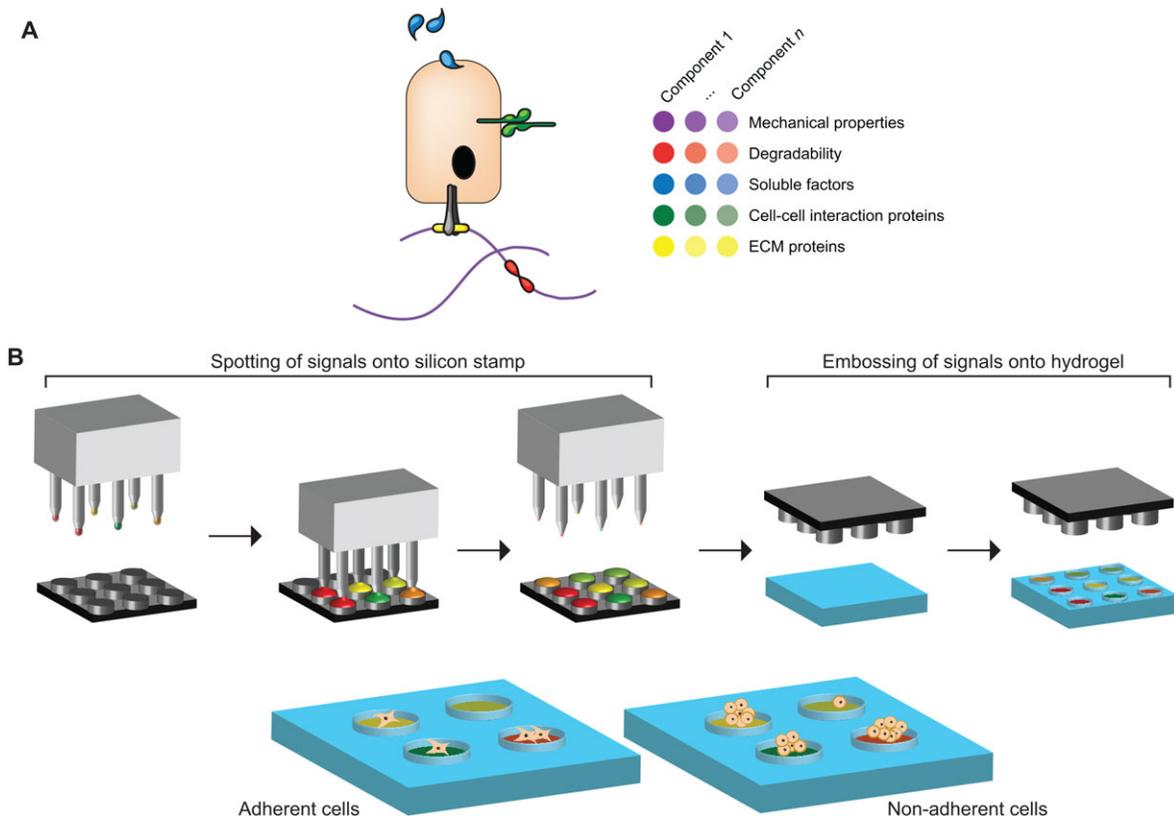
Fig. 1. Bioengineering tailored cell microenvironments. (A) The cellular microenvironment can be modulated and controlled when contained within a synthetic matrix. Key aspects amenable to manipulation include mechanical properties, degradability, ECM proteins, cell-cell interaction proteins and soluble factors. Further manipulation of the individual components of each of these can allow for a theoretically infinite variability in the cell microenvironment. (B) Example of a state-of-the-art technology used to create combinatorial microarrays of biophysical and biochemical signals. The microfabrication process involves spotting of the desired signals onto the posts of a rigid silicon stamp which is then used to emboss a partially polymerized hydrogel. The resulting microwell array presents distinct combinations of signals to adherent or non-adherent cells. Modified from Gobaa et al. (Gobaa et al., 2011) with permission from the Nature Publishing Group.

stimulatory cues identified by the screen into a single hydrogel would afford custom-designed, organ-specific synthetic matrices, which can be used to study morphogenesis *ex vivo* in a highly defined and reproducible environment. Importantly, aside from their value in investigating fundamental developmental phenomena, such matrices hold translational promise: stem cell-derived organoids are considered feasible sources of stem cells for use in regenerative therapy (Sasai, 2013; Sato and Clevers, 2013). Indeed, intestinal organoids generated from single Lgr5^{+} stem cells have been transplanted into the mouse colon, where they efficiently restored epithelial architecture and function (Yui et al., 2012). However, current protocols for the generation and expansion of self-assembling tissues rely upon Matrigel, which is murine-derived, potentially immunogenic and, as such, unsuitable for transplantation in human patients. Synthetic matrices, by contrast, are inherently chemically defined and thus can be rendered clinical grade, which, along with their highly pliable physical properties, makes them ideal candidates for cell-based therapies in humans.

Bioengineering approaches to guide self-organization

A major difference between organogenesis *in vivo* and *in vitro* lies in the mode by which signals are presented to cells. Whereas the native microenvironment delivers cues with a high degree of spatiotemporal control, traditional 3D culture floods cells with biochemical and biophysical signals that are uniform in space and static in time. Advances in biomaterials technologies that mimic the

spatiotemporal complexity of the *in vivo* microenvironment can potentially be used to reconcile these differences and to modulate tissue formation with a greater degree of control than previously achieved.

3D spatial and temporal patterning of mechanics

The mechanical nature of morphogenesis and organogenesis is well-established (Nelson and Gleghorn, 2012). The mechanical properties of the microenvironment, as well as active mechanical influences, for example mechanical strains and stresses, regulate a range of basic cellular phenomena, including cell proliferation, apoptosis, epithelial-to-mesenchymal transition and stem cell fate decisions (Chen et al., 1997; Gilbert et al., 2010; Lee et al., 2012; Nelson et al., 2005). Importantly, different organs (Engler et al., 2006) and even separate tissue components within the same organ (Lopez et al., 2011) are characterized by distinct mechanical properties, and this mechanical modularity may serve to pattern cellular behaviors, giving rise to the regional differences that ultimately drive morphogenesis. In fact, individual organs almost never develop in isolation, but rather concurrently with surrounding tissues and organs, which mechanically confine, impinge upon or pull on them. These mechanical influences originating from surrounding tissues, which are notably absent in organoid culture, have been postulated to affect the development of the optic cup (Sasai et al., 2012), the intestine (Shyer et al., 2013) and, very recently, the entire early mouse embryo (Hiramatsu et al., 2013).

Modulating the mechanical properties of traditional, collagen- or Matrigel-based matrices leads to concomitant changes in the availability of ECM adhesion sites with which cells can interact via surface receptors, and directly affects adhesion-based signaling. Hence, the role of the mechanical environment per se in 3D processes has been difficult to ascertain in culture. Synthetic ECM analogs have afforded the possibility of varying the mechanics of the matrix, while maintaining a constant concentration of adhesive proteins (Ehrbar et al., 2011; Gill et al., 2012). Moreover, recent advances in light-mediated patterning (see Glossary, Box 1) have enabled researchers to manipulate the physical properties of the ECM with micrometer resolution, thus recapitulating mechanical non-uniformities observed *in vivo*. In a 'subtractive' strategy, 3D hydrogels are formed by crosslinking PEG precursors conjugated with photolabile chemical groups (see Glossary, Box 1) (Kloxin et al., 2009). Shining light on the desired region results in cleavage of the photo-sensitive moiety (see Glossary, Box 1) and local softening of the matrix (Fig. 2A). Conversely, light shone on a specific region within the gel in the presence of a photoinitiator (see Glossary, Box 1) can trigger additional crosslinking, resulting in local stiffening (Guvendiren and Burdick, 2012). Stiffness gradients have also been achieved using specially designed photomasks to control spatially the light exposure experienced by UV-crosslinked materials. This approach has been used to reconstitute natural stiffness variations present in normal or infarcted myocardial tissue, and to investigate mesenchymal stem cell durotaxis in culture (Tse and Engler, 2011; Vincent et al., 2013).

Importantly, a key feature of these platforms is that they allow not only spatial but also temporal control of matrix mechanics, and, as such, can be used to dynamically tune the mechanical properties of the microenvironment (Lim et al., 2011). For example, manipulating the stiffness of discrete matrix regions surrounding an organoid could be used to mimic expansive growth of neighboring tissues and consequent mechanical confinement.

3D spatial and temporal patterning of biochemical signals

Aside from patterned mechanical cues, developing tissues and organs receive and interpret a range of biochemical signals that are likewise controlled in space and time. Both retinal and intestinal development *in vivo* proceed amidst complex spatiotemporal fields of extracellular signals, including Wnts, BMPs, Shh and their various antagonists. For example, localized Wnt signals, which emanate from retinal and non-retinal sources alike, direct retinal differentiation towards the RPE fate *in vivo* (Fuhrmann, 2010; Fujimura et al., 2009), whereas lens-derived soluble cues, including FGFs, are thought to provide the positional information necessary for neural retina development (Martinez-Morales et al., 2005). In the intestine, the restriction of Wnt signaling to the crypt regions (Gregorieff et al., 2005) is crucial for stem cell maintenance and proliferation (Pinto et al., 2003). Localized expression of Shh is required for the formation of villi and their demarcation from adjacent crypts (Madison et al., 2005). Crypt-villus separation is reinforced by BMP signaling, which, owing to the crypt-specific expression of the BMP antagonist noggin, is confined
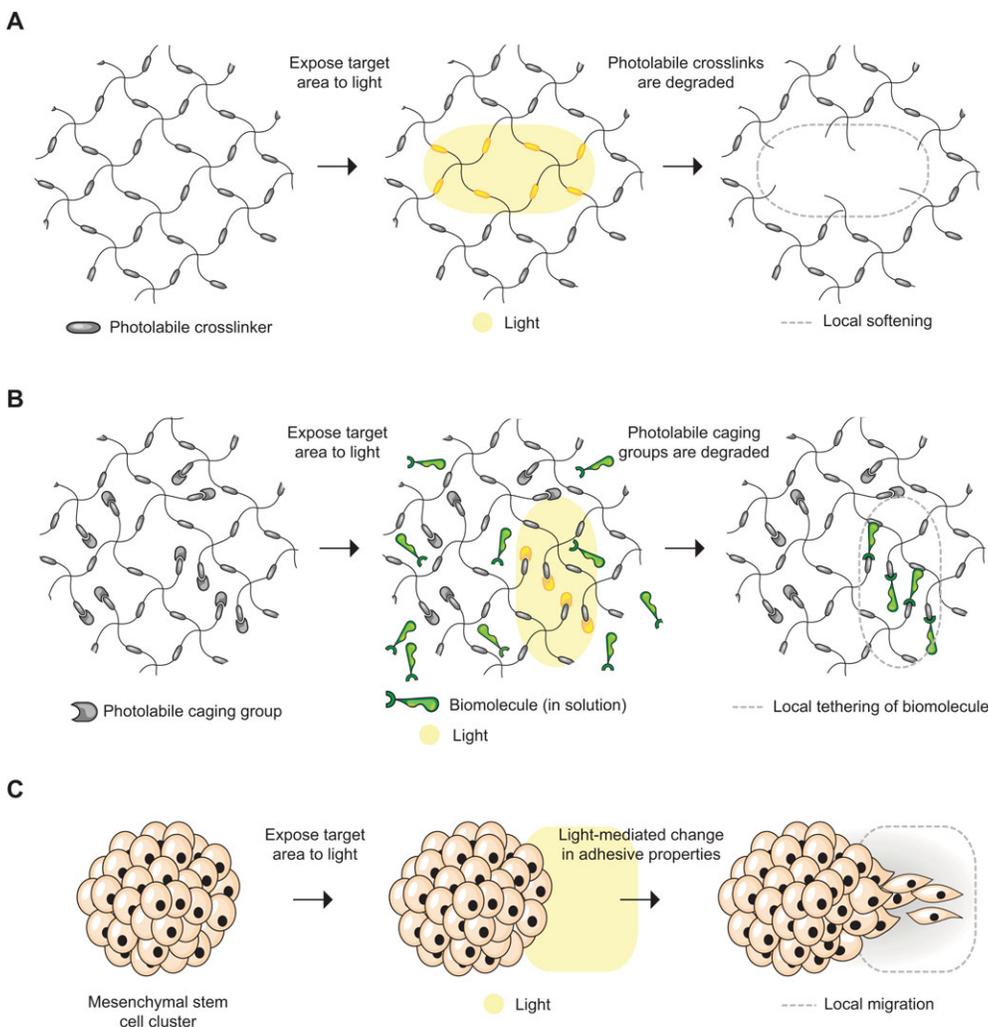
Fig. 2. Concept of light-controlled patterning of mechanical and molecular signals within 3D matrices. (A) Shining light on specific regions of a hydrogel held together by photo-sensitive crosslinks results in local degradation and softening of the matrix. (B) A hydrogel can contain biomolecule-binding sites initially protected by a photo-sensitive caging group. Exposing specific regions of the gel to light leads to local uncaging of the binding sites and patterned immobilization of a biomolecule. (C) For example, light-mediated patterning of adhesive peptides can be used to spatially orient the migration of mesenchymal stem cells within a 3D matrix.

A

Photolabile crosslinker | Expose target area to light | Light | Photolabile crosslinks are degraded | Local softening

B

Photolabile caging group | Expose target area to light | Light | Photolabile caging groups are degraded | Biomolecule (in solution) | Local tethering of biomolecule

C

Mesenchymal stem cell cluster | Expose target area to light | Light | Light-mediated change in adhesive properties | Local migration

to the villus regions (Haramis et al., 2004). All of these signaling events are subject to tight regulatory control in space and time in the developing intestinal tissue. By contrast, intestinal organoid culture uniformly floods cells with soluble cues, including noggin, thus probably removing the positional information necessary for proper organization of the crypt-villus system. The absence of villus structures in culture is thus unsurprising. We believe that recent sophisticated biomaterials approaches could be used to circumvent these issues and to present biochemical cues with greater spatial and temporal precision.

We recently introduced a platform for the rapid generation of protein gradients of arbitrary size and shape tethered on the surface of soft hydrogels using hydrodynamic flow focusing and bioconjugation strategies (see Glossary, Box 1) (Cosson et al., 2013). We used this technique to explore how gradients of leukemia inhibitory factor (LIF) influence ESC behavior. However, in their current form, these immobilized protein gradients are formed before the introduction of a cellular component, and are hence static. Furthermore, the gradient is effectively two-dimensional, because it is confined to the surface of the gel. Gel microfluidic approaches, which are becoming ever more sophisticated, promise to deliver soluble signals dynamically and in three dimensions (Choi et al., 2007a; Zheng et al., 2012). Here, soft lithography (see Glossary, Box 1), for example, used to form channels within alginate or type I collagen gels, and the channels are subsequently perfused with a concentrated solution of a given biomolecule. The biomolecule diffuses away from its source into the permeable hydrogel, forming a gradient, the spatial and temporal profile of which can be controlled by adjusting the flow rate of the solute within the channels. Interfacing these types of techniques with chemically and physically versatile synthetic matrices would afford additional control over the cellular microenvironment. Light-mediated patterning approaches provide yet finer control over the spatial and temporal distribution of signals available to cells by tethering biomolecules at a desired location and at a desired time within 3D matrices (DeForest and Anseth, 2012; Wylie et al., 2011). We recently described a technique whereby highly localized bio-tethering of specific molecules within a PEG hydrogel can be used to control cell migration. In this study, the peptide substrate of active transglutaminase factor XIII (FXIIIa) was rendered photo-responsive by masking its active site with a photo-degradable moiety, and this photo-responsive domain was incorporated within a cell-containing 3D hydrogel. Shining light on a desired region within the gel at a desired time lead to local uncaging of the FXIIIa substrate and site-specific tethering of a biomolecule of interest (Fig. 2B). We used this platform to show that the migration of human mesenchymal stem cells can be controlled in space and time (Fig. 2C), by manipulating the distribution of ECM proteins and growth factors within the matrix (Mosiewicz et al., 2013).

The ability to sculpt the biophysical and biochemical microenvironment with spatial and temporal control may help increase the faithfulness of stem cell-derived organoids to real organs, and open up the possibility to guide morphogenesis and organogenesis in culture. As one example of the latter, the appearance of crypt-like buds could be induced at specific positions within intestinal organoids using the strategies described above. Organoids derived from single ISCs are initially spherical and symmetrical (Sato et al., 2009). The stochastic appearance of a Paneth cell is considered to be the symmetry-breaking event that ultimately transforms the cystic structure into a complex organoid. More specifically, Paneth cell-derived focal Wnt and Notch sources are thought to transform non-differentiated adjacent cells into Lgr5^{+}

ISCs, which then push themselves outwards into crypt-like invaginations (Sato and Clevers, 2013). Instead of relying on chance, it is possible that Wnt and Notch signals may be delivered focally using microfluidics or light-mediated patterning, to attempt to instruct crypt formation at a desired location. Such stereotyped and extrinsically controlled crypt formation would both rigorously test the aforementioned symmetry-breaking hypothesis and also allow a closer look at the appearance and maintenance of the stem cell niche within the intestine.

Establishing and assessing function: next-generation organs-on-a-chip

Organoids formed by self-organizing stem cells promise to revolutionize the study of organogenesis, and offer highly relevant and patient-specific disease models, drug-screening platforms and sources of cells for transplantation therapies. The extent to which these tissues contribute at the basic or translational level vitally depends on how closely they approximate real organs, and this fidelity has thus far been evaluated based upon the presence of specific types of cells, arranged into the correct 3D architectures. Whereas the need for confirming cellular and histological aspects is clear, real tissues are more than the requisite cell types and multicellular shapes. Above all, they serve a particular physiological function: the intestine absorbs nutrients, and the brain deploys and interprets electrochemical signals to provide centralized control over the body. Quantifying these organ-specific functions within stem cell-derived organoids is not straightforward, owing mainly to their isolation from the exterior. For example, determining the barrier function and paracellular permeability of the intestinal epithelium requires physical access to the lumen for perfusion of chemicals, placement of electrodes and so on. However, given the current closed spheroid structure of the mini-guts, the lumen is only accessible by microinjections, which are tedious, imprecise and poorly reproducible. Likewise, the poor perfusability of the cerebral organoids both limits growth, owing to a shortage in oxygen and nutrients, and hampers electrophysiological studies.

A recent number of microengineering studies have developed biomimetic microsystems termed 'organs-on-a-chip', which aim to recreate the minimal functional units of living organs by replicating key structural and physiological features. Miniaturized and reductionist models of several organs, including the lung (Huh et al., 2010), the liver (Nakao et al., 2011), the kidney (Jang and Suh, 2010) and the gut (Kim et al., 2012), have thus far been created. For example, the 'human gut-on-a-chip' comprises two microfluidic channels, separated by a porous membrane, which is coated with ECM proteins and lined with a human intestinal epithelial cell line (Fig. 3). The microchannels permit perfusion at low flow rates, matched to those observed *in vivo*. Furthermore, physiological peristaltic contractions are mimicked by exerting exogenous cyclic strain, and symbiotic intestinal bacteria are cultured on the luminal side of the epithelium. The physical accessibility to both the luminal and basal epithelial surface enable the assessment of intestinal barrier function and permeability. Of note, incorporating a peristaltic component enhanced the paracellular permeability of the epithelium, and led to the formation of folds and creases, resembling rudimentary villous structures (Kim et al., 2012). These results appear to be in line with a recent report suggesting that mechanical contractions produced by the outer layer of smooth muscle actin-expressing cells are required for villi formation in the mouse intestine (Shyer et al., 2013). Translating the mechanical stimulation technology used in the 'gut-on-a-chip' to the mini-gut and testing whether it may potentially lead to villus formation within this system would certainly be exciting.
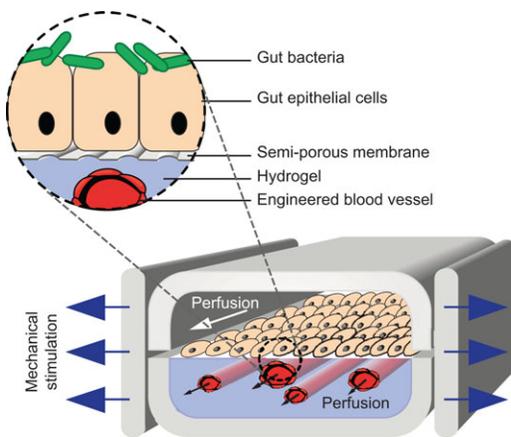
Fig. 3. Concept of a gut-on-a-chip. Aspects of the human intestine can be mimicked by culturing intestinal epithelial cells on a semi-porous membrane between two chambers representing the intestinal lumen and the sub-epithelial mesenchyme. Perfusion of both chambers enables functional measurements, including nutrient and drug absorption and trans-epithelial permeability. Hydrogels containing engineered blood vessels can be incorporated in the lower chambers to mimic the epithelial-vascular interface, although this can also be mimicked using microfluidic technologies to minimize biological variables (not shown). Additional biological components present in the human gut *in vivo* can also be incorporated, such as the bacterial microflora. Attached side-chambers can be used to impose cycles of mechanical stretch to mimic physiological peristaltic contractions.

These microengineering approaches can also be used to side-step the vascularization and nutrient delivery problem that constrains the growth of cerebral organoids. Although strategies to build vascular networks in culture appeared more than three decades ago (Folkman and Haudenschild, 1980) and are becoming ever more sophisticated (Arnaoutova and Kleinman, 2010; Ghajar et al., 2008; Miller et al., 2012; Zheng et al., 2012), an endothelial cellular component introduced into the system would bring along its own set of biochemical and mechanical parameters, thus undermining our attempts to establish a well-defined and controlled microenvironment. Instead, microfluidic features can be included to mimic the essential function of blood vessels: delivery of oxygen and nutrients, and removal of waste. In fact, this technology was initially developed for similar reasons – enhancing the core viability of thick brain slices classically used in neuroscience research (Huang et al., 2012). In one example, photolithography-based microfabrication (see Glossary, Box 1) was employed to make arrays of silicon microneedles (Choi et al., 2007b) or microposts (Rajaraman et al., 2007). While simultaneously physically anchoring the tissue, these micro-devices create direct tunnels for oxygen- and nutrient-rich solutions to be delivered to the inner layers of the slice. These technologies are readily transferable to cerebral organoid culture, where, aside from enhancing growth and viability, they can also be advantageous in conducting functional studies.

Perspectives and conclusions

Our decades-long attempts to recreate morphogenesis *ex vivo* have been motivated by interests in both examining the process itself more closely, and by the potential application of the final product, whether for drug screening or for tissue regeneration in the clinic. Over the past five years, it has become clear that harnessing the pluri- or multipotency and self-organizing potential of stem cells is an unmatched approach for reproducing the histological and architectural complexity seen *in vivo*. However, the self-assembling nature of such systems, despite being one of their greatest advantages,

is also one of their greatest drawbacks: we currently have little control over the course of the morphogenetic process and the resulting organoid. In addition, organoid formation currently occurs in complex, animal-derived matrices, which are largely chemically and physically opaque. Thus, we have not fully surpassed the main limitations of *in vivo* models: complexity and lack of control.

Despite these limitations, stem cell-derived organoids recreate the complexities of morphogenesis to an extent unmatched by any other *in vitro* approach, although they currently fall short of replicating full physiological function. Organs-on-a-chip approaches, by contrast, have had a great deal of success in establishing organ-level function *ex vivo*, but suffer from an overly simplistic cellular component, typically a monolayer of immortalized cells, and hence approximate aspects of the adult rather than the developing tissue. We propose that combining the exquisite self-organizing potential of stem cells with the physical accessibility afforded by micro-engineering approaches could give rise to a new generation of histologically and functionally more realistic organs-on-a-chip. The interface with the exterior would facilitate the exchange of nutrients and waste, as well as the delivery of biomolecules and pharmacological agents, thus improving growth efficiency and making the tissues amenable to both developmental and physiological studies.

It is important to note that merging stem cell-derived organoids with engineered platforms comes with a set of challenges that must be overcome in order to take full advantage of the combined systems. For example, whereas image-based and immunohistochemical analyses are readily performed *in situ*, cell retrieval for rigorous downstream characterization or further culture is difficult and represents a major drawback of organs-on-a-chip models. In the past, micromanipulation (Jin et al., 2009) and laser microdissection (Revzin et al., 2005) have been used to circumvent this problem, but these approaches are not conducive to automated, parallelized and high-throughput analyses. The recently described inverted microwells, wherein cells are cultured at the air-liquid interface and released on-demand pneumatically (Bocchi et al., 2012; Faenza et al., 2013) are promising alternatives.

Furthermore, whereas synthetic matrices have been used extensively to culture endothelial and various types of mesenchymal adult or stem cells, success in culturing multicellular polarized epithelia is lower by comparison. This problem may be attributed to the reductionist nature of synthetic ECMs, which differ from native matrices in several structural, biophysical and adhesive features. For example, the native ECM has a fibrillar and porous structure, which is highly permissive to cellular movement, and gives rise to complex, emergent physical behavior thought to influence biological processes such as morphogenesis (Brownfield et al., 2013; Gjorevski and Nelson, 2012; Guo et al., 2012). By contrast, polymer-based synthetic matrices are typically uniform in structure. Nonetheless, efforts are underway to capture both the porous (Viswanathan et al., 2012) and fibrillar (Prabhakaran et al., 2009; Silva et al., 2004) aspects of natural ECMs within synthetic materials.

Reconstituting basement membrane-derived adhesive cues within synthetic materials is another key step towards maintaining polarized and functional epithelia, and for this laminin-based adhesion is particularly important (Bissell and Bilder, 2003; Bryant and Mostov, 2008). Although individual peptides typically originating from the α1 laminin chain have been incorporated in synthetic matrices (Silva et al., 2004; Yamada et al., 2011), the field has failed to identify a single short sequence that approximates the full length protein to the same extent as the RGD epitope (a tripeptide composed of arginine, glycine and aspartic acid), for example, mimics adhesion to fibronectin. It is likely that simultaneous engagement with multiple sequences from the different laminin chains is necessary to

recapitulate adhesive interactions with the basement membrane, and it is also possible that the combination is cell type and context dependent. The 3D high-throughput combinatorial screening methods discussed earlier may help identify the minimal set of laminin-derived adhesive motifs and build a 'synthetic basement membrane'.

In summary, we believe that by combining self-organizing stem cells with the biomaterials and microtechnology approaches discussed in this Review it will be possible to increase the fidelity with which *in vitro*-derived organoids replicate their corresponding native organs, while also enabling additional control over the process. Instead of uniformly exposing tissues to the hundreds of signals present in a poorly controlled naturally derived 3D matrix, many of which may be unnecessary or even inhibitory, we could use high-throughput screening to identify the minimal microenvironmental cues that govern morphogenesis and combine them into a highly defined, reproducible and tissue-specific synthetic matrix. Additionally, microfluidics and light-mediated patterning can be used to spatially and temporally fine-tune the chemical and physical environment of the tissues, and technologies borrowed from the organs-on-a-chip field can help interface them with the exterior to establish and assess physiological function.

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