Towards Organogenesis and Morphogenesis In Vitro: Harnessing Engineered Microenvironment and Autonomous Behaviors of Pluripotent Stem Cells

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Insight Box

Stem cells derived organoids significantly advance the modeling of development and diseases, while the consistency and robustness of current organoids biofabrication need to be improved. This review article highlights recent advances in the applications of bioengineering tools and biomaterials for reproducible fabrication of organoids from stem cells. These emerging approaches may revolutionarily change the way organoids are produced and provide customized functionalities for addressing specific biological questions. We call for more research in this area to fully leverage the achievements in bioengineering to take the organoids research to the next level.

Towards Organogenesis and Morphogenesis In Vitro: Harnessing Engineered Microenvironment and Autonomous Behaviors of Pluripotent Stem Cells

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ABSTRACT

Recently, researchers have been attempting to control pluripotent stem cell fate or generate self-organized tissues from stem cells. Advances in bioengineering enable generation of organotypic structures, which capture the cellular components, spatial cell organization and even some functions of tissues or organs in development. However, only a few engineering tools have been utilized to regulate the formation and organization of spatially complex tissues derived from stem cells. Here, we provide a review of recent progress in the culture of organotypic structures *in vitro*, focusing on how microengineering approaches including geometric confinement, extracellular matrix (ECM) properties modulation, spatially controlled biochemical factors, and external forces, can be utilized to generate organotypic structures. Moreover, we will discuss potential technologies that can be applied to further control both soluble and insoluble factors spatiotemporally *in vitro*.

In summary, advanced engineered approaches have a great promise in generating miniaturized tissues and organs in a reproducible fashion, facilitating the cellular and molecular understanding of embryogenesis and morphogenesis processes.

1 INTRODUCTION

Recent advances in stem cells engineering have enabled generation of organoids in *in vitro* systems. These organoids are 3D structures formed by cells *in vitro* that can capture the cellular components, spatial cell organization and some functions of organs [1]. They demonstrated great promises as human-specific models for diseases that are lack of animal models (*e.g.*, infectious diseases) [2-3]. As organoids are high-throughput and cost-effective, they can also be applied to anti-cancer drug sensitivity testing [4-5], toxicology [6], and personalized medicine [7]. Moreover, organoids are ideal systems for investigation of the embryogenesis mechanism in cellular and molecular levels, which will potentially contribute to the discovery of treatments for birth defects. For the case of human embryogenesis, the culture of human embryos *in vitro* beyond 14 days, the onset of the primitive streak, is currently not only technically challenging but also generally considered unethical around the world [8-10]. Therefore, organoids derived from human pluripotent stem cells (PSCs) provide a perfect alternative to study early-stage development events such as gastrulation and neurulation. Of note, miniaturized tissues derived from primary or progenitor cells is sometimes referred as organoids. This review, however, will only focus on organoids and other organotypic structures developed from PSCs.

Conventionally, organoids are generated from aggregates of stem cells. These aggregates, termed embryoid bodies (EBs), are usually spherical and derived by suspending stem cells in growth medium or Matrigel. Soluble biochemical factors are sequentially introduced to the growth medium at different time points to guide stem cell differentiation. Using this method, organoids resembling various organs, including livers [11], intestines [12], kidneys [6, 13], hearts [14], retina [15] and brains [16-18], have been successfully derived from human stem cells. However, cells in the suspended EBs are usually exposed to micro-environments that are vastly different from those *in*

vivo, where the chemical and mechanical properties of stem cell niches are spatiotemporally controlled by surrounding tissues, ECM, and fluids [19-20]. The EB-based organoids culture system provides limited controls over those factors and relies on the self-organization of stem cells. As a result, such organoids culture models, hereafter referred to as self-organized development models, often lead to organoids that are inconsistent batch-to-batch and show structural discrepancies with developing embryos.

More recently, a few groups, including ours, are developing new strategies to combine advanced micro/nanotechnologies and tissue engineering approaches with embryonic stem cells (ESCs) to model development. Due to improved controls of soluble and insoluble cues in the stem cell microenvironment, these engineered organotypic models can robustly recapitulate certain stages of development in a high-throughput fashion. In this review, we will first briefly discuss the techniques used in self-organized development models and existing challenges in organotypic cultures engineering. We will then highlight recent progress in engineered organotypic culture models. Finally, we will provide our perspectives on the potentials of several existing tools to further advance engineered organotypic culture.

2 SELF-ORGANIZED DEVELOPMENT MODELS

Organoids have been conventionally generated without engineered microenvironments, but by leveraging the capabilities of stem cells to differentiate, migrate, orient and arrange into structures mimicking organs under minimal guidance. Experimentally, stem cell colonies or aggregates were cultured in suspension, in some cases within bioreactors, and their fates were controlled by biochemical factors introduced to the culture medium at different time points (**Fig. 1a**). The mechanism and results of these studies have already been reviewed in detail in other

works [1, 19, 21]. In this review, we will focus on the methods and techniques used in these studies, as well as controllable factors or lack thereof.

In adherent monoculture of mouse and human PSCs, it has been observed that under the neural differentiation condition, they simultaneously aligned radially to form circular structures, the so-called neural rosettes (**Fig. 1b**) [22]. Although not defined as organoids, these rosettes resemble the cross-sectional cell arrangement of a neural tube and have been considered to be an *in vitro* model to study neural tube development. However, the 2D structure of such neural rosette lacks the complexity and degree of freedom regarding cell movement and rotation compared to 3D cell structures *in vivo*.

In recent years, numerous studies have been dedicated to the formation of organoids in 3D by using EBs and aggregates of stem cells. In some cases, EB sizes were roughly controlled by seeding fixed number of cells in 96-well plates with V-shaped conical bottom [18, 23-24]. More precise size control of EBs was also achieved by using micro-fabricated wells[25-26], and forced aggregation combined with rotary orbital suspension culture[27]. Matrigel was also used in some of these studies, encapsulating the cell aggregates to mimic the chemical and mechanical environment of ECM. Similar to 2D culture, the fate of the stem cell is controlled by addition of biochemical factors homogeneously to growth media at different growth stages. Using this method, researchers have successfully generated 3D structures consist of multiple cell types, mimicking the cell components of several tissues *in vivo*, including but not limited to neural retina [15, 24], neural tube [28] and hippocampus [16]. In addition to more closely mimicking the *in vivo* environment, 3D culture also produces a larger number of cells compared to 2D culture, increasing its efficiency.

Occasionally, organoids generated using such method resemble not merely cell components, but also, more importantly, the shapes, structures, and patterns of organs *in vivo*. For example, in the works of Sasai and colleagues, optic cups, a stage during eye formation, were successfully fabricated *in vitro* from suspension of mouse ESCs [24] and human ESCs [15] EBs. Directed by the retinal differentiation inducing chemical environment, suspended EBs (*diameter* ~ 400 μm) simultaneously turned into hollow spheres, where retinal anlage cells and non-retinal cells simultaneously segregated. The retinal region of the EB subsequently invaginated to form a two-wall cup-like structure, where neural retina cells were found inside the cup, and cells forming the outer shell were found to be resembling retinal pigment epithelium, recapitulating the cell component and arrangement of the optical cups *in vivo* (**Fig. 1c**) [24].

While such 3D culture method is promising for modeling optical cups *in vitro*, it may not be as ideal in the fabrication of other organs. Organoids resembling the dorsal-ventral polarized structure of neural tube have been realized in self-organized development models. Suspended in Matrigel[28] or synthetic gel with controllable stiffness and chemical compositions [29], mouse ESCs were induced to differentiate into NE (neuroepithelial) cells and self-organized into spherical or ellipsoidal NE cysts. Inside such NE cysts, the NE cells polarized apicobasally, and aligned radially, forming a single lumen at the center. Subsequently, cells within the NE cyst polarized dorsoventrally, where the cross-sectional views of such cysts resembled the ones of neural tubes (**Fig. 1d**). However, the formation process of such NE cysts does not resemble that of a neural tube *in vivo*, which originates from folding of a planar neural plate [30]. Although patterned neural tube-like spherical structures can be made from this system, it is not ideal for studying the processes of neural tube folding and closure. In addition, another study showed that the diversity

of cell components in brains could be found in self-organized brain organoids. However, the tissue structures of these brain organoids are still far away from those *in vivo*[18].

In summary, self-organized development models offer a promising platform for harnessing stem cells to recapitulate many important features of developing tissues, although gaps between *in vitro* and *in vivo* remain. Such models only allow control over the timing of biochemical factors introduction. For most parts, it relies on autonomous cell differentiation, segregation and morphology change in relatively homogenous natural or synthetic matrices. For different tissues, the microenvironment *in vivo* can be entirely different, and it has been shown that various factors including ECM stiffness, cell shapes, colony size, substrate topography and local biochemical factors gradients are crucial for stem cell fates. The lack of control over these factors makes culturing certain types of tissues difficult, and outcomes from different batches inconsistent. For example, in the work by Ranga *et al.*, only 40% of neural cyst successfully polarized apicobasally when cultured in Matrigel. The percentage of cyst polarized increased to 70% when the stiffness and chemical composition of the gel was optimized[29]. Therefore, we reason that, in addition to existing organotypic cultures generation methods, more complex structures can be generated consistently if the surrounding microenvironment can be precisely controlled. Some pioneer works in engineered microenvironment will be discussed in the next section.

3 ENGINEERED PLATFORM FOR ORGANOTYPIC CULTURE

Although various approaches have long been used to engineer the microenvironment of stem cells to modulate their self-renewal or differentiation towards a specific lineage [31-35], it is until very recently that these tools are applied to generate organotypic 2D or 3D structures. In this section, we will discuss how engineering approaches are utilized to modulate EB size and shape, ECM properties, and tissue geometry to generate organotypic cultures.

3.1 EB size and shape

Inside EBs, the diffusive behaviors and concentration profiles of both biochemical factors from growth media and molecules secreted by cells are regulated by EB shapes and sizes [36]. Since stem cell fate greatly depends on concentrations of such substances, control over EB morphology is a crucial step towards efficient and consistent organoid generation.

As mentioned earlier, EB radius can be adjusted by numbers of cells seeded in a V-shaped conical bottomed well as well as micro-wells. However, merely controlling the radius of spherical EBs is not sufficient to promote the efficiency of organoids fabrication. In a recent work by Lancaster *et al.*, the authors discovered that, in the process of cerebral organoids generation, the high surface-to-volume ratio of the tissue is required for efficient formation of polarized neural ectoderm in EBs [37]. While one way to increase the surface-to-volume ratio of spherical EBs is to decrease their diameters, small EB size also results in a small number of cells, making spherical EB a poor system for brain organoids generation. To increase surface-to-volume ratio without compromising cell number, the authors generated EBs with elongated shapes, termed microfilament-engineered cerebral organoids (enCORs), by using polymer fibers as templates for cell adhesion (**Fig. 2a**). The length of the fibers was in micrometer-scale, and the fibers were only in contact with the innermost cell layer. Since the enCORs generated were large with the long axis of around 200 μm, the authors reasoned that the majority of the cells had undisturbed organization and cell-cell interaction. Results showed that the ratio of EBs displaying radial neural epithelium increased from around 50% in spheroids to above 80% in enCORs (**Fig. 2b**). Further, radially aligned neurons were found reproducibly in enCORs, resembling the structures of neurons in developing cortical plate *in vivo*, while such a phenomenon could not be found in spherical organoids.

3.2 ECM stiffness

Due to advances in material engineering, natural or synthetic hydrogels with stiffness in the range of 0.1-100 kPa are increasingly used to mimic the soft *in vivo* ECM[20]. However, the mechanical properties of ECM change spatially and dynamically during development, making materials with homogeneous and static stiffness insufficient to accurately recapitulate such microenvironment *in vivo*. For example, *in situ* measurement revealed a stiffness gradient along the anteroposterior axis in the tail bud of zebrafish, ranging from 0.2 kPa at the posterior and 0.7 kPa at the anterior [38]. Moreover, in the earliest stage of development, pre-implant embryos moving in the oviduct are exposed to mainly fluidic environment, while initiation of differentiation happens around day 6 when embryos implant on the collagen-rich uterine wall [39]. Poh *et al.* demonstrated that by mimicking this critical transition of matrix stiffness, organized germ layer patterns could be replicated *in vitro*. The authors showed that 3D colony of mouse ESCs grown from a single cell embedded in soft fibrin gels (90 Pa) successfully differentiated into structures resembling the germ layers when transferred to collagen I coated polyacrylamide (PA) gel substrate (1 kPa) (**Fig. 2c**) [40]. During the colony growth stage, gel stiffness affected cell division and colony morphology. It was found that in softer fibrin gel (90 Pa), cells divided faster and moved outward more easily compare to stiffer fibrin gel (420 or 1050 Pa), leading to larger and less spherical colonies. Moreover, when induced, more cells in colonies grown in softer gel differentiated. When the colonies were transferred to collagen I coated PA gel substrates, stiff PA gel (3.5 or 8 kPa) can disrupt the germ layer formation. While remained spherical on the soft PA gel, the colony spread on stiff PA gel, losing the spherical morphology and eventually failed to form the properly organized germ layers.

3.3 Confinements

Another feature of embryo development that is missing in self-organized organotypic culture conditions is the boundaries of tissues. Confinement of tissues provides definitive boundaries that directly influence the distributions of mechanical strain and morphogens. A variety of micropatterning approaches have been developed to confine single cells or a colony of cells [41-44]. They are realized by printing proteins with a stamp of designed pattern named micro-contact printing, or by photo-oxidation of a cell-repellent substrate through a photomask [45-47]. Cells confined by such patterns cannot expand beyond the boundaries, and therefore experience additional mechanical strain comparing to EBs that can expand during development.

Micropatterning techniques have been widely used to investigate the migration, chirality, polarity and stem cell differentiation [48-52], and have the potential for high-throughput screening [53-60]. Recently, a few groups, including ours, demonstrated that such confinement could induce spatial patterning of cells differentiated from human PSCs, leading to miniaturized models for gastrulation, neurulation, and cardiac development under different chemical induction conditions. In this section, we will highlight new insights provided by these *in vitro* models for human development.

3.3.1 Gastrulation:

Warmflash *et al.* reported that human ESCs could reproducibly generate radial symmetric patterning of the three germ layers and trophectoderm-like embryonic tissue (**Fig. 2D**) in culture with large circular micro-patterns (*diameter* = 1000 μm) in the presence of bone morphogenic protein 4 (BMP4) [61]. The gene expression profile was consistent with that of germ layer formation in gastrulation *in vivo*. Specifically, they first demonstrated that human ESCs exhibited pre-patterned pluripotency markers (Nanog, OCT4, and SOX2) expression, with

greater expression in peripheral cells. They then showed that upon BMP4 stimulation, human ESCs could self-organize into gastruloids, with a trophectoderm-like ring ($Cdx2^+$) on the outer edge, an endodermal/mesodermal mixture ($Sox17^+$/Brachyury$^+$, Bra^+) in the middle, and an innermost circular ectodermal layer ($Sox2^+$).

During gastrulation, the organization of the three germ layers is mainly regulated by a group of signaling pathways including transforming growth factor β (TGF-β), Nodal, and Wnt/β-catenin [62]. Determining how micropatterning interacts with these signaling molecules can provide unique perspectives for the fundamental understanding of pattern formation in development.

In their follow-up study, Etoc *et al.* observed that the gastruloids showed patterned phosphorylated SMAD1 activity, which was higher at the periphery of the micropatterns (*diameter* = 500 μm) [63]. The authors further demonstrated that cell fate patterning was a combined result of two independent mechanisms. Firstly, in the center of the colony where cell density was high, the TGF-β receptors re-localized from apical to basolateral regions, while the receptors near the edge remained apical where cell density was low. Receptors of center cells become inaccessible and therefore insensitive to the BMP4 signaling pathway in a cell density-dependent manner, while cells on edge remain sensitive regardless of cell density. Secondly, the authors discovered that NOGGIN, a BMP4 inhibitor, is essential for BMP4 activity gradient establishment. Since the chemical environment at the confinement boundary should be the same as the bulk, the authors assumed that NOGGIN concentration quickly dissipated to zero at the boundary. Via numerical simulation, the authors found out that a gradient of NOGGIN concentration, decreasing from the confinement center towards the edge, could be sustained under fast diffusion and degradation rate. Therefore, the activity of BMP4 signaling is reduced towards the colony center due to the increasing NOGGIN concentration, inducing a gradient of

Activin-Nodal signaling that patterns mesendodermal fates. Considering the pattern edge played an essential role in the establishment of gradients in both mechanisms, the authors concluded that cell fates were defined relative to the boundary with a fixed distance, and therefore inner cell fates do not arise in small colonies (diameter = 250 μm) as seen in Warmflash *et al.*[61].

More recently, Tewary *et al.* also performed similar gastruloids induction experiments using confined culture of human PSCs. In contrast to the mechanisms proposed by Etoc *et al.*, Tewary *et al.* proposed a two-step model for peri-gastrulation-like fate patterning based on the reaction-diffusion (RD) model and Positional-Information (PI) paradigm [64]. Here, the PI paradigm assumes that the pattern formation is not due to prepatterns in the embryos. Instead, differential gene expressions are achieved through diffusive signaling gradients or timing[65-66]. The classic RD model, a most commonly used model that quantifies the setting up of positional gradients first proposed by Alan Turing in 1952, describes how reactions and diffusions of the initially homogeneous morphogens can lead to complicated pattern formations in tissues[67-68]. For example, the two-component RD model requires the presence of an interaction between two closely related morphogens (an activator and inhibitor pair). The activator induces the expression of both itself and the inhibitor, while the inhibitor inhibits the expression of the activator. This interaction between the pair, combined with their different diffusivities, is sufficient to form periodic distributions of morphogens.

In addition to gastruloids formation similar to Warmflash *et al.*, Tewary *et al.* also observed periodically distributed domains with high activity of BMP4 signaling in large patterns (*diameter* = 3 mm). Such observations cannot be explained by the model proposed by Warmflash *et al.* and Etoc *et al.*, where the positive feedback of BMP4 is absent, and BMP4 signaling activity is predicted to depend only on the distance from the boundary. On the other hand, the two-step

model proposed by Tewary *et al.* showed that BMP4 elicited both positive and negative feedback. BMP4 autocrine and paracrine can induce activation of BMP signaling [69], while BMP4 induced NOGGIN expression can result in long-range inhibition of BMP signaling [70]. Using this two-step model, the authors recapitulated the periodically located high BMP4 activity domains in simulation. Tewary *et al.* also observed regions with varying cell densities at 48 hours after BMP4 introduction for 1000 μm patterns, contradicting to the model proposed by Etoc *et al.*, where cell density was assumed to be high at the center and low on edge. It is therefore likely that the high density-dependent receptor relocalization is, in fact, a result of the periodic BMP4 gradient predicted via the RD model rather than its cause. Moreover, the two-step model predicted that lower concentration of BMP4 could rescue cell fate patterning of all three layers in small patterns (*diameter* = 250 μm), and this result was confirmed in experiments. In conclusion, Tewary *et al.* claimed that the more sophisticated two-step model could better explain cell fate patterning observed in confinements of various sizes.

Lastly, we want to point out that mechanisms other than RD and PI may also contribute to cell pattern formation during organogenesis. For instance, in the study of Tewary *et al.*, many sporadic spots of cell condensation areas appeared in colonies of 3 mm in diameter. This condensation may be a result of directed migration of cells, position-dependent cell proliferation, and adhesion-based cell sorting, etc. A detailed study of the involvement of such cellular processes will lead to a more comprehensive understanding of pattern formation. In addition, gastrulation is a 3D process *in vivo* that requires invagination of mesodermal cells, which has not been fully captured in these 2D models. Modeling gastrulation in 3D *in vitro* will be an interesting future direction to pursue.

3.3.2 Neurulation

We recently developed a human ESCs-based *in vitro* model that can recapitulate neural induction, the first stage of neurulation, using a similar micropatterned platform [71]. Using dual Smad inhibition and transient Wnt activation culture condition, geometrical confinement induced emergent patterning of NE and neural plate border (NPB) cells, mimicking neuroectoderm regionalization during neural induction (**Fig. 2e**). Similar to the observations in the gastrulation model mentioned above, we also found more prominent Smad 1/5 phosphorylation at the periphery of micropatterns compared to the center. However, detailed investigations revealed that surprisingly, mechanisms mentioned above, *i.e.*, diffusion reactions of morphogens and lateralization of BMP receptors, were not dominant mechanisms that led to neuroectoderm regionalization. This is supported by the evidence that silencing BMP4 or NOGGIN using siRNAs did not affect neuroectoderm regionalization in micropatterned colonies, and proper neuroectoderm patterning could still be achieved when cell-cell contacts were disrupted. Instead, we found that two tissue-scale morphogenetic signals, cell shape and cytoskeletal contractile force, instructed NE / NPB patterning via BMP-SMAD signaling. We consistently observed larger contractile forces and projected cell area in NPB cells. To test whether p-Smad 1/5 can be activated via enhancing the cell area and contractile force, we used a custom-designed microfluidic cell stretching device (**Fig. 2f**) that expands only the central regions of micropatterned human ESC colonies. Indeed, we found activation of p-Smad 1/5 upon stretching, and a significant number of NPB cells at colony center. Immunocytochemistry and qPCR analysis of patterned single human ESCs with prescribed spreading areas confirmed that Smad-BMP signaling could be activated by solely changing cell shape. This study suggests an alternative self-organizing principle in autonomous patterning in the context of neural induction and highlights the mechanical cues interplay with established cellular and genetic regulatory

mechanisms to directly control cell fate during neural development. Notably, these studies are uniquely enabled by a series of engineered tools that allow quantification and manipulation of mechanical cues, which is very challenging to apply to animal models. Engineered organotypic models will not only increase the reproducibility and efficiency for the generation of organotypic structures but also provide new tools for fundamental studies of developmental principles.

Following neural induction, the next step in neurulation is where neuroectoderm polarizes and folds into a neural tube. Conventional neural induction of human ESCs usually leads to neural rosettes with multiple centers. Knight *et al.* found that under Essential 6 induced neural induction condition [72], micropatterns with diameters of 200-250 μm and 150 μm were optimal for inducing singular forebrain and spinal neural rosettes emergence with efficiencies of 80-85% and 73.5%, respectively [73]. Further, using a micropatterned surface based on the poly(ethylene glycol) (PEG) brush chemistry that allows confinement release by simply adding cell adhesive peptides to the media [74], Knight *et al.* found that such singular neural rosette cytoarchitecture was maintained during outgrowth radial tissue. Compared with organoids grown in suspension culture, these neural tissues initiate as a 2-D monolayer and then become multilayered, and the arrayed approach enables the continuous monitoring tissue morphogenesis using confocal microscopy, without losing the benefits of 3D culture.

3.3.3 Cardiac development

Ma *et al.* developed an in vitro model for cardiac development using a different patterning method. Instead of generating flat patterns, the authors coated PEG-based non-fouling polymer films to tissue culture dishes and then patterned the substrates to generate microchambers subsequently coated with Matrigel [75]. Activation of WNT/β-catenin pathway via CHIR promotes spatial patterning with the cell condensation process mediated by biased cell migration and

differential cell proliferation, forcing cells at the perimeter to express an $OCT4^+$ annulus. When exposed to cardiac differentiation media for 15 days, cells in the center of the chamber differentiated to beating cardiomyocytes, and cells at the periphery differentiated to myofibroblasts. As a proof of concept, Ma *et al.* treated cardiac microchambers with Thalidomide, which is known to induce cardiac birth defects, and found that Thalidomide treatment led to lower cardiac differentiation efficiency and compromised cardiac microchambers. Compared to the conventional 2D culture of human PSCs that has been used to examine drug toxicity and effectiveness on cells, such *in vitro* model can also predict the effects of drugs on tissue morphogenesis.

4 POTENTIAL TOOLS FOR ORGANOTYPIC STRUCTURES ENGINEERING

A central question in developmental biology research is how the cell fate patterning and drastic changes in cell shape are achieved during development. Although those questions have yet been fully addressed, there are a few well-established mechanisms to explain the embryogenesis and morphogenesis processes. Taking the neurulation as an example, the first stage of neurulation, termed neural induction, is believed to be initiated by a BMP gradient established by a gradient of BMP antagonist secreted by the notochord[76]. The NE cells then establish both apicobasal and planar cell polarity, and directional contraction of the actomyosin network leads to apical constriction and then bending of the neural plate[77]. Emerging evidence has shown that mechanical forces are also involved in this morphogenesis process[78-80], and other processes such as branching morphogenesis in lung development[81-82]. Together, precise control of chemical factors, extracellular matrices, and mechanical forces are essential for proper embryogenesis and morphogenesis.

Bioengineering tools have been broadly applied to control various cell functions by providing highly controllable cell microenvironment[83]. For example, substrates with tunable mechanical properties have been used to regulate the self-renewal and differentiation of pluripotent stem cells[32-34, 84] and adult stem cells[85-86]. Micro/nanofabricated substrates have been used for generating highly organized tissues with proper cell organizations[87-89], and directing cell migrations[90-91]. Integrated microfluidic devices have also been widely applied for modulating shear stresses and generating chemical gradients[92-93]. Organ-on-a-chip devices that combine microfluidic co-culture of primary cells and mechanical stimulations receive great successes in recapitulating certain tissue/organ functions and are promising platforms for drug screening[94-95].

Only a few techniques mentioned above have been applied to date for the organotypic culture of stem cells. In this section, we suggest potential applications of several recently developed biomaterials and micro-devices for advanced organotypic cultures. These approaches, together with those mentioned above, improve the spatiotemporal control of both soluble and insoluble factors to mimic stem cell niche (**Fig. 3**).

Morphogen gradient is considered as the major cause for cell fate patterning during development. Although such gradient can be self-generated by cells, more reproducible results are expected if it can be maintained externally, where complex spatial patterning can be achieved by design. Microfluidic devices have been used to generate and maintain concentration gradients by flowing streams containing molecules with different concentrations through interconnected channels [96-97]. Alternatively, morphogen gradients can be generated through diffusion. For example, diffusible channels have been generated in enzymatically cross-linked gelatin hydrogel as inserts to standard tissue culture plate to precisely deliver biomolecules [98]. By using this system, neuronal differentiation of mouse ESCs can be spatiotemporally controlled via the delivery of retinoic

acid gradients. More local biochemical gradient, as well as slow-release source, can also be achieved by using functionalized micro- or nanomaterials [99]. In addition, most *in vitro* development systems we have discussed above only allow manually introducing biochemical factors at different time points to realize the temporal control of chemical cues. Automated microfluidic systems hold promise to identify the ideal combination of growth factors and small molecules to optimize organotypic cultures induction in a high-throughput manner.

To mimic heterogeneous and dynamic ECM in development, a variety of techniques have been developed to spatiotemporally control ECM stiffness, such as thermal gradients within polymerizing (poly)dimethylsiloxane (PDMS) [100], inclusion of rigid particles in a soft hydrogel [101], polyelectrolyte monolayers with a patterned cross-linker [102], PA gel based diffusion-driven methods [103], and photoinitiator and photomask based methods [90, 104]. While a stiffness gradient has been applied to study durotaxis, recent work by Hadden *et al.* demonstrated a shallow gradient that is nondurotactic for human adipose-derived stem cells could regulate their morphology, differentiation, and expression of mechanosensitive molecules [105]. In addition to spatial controls, recent advances in biomaterials also enable temporal control of ECM stiffness. PEG hydrogel with photolabile crosslinks or photo-initiators in the gel can become softer or stiffer when exposed to light via degradation or crosslinking, respectively [106]. Moreover, on-demand activation of the desired signaling can be achieved by modulating biomolecular-binding site presentation by incorporating signaling proteins into a hydrogel and masking their active sites with a photo-degradable moiety [90, 107-109], or creating 3D scaffolds decorated with growth factors or signaling proteins via chemical/enzymatic crosslinking through adhesive or proteolytically cleavable sites [110]. ECM degradation and remodeling in response to cell-secreted matrix metalloproteinases have also been modeled in synthetic systems [111-112].

5 CONCLUSION AND PERSPECTIVE

In summary, engineering cell microenvironment, including ECM properties, geometrical confinement, and morphogen distribution, can be effective tools in addition to existing organotypic structure fabrication techniques, and potentially can promote directed stem cell differentiation and patterning towards complex 2D or 3D structures. For example, morphogenesis processes such as neurulation have not been recapitulated *in vitro* using self-organized models. By applying engineering tools to mimic the mechanical and chemical *in vivo* environment, *in vitro* models for such complex processes can be achieved. Combining engineered systems and autonomous behaviors of stem cells will further enhance our understanding of stem cell biology and provide reproducibility and parallelization advantages for drug or toxin screening applications.

While the formation of organotypic structures for most major organs has been studied using standard self-organized differentiation approaches, engineered models have only been developed for very few stages as discussed above despite their importance. The immediate next step is to develop a library of engineered organotypic culture models that mimic all major processes during embryogenesis, including neurulation, somite formation, gut tube development, *etc*. A major challenge of tool development is the difficulty to control cell/tissue shape, mechanical forces, chemical environment, and ECM properties in 3D, especially within millimeter-sized tissues. Also, as cells continuously secrete, deposit, and degrade ECM and/or signaling molecules, a fine balance between the external signals and autocrine/paracrine signals need to be defined. It is possible that external signals are needed only to initiate the development and can be withdrawn later. Collectively, combining engineering approaches with autonomous behaviors of

stem cells could lead to organotypic cultures that better mimic the development processes *in vivo* both anatomically and physiologically.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

FIGURE CAPTIONS

Figure 1.

Self-organized development models. (A) Schematic of methods used in the self-organized development of organotypic cultures. (B) Neural rosettes formation. Cells were stained for Pax6 (red) and N-cadherin (green). The inset shows the magnified structure of a rosette. Scale bar,100 μm. Adapted with permission[22]. (C) A two-wall optic cup from derived from mouse embryonic stem cells. Red: Mitf, green: Rx-GFP. Adapted with permission[24]. (D) A dorsoventrally polarized neural cyst; red: SHH, green: Sox1, blue: DAPI. Scale bar, 50 μm. Reprinted with permission [29] .

Figure 2.

Engineered development models. (A) Bioengineered EBs from H9 cells at day 11, during neural induction. Arrows mark polarized neural ectoderm. (B) Formation of spheroid cerebral organoids with elongated shapes termed microfilament-engineered cerebral organoids (enCORs). Cells were stained for Foxg1(Red) and Nuclear (DAPI, blue). Arrows indicate radial neural epithelium. Adapted with permission [37]. (C) Phase and fluorescence images of a differentiated 3D colony. Scale bar, 50 μm. Adapted with permission [40]. (D) Formation of germ layers in vitro as a model of gastrulation. Scale bars, 100μm. Adapted with permission [63]. (E) Developmental patterning of neural ectoderm from geometric confined human ESCs. (F) A custom-designed microfluidic cell stretching device that expands only the central regions of micropatterned human ESCs colonies to enhance cell area and contractile forces in situ, we found activation of p-Smad 1/5 upon stretching, and a significant number of NPB cells at colony center. Adapted with permission [71].

Figure 3.

Potential engineering approaches for improved organotypic cultures. The potential methods for reproducible organotypic culture of stem cells via engineering ECM stiffness, chemical gradients, colony shape, confinement, and external forces. These approaches will improve the spatiotemporal control of both soluble and insoluble factors to mimic the stem cell niche.

FIGURES

Figure 1.

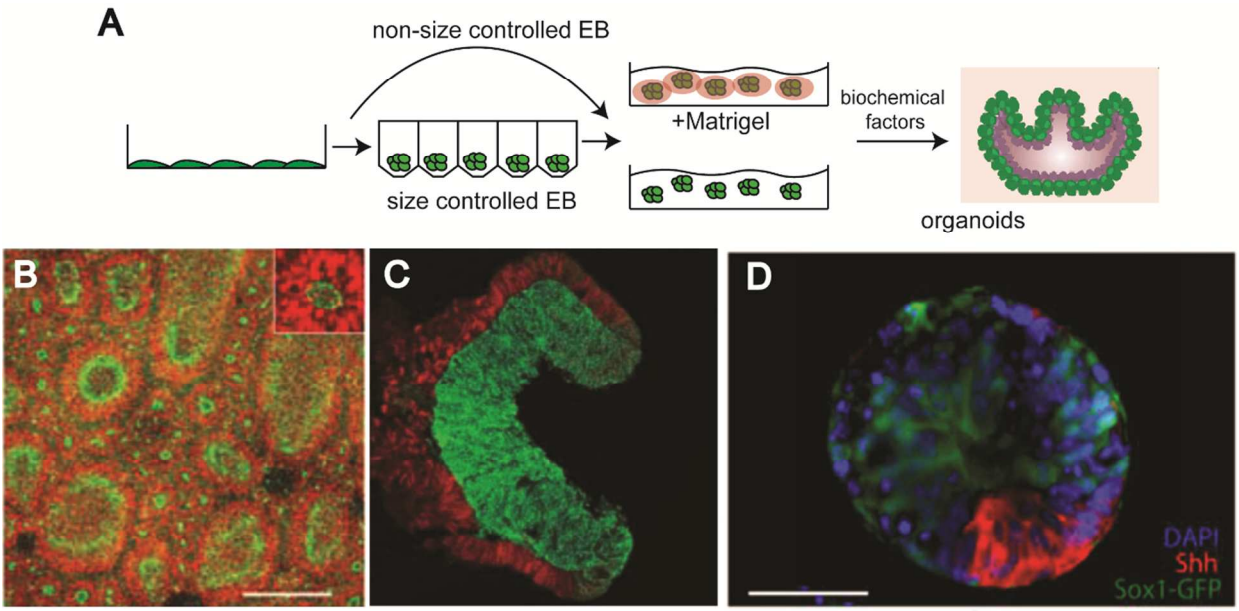


Figure 2

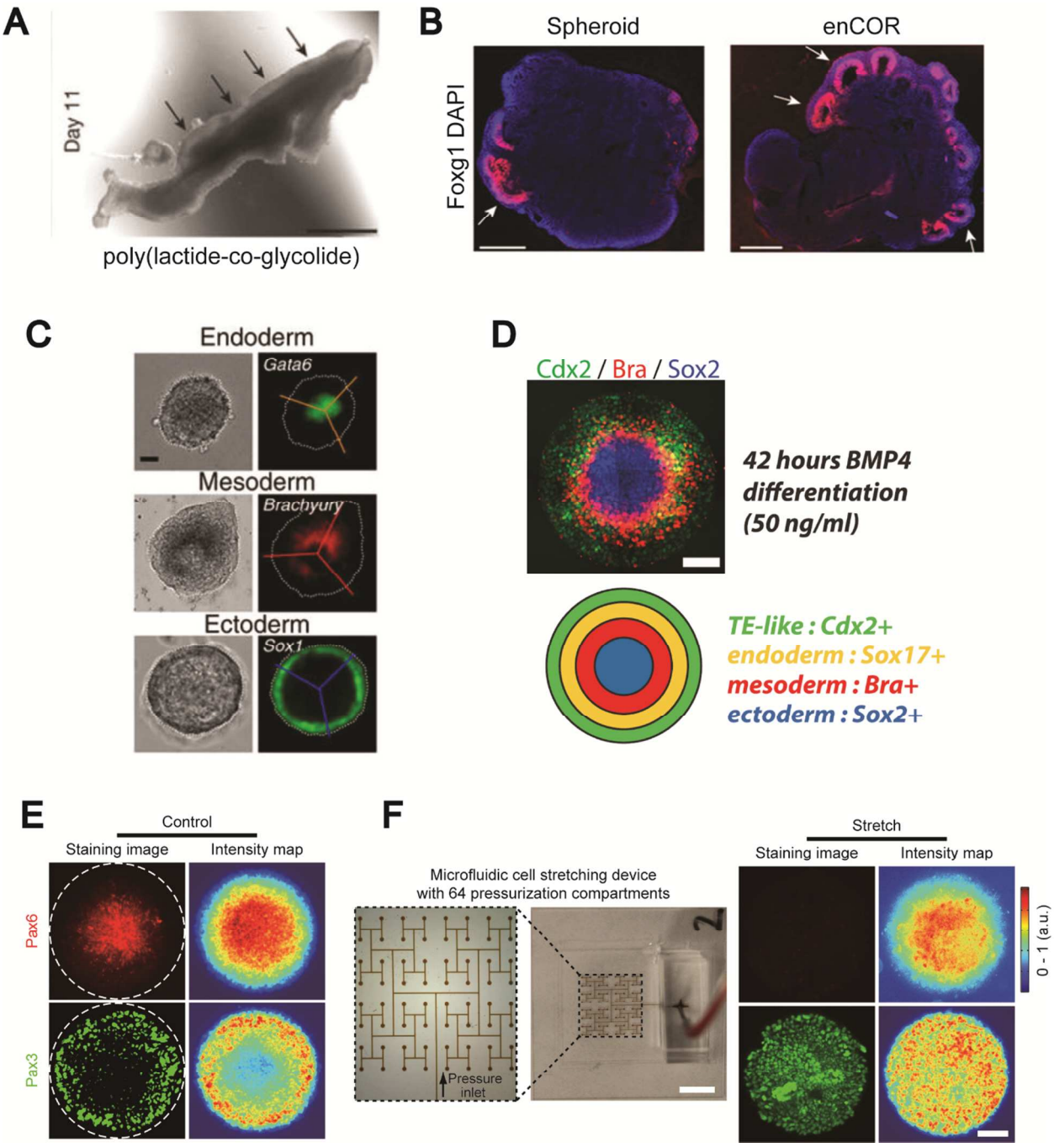
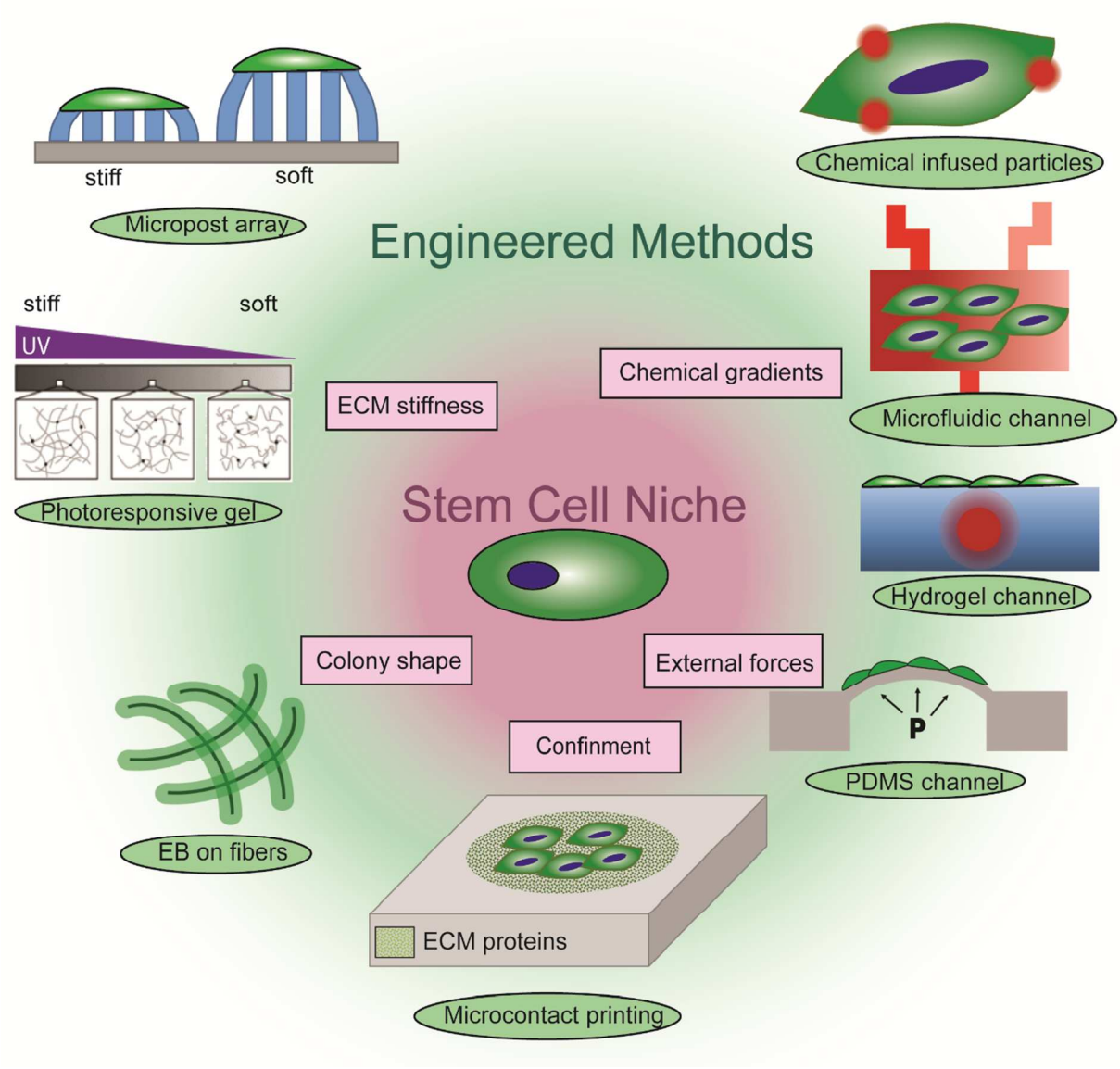

A
Day 11
poly(lactide-co-glycolide)
B
Spheroid
enCOR
Foxg1 DAPI
C
Endoderm
Gata6
Mesoderm
Brachyury
Ectoderm
Sox1
D
Cdx2 / Bra / Sox2
42 hours BMP4 differentiation (50 ng/ml)
TE-like : Cdx2+
endoderm : Sox17+
mesoderm : Bra+
ectoderm : Sox2+
E
Control
Staining image
Intensity map
Pax6
Pax3
F
Microfluidic cell stretching device with 64 pressurization compartments
Pressure inlet
Stretch
Staining image
Intensity map
0 - 1 (a.u.)

Figure 3


stiff
soft
Micropost array
Chemical infused particles
Engineered Methods
stiff
soft
UV
ECM stiffness
Chemical gradients
Microfluidic channel
Photoresponsive gel
Stem Cell Niche
Hydrogel channel
Colony shape
External forces
P
Confinment
PDMS channel
EB on fibers
ECM proteins
Microcontact printing

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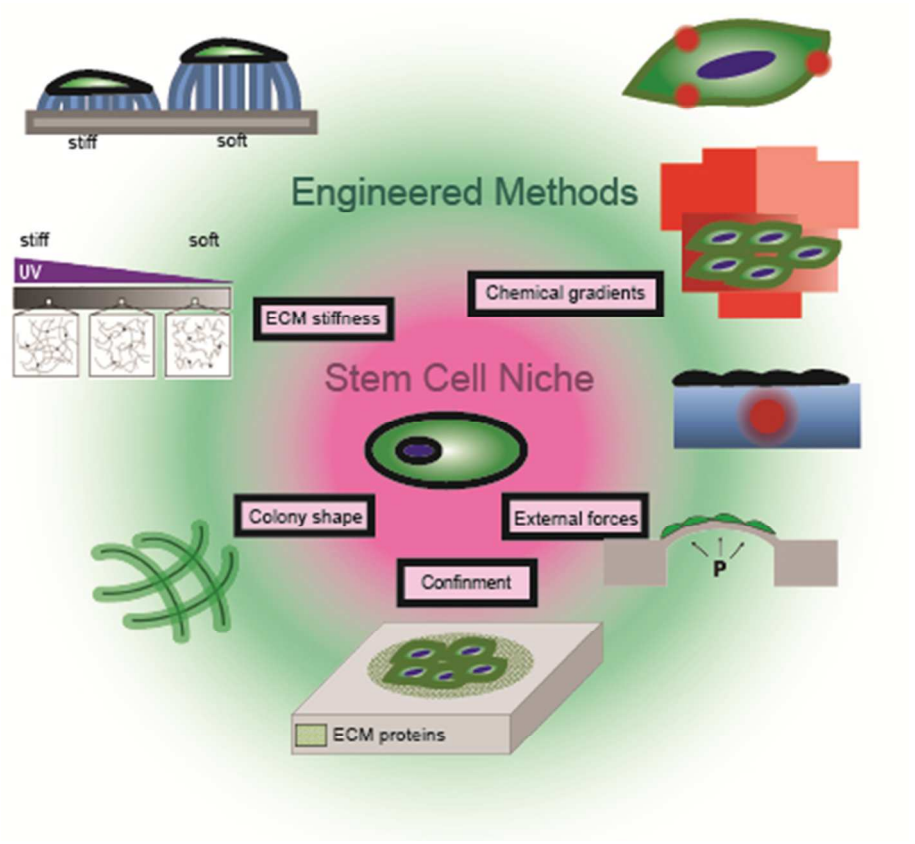

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