Cultivating advanced embryo models through bioengineering mastery

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ABSTRACT

Stem cell-based embryo models, which recapitulate symmetry breaking, pattern formation and tissue morphogenesis during early development, provide promising experimental tools to study development of mammalian species, including humans. Despite considerable progress in embryo modeling using cultured stem cells, it remains challenging to construct embryo models with high fidelity, efficiency, controllability, and *in vivo*-like cellular organization and tissue architecture. This is largely due to intrinsic variabilities in self-organization and differentiation of mammalian stem cells in uncontrolled culture environments utilized in current embryo modeling. In this review, we argue that bioengineering tools, which are powerful for controlling topological boundaries and dynamic chemical and mechanical signals and thus are efficient in guiding symmetry breaking, pattern formation, tissue morphogenesis and tissue-tissue interactions, should be utilized for constructing high-fidelity, high-efficiency embryo models. In this review, we first discuss pattern formation and morphogenesis during embryonic development, and selectively examine different embryo models to highlight the importance of bioengineering controls in developing these models. We then explore how different bioengineering approaches useful for guiding pattern formation, morphogenesis, cell fate decisions and cell-cell interfaces can be utilized in stem cell-based embryo modeling to promote their efficiency, reproducibility, controllability, complexity, and *in vivo* relevance.

1. INTRODUCTION

During development, patterned multicellular structures with stereotypical, three-dimensional tissue architecture emerge from a single fertilized egg. Mammalian development involves coordinated developmental processes, including pattern formation, tissue morphogenesis, cell differentiation, and growth[1]. Pattern formation establishes body axes in mammalian embryos, including anterior (head)-posterior (tail) (A-P) and dorsal (back)-ventral (belly) (D-V) axes. Mammalian embryos undergo substantial morphological changes in form, or morphogenesis, to achieve complex tissue and organ anatomies. Mammalian development also encompasses cell differentiation, giving rise to specialized cell types with tissue- or organ-specific functions, and tissue growth. These developmental processes are intricately linked during dynamic, progressive development. Precisely how the mammalian embryo develops from a fertilized egg through these coordinated developmental processes remains one of the most fundamental questions in biology with enormous biomedical implications.

Recent advances in stem cell biology, developmental biology and bioengineering have fostered the emergence of an exciting interdisciplinary field termed stem cell-based embryo modeling[2-5]. This field aims to apply developmental biology principles to create embryo models using mammalian pluripotent and extraembryonic stem cells *in vitro*. When differentiated *in vitro*, these stem cells largely follow the natural developmental programs of their *in vivo* counterparts[6]. Over the last decade, numerous studies have showcased the intrinsic self-organizing properties of these cells to differentiate and self-assemble into embryonic- or extraembryonic-like structures *in vitro* (see examples discussed in two excellent reviews[7,8]). Thus, stem cell-derived embryo models have successfully been developed to recapitulate major developmental processes such as tissue patterning and morphogenetic events during the pre-implantation, gastrulation, neurulation, and somitogenesis[3,4,9] (**Table 1**). Nonetheless, continuous development and future applications of stem cell-based embryo models hinge on the premise that these

models can serve as useful experimental tools to disentangle the genetic, molecular and cellular events involved in human embryogenesis.

Given their high tractability and ease with which mammalian stem cells can be genetically modified, stem cell-based embryo models have been hailed as promising experimental tools for studying mammalian development and associated disorders[3,10,11] (**BOX 1**). However, despite considerable excitement and success in these models, they have rarely been used to reveal fundamental new insights into mammalian development or in translational applications. One major hindrance to such important applications of embryo models is that it remains a significant challenge for these models to faithfully recapitulate tissue patterning or morphogenetic events occurring in natural embryonic development (or fidelity) with a sufficiently high level of successful rate (or efficiency). This difficulty arises largely because approaches for embryo modeling have been heavily relying on spontaneous differentiation and self-organization of mammalian stem cells and their progenies in uncontrolled culture environments. Consequently, their developments are critically affected by stochastic genetic noises or random cellular interactions with local culture microenvironment. Genetic noises, which arise from stochastic variations in the synthesis and degradation of mRNA and proteins[12], could contribute to phenotypic variabilities observed in embryo models. Random interactions between stem cells using in embryo modeling and their local microenvironment could further compound the efficiency or fidelity of embryo models[13].

So far, a variety of mammalian stem cells, including pluripotent stem cells (PSCs) and stem cells of extraembryonic lineages, such as trophoblast stem cells (TSCs)[14,15] and extraembryonic endoderm cells (XENs)[16], have been utilized for building embryo models. However, culture conditions for these stem cells have not yet been standardized. Furthermore, human extraembryonic stem cells remain to be fully established. Additionally, human PSCs (hPSCs) generated using different culture protocols might represent distinct pluripotency states with varied differentiation potentials[17]. These variabilities of

mammalian stem cells used in embryo modeling further complicate robust development and, consequently, limit the usefulness and broad applications of embryo models in fundamental or translational applications.

In this review, we aim to highlight bioengineering tools and their promising applications for constructing high-efficiency and high-fidelity embryo models. Bioengineering approaches developed over the last few decades are powerful in designing synthetic cells to regulate their responses to external stimuli or controlling dynamic external microenvironmental signals critical for cellular behavior and fate regulation[13]. In this review, we first discuss the two prominent developmental processes involved in embryonic development: pattern formation and morphogenesis. We selectively examine different embryo models, each clearly manifesting one or more of these embryonic developmental processes, with an emphasis on the importance of bioengineering controls implemented in their development. We then discuss how bioengineering approaches for guiding pattern formation, morphogenesis, cell fate decisions and cell-cell interactions can enable precise control of the formation and development of embryo models, enhancing their efficiency, reproducibility, complexity and *in vivo* relevance. We conclude by discussing the limitations of current embryo models before highlighting design principles that should be considered to promote the widespread applications of stem cell-based embryo models.

2. PATTERN FORMATION

In development, pattern formation is the process by which cellular activity is organized both spatially and temporally such that a well-ordered tissue structure develops[18]. Pattern formation can be either self-organized[19] or guided by external cues[20]. Self-organized pattern formation involves patterning of cell fates through spontaneous cell-cell interactions among a seemingly homogeneous cell population[19]. One such system, proposed by Alan Turing, is based on the reaction-diffusion model, in which stable patterns

emerges *via* cell-cell interactions through diffusion of activator-inhibitor pairs[21]. Turing's reaction-diffusion model has successfully explained pattern formation in various *in vivo* (*e.g.*, hair follicle patterning[22]) and *in vitro* (*e.g.*, two-dimensional or 2D gastrulation models[23]) contexts. The initial and boundary conditions of pattern formation morphogenetic fields are critical for generating a reproducible pattern in self-organized pattern formation[19]. As such, bioengineering approaches effective in controlling initial cell number and geometries of cell colonies have been used for generating robust embryo models whose developments rely on self-organized pattern formation[24,25].

For external cue-guided pattern formation, spatially patterned cell fates are instructed by external signals, such as diffusible molecules, including both morphogens and their antagonists, secreted from adjacent pre-formed tissues that function as signaling centers. External cue-guided pattern formation can be explained by the positional information theory proposed by Lewis Wolpert[26]. This theory proposes that concentrations of external morphogens provide positional values as in a coordinate system for cell fate decisions[26]. Recent studies, however, also support the important roles of other features of morphogen gradients (gradient slope, duration and time integral) in providing positional information for spatial cell fate patterning[27,28]. It should be noted that morphogen gradients can also arise through intrinsic cell-cell interactions per Turing's reaction-diffusion model, which then provides positional information to guide pattern formation. For example, a stepwise reaction-diffusion and positional model was proposed to explain pattern formation in 2D human gastrulation models[29]. There are effective bioengineering approaches for controlling exogeneous soluble culture environments, such as bioengineered co-culture systems for controlling paracrine signals and microfluidic devices for generating dynamic morphogen gradients[13,30]. Importantly, such bioengineering tools have been successfully employed in embryo models to recapitulate external cue-guided pattern formation[31-34].

Even though pattern formation *in vivo* often involves both self-organized cell-cell interactions and external cue-guided spatial fate patterning[35], there are some well-studied pattern formation events in development in which each of these mechanisms plays an essential role. In this section, we first discuss three such events and related embryo models successfully developed to recapitulate them: 1) Specification of trophectoderm (TE), hypoblast and epiblast (EPI) during blastocyst formation through self-organized pattern formation, 2) A-P symmetry breaking in the EPI at the onset of gastrulation guided by extraembryonic tissues, and 3) neural tube (NT) patterning *via* morphogen gradients. We next discuss how bioengineering approaches have been implemented in embryo models to enhance the efficiencies of recapitulating these pattern formation events by guiding cell-cell interactions and controlling external bio-physical and -chemical signals.

2.1. Pattern formation *in vivo* and in embryo models

Blastocyst formation and modeling through self-organized pattern formation. The first major symmetry breaking event in mammalian development takes place during blastocyst formation[36,37]. During blastocyst development, cells of the morula undergo an ordered series of cell-fate decisions and symmetry-breaking events. This developmental sequence results in the formation of a blastocyst, composed of TE surrounding the blastocoel and inner cell mass (ICM) (**Fig. 1a**). After implantation, the TE contributes to placental development. Within the blastocyst, the ICM differentiates into two distinct cell lineages: EPI, forming the embryo proper, and hypoblast, or called primitive endoderm (PrE) in mice (**Fig. 1a**).

Most of the current knowledge about lineage segregations during blastocyst formation comes from studies of mouse embryos. We now know that lineage segregation between TE and ICM cells occurs first during blastocyst formation. At the 8-cell stage, driven by cortical tension, the mouse

embryo becomes compacted, and each individual cell acquires an apical domain[38] (**Fig. 1a**). As mouse morula cells divide, cells in the outer layers inherit apical components and become polarized, whereas those in inner layers become apolar[39] (**Fig. 1a**). The distinct polarity status leads to differential Hippo signaling and subsequent differential expression of TE- and EPI-related transcription factors in the outer and inner cells of the mouse morula to specify TE and EPI fates, respectively[40,41]. Thus, embryo geometry, coupled with cortical tension, cell position, and division pattern, defines the polar to apolar cell ratio and thus TE and ICM lineage allocation[38,42-44] (**Fig. 1a**). After TE and ICM lineage bifurcation, transmembrane pumps in TE cells pump fluids into extracellular space in the mouse blastocyst, leading to formation of small fluid-filled cavities[45]. These cavities coalesce into a single large cavity, the blastocoel, due to pressure imbalance[46]. After blastocoel formation, ICM cells become segregated into EPI and PrE[36,37]. Specification of EPI and PrE in mice is regulated by heterogeneous FGF signals in the ICM, with EPI precursors secreting FGF ligands to drive PrE fate specification in neighboring cells[47] (**Fig. 1a**). Thus, PrE and EPI cells initially form a "salt-and-pepper" pattern, before cell sorting to establish a well delineated spatial pattern of PrE and EPI cells, with PrE layer lining the blastocoel and EPI surrounded by PrE and TE[48]. Molecular mechanisms underlying human hypoblast development remain elusive. There are two studies showing that FGF inhibition does not have a significant effect on hypoblast induction in human blastocysts[49,50]. However, more recent studies suggest that FGF inhibition could suppress specification of hypoblast or hypoblast-like cells in human blastocysts or from naïve hPSCs, respectively[51,52]. Thus, additional studies are needed to reconcile these discrepancies to fully elucidate the role of FGF signaling in human hypoblast specification.

Stem cell-based models of blastocysts, or blastoids, were first generated using mouse stem cells[53-55] (**Fig. 1b**, **Table 1**). Since then, numerous human blastoid systems have been reported[24,56-60] (**Fig. 1b**, **Table 1**). Human blastoids resemble human blastocysts in terms of morphology, transcriptome

and lineage composition. Given the potency of naïve hPSCs for both embryonic and extraembryonic lineage developments[61], they have been the most commonly used starting cell populations for developing human blastoids[24,58,59,61]. Regardless the initial cell types being used, human blastoid protocols commonly involve seeding and aggregating hPSCs in confining microwell plates to initiate and promote self-organized tissue patterning[24,56,58-61]. In such blastoid formation protocols, initial cell number in each cell aggregate, which is controlled by cell seeding density, appears to be an important factor affecting blastoid formation[24,58,61]. Low cell numbers in initial aggregates of hPSCs often lead to formation of trophoblast spheroids lacking ICM-like cells[61]. How the initial cell number in hPSC aggregates affects human blastoid formation remains unclear. After initial cell aggregate formation, cell clusters are then exposed to ERK, ROCK and NODAL inhibitors to induce TE lineage differentiation[24,58,59,61]. Similar to TE and ICM lineage segregation during human blastocyst development, the outer and inner cells in human blastoids acquire differential polarity status and Hippo signaling activity, leading to the outer and inner cells being specified into TE-like and ICM-like cells, respectively[24,58,59,61]. Notably, similar to natural human blastocyst development, inhibition of Hippo signaling promotes TE differentiation and increases the efficiency of human blastoid generation[24,59]. Although hypoblast-like cells are present in human blastoids, they are not as developed or organized as their *in vivo* counterpart. This deficiency underscores our current limited understanding of human hypoblast lineage development.

Given that human blastoids can be generated with high efficiency (~ 80%) and in large quantities, they offer a useful experimental tool to study human pre- and peri-implantation development. For example, there are implantation modeling studies by co-culturing human blastoids with endometrial epithelial cells and stromal cells[24,59,62]. These studies support that in human blastoids maturation of TE cells and their interaction with endometrial epithelial cells are both regulated by signals emanated from

EPI-like cells[24]. The presence of endometrial stromal cells also seems to promote progressive development of human blastoids into early peri-implantation stages[59].

Epiblast symmetry breaking during peri-gastrulation development and its modeling. After implantation, the next major milestone of mammalian development is the gastrulation, through which the single layer of pluripotent EPI cells transforms into a multilayered and multidimensional structure with fully defined body axes. Human gastrulation remains mysterious. Current knowledge about mammalian gastrulation is mainly derived from studies of mouse embryos, revealing the important role of extraembryonic tissues in inducing symmetry breaking of the EPI and establishing the A-P axis at the onset of gastrulation[36]. Specifically, in pre-gastrulation mouse egg cylinder, the EPI is surrounded by the extraembryonic ectoderm (ExE) derived from TE and visceral endoderm (VE) derived from PrE. Regionalized BMP signals from the ExE leads to formation of a distal VE (DVE) at the distal region of VE[63,64] (**Fig. 1c**). Involving NODAL and WNT signaling, DVE subsequently migrates towards one side of the embryo and specifies into anterior VE (AVE), thereby marking the future anterior domain of mouse embryos[65] (**Fig. 1c**). The AVE secrets WNT, NODAL and BMP antagonists, shielding anterior EPI from posteriorization and thus restricting the primitive streak formation and gastrulation to posterior EPI[66,67] (**Fig. 1c**).

In contrast to a cup shape of the pre-gastrulation mouse EPI, pre-gastrulation human EPI exhibits a disc-like morphology, and it is juxtaposed with the dorsal amniotic ectoderm and ventral hypoblast[68] (**Fig. 1c**). How the amniotic ectoderm and hypoblast regulate symmetry breaking and the A-P axis formation in the EPI during human gastrulation remains unclear. Recent studies of *in vitro* cultured human embryos reveal a portion of hypoblast cells at the putative anterior region expressing BMP, WNT and NODAL antagonists[69,70], similar to the mouse AVE. Nonetheless, whether these cells act as the

anterior signaling center to induce symmetry breaking and establish the A-P axis in the human EPI remains to be fully elucidated.

Stem cell-based embryo models that recapitulate symmetry breaking and the formation of A-P axis in the EPI offer promising tools to study these important developmental events and the underlying genetic, molecular and cellular mechanisms. Towards this goal, peri-gastrulation mouse embryo models have been generated by aggregating mouse embryonic stem cells (mESCs) and mouse TSCs (mTSCs) in 3D Matrigel culture[71] (**Table 1**). Through spontaneous self-organization, some cell aggregates formed organized egg cylinder-like structures, termed ESC- and TSC-derived embryo model, or ETS embryo model[71]. ETS embryo model comprises mESC-derived pseudostratified EPI-like epithelium juxtaposed with mTSC-derived ExE-like cells[71]. Even though lacking VE-related lineages, some ETS embryo models could undergo symmetry breaking and give rise to regionalized mesoderm tissues[71]. How this occurs in ETS embryo models without VE-related lineages is unclear, possibly depending on genetic noises or random cellular interactions with local culture microenvironments. Whether such a mechanism plays a role in symmetry breaking and A-P patterning of the mouse EPI *in vivo* warrants future investigation. Follow-up studies incorporated mouse XENs, the *in vitro* counterpart of PrE, into ETS embryo models, resulting in a more complete peri-implantation mouse embryo model, termed ETX embryo models (ESC-, TSC-, XEN-derived embryo models)[72,73] (**Fig. 1d**, **Table 1**). A-P patterning was also achieved in ETX embryo models, evidenced by the development of AVE-like cells expressing signaling antagonists and graded expression of NODAL in posterior EPI-like cells[72,73]. ETX embryo models further exhibited cellular features of the gastrulation, including epithelial-mesenchymal transition (EMT) of the EPI and formation of mesoderm and endoderm lineages[72,73]. Both ETS and ETX embryo models show limited efficiencies (20 - 30%) due to random, uncontrolled interactions between embryonic and extraembryonic cells in the models[71-73]. Notably, the ETX embryo model presents the

potential for development beyond gastrulation (**Table 1**). To this end, *in vitro* studies of natural embryos are very useful in guiding the culture protocol development for stem cell-based embryo models. When cultured in an *ex utero* mouse embryo roller culture system under optimized medium conditions[74], ETX mouse embryo models develop structures mimicking the neural tube (NT), heart, gut tube, somites and a tail bud, albeit with a very low efficiency and organ primordia exhibiting notable defects[75-77].

Recently, assembloid approaches have been utilized for scalable and controllable generations of peri-gastrulation mouse embryo models[31,78] (**Table 1**). Assembloids are 3D structures formed through fusion of two or multiple spheroids to recapitulate structural and functional properties of an organ[79,80]. Assembloids are instrumental in modeling tissue-tissue interactions. However, due to its simplistic assembly process, assembloids might be limited in modeling the complex tissue cytoarchitecture formed through dynamic pattern formation processes in natural embryos. To model peri-gastrulation mouse development, mESC and mTSC spheroids were assembled to guide their interaction, leading to the formation of regionalized mesodermal cells in mESC spheroids with an efficiency of 50 - 70%[78]. Similarly, assembling of mESC clusters and BMP4-treated mESC clusters, which served as an engineered signaling center secreting WNT and NODAL signals, resulted in peri-gastrulation mouse embryo models showing well-delineated orthogonal A-P and D-V body axes and patterned germ layer lineages with a efficiency greater than 90%[31]. Progressive development of this mouse embryo model showed organ primordia similar to those in neurula-stage mouse embryos, including the NT, gut tube, somitic and intermediate mesodermal tissues, cardiac tissues, and a vasculature network[31]. These examples highlight how bioengineering approaches can be applied to control embryonic-extraembryonic interactions to improve the efficiency of peri-gastrulation embryo model development.

Peri-gastrulation human embryo models were first developed from primed hPSCs without involving extraembryonic cells[81-83] (**Table 1**). In one model exhibiting drastic symmetry breaking

events, a subset of primed hPSC clusters in 3D Matrigel culture spontaneously underwent epithelization and lumenogenesis, giving rise to a lumenal epithelial structure, before spontaneously breaking symmetry to form a bipolar structure mimicking early post-implantation EPI and amniotic ectoderm patterning[83]. This model was termed post-implantation amniotic sac embryoid (PASE)[83]. Some of the PASE structures progressively developed, with the EPI-like compartment undergoing EMT and giving rise to mesodermal lineages[83]. Follow-up studies using controllable microfluidic devices to provide asymmetric external morphogen stimulations to primed hPSC clusters showed significantly enhanced efficiencies of PASE formation[32]. Further mechanistic studies identified the role of paracrine signaling, likely involving BMP signals downstream of *ISL1*, from amniotic cells in inducing gastrulation-like events in the EPI at the onset of human gastrulation[32,84]. This finding was further corroborated from studies of peri-gastrulation primate monkey embryos[84].

Recently, there have been numerous peri-gastrulation human embryo models reported that incorporated both embryonic and extraembryonic compartments, such as a yolk sac-like structure and TE derivatives[85-91] (**Fig. 1d**, **Table 1**). Some of these models showed evidence suggesting that yolk sac endoderm-like cells might secret BMP and WNT antagonists to promote anterior ectoderm formation in these models[85-91], supporting the notion that in humans there are yolk sac endoderm cells functioning similarly as the mouse AVE to induce symmetry breaking and A-P patterning of peri-gastrulation human EPI. Even though these recently reported human embryo models are promising for studying embryonic-extraembryonic interactions in the peri-gastrulation human development, their limited efficiencies in modeling EPI symmetry breaking and A-P patterning and forming *in vivo*-like tissue structures remain a significant issue. Continuous efforts will be needed to improve the efficiencies of these models to promote their applications for fundamental studies to understand tissue-tissue interactions and patterning in human development.

Morphogen-mediated NT patterning and its modeling. During gastrulation, embryonic germ layer subpopulations in the mammalian embryo come together, facilitating interactions that shape tissue layers, specify cell types, and initiate organ rudiment development. A critical event in the embryonic ectoderm after gastrulation is the NT formation and patterning, laying the foundation of the functional complexity and anatomical organization of the human nervous system. Patterning of NT along the A-P axis leads to the formation of four main NT subdivisions: forebrain, midbrain, hindbrain and spinal cord (**Fig. 1e**). D-V patterning of each NT subdivision gives rise to various neuronal progenitor cells along the D-V axis. For example, D-V patterning of the spinal cord results in the formation of eleven neural progenitor domains - 5 ventral and 6 dorsal – each expressing a specific combination of transcription factors that constrain their lineage development.

Knowledge of NT patterning mostly comes from studies of chick and mouse embryos. It is well established that axial patterning of NT involves external morphogen gradients, which establish positional information across the NT along both the A-P and D-V axes. A-P patterning is mainly governed by P-A gradients of posteriorizing signals including WNT, retinoic acid (RA) and FGF[92-94] (**Fig. 1e**). A-P patterning of the NT is further refined by developmental signals emanated from secondary organizers, such as FGF signals from the anterior neural ridge and the isthmic organizer at the midbrain-hindbrain boundary[95] (**Fig. 1e**). D-V patterning of the NT is mainly mediated by antiparallel gradients of BMP / WNT and SHH along the D-V axis[96,97] (**Fig. 1e**). BMP and WNT molecules are secreted by dorsal non-neural ectoderm and subsequently by the roof plate, whereas SHH is emanated from the ventral notochord and subsequently by the floor plate[96,97] (**Fig. 1e**). RA secreted from the paraxial mesoderm and somites flanking the NT is also important for ventral patterning of the NT[98].

Stem cell-based NT patterning models have been successfully established with both mESCs and hPSCs, leveraging their self-organizing pattern formation properties[99-101] (**Table 1**). Seeded in Matrigel or synthetic poly(ethylene glycol) (PEG)-based hydrogels under neural induction culture conditions, both mESCs and hPSCs formed spherical lumenal neuroepithelial cysts containing a single central lumen[99-101]. When ventralizing signals RA and / or SHH were supplemented into culture medium, D-V patterning of neuroepithelial cysts was evident, featuring sequential emergence of the ventral floor plate, P3, and pMN domains in discrete, adjacent regions and a dorsal territory progressively restricted to the opposite dorsal pole[99,100]. It remains unclear how global applications of exogenous ventralizing morphogens lead to self-organized, D-V patterning of neuroepithelial cysts. Experimental data showed that at the initial phase of D-V patterning, FOXA2+ floor plate progenitor cells emerged first in neuroepithelial cysts, but in a scattered fashion[99,100]. Soon thereafter, FOXA2 expression became restricted to a local floor plate-like region, defining the ventral pole of neuroepithelial cysts[99,100]. It was suggested that this FOXA2+ floor plate-like domain might function as a local signaling center to induce and pattern other ventral regions in both the mouse and human neuroepithelial cysts[99,100]. Further mechanistic studies revealed that self-organized D-V patterning of mouse neuroepithelial cysts might involve interactions of BMP and Noggin with a reaction-diffusion-based Turing patterning mechanism[102,103]. Whether such self-organized patterning mechanisms operate *in vivo* for axial patterning of the NT is unclear. PEG-based hydrogel systems with tunable degradability, stiffness and matrix composition were also utilized in D-V patterning of mouse neuroepithelial cysts to disentangle the contributions of individual biochemical and physical components of the microenvironment[101]. Nevertheless, D-V patterning of mouse and human neuroepithelial cysts under uniform culture conditions remains inefficient.

Microfluidics technologies, which can provide precise spatiotemporal controls of chemical signals, have been successfully implemented to create morphogen environments for different neural tissue engineering applications, including improving patterning efficiency of stem cell-based NT development models[33,34] (**Table 1**). For example, D-V patterned human forebrain organoids derived from hPSCs were generated by culturing embryonic bodies under a SHH gradient generated in a passive diffusion-based microfluidic gradient generator[104]. Another microfluidic device that employed a series of splitting and mixing of microfluidic flows was utilized to generate a linear WNT signal gradient across 2D layers of hPSCs[33]. This microfluidic device was shown effective in generating an A-P patterned human NT development model that exhibited progressive caudalization from forebrain to midbrain to hindbrain[33]. Interestingly, an isthmic organizer-like domain spontaneously emerged in this human NT model between the midbrain- and hindbrain-like regions, thus highlighting the autonomous and self-organizing properties of NT development, leading to the formation of local secondary organizers at NT subdivision boundaries[33].

More recently, we reported a hPSC-based, microfluidic NT-like structure (or μNTLS), whose development recapitulated some critical aspects of human neural patterning in both the brain and spinal cord regions and along both A-P and D-V axes in a 3D tubular geometry[34] (**Table 1**). To generate A-P and D-V patterned μNTLS, tubular-shaped hPSC tissues were generated in a microfluidic chamber, in which morphogen gradients were established through passive diffusion of different morphogens added to medium reservoirs connected to the chamber. Orthogonal morphogen gradients were established, one of WNT, RA and FGF8 signals along the length of μNTLS to induce its A-P patterning and the other of antiparallel BMP4 and RA / SHH signals perpendicular to the μNTLS length to induce its D-V patterning (**Fig. 1f**). Importantly, the μNTLS was utilized for studying neuronal lineage development, revealing pre-patterning of axial identities of neural crest progenitors and functional roles of

354 neuromesodermal progenitors (NMPs) in spinal cord and trunk neural crest development. Thus, stem
355 cell-based NT development models, developed using microfluidic tools with an *in vivo*-like
356 spatiotemporal cell differentiation and organization, are promising for studying human
357 neurodevelopment and disease.
358
359 **2.2. Bioengineering tools to guide pattern formation**
360 As discussed, pattern formation can be either self-organized[19] or guided by external cues[20]. Self-
361 organized pattern formation depends on spontaneous cell-cell and cell-microenvironment interactions,
362 whereas for external cue-guided pattern formation, precisely controlled dynamic external signals, such
363 as diffusible molecules, are of critical importance. Bioengineering approaches that are efficient in
364 controlling cell number and geometries of morphogenetic fields, tissue-tissue interactions (including
365 both paracrine and juxtacrine interactions), and external chemical gradients are of most significant
366 relevance to improve the efficiency and fidelity of modeling tissue patterning using stem cells *in vitro*.
367
368 *Controlling initial cell cluster formation.* Commercial AggreWell and micropatterned agarose microwell
369 plates have been utilized in blastoid and other embryo model developments, including ETS and ETX
370 embryo models[24,56,58-61,71-73] (**Fig. 2a**). These microwell plates are useful for improving the efficiency
371 and throughput of these embryo models, since confining microwell environments promote cell
372 aggregation and cell-cell interactions. However, microwell plates have some notable limitations. Firstly,
373 AggreWell only allows control over the size but not the geometry of initial cell aggregates. This issue
374 can be addressed by using 3D hydrogel microwells fabricated by molding or laser ablation of hydrogels
375 such as collagen or Matrigel[105,106]. In this way, the initial cell cluster geometry can be fully defined by
376 the hydrogel microwell shape. The pyramidal shape of AggreWell also prevents high-quality *in situ*

imaging of embryo models. High-resolution imaging of free-floating embryo models requires two-photon light sheet microscopy. Microfluidics, which is compatible with high-quality *in situ* imaging, provides an alternative approach to control initial cell cluster formation for embryo modelling. For developing the PASE, microfluidics was used to precisely control the size and location of hPSC clusters with defined cell numbers by aggregating hPSCs in preformed concave hydrogel pockets[32]. Using the microfluidic PASE system, live imaging was conveniently conducted to track tissue morphogenetic events, including symmetry breaking, and signaling dynamics using reporter lines[32].

In development, pattern formation is often followed by tissue growth and organ functional maturation. Microfluidic tools, even though convenient for controlling the formation of initial cell clusters and their positions in prescribed locations inside microfluidic environments, are intrinsically not ideal for long-term culture and continuous growth of embryo models. It is foreseeable that future developments of this direction would involve integrated applications of microfluidics and conventional tissue cultures to promote long-term growth of spatially patterned embryo models, with initial cell cluster formation and tissue patterning conducted in microfluidic devices, followed by transferring embryo models to conventional culture systems for continuous development and tissue growth.

Bioengineered co-culture systems to guide tissue-tissue interactions. A successful co-culture system should satisfy three key criteria: 1) the system should allow precise controls of the proportions of each cell type within specific regions of the co-culture, 2) the co-culture system should allow efficient cell-cell interactions (paracrine or juxtacrine interactions), and 3) the culture environment should facilitate co-culturing of different cell types. The last criterion relies on improved knowledge from stem cell biology and developmental biology. The first two criteria, however, could benefit from the development and application of bioengineering approaches.

Micropatterning-based co-culture platforms, which rely on PDMS stencils or microcontact printing, have been well developed to precisely control spatiotemporal organizations of two or more different adherent cell types in 2D cultures[107-110]. Stencil-based co-culture platforms, however, are unable to physically separate distinct cell types[108-110], making it unsuitable for decoupling juxtacrine and paracrine interactions. In contrast, microcontact printing-based co-culture methods could control the distance between different cell types, rendering them suitable for studying paracrine interactions[107]. There are also micromachined silicon substrates with controllable moving parts to dynamically control tissue organization and composition through mechanical repositioning, thus enabling investigations of dynamic cell-cell interactions[111]. This micromachined silicon substrate system was successfully applied to investigate intercellular communication in co-cultures of hepatocytes and non-parenchymal cells[111]. Owing to their superior controllability and compatibility with live imaging, these 2D co-culture platforms show considerable promise for generating advanced 2D embryo models to study tissue-tissue interactions. Micropatterning-based co-culture platforms are also compatible with many other microengineering and mechanobiology tools, such as microfluidic gradient devices[112], tissue stretching devices[113], microfabricated pillar force sensor arrays[113], and surface biosensing techniques[114]. Integrated applications of these bioengineering tools will enable control, perturbation and quantification of both bio-chemical and -physical factors involved in tissue-tissue interactions during embryo modeling. For example, amnion has been suggested to function as a signaling center secreting BMP and/or WNT molecules to drive the primate gastrulation[32,84]. However, the molecular mechanism(s) underlying the amnion-EPI interaction remains unconfirmed, and it is difficult if not impossible to use primate embryonic tissues for such mechanistic investigations. An amnion-EPI co-culture model can be developed using micropatterning-based co-culture methods to study the role and nature of their interactions in driving primate gastrulation. Additionally, integration of such 2D amnion-EPI co-culture

platforms with surface biosensing techniques could provide concrete information about paracrine or juxtacrine interactions between the two tissues during primate gastrulation. Direct measurements of BMP and WNT ligands, in combination with genetic and chemical perturbations, in the amnion-EPI co-culture model could shed light on molecular mechanism(s) underlying inductive effects of amnion on human EPI development.

2D co-culture platforms are limited to studying intercellular and tissue-tissue communications in 2D settings. Transitioning from 2D to 3D cell culture, 3D bioprinting is emerging as a promising technology to control and study tissue-tissue communications in 3D settings. 3D bioprinting allows precise and flexible assembly of living cells, tissues, biomaterials, and other bioactive factors into complex 3D structures, replicating the intricate architecture of biological tissues or organs[115] (**Fig. 2b**). Even though 3D bioprinting has not yet been applied for embryo modelling, it has been utilized for the development of some promising high-cell density heterogeneous tissue models[116]. For example, magnetic- and aspiration-assisted bioprinting is one of the embedded 3D bioprinting methods that uses aspiration forces to transfer tissue spheroids into self-healing support hydrogels at high resolution, which enables their patterning and fusion into high-cell density microtissues of prescribed spatial organization[117,118] (**Fig. 2b**). This bioprinting method was recently applied to develop co-cultures of hPSC-derived cardiomyocytes and cardiac fibroblasts to recapitulate structural and functional features of cardiac tissues[117]. In another example of embedded 3D bioprinting, perfusable vascular channels were printed into large quantities of organ-specific spheroids to promote their long-term growth and maturation[119].

We envision that controllable and reproducible tissue co-culture platforms with prescribed spatial tissue organizations, based on either 2D systems or 3D bioprinting, will have immense potentials for building advanced embryo models that could be utilized for advancing our understanding of

446 intercellular communications and tissue-tissue interactions in human development, which remains very
447 difficult to study.
448
449 *Engineering signaling centers and morphogen gradients.* Chemical signals, often in the form of
450 signaling molecules such as growth factors and morphogens, guide cellular behaviors, ensuring proper
451 organization and specialization of cells for the formation of functional tissues and organs during
452 development. Understanding and manipulating chemical signals are fundamental to studying
453 developmental biology and hold important implications for advanced embryo modeling. *In vitro*
454 methods for spatiotemporal control of chemical signals involve various techniques to precisely
455 manipulate the distribution and timing of signaling molecules in cell culture environments. These *in*
456 *vitro* methods include chemical-soaked microbeads[120,121], microfluidics[30,122,123], engineered morphogen-
457 secreting signaling centers[31,124,125], and optogenetics[126,127] (**Fig. 2c**).
458 Chemical-soaked beads are often used in developmental biology studies to create controlled
459 microenvironments and study the effects of localized chemical signals and their gradients on developing
460 tissues or organisms. Porous beads are loaded with growth factors, hormones or morphogens, before the
461 beads are placed near developing tissues or embryos. Passive diffusion of chemicals loaded in the beads
462 leads to the formation of their local gradients, allowing studies of how the presence of specific
463 chemicals and their varying concentrations influence cell behaviors including cell fate changes (**Fig. 2c**).
464 Chemical-soaked microbeads were used successfully in studying morphogen-guided pattern formation
465 in amphibian embryos[128,129]. In a recent study, CHIR- and BMP-soaked agarose beads were placed
466 adjacent to human brain organoids to induce localized dorsal cortical hem-like identities in the
467 organoids[120].

Localized signaling centers can also be generated by engineering cells either chemically or genetically. Since treating mESCs with BMP4 upregulates *Nodal* and *Wnt3* expression in the cells, clusters of BMP-treated mESCs could serve as a signaling center emanating NODAL and WNT signals. Assembly of BMP-treated mESC clusters with wild-type mESC clusters led to the development of an mouse embryo model that recapitulated the formation and organization of the three embryonic germ layers[31] (**Fig. 2c**). In another study, genetically modified, chemically inducible SHH-secreting hPSCs were placed on one side of human brain organoids. Under chemical induction, these modified hPSCs secreted SHH to guide patterning of human brain organoids to form spatially restricted forebrain subregions, including regions resembling the ganglionic eminence, hypothalamus, thalamus and dorsal forebrain[124].

Chemical-soaked beads and engineered signaling centers can easily be adopted in biological labs owing to their simplicity. However, it is difficult to precisely control the range, slope and temporal dynamics of morphogen gradients using these methods, thus limiting quantitative studies of morphogen signals and their roles in pattern formation and other developmental processes. These limitations can be addressed by using microfluidics. Microfluidics can control spatiotemporal distributions of signaling molecules and thus create signaling gradients with precise ranges and slopes (**Fig. 2c**). As discussed earlier, a linear microfluidic WNT signal gradient was utilized to generate an A-P patterned human NT model[33]. D-V patterned human forebrain organoids were generated by culturing embryoid bodies under a linear SHH gradient in microfluidic devices[104]. Another advantage of microfluidics is its versatilities and capabilities for different bioengineering applications, including generating 3D orthogonal morphogen gradients useful for inducing A-P, D-V, medial-lateral and left-right patterning along the three body axes. As discussed earlier, we recently developed a microfluidic device that could establish two orthogonal morphogen gradients through passive diffusion of morphogens in a microfluidic

chamber[34] (**Fig. 2c**). A-P and D-V patterned μNTLS were generated by exposing hPSC-derived tubular neuroepithelial tissues to such orthogonal morphogen gradients in the microfluidic chamber[34].

Despite its technical versatility and precision in controlling morphogen dynamics, microfluidics remains challenging to be adopted in common biology and biomedical research laboratories. This is mainly due to the fact that microfluidic device design and fabrication can be complex and require specialized knowledge in microfabrication techniques. The intricate structures and precise features necessary for microfluidic functionalities can also be challenging to produce and troubleshoot. Biological laboratories may not always have personnel with the diverse skill set needed to design, fabricate and operate microfluidic systems. Efforts are being made to address these challenges through the development of user-friendly microfluidic platforms, improved standardization, and increased collaboration between engineers and biologists[30,130]. As microfluidic technologies continue to advance and mature, it is likely that some of these barriers will be overcome, making microfluidic devices more accessible and widely adopted in biological laboratories for stem cell research and embryo modeling.

Optogenetics is another useful approach for establishing exogeneous morphogen gradients. Some optogenetic approaches have been developed to enable photoactivation of transcription factors for controlling gene expression with high spatiotemporal precision, and as such are promising for generating localized signaling centers by inducing subpopulations of cells in a large cell population to produce specific morphogen signals[131] (**Fig. 2c**). Compared to microfluidics, potentially more precise temporal control over morphogen signals can be achieved using optogenetics. Local activation of SHH through optogenetics in a human spinal cord organoid was shown to robustly induce spatially organized ventral spinal cord domains surrounding SHH secreting cells[126,132]. Nonetheless, there are still some important technical limitations of optogenetic approaches for transcriptional controls, including off-target effects, limited tissue penetration, phototoxicity and limited dynamic range[133]. Optimization and validation steps

514 are crucial for obtaining reliable and interpretable results using optogenetic approaches for
515 transcriptional controls.

516 We envision that advances in different bioengineering approaches, particularly those based on
517 microfluidics and optogenetics, and continued refinement of existing systems to precisely control
518 spatiotemporal dynamics of chemical environments and cellular signaling, will enable the development
519 of more advanced and sophisticated embryo models that can faithfully and reproducibly model pattern
520 formation as well as progressive tissue development.

521

522 **3. TISSUE MORPHOGENESIS**

523 Proper morphogenesis processes are required for tissues and organs to acquire their 3D structures during
524 development. Morphogenesis is achieved by coordinated cellular activities, such as cell division,
525 adhesion, migration, and changes in cell shape. Cell-cell adhesive interactions maintain tissue
526 boundaries and enable tissue-level cell arrangements and movements. Loss of cell-cell adherens
527 junctions between epithelial cells leads to EMT, as in the primitive streak formation during
528 gastrulation[134]. The formation of adherens junctions between mesenchymal cells results in their
529 epithelization; this process, termed mesenchymal-to-epithelial transition (MET), is involved in
530 condensation and segmentation of presomitic mesoderm (PSM) to form somites[135]. *In vitro,* cell-cell
531 adhesions can be engineered using synthetic biology approaches to facilitate tissue assembly to build
532 multicellular structures (see Section 4 for more discussions). Cell shape change and cell migration,
533 which are regulated by dynamics of the intracellular cytoskeleton, are crucial in many morphogenetic
534 events, such as the gastrulation and neurulation[134,136]. Cell shape can be modulated by geometrical
535 confinement (*e.g.*, micropatterning) and mechanical perturbations (*e.g.*, tissue stretching)[25,113]. These
536 bioengineering approaches have been applied to build embryo models such as 2D micropatterned

gastrulation and neural development models and to study the roles of cell mechanics and the cytoskeleton in embryonic development[25,113].

Cell-ECM interactions play crucial roles in tissue morphogenesis by influencing cell shape, polarity and organization[137,138]. Cell-ECM interactions are also essential for cell migration during processes such as the gastrulation and organogenesis[137,138]. Besides providing mechanical support and promoting tissue integrity, cell-ECM interactions modulate various signaling pathways, influencing processes such as proliferation, survival and differentiation[137,138]. There are recent studies in which different embryo models were embedded in natural or synthetic hydrogels; this step was critical for triggering morphogenetic events mimicking neurulation and somitogenesis, supporting the importance of controlling cell-ECM interactions in modeling proper tissue morphogenesis in embryo modeling[139-141]. Over the last decade, there has been remarkable progress in developing various embryo and organ models that recapitulate key morphogenetic events during mammalian development.

3.1. Morphogenesis *in vivo* and in embryo models

Gastrulation: forming organized body plan. As a highly orchestrated process, gastrulation lays the foundation of the basic body plan[36,68,134]. Besides germ layer lineage fate specification, gastrulation involves a myriad of morphogenetic events to organize the topology of the three definitive germ layers. These morphogenetic events include EMT, cell migration, convergent extension, and elongation along the A-P axis[134,142] (**Fig. 3a**). Much attention has been paid to mimicking germ layer lineage differentiation and patterning in peri-gastrulation embryo models established using 2D micropatterned hPSC colonies treated with exogeneous BMP4[25] (**Fig. 3b**, **Table 1**). These 2D human gastrulation models are compatible with live imaging and can be generated robustly, thus allowing quantitative mechanistic studies of how self-organized signaling events drive lineage fate patterning during

gastrulation. Several signaling mechanisms were identified using these 2D micropatterned human gastrulation models, including dynamic waves of BMP, WNT and NODAL signaling, interaction of endogenous activators and inhibitors in a diffusion-reaction mechanism, and basolateral localization of BMP receptors[23,28,29]. The latter mechanism was further shown to play a role in the formation of a robust BMP signaling gradient in peri-gastrulation mouse embryos[143].

3D gastrulation models, or gastruloids, have been generated by treating 3D clusters of mESCs or hPSCs with a pulse of WNT agonist CHIR99021, leading to the formation of an A-P axis and differentiated germ layer derivatives spatially organized along this axis[139,144-146] (**Table 1**). More advanced gastruloids were demonstrated to recapitulate the formation of organ primordia during early organogenesis, such as the NT, bilateral somites, gut tube, and cardiac tissues, by optimizing chemical and mechanical signals in gastruloid culture environments[141,147,148] (**Table 1**). Tissue organization and patterning in gastruloids were achieved without the presence of extraembryonic lineages, suggesting self-organized pattern formation mechanisms in play. Recently, numerous post-implantation and peri-gastrulation human embryo models that incorporated putative extraembryonic signaling centers, such as the amnion, yolk sac and TE derivatives, were reported[85-91] (**Table 1**). These models are promising for studying how self-organized patterning mechanisms function synergistically with embryonic-extraembryonic interactions to ensure the robustness of the gastrulation process and embryonic germ layer organization.

There is emerging evidence suggesting that cell fate patterning and morphogenesis are possibly regulated independently during gastrulation[134,149]. This observation is corroborated by findings from 2D micropatterned gastrulation models and gastruloids, which recapitulate spatially patterned cell fate organization during gastrulation but with limited morphogenetic events. However, how cell fate patterning and morphogenesis are coordinated to establish the body plan during gastrulation remains ill-

583 understood. Stem cell-based embryo models that can mimic both cell fate patterning and morphogenetic
584 events during gastrulation are thus promising for elucidating this important question.

585 To promote morphogenetic events, bioengineering approaches have been integrated with
586 gastrulation models. For example, culturing micropatterned 2D human gastrulation models on a soft
587 polyacrylamide hydrogel that recapitulated the biophysical properties of early embryos promoted a
588 "gastrulation-like" morphogenesis of hPSCs, including self-organization of "gastrulation-like" nodes
589 and ingression and migration of mesoderm cells through the nodes[150] (**Fig. 3b**, **Table 1**). This study
590 further showed that tissue geometries dictated the formation of "gastrulation-like" nodes in regions
591 where high cell-adhesion tension would arise, which directed spatial patterning of BMP4-dependent
592 "gastrulation-like" phenotype by promoting WNT signaling and mesoderm specification. Further
593 integration of this micropatterned 2D human gastrulation model with a bioengineered tissue stretching
594 device showed that direct force application *via* mechanical stretching could promote BMP-dependent
595 mesoderm specification[150], confirming the role of tissue-level forces in directly regulating lineage
596 differentiation during gastrulation[151,152].

597 Without the presence of extraembryonic tissues, there is no structure formation in gastruloids
598 that mimics the primitive streak[142,146]. Other morphogenetic events associated with the gastrulation,
599 including collective cell migration, also don't seem to occur in gastruloids[142,146]. However, when mESCs
600 were co-cultured with extraembryonic cells such as mTSCs and/or XENs in ETS and ETX embryo
601 models, a primitive streak-like structure emerged in the compartment of mESCs adjacent to mTSCs[72]
602 (**Table 1**). Similarly, in a 3D human peri-gastrulation model encompassing both embryonic and
603 extraembryonic tissues, a putative primitive streak-like structure emerged at the posterior end of the
604 model, characterized by cells at this region undergoing EMT and inward migration[153] (**Table 1**).
605 Together, these studies suggest that the primitive streak might be a transient structure whose formation

involves the mechanics and geometrical boundary constraints imposed by adjacent extraembryonic tissues[134,142]. Using bioengineering approaches to control tissue mechanics and topology as well as cell-cell and cell-ECM interactions during embryo modeling will be an important direction to recapitulate and study morphogenetic events that occur during the gastrulation.

One morphogenetic event that is well recapitulated in gastruloids is axial elongation. Along the A-P axis, peri-gastrulation embryo elongates, with progenitors in the tail bud proliferating and differentiating to give rise to tissues that generate posterior spinal cord, skeleton and musculature[154]. In gastruloids, posterior progenitors, likely including NMPs, emerge at their posterior ends[139,141,144-146]. These cells have been shown to give rise to both neural and mesodermal lineages and contribute to posterior elongation of gastruloids[139,141,144,145]. In mouse gastruloids, P-to-A gradients of Erk and Akt signals were shown to play important roles in controlling the unidirectional tissue growth at their posterior ends by modulating cell proliferation, motility and adhesion[155]. Tissue elongation and narrowing along the A-P axis, which resembles convergent extension during mouse gastrulation, was also observed in mouse gastruloids[156]. Consistently, an *in silico* model showed that convergent extension could help explain the elongation of gastruloids[156]. In embryonic development, convergent extension is a conserved complex process involving a series of molecular and cellular events that coordinate cell movements and rearrangements, including cell polarization, dynamic changes of cell adhesion and cytoskeleton, cell intercalation, and planar cell polarity signaling[157]. It will be important for future studies to examine whether similar molecular and cellular events are involved in axial elongation of mouse and human gastruloids.

Neurulation: tissue bending morphogenesis. Neurulation describes the developmental process through which the NT is formed from the neural plate (NP) specified in the ectoderm. After gastrulation, the

ectoderm is specified into the neuroectoderm, containing the NP, flanked by the neural plate border (NPB) and non-neural ectoderm (NNE), through the process of neural induction. As the neurulation progresses, the NP undergoes structural changes, with the lateral edges of the plate elevating to form two parallel ridges called neural folds (**Fig. 3c**). The neural folds eventually meet in the dorsal midline of the embryo and fuse to form the hollow NT, containing a central lumen called neural canal (**Fig. 3c**). In mammals, NP bending is primarily driven by mechanical forces generated by apical constriction in NP cells at the median and dorsolateral hinge points[136] (**Fig. 3c**). There is also evidence suggesting the role of proliferation of NNE tissues in generating mechanical pushing forces to facilitate NP bending and closure[136]. Once the two opposing neural folds meet at the dorsal midline, cells at the tips of the neural folds develop cellular protrusions to attach to the opposite neural folds. The NT then becomes separated from the dorsal NNE tissue due to differential adhesions.

Neurulation is a tightly regulated process, disruption of which can lead to NT closure defects (NTDs), one of the most common congenital disorders affecting the nervous system. Genetic mutations affecting BMP, SHH, WNT, and planar cell polarity signaling pathways, impaired cell proliferation, apoptosis and ECM formation, abnormal cell adhesion, migration and actomyosin turnover, oxidative stress and inflammation, and environmental factors have all been implicated in NTDs[158-161]. Dynamics of the neurulation depends on the A-P axial level[136]. Fusion of neural folds begins in the middle of the embryo and moves anteriorly and posteriorly[136]. Disruption of several actin-associated proteins prevents bending of the NP only in anterior regions but not in spinal regions[161-164], supporting different dynamics and regulatory mechanisms of the neurulation at different A-P axial levels. Understanding the molecular and cellular mechanisms underlying neurulation is pivotal for providing insights into the etiology of NTDs. Most of the knowledge about neurulation comes from studies of chick and mouse embryos, which remains to be validated in human-relevant models. Recently, various stem cell-based human

652 neurulation models have been developed using hPSCs[113,140,165-168] (**Table 1**). These models offer
653 accessible and tractable experimental tools to study different morphogenetic events involved in human
654 neurulation.

655 The lumenal morphology of human NT was initially modeled successfully using 2D neural
656 rosettes containing a single central lumen[165,169] (**Table 1**). Neural rosettes are 2D neuroepithelial tissues
657 derived from hPSCs. The initial tissue geometry of 2D neural rosettes plays an important role in lumen
658 formation. Thus, using micropatterning tools to control the geometry of 2D neuroepithelial tissues,
659 neural rosettes with a single lumen were derived reproducibly from hPSCs with a high efficiency[165].
660 More recently developed 2D micropatterned neurulation models using hPSCs recapitulated
661 neuroectoderm patterning and even the single lumen morphology of the NT[113,166,167] (**Table 1**). Studies
662 using these models demonstrated the important roles of both bio-mechanical and -chemical cues in
663 neuroectoderm patterning[113,166,167]. Importantly, these studies further supported the self-organization of
664 morphogenetic cues, including cell shape and cytoskeletal contractility, in neuroectoderm patterning[113].
665 Self-organized morphogenetic cues could directly feedback to mediate BMP activity to guide spatial
666 regulation of neuroectoderm patterning[113]. Thus, mechanistic studies of neuroectoderm patterning using
667 these 2D micropatterned neurulation models support intricate patterning signaling crosstalk involving
668 both biophysical and biochemical determinants, which may be important in controlling patterning
669 networks to ensure the remarkable robustness and precision of neuroectoderm patterning *in vivo*.

670 Since 2D micropatterned neurulation models are confined to 2D surfaces, they are intrinsically
671 not suitable for recapitulating the dynamic NP folding process during neurulation, which might limit
672 their applications for studying NTDs. A recent study combining micropatterned 2D hPSC colonies with
673 3D culture successfully recapitulated neuroectoderm patterning followed by the dynamic NP folding and
674 fusion process, leading to the formation of a 3D tubular structure mimicking the cranial NT with a high

efficiency[140] (**Fig. 3d**, **Table 1**). This 3D human neurulation model was further utilized for modeling NTDs, specifically demonstrating the impacts of chemicals perturbing apical constriction and basal ECM synthesis on folding and closure of the NP.

Spinal cord organoids derived from hPSCs have also been reported that recapitulate neurulation-like tube-forming morphogenesis of the early spinal cord[168] (**Table 1**). Specifically, to develop these spinal cord organoids, caudalized 2D hPSC colonies, likely through a transient NMP stage, were detached from 2D cultures to form 3D spheroids in a free-floating culture. These spheroids were induced with a neuroepithelial fate. NP-like structures spontaneously emerged at the surfaces of these 3D spheroids, and they underwent folding morphogenesis to form NT-like structures within the 3D spheroids, likely involving apical constriction in neuroepithelial cells[168]. Neurulation-like tube-forming morphogenesis in the spinal cord organoids occurred in the absence of NPB or NNE tissues, supporting a role of innate self-organizing properties of NP cells in driving their folding dynamics[168].

Somitogenesis: tissue segmentation morphogenesis. Somitogenesis is the process by which somites are formed in the embryo. Somitogenesis establishes the segmented pattern of the vertebrate body plan, essential for proper formation and function of the musculoskeletal system. Defects or disruptions in somitogenesis lead to congenital abnormalities such as scoliosis. Current knowledge about somitogenesis is mainly derived from chick and mouse studies. During somitogenesis, the PSM, a bilateral strip of mesenchymal tissue flanking the forming NT, progressively segments into bilaterally symmetrical epithelial somites in the A-P direction[135] (**Fig. 3e**). Somitogenesis can be explained by the clock-and-wavefront model, which proposes that somitogenesis is regulated by the combined action of a molecular clock mechanism within the PSM and a moving wavefront of signaling activity along the A-P

697 axis[135] (**Fig. 3e**). This model provides a conceptual framework for understanding how the periodic
698 segmentation of somites is controlled during embryonic development.

699 Somitogenesis has provided an excellent model to study the formation of morphological
700 boundaries between developing tissues. New somites are formed at the anterior end of the PSM, where
701 cells undergo MET and coalesce into a rosette-like structure that pulls apart from the PSM. Specifically,
702 cells in forming somites at the anterior PSM undergo rearrangements, which involves changes in cell
703 adhesion, cell shape and cell-cell interactions, contributing to the morphological formation of an
704 intersomitic boundary (also called a fissure) and separation of forming somites from the PSM[170]. Fissure
705 formation in somitogenesis has been suggested as an intrinsic property of PSM cells and does not rely
706 on surrounding tissues[171]. After its formation, PSM cells in the somite undergo MET, resulting in the
707 conversion of loosely packed mesenchymal cells into tightly packed epithelial cells, which contribute to
708 the structural integrity of somite boundaries[170]. Besides classic morphogenetic studies of the
709 somitogenesis, recent studies revealed decreasing cell stiffness, polarity, and adhesion along the A-P
710 axis in the chick PSM[172]. Applying external mechanical stress along the A-P axis of the chick PSM also
711 induced the formation of new somite boundaries that were not determined by the clock-and-wavefront
712 model, suggesting mechanical cues could be instructive for somite formation[173,174].

713 Somitogenesis was first recapitulated in gastruloids. When embedded in Matrigel, mouse
714 gastruloids, termed trunk-like structures, developed a NT-like structure flanked by the PSM and
715 epithelial somite-like tissues[141] (**Fig. 3f**, **Table 1**). Recently, similar human somitogenesis models were
716 developed by culturing hPSC clusters in PSM differentiation environments[175-178] (**Table 1**). These
717 models exhibited oscillatory expression of clock genes in the PSM, the periodic segmentation and
718 epithelialization of somites, and A-P polarity of each single somite. Novel biological insights into
719 somitogenesis were revealed from these models, including an unexpected role of cell sorting during

somite formation and the role of RA in epithelialization of somites[177,178]. Matrigel appeared to be critical for epithelialization of somitic cells but was not required for somitic differentiation of PSM cells in either mouse or human somitogenesis models, highlighting the importance of the ECM environment in morphogenetic events involved in somitogenesis[141,177,178].

To study the biochemical-biomechanical interactions that drive somitogenesis, another somitogenesis model was recently developed in a microfluidic device by controlling exogenous anti-parallel RA and WNT / FGF signaling gradients and mechanical boundaries in hPSC-derived PSM tissues[179] (**Table 1**). A fracture mechanics-based theoretical model, which considered strain energy induced by compaction of a forming somite and surface energy resulted from a new somite-PSM boundary, was proposed to explain the role of mechanics in regulating somite size. Despite its simplistic nature, this theoretical model revealed a scaling law connecting the dimension of nascent somite with the length of PSM. The primary correlation between nascent somite sizes and the PSM length agreed with data from mouse, chick, zebrafish and human embryos quantitatively, supporting a common mechanics-based mechanism in play in regulating somite boundary formation in different species.

Co-morphogenesis of NT- and somite-like structures was also achieved in mouse and human trunk-like structures, under a low dose of WNT signal stimulation[141,180] (**Fig. 3f**, **Table 1**). *In vivo*, posterior NT and somites originate from a common caudal progenitor population, including NMPs. Mouse and human trunk-like structures, recapitulating the spatiotemporal development of these three lineages, could be very useful for studying their lineage relationships and the genetic and molecular mechanisms underlying caudal lineage diversifications.

3.2. Bioengineering tools to guide morphogenesis

742 During development, morphogenesis is brought about by the coordination of intra- and inter-cellular
743 forces and their interactions with surrounding tissues and ECM. Intracellular forces are generated by the
744 contractile cytoskeleton, which are transmitted to the local cell microenvironment through cell-cell and
745 cell-ECM adhesions. Surrounding tissues and ECM with specific geometries and mechanical and
746 biochemical properties provide additional boundary conditions that can influence tissue morphogenesis.
747 Through both *in vivo* and *in vitro* studies, it has become clear that the geometrical tissue boundary
748 conditions provide a ground state for morphogenetic events[134,142]. Thus, bioengineering approaches
749 effective in controlling geometrical boundaries of 2D and 3D tissues are useful for engineering tissue
750 morphogenesis and improving the efficiency and robustness of embryo and organ model developments.
751 Furthermore, synthetic biomaterials engineered with specific mechanical properties and / or
752 functionalized with growth factors and signaling molecules can be promising for guiding tissue
753 development and organization in embryo models. In this section, we discuss bioengineering approaches
754 for controlling geometrical boundaries of 2D and 3D tissues and different biomaterial systems for
755 embryo models. Discussions of synthetic biology approaches to control intracellular contractility and
756 cell-cell and cell-ECM adhesions are in Section 4.
757
758 *Engineering tissue geometry and boundary.* Micropatterning allows the creation of 2D cell colonies with
759 controlled size, shape, and physical boundary conditions. In micropatterning, patterns of adhesive
760 molecules at the micro- and nanoscale resolutions are created on 2D tissue culture surfaces (either tissue
761 culture plates or glass coverslips) through a variety of bioengineering tools, including microcontact
762 printing[181], stencil micropatterning[182,183], and photopatterning[25] (**Fig. 4a**). Thus, selective adhesion of
763 mammalian cells to micropatterned adhesive regions leads to formation of 2D cell colonies with
764 prescribed sizes and shapes (**Fig. 4a**).

765 Microcontact printing, the process to print adhesive islands onto substrates using stamps, is
766 currently the most widely used technique to generate micropatterned 2D cell colonies in cell biology and
767 tissue engineering laboratories[181]. In microcontact printing, adhesion properties, surface energy, and
768 chemical compatibility between the stamp, printed adhesive molecules and substrates must be carefully
769 considered to ensure successful pattern transfer. Furthermore, stamp reusability and surface
770 homogeneity can be issues. In microcontact printing, stamps are often generated using microfabrication,
771 which requires access to cleanroom facilities, thus limiting wide applications of this convenient and
772 powerful technology in biology and biomedical research laboratories.
773 Stencil micropatterning involves the use of stencils, which are essentially masks with specific
774 patterns or designs cut out of them. These stencils are placed onto a substrate, and material is deposited
775 or removed through the openings in the stencil to create desired patterns on the underlying surface.
776 Thus, in stencil micropatterning, the stencil-to-substrate contact is important in determining pattern
777 fidelity and impacting experimental reproducibility. Stencil micropatterning was originally developed
778 for microelectronics applications[184,185]. However, it has also found applications in generating
779 micropatterned 2D cell colonies by applying stencils onto cell culture substrates before cell
780 seeding[182,183]. After cell seeding, stencils can be removed to release geometrical constraints to the
781 growing cell colonies, to promote their long-term development. Stencil micropatterning is best suited for
782 simple geometric patterns with well-defined shapes and features. Similar to microcontact printing,
783 stencil micropatterning typically provides static, fixed patterns on substrates. The lack of dynamic
784 control over pattern geometry and cell behaviors limits the applicability of stencil micropatterning and
785 microcontact printing for certain tissue engineering studies.
786 In photopatterning, cell culture surfaces coated with a layer of cytophobic or cell-repellent
787 molecules are patterned using UV light passing through a photomask[186-188]. Localized UV light exposure

of cytophobic molecules promotes their degradation and exposes underlying cell culture surfaces for subsequent functionalization with adhesive molecules to promote local cell attachment[186-188]. Similar to microcontact printing and stencil micropatterning, photopatterning typically involves patterning cells at a single time point, resulting in static, fixed patterns. Photopatterning also relies on the use of photosensitive materials whose long-term cytotoxic effects and degradation properties need to be carefully evaluated. Nonetheless, micropatterned cell culture surfaces generated using photopatterning are commercially available, which promotes broader applications of micropatterning techniques for 2D cell culture studies, especially in research labs that lack access to microfabrication tools[25].

3D morphogenetic events cannot be fully recapitulated in 2D cell culture systems. Nonetheless, integration of 2D micropatterning tools and 3D culture expands the application of 2D micropatterning to model 3D morphogenetic events, such as tissue folding and fusion[140]. In addition, 3D structures made of PDMS or hydrogels containing channels or wells of defined sizes and shapes, which are fabricated by soft lithography or laser ablation, have also been utilized for guiding morphogenetic events involved in the somitogenesis and intestinal morphogenesis[105,106,179] (**Fig. 4a**). Given their superior reproducibility, scalability, and compatibility with live imaging, micropatterned 2D or 3D culture systems are promising for translational applications such as drug and toxicity screens.

Bioengineering hydrogels. In vivo, the ECM provides structural support, mechanical stability, and biochemical signaling cues for guiding morphogenetic events during embryonic development. Matrigel, a gelatinous protein mixture extracted from Engelbreth-Holm-Swarm mouse sarcoma, is the most widely used natural ECM for 3D cultures, including embryo and organ model cultures. However, Matrigel is ill-defined with significant batch-to-batch variations, which limits the reproducibility of 3D cultures using Matrigel[189]. In addition, Matrigel offers no direct control over its molecular composition

or components, making it unsuitable for studying the role of individual ECM components in regulating embryonic development[189]. Since Matrigel is derived from animals, there is a risk of contamination with animal-derived factors, hindering clinical applications of Matrigel[189].

Over the last few decades, there have been tremendous advances in fully defined synthetic biomaterials and their applications in 3D tissue cultures. By incorporating bioactive molecules, cell-adhesive motifs, and tissue-specific cues into synthetic biomaterials, these synthetic biomaterials are designed to mimic the structure and function of natural ECM. Synthetic matrices such as PEG-based hydrogels have already been successfully utilized for reproducible development of mouse and human intestinal organoids and D-V patterned mouse NT development models[101,190-192]. Besides biochemical properties, synthetic matrices also offer the advantageous feature of convenient and independent modulations of their biophysical properties, such as degradability, porosity, and viscoelastic properties, thus making them suitable for studying the independent effects of these factors on embryo and organ model development[101,190-192]. Systematically screening synthetic matrices of different properties will lead to the discovery of optimal hydrogel conditions for the development and growth of embryo and organ models, which will facilitate their scalable and reproducible production for both fundamental and translational applications.

Another important future direction is to apply synthetic biomaterials with spatially patterned biochemical and biomechanical properties, such as dynamically controllable growth factor release and matrix stiffness changes, for guiding symmetry breaking, pattern formation, and morphogenesis of embryo models[138,193] (**Fig. 4b**). Hydrogels containing photoreversible immobilization of growth factors were developed to achieve spatially localized growth and differentiation of human mesenchymal stem cells[194-196]. Spatial patterning of cell adhesive ligands within 3D synthetic matrices was shown to guide directional cell migration[197,198]. Additionally, photo-induced hydrogel cross-link exchange reaction was

834 employed to spatiotemporally alter the local viscoelasticity of a synthetic hydrogel system formed
835 through reaction between PEG-dibenzylcyclooctyne and allyl sulfide bis(PEG3-azide). Photo-induced
836 local changes in the viscoelasticity of the hydrogel were utilized to control epithelial tissue curvatures
837 and study how dynamic changes in tissue curvatures influence mechanotransduction pathways to
838 instruct crypt morphogenesis in intestinal organoids[199]. The spatiotemporal control of extracellular
839 signals, offered by such synthetic hydrogels, offer another layer of external regulation that can be
840 integrated with other bioengineering approaches, such as microfluidics and optogenetics, for future
841 development of controllable and programmable embryo models.
842
843 **4. ENGINEERING CELL FATE AND CELL-CELL INTERFACES USING SYNTHETIC**
844 **BIOLOGY**
845 Synthetic biology offers powerful molecular tools to design and construct new biological systems or
846 redesign existing biological systems for useful purposes. Over the last decade, synthetic biology
847 provides various approaches to control transcription factor expression, modulate signaling pathways,
848 manipulate epigenetic modifications, direct cell fate specification, and regulate cellular interactions and
849 organization[200-203]. Thus, synthetic biology offers attractive approaches orthogonal to microfluidics,
850 micropatterning and synthetic biomaterials to achieve programmable cellular complexity, architecture
851 and function in bioengineered embryo and organ models. Synthetic biology could contribute to the
852 construction of embryo models through engineering fate (**Fig. 5a**), engineering cell adhesion (**Fig. 5b**),
853 optogenetics (**Fig. 5c**), and engineering morphogen gradients (**Fig. 5d**). Optogenetics and bioengineered
854 signaling centers have been discussed in the section of "*Engineering signaling centers and morphogen*
855 *gradients*". Thus, here we focus on discussing how transcription factor overexpression and synthetic cell

adhesions could be utilized for engineering cell fate and cell-cell interfaces, respectively, for improving cellular complexity, composition and organization in embryo and organ models.

Engineering cell fate. Overexpression of transcription factors through genetic engineering has been commonly employed for inducing cell differentiation or transdifferentiation (**Fig. 5a**). Compared with chemical induction, transcription factor overexpression can produce specific cell lineages more quickly and efficiently. Genetically engineering PSCs by overexpressing transcription factors could produce additional cell sources for constructing embryo and organ models. As mentioned earlier, ETX embryo models, developed by assembling mESCs, TSCs and XENs, resemble the mouse egg cylinder. ETX embryo models, however, could not develop much further beyond the onset of gastrulation[71,72] (**Table 1**). This limited developmental potential of ETX embryo model is likely resulted from heterogeneous TSC and XEN populations, which contain subpopulations resembling different stages of their *in vivo* counterpart development[76,204]. When mouse TSCs and XENs were replaced with mESCs transiently overexpressing *Cdx2* and *Gata4*, respectively, resulting mouse embryo models could progressively develop beyond the gastrulation and reaching early organogenesis stages[75-77], albeit with a very low efficiency and containing organ primordia often showing notable structural defects (**Table 1**). More recently reported, integrated peri-implantation human embryo models also utilized similar transcription factor overexpression strategies to generate extraembryonic lineages, before assembling them with either naïve or primed hPSCs for constructing peri-implantation human embryo models[85,86,88-91] (**Table 1**). These examples highlight the application of engineering cell fate through overexpressing transcription factors to obtain homogenous stem cell or progenitor populations, promoting cellular complexity, composition and organization in embryo models.

In another example, genetic engineering tools were combined with 3D bioprinting to construct organoids with controlled cellular composition and organization. Specifically, an orthogonally induced differentiation (OID) method was developed, by overexpressing different transcription factors in the same hPSC population, to simultaneously differentiate the cells into various organ-specific lineages independently of culture medium conditions[205]. This OID method was applied to generate endothelial cells and neurons from hPSCs simultaneously under the same culture to produce vascularized cortical organoids[205]. By integrating OID with multi-material bioprinting of hPSCs with inducible-transcription-factor-overexpression, patterned neural tissues with layered regions composed of neural stem cells, endothelium and neurons were generated, highlighting the promise of integrating 3D bioprinting and orthogonally induced differentiation of stem cells for building multicellular tissue structures with controlled cellular composition, organization and function[205].

Engineering cell adhesion. Cell-cell adhesion is critical for tissue development by regulating tissue architecture, cell behavior and signaling processes. Cadherins form adherens junctions between adjacent cells, which play an important role in regulating tissue morphogenesis by maintaining tissue integrity, shape and organization[206,207]. Varying expression levels and types of cadherins could result in autonomous cell sorting and segregation during tissue development, a phenomenon termed differential adhesion[207]. Differential adhesion plays an important role in establishing tissue boundaries and spatial organization during development[206,207]. Interestingly, by overexpressing cadherin molecules specific to the mouse EPI, TE and PrE in mESCs, TSCs and XENs, respectively, the efficiency of ETX embryo model was improved through autonomous cell sorting and segregation similar to those in mouse blastocyst formation[208].

Over the past two decades, synthetic biologists have repurposed physical parts and concepts from natural receptors to engineer synthetic receptors[200]. These technologies implement customized sense-and-respond programs that link a cell's interaction with extracellular and intracellular cues to user-defined responses, including rationally programmed assembly of multicellular architectures[202,209] (**Fig. 5b**). In recent years, the library of synthetic receptors and their capabilities has substantially evolved. In one recent study, synthetic cell adhesion molecules were demonstrated by combining orthogonal extracellular interactions with intracellular domains from native adhesion molecules, such as cadherins and integrins[209]. The resulting synthetic molecules were shown to yield customized cell-cell interactions with adhesion properties that are similar to native interactions, including eliciting intracellular actin cytoskeleton dynamics and tension[209]. How synthetic receptors could be utilized for improving the efficiency of embryo and organ models remains to be demonstrated. This effort might further lead to the development of programmable embryo and organ models endowed with certain enhanced functionalities.

5. PERSPECTIVE AND FUTURE DIRECTIONS

In this review, we have discussed recent progress in stem cell-based embryo modeling and how bioengineering approaches useful for engineering pattern formation and morphogenesis can be employed to construct advanced embryo models with enhanced efficiency and controllability and heightened cellular complexity, composition and organization. The heterogeneity of initial stem cell populations used in embryo modeling and their less characterized functions are also important issues that should be fully addressed. To this end, synthetic biology approaches to engineer cell fate have shown significant promises to generate more uniform cell sources to construct embryo models.

Stringent validation of embryo models is important for their downstream applications, as how faithfully an embryo model can recapitulate *in vivo* development determines its usefulness as a model system to study embryo development. While it is desirable to compare embryo models with *in vivo* embryos of the same species and compatible stages, this is a challenge when it comes to human embryo modeling. Recent significant advancements in studies of *in vivo* and *ex vivo* cultured human and non-human primate embryos provide important molecular and cellular knowledge for authenticating and benchmarking human embryo models[210-217]. When comparing embryo models with *in vivo* or *ex vivo* cultured embryos, we should not only consider molecular signatures, cell lineage identities or multicellular tissue organization at isolated time points, but also examine the dynamic and progressive tissue developmental events that cultivate their morphology, architecture and function.

Single-cell multiome, including single-cell RNA-sequencing (scRNA-seq), provides a wealth of information about the molecular characteristics and functions of single cells, allowing researchers to gain insights into cellular heterogeneity, cell-to-cell variability, and complex biological processes. Over the past decade, numerous scRNA-seq datasets have been generated from *in vivo* and *ex vivo* cultured human and non-human primate embryos at different development stages[214-217]. This rich information provides important molecular references for authentication and identification of cell types, characterization of their transcriptional states, detection of rare cell populations, and study of intracellular signaling and tissue-tissue interactions in embryo models. When examining embryo models and how useful they will be for fundamental and translational applications, great care must be taken to understand the specific developmental stages and associated cell lineage developments each embryo model is capable of recapitulating. There are many embryo models now available, with some considered as integrated with both embryonic and extraembryonic compartments and others as non-integrated. For many embryo models, there remains significant ethical challenges. There are ongoing discussions that

human embryo models should warrant careful regulations, and particularly those considered as integrated ones should follow regulations similar to those for natural human embryos[218-220]. Thus, we need to be very cautious in choosing the most relevant embryo models for studies, while also considering their ethical implications. It is desirable to choose ethically less challenging embryo models if possible, when different models are available to recapitulate the developmental stages and associated cell lineage developments of interest (**BOX 2**).

Appropriate embryo models need to be selected for specific applications. Increased cellular complexity and composition in embryo models often come with decreased tissue organization and/or model controllability and reproducibility. For example, studies of individual germ layer development likely do not need the use of integrated embryo models that contain certain extraembryonic cell lineages. In contrast, studies of tissue-tissue interactions, such as interactions between embryonic and extraembryonic lineages, will require more complex embryo models that contain all relevant cell types[85-91,153,221]. For fundamental mechanistic studies to understand human development, embryo models that closely resemble *in vivo* development in terms of lineage diversification, tissue patterning, morphogenetic events, and tissue architecture will be desired. However, this might not be the case for translational applications, especially high-content screening applications, which favor embryo models with high reproducibility and scalability. For example, the 3D NT folding model faithfully recapitulates the dynamic NP folding and provides a promising experimental tool to understand biomechanics of NT closure[140]. In contrast, 2D micropatterned neural rosette arrays, while they do not recapitulate the NP folding morphogenesis, demonstrate high reproducibility, scalability and easy manipulation. As such, 2D micropatterned neural rosette arrays have already been successfully utilized for high-content screens of factors that might disrupt neurulation and cause NTDs[165,222,223].

The field of stem cell-based embryo models is progressing rapidly, with tremendous potentials for both fundamental and translational applications. Continuous development and broad applications of embryo models will require the integrations of bioengineering approaches for improving cellular complexity, composition and organization in embryo models and enhancing efficiency, controllability, reproducibility, scalability and thus usefulness of the models. As this field moves forward, we should keep in mind social responsibility as an essential part of the responsible conduct of research. Transparency and effective engagement with all stakeholders including the public is essential to ensure that promising avenues for research proceed with due caution, especially given the complexity and rapid progress of this field.

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AUTHOR CONTRIBUTIONS

X.X., Y.L. and J.F. wrote article text. X.X. generated figures. All authors contributed to the conceptualization of the article.

COMPETING INTERESTS

The authors declare no competing interests.

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KEY POINTS

- Stem cell-based embryo models can recapitulate essential developmental processes such as tissue patterning and morphogenetic events from pre-implantation to early organogenesis.
- Bioengineering approaches, which are efficient in controlling tissue geometrical boundaries and interactions, chemical gradients and extracellular matrix signals, are instrumental to improve the efficiency and fidelity of stem cell-based embryo modeling.
- Synthetic biology offers attractive approaches orthogonal to other bioengineering approaches to achieve programmable cellular complexity, architecture and function in bioengineered embryo models.
- Bioengineered embryo models with enhanced efficiency, controllability, reproducibility, scalability and usefulness hold promise in fundamental research and biomedical applications.

Table 1. Stem cell-based embryo models discussed in the paper.

Cell lineages: EPI, epiblast. TE, trophectoderm. PrE, primitive endoderm. ExE, extraembryonic ectoderm. VE, visceral endoderm. AVE, anterior visceral endoderm. PGC, primordial germ cell. NP, neural plate. NPB, neural plate border. NNE, non-neural ectoderm.

Stem cells: (mouse) mESC, mouse embryonic stem cell. mTSC, mouse trophoblast stem cell. mXEN, mouse extraembryonic endoderm stem cell. mEPSC, mouse extended / expanded potential stem cell. i*Gata4* mESC, mESCs transiently expressing *Gata4*. i*Cdx2* mESC, mESCs transiently expressing *Cdx2*. (human) hPSC, human pluripotent stem cell. hEPSC, human extended / expanded potential stem cell. i*GATA6* hPSC, hPSCs transiently expressing *GATA6*. i*GATA6-SOX17* hPSC, hPSCs transiently expressing *GATA6* and *SOX17*. i*GATA3-TFAP2C* hPSC, hPSCs transiently expressing *GATA3* and *TFAP2C*.

Embryo model names: ETS embryoid, ESC-, TSC-derived embryo model. ETX embryoid, ESC-, TSC-, XEN-derived embryo model. PASE, post-implantation amniotic sac embryoid. SEM, post-implantation stem-cell-based embryo model. μNTLS, microfluidic neural tube-like structure. μSDM, microfluidic somite development model.

Mouse embryo models					
Model names	**Starting cell population**	**Approach**	**Developmental events modeled**	**Limitations**	**Ref.**
Blastoid	mESC + mTSC; mEPSC; mEPSC + mTSC	Cell aggregation in microwell	Blastocyst formation; TE, EPI and PrE lineage segregation	Underdeveloped PrE; Limited developmental potential	[53-55]
ETS embryoid	mESC + mTSC	Cell aggregation in microwell	Post-implantation ExE and EPI compartment formation; EPI patterning	Low efficiency; Lack of VE	[71]

ETX embryoid	mESC + mTSC + mXEN; mESC + mTSC + i*Gata4* mESC	Cell aggregation in microwell	Post-implantation ExE, VE and EPI compartment formation; AVE and A-P axis formation; Initiation of EMT and gastrulation	Low efficiency	[72,73,204]
Advanced ETX embryoid	mESC + mTSC + i*Gata4* mESC; mESC + i*Cdx2* mESC + i*Gata4* mESC	Cell aggregation in microwell; *Ex utero* roller culture system	Post-implantation development up to neurulation and early organogenesis	Low efficiency; Structural defects in organ primordia	[75-77]
EpiTS embryoid	mESC + mTSC	Assembloid	Post-implantation EPI symmetry breaking and initiation of EMT	Lack of *in vivo*-like tissue morphology	[78]
Peri-gastrulation assembloid	mESC + BMP4-treated mESC	Assembloid	Post-implantation A-P and D-V patterned three germ layer derivatives	Lack of forebrain; Limited morphogenesis; Structural defects in organ primordia	[31]
Gastruloid	mESC	Cell aggregation in microwell	Post-implantation A-P patterning of germ layer lineages; Axial elongation	Limited morphogenetic events	[144]
Gastruloid with ECM	mESC	Cell aggregation in microwell; Matrigel embedding	Post-implantation A-P patterning of germ layer lineages; Axial elongation; Somite formation	Low efficiency	[139]
D-V patterned neural cyst	mESC	Matrigel embedding; Synthetic hydrogel	Spinal cord D-V patterning	Low efficiency; Lack of control of biochemical gradients	[99,101]
Trunk-like structure	mESC	Cell aggregation in microwell; Matrigel embedding	Post-implantation A-P patterning of germ layer lineages; Axial elongation; Somite and neural tube co-development	Lack of control of biochemical gradients	[141]

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Human embryo models					
Model names	**Starting cell population**	**Approach**	**Developmental events modeled**	**Limitations**	**Ref.**
Blastoid	Naïve hPSC; hEPSC; Fibroblast reprogramming intermediate	Cell aggregation in microwell	Blastocyst formation; TE, EPI and PrE lineage segregation	Underdeveloped PrE; Limited developmental potential	24,56,58-61,85
PASE	Primed hPSC	Microfluidics	Post-implantation amnion-EPI patterning; PGC development; Initiation of EMT and gastrulation	Lack of A-P patterning in EPI	32,83
heX embryoid	Primed hPSC + i*GATA6* hPSC	Co-culture	Post-implantation amnion-EPI patterning; Bilaminar disc; A-P patterning of EPI; York sac formation; Haematopoiesis	Unclear identity of i*GATA6* hPSCs; Limited development of embryonic germ layers	86
Bilaminoid	Naïve hPSC + i*GATA6* hPSC	Cell aggregation in microwell	Post-implantation amnion-EPI patterning; Bilaminar disc; A-P patterning of EPI	Low efficiency	91
Extra-embryoid	hPSC with intermediate pluripotency	Cell aggregation in microwell	EPI and hypoblast segregation; Amnion-EPI patterning; A-P patterning of EPI	Low A-P patterning efficiency	89
Inducible embryoid	Naïve hPSC + i*GATA6-SOX17* hPSC + i*GATA3-TFAP2C* hPSC	Cell aggregation in microwell	Post-implantation amnion-EPI patterning; A-P patterning of EPI; PGC specification	Low efficiency; Lack of yolk sac-like structure	90
Peri-gastruloid	hEPSC	Cell aggregation in microwell	Bilaminar disc; Amnion-EPI patterning; A-P patterning of EPI; Initiation of EMT and gastrulation; PGC specification; Yolk sac formation	Low efficiency	87
SEM	Naïve hPSC + chemically induced PrE- and trophoblast-like cells	Cell aggregation in microwell; *Ex utero* roller culture system	Bilaminar disc; Amnion-EPI patterning; A-P patterning of EPI; PGC specification; Yolk sac formation; Development of ExEM and TE lineage	Low efficiency	88
2D gastrulation model	Primed hPSC	Micropattern	Patterning of three germ layer lineages	Limited morphogenetic events	25
2D gastrulation model on soft gel	Primed hPSC	Micropattern; Synthetic hydrogel	EMT and mesoderm ingression	Limited germ layer patterning	150
Gastruloid	Primed hPSC	Cell aggregation in microwell	A-P patterning of germ layer lineages; Axial elongation	Limited morphogenetic events	145

2D neural rosette	Primed hPSC	Micropattern	Neural tube morphology	Limited cell fate patterning or morphogenetic events	[191,195]
2D ectoderm model	Primed hPSC	Micropattern	NP and NPB patterning	Limited morphogenetic events; Lack of NNE	[113]
	Primed hPSC	Micropattern	Ectoderm patterning	Limited morphogenetic events	[166]
	Primed hPSC	Micropattern	Ectoderm patterning; neural cyst formation	Limited morphogenetic events	[167]
Cranial neurulation model	Primed hPSC	Micropattern; Matrigel embedding	Cranial neural tube folding and closure	Lack of A-P or D-V axis	[140]
Spinal cord neurulation model	Primed hPSC	Cell aggregation in microwell	Spinal cord neural tube folding and closure	Heterogeneity; Lack of D-V axis	[168]
D-V patterned neural cyst	Primed hPSC	Matrigel embedding; Synthetic hydrogel	Spinal cord D-V patterning	Low efficiency; Lack of control of biochemical gradients	[100]
A-P patterned neural tube model	Primed hPSC	Microfluidics	Brain region A-P patterning	Lack of 3D tubular morphology; Lack of D-V axis	[33]
μNTLS	Primed hPSC	Microfluidics	Neural tube A-P and D-V patterning	Lack of neural tube folding morphogenesis	[34]
Somitoid	Primed hPSC	2D culture of spheroids	Somitogenesis	Lack of sequential formation and A-P patterning of somites	[177]
Segmentoid	Primed hPSC	Cell aggregation in microwell; Matrigel embedding	Axial elongation; A-P patterning; Somitogenesis	Lack of control of biochemical gradients	[177]
Axioloid	Primed hPSC	Cell aggregation in microwell; Matrigel embedding	Axial elongation; A-P patterning; Somitogenesis	Lack of control of biochemical gradients	[178]
μSDM	Primed hPSC	Microfluidics; Matrigel embedding	Somitogenesis	Lack of sequential formation and A-P patterning of somites	[179]
Trunk-like structure	Primed hPSC	Cell aggregation in microwell; Matrigel embedding	Axial elongation; A-P patterning; Somite and neural tube co-development	Lack of control of biochemical gradients	[180]

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FIGURES AND FIGURE CAPTIONS

Figure 1

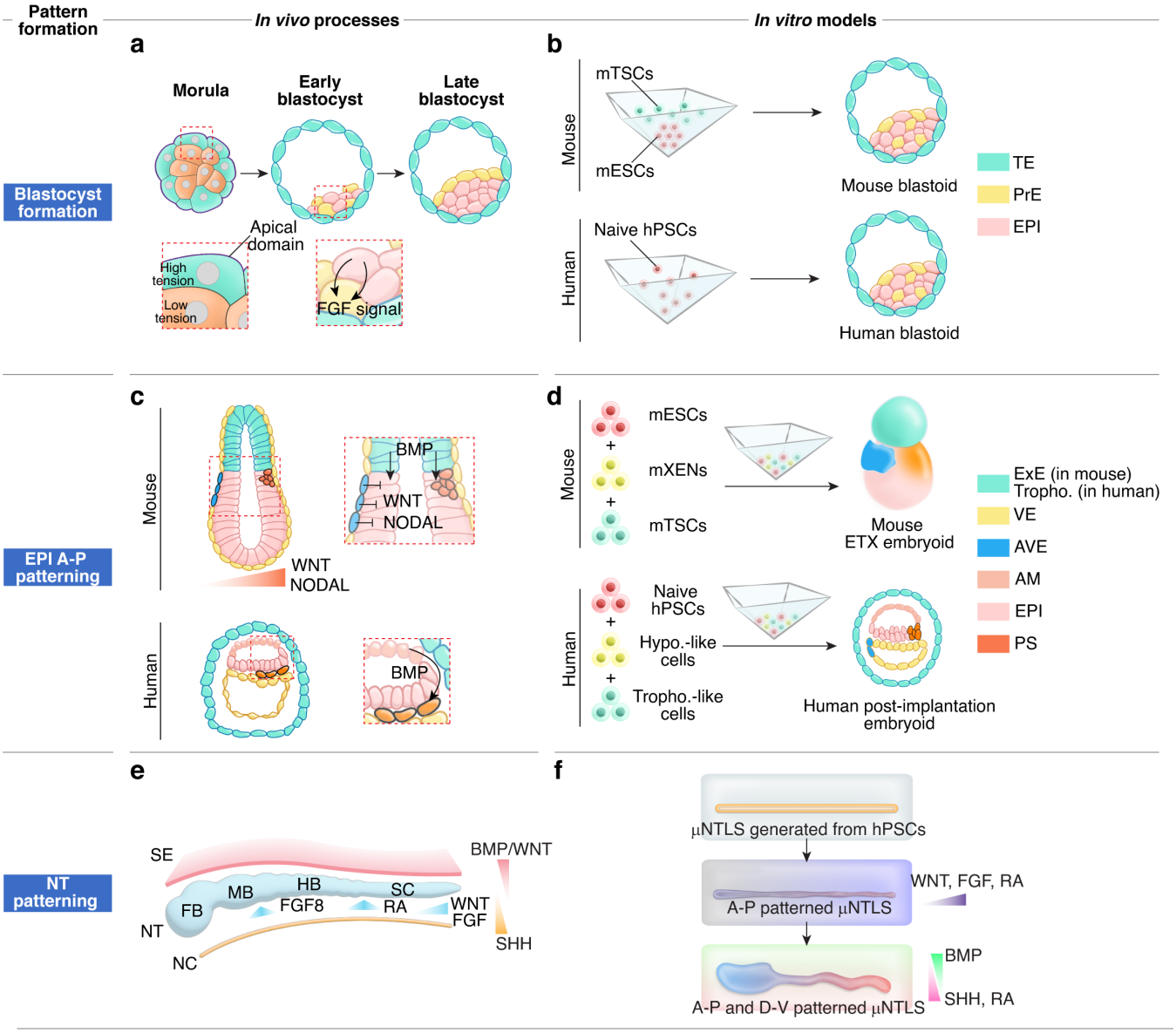

Figure 1. Pattern formation *in vivo* and in embryo models. *In vivo* knowledge and *in vitro* modeling of blastocyst formation (**a**&**b**), epiblast symmetry breaking at the onset of gastrulation (**c**&**d**), and NT regional patterning (**e**&**f**). (**a**) Formation of mouse and human blastocysts as a self-organized pattern formation process, involving two rounds of cell lineage specification. The first cell fate decision leads to lineage segregation between TE and ICM. ICM cells then become segregated into EPI and PrE (or

hypoblast in humans), a process that is suspected to be regulated by FGF signaling. (**b**) Aggregation and self-organization of mouse or human stem cells leads to the formation of mouse or human blastoids, respectively[24,53-56,58,60,61]. (**c**) After blastocyst implantation, mouse embryo develops into an elongated structure with a cup-shaped EPI juxtaposed to ExE, surrounded by VE. In contrast, post-implantation human EPI exhibits a disc-like morphology, and it is juxtaposed with AM and hypoblast. Gastrulation is initiated in posterior EPI. Symmetry breaking and AP patterning of mouse EPI are guided by extraembryonic tissues. Similar mechanisms might be conserved in human gastrulation. (**d**) Aggregation of mouse or human embryonic and extraembryonic cells leads to mouse or human peri-gastrulation embryo models, respectively, recapitulating embryo morphology and A-P patterning of the EPI[72,88]. (**e**) NT patterning *via* external morphogen gradients. A-P patterning of NT is mainly governed by WNT, RA and FGF signal gradients. D-V patterning of NT is mediated by BMP / WNT and SHH gradients secreted by SE and NC, respectively. (**f**) An A-P and D-V patterned NT model is achieved by exposing hPSC-derived tubular structures to stepwise orthogonal morphogen gradients[34]. Cell lineages: TE, trophectoderm; PrE, primitive endoderm; EPI, epiblast; ExE, extraembryonic ectoderm; Tropho., trophoblast; Hypo., hypoblast; VE, visceral endoderm; AVE, anterior visceral endoderm; AM, amnion; PS, primitive streak; NT, neural tube; FB, forebrain; MB, midbrain; HB, hindbrain; SC, spinal cord; SE, surface ectoderm; NC, notochord. Stem cells: mESCs, mouse embryonic stem cells; mTSCs, mouse trophoblast stem cells; mXENs, mouse extraembryonic endoderm stem cells; hPSCs, human pluripotent stem cells. Embryo model names: ETX embryoid, ESC-, TSC-, XEN cell-derived embryo models; μNTLS, microfluidic neural tube-like structures. Others: A-P, anterior-posterior; D-V, dorsal-ventral; BMP, bone morphogenetic protein; FGF, fibroblast growth factor; RA, retinoic acid; SHH, sonic hedgehog.

Figure 2

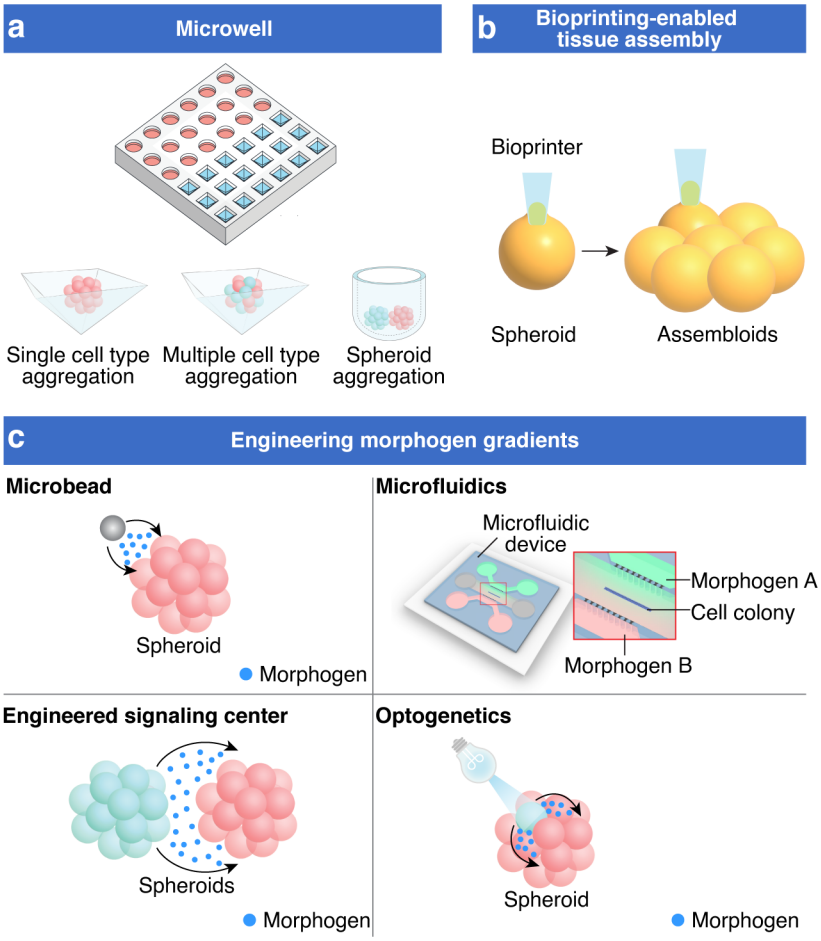

Figure 2. Bioengineering approaches for guiding pattern formation in embryo models. (**a**) Microwells to promote cell and spheroid aggregation. (**b**) 3D bioprinting to assemble spheroids into complex structures. (**c**) Engineering signaling centers and morphogen gradients through porous microbeads, optogenetics, engineered signaling centers, and microfluidics, as indicated.

Figure 3.

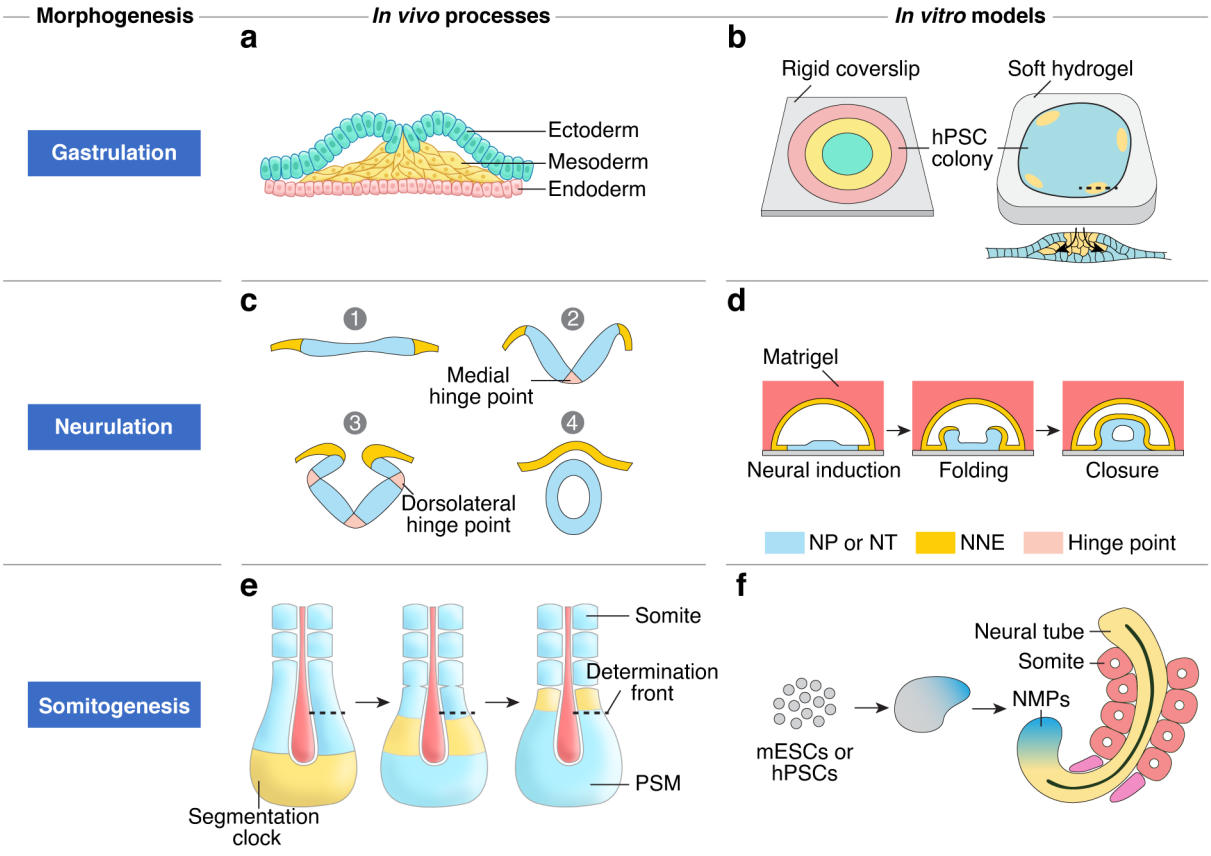

Figure 3. Morphogenesis *in vivo* and in embryo models. *In vivo* knowledge and *in vitro* modelling of gastrulation (**a**&**b**), neurulation (**c**&**d**), and somitogenesis (**e**&**f**). (**a**) Gastrulation involves different morphogenetic events, such as EMT, cell migration and axial elongation, to organize the topology of three embryonic germ layers. (**b**) 2D micropatterned hPSC colonies treated with BMP4 develop into concentric rings of three germ layers, recapitulating cell fate patterning during gastrulation[25]. Culturing micropatterned 2D gastrulation models on a soft hydrogel promotes gastrulation-like morphogenesis, such as ingression and migration of mesoderm cells[150]. (**c**) Bending and closure of the NP during neurulation. NP bending is primarily driven by mechanical forces generated by apical constriction in NP cells at the median and dorsolateral hinge points. (**d**) A neurulation model established by combining micropatterned 2D hPSC colonies with 3D culture to recapitulate NP folding and closure[140]. (**e**) Somite

formation during somitogenesis, which can be explained by the clock-and-wavefront model. In this model, somitogenesis is regulated by the combined action of a molecular segmentation clock and a moving determination front of FGF and WNT signaling activities. (**f**) Co-morphogenesis of NT- and somite-like structures in mouse or human embryo models[141,180]. NP, neural plate. NT, neural tube. PSM, presomitic mesoderm. NMPs, neuromesodermal progenitors. mESCs, mouse embryonic stem cells. hPSCs, human pluripotent stem cells.

Figure 4

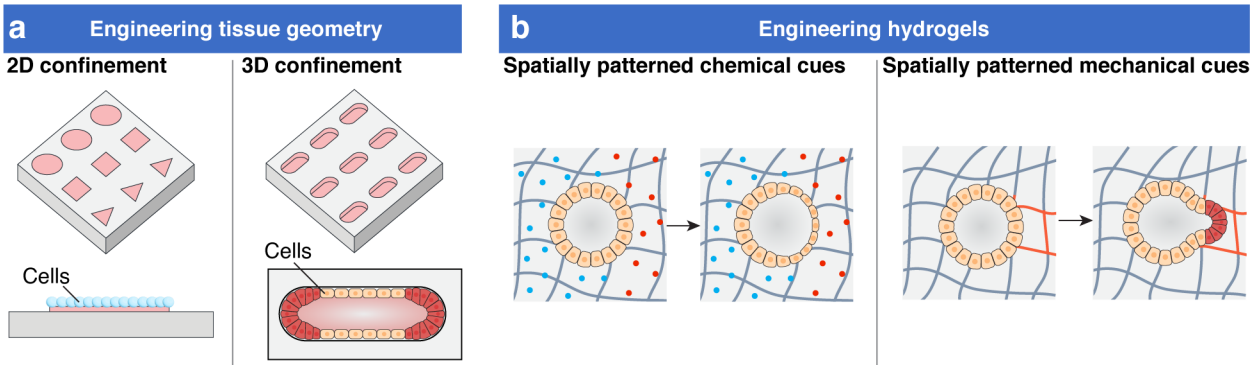

Figure 4. Bioengineering approaches to guide morphogenesis in embryo models. (**a**) Engineering tissue geometry and boundary using micropatterning tools. (**b**) Synthetic hydrogels with engineered spatially patterned chemical and mechanical cues.

Figure 5.

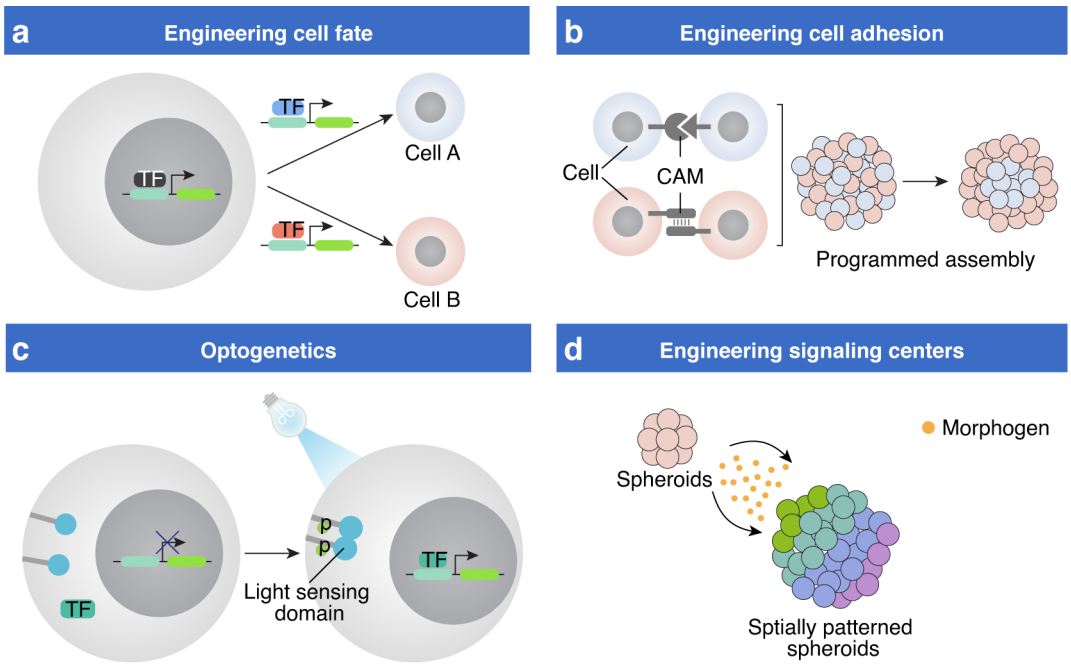

Figure 5. Engineering cell behaviors using synthetic biology. (**a**) Engineering cell fate by controlling transcription factor expression to produce specific cell lineages. (**b**) Engineering cell adhesion using synthetic receptors, allowing programmed generation of spatially patterned multicellular structures[209]. (**c**) Optogenetics to modulate intracellular signaling pathways, for example, through fusing a light sensing domain with ligand receptors. (**d**) Engineering signaling centers to regulate fate specification and tissue patterning. Localized signaling centers can be generated by genetically engineering cells to express signaling molecules, which will guide spatial patterning of adjacent spheroids. Different colors represent different cell types. TF, transcription factor. CAM, cell adhesion molecule.

BOX 1. Applications of embryo models.

Stem cell-based embryo models provide an accessible and experimentally tractable approach to study mammalian development. Together with genome editing, live-cell imaging and multi-omics, stem cell-based embryo models are becoming attractive tools to elucidate molecular and cellular mechanisms in mammalian development. For example, owing to its controllability and compatibility with live-cell imaging, the microfluidic PASE model has been used to unveil a human-specific role of amniotic cells in triggering gastrulation-like events[32]. Pre-implantation embryo models, such as blastoids, highlight the important role of the interactions between embryonic and extraembryonic tissues, and could serve as a useful tool to study pregnancy loss arising from defects in embryonic-extraembryonic interactions. Recently, a co-culture system of human blastoids with endometrial organoids provides a valuable platform to study human implantation and understand implantation failure[62]. When combined with patient-derived hPSCs and genome-editing technologies, human embryo models could potentially provide novel insights into pathological conditions. For example, studies using neural rosettes have identified the roles of autism risk genes in early human neural development[224]. Embryo models have also been used to study the embryonic origin of late-onset neurodegenerative diseases, such as Huntington's disease[167]. Finally, bioengineered embryo models, with enhanced efficiency, reproducibility and scalability, could potentially be applied in high-throughput teratogenicity and drug screens. Together, stem cell-based embryo models hold promise for advancing fundamental research in developmental and reproductive biology, as well as in biomedical applications for preventing and treating pregnancy loss, birth defects and developmental disorders.

BOX 2. Ethical considerations of embryo models.

The similarity of stem cell-based embryo models to natural embryos, especially human ones, raises ethical concerns. In most countries, stem cell-based embryo models currently do not explicitly fit into existing legal regulatory categories. Historically, scientific societies such as the International Society for Stem Cell Research (ISSCR) have played a significant role in establishing ethical frameworks for stem cell research. ISSCR provided updated ethical guidelines for stem cell research in 2021[220], which included considerations on embryo modelling research. ISSCR proposed that integrated embryo models should require stricter oversight than non-integrated ones. ISSCR guidelines also proposed prohibiting the transfer of human embryo models into any uterus or the use of human embryo models for reproduction purposes. Although none of the current embryo models are considered equivalent to human embryos or have shown the potential to develop into fetuses, as this field progresses further, embryo models are expected to mimic the entire natural embryo, or a considerable portion thereof, more closely in terms of molecular signatures, tissue structures, and development potential. Such similarity between human embryo models and natural ones inevitably raises ethical concerns. What should the legal definition of human embryos be? At what point should human embryo models be regulated as natural ones? There are ongoing discussions on these important questions, and readers are referred to other recent excellent reviews[219,225,226]. Many scientists working in the field agree that for different scientific studies or biomedical applications, the least ethically challenging embryo models should be used. For example, while studying how the whole complex embryo develops might require integrated embryo models, studies of individual germ layer development or the formation of specific organs likely do not need the use of integrated embryo models. Another consideration is that there are many ethically less challenging, non-integrated embryo models available that mimic some aspects of human development, including gastrulation and early organ formation. These non-integrated embryo models are easier to study since they contain clearly defined cell types, with the cells organized into regular patterns, unlike

the often-disorganized tissues found in most current integrated models. Since non-integrated embryo models are often built using bioengineering tools, they often are more efficient, reproducible and scalable. Continuous development of human embryo models calls for public conversations on the scientific significance of such research, as well as on the societal and ethical issues it raises.

SHORT SUMMARY (40 words)

Stem cell-based embryo models hold great promise to advance fundamental research and reproductive and regenerative medicine. This review discusses how bioengineering approaches can be utilized to construct embryo models with enhanced efficiency, controllability, reproducibility, scalability and usefulness.