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Stem Cell Based Embryo Models for Fundamental Research and Translation

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Abstract

Despite its importance, understanding the early phases of human development has been significantly limited by availability of human samples. The recent emergence of stem cell-derived embryo models, a new field aiming to use stem cells to construct *in vitro* models to recapitulate snapshots of the development of the mammalian conceptus, opens up exciting opportunities to promote fundamental understanding of human development and advance reproductive and regenerative medicine. This review provides a summary of the current knowledge of early mammalian development, using mouse and human conceptuses as models, and emphasizes their similarities and critical differences. We then highlight existing embryo models that mimic different aspects of mouse and human development. We further discuss bioengineering tools used for controlling multicellular interactions and self-organization critical for the development of these models. We conclude with a discussion of the important next steps and exciting future opportunities of stem cell-derived embryo models for fundamental discovery and translation.

Summary:

This Review highlights the recent emergence of stem cell-derived embryo models and opportunities of using these models for advancing human embryology and reproductive and regenerative medicine.

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The development of a multicellular organism from a single fertilized egg is a brilliant triumph of evolution that has fascinated generations of scientists (Box 1). Understanding our own development is of particular fundamental and practical interest; however, it poses a unique set of technical and ethical challenges. Our current knowledge of embryonic development is derived from a number of animal species, chosen because they are convenient to study and amenable to experimental manipulation or genetic analysis¹. These studies have revealed developmental principles (Box 2) and signaling and transcriptional networks that underlie cell fate patterning and tissue morphogenesis. In particular, most of our knowledge of mammalian development derives from the mouse model. However, it is becoming evident that there are morphological and genetic differences between mice and humans that make cross-species comparisons problematic².

Knowledge of human embryogenesis, which is critical for assisted reproductive technologies and prevention of pregnancy loss, birth defects and teratogenesis (Box 3), should ideally be learned from studying the human embryo *per se*; however, such studies have been challenging, due to limited access to and bioethical constraints on human embryo specimens. Excess pre-implantation human embryos generated in *in vitro* fertilization (IVF) clinics are available for research^{3,4}; however, once a human embryo implants into the uterus, subsequent development is hidden from direct observation. Recent progress in prolonged *in vitro* culture of IVF human embryos has opened the door for genetic and molecular studies of human embryos directly⁵⁻⁷. However, international guidelines prohibit *in vitro* culture of human embryos beyond 14 days post-fertilization (embryonic day 14, E14) or reaching the onset of primitive streak (PS) development (“the 14-day rule”)^{8,9}, which marks the outset of gastrulation. The bioethical regulation of human embryo culture has significantly limited studies of IVF human embryos for understanding post-implantation human development. There is significant progress in studying non-human primate (NHP) monkey embryos¹⁰⁻¹², whose developments are similar to humans. However, NHP monkey models remain costly, are difficult to modify genetically, and have their own ethical challenges.

Recent advances in mammalian embryology, stem cell biology, organoid technology, and bioengineering have contributed to a significant interest in the development of multicellular systems based on emergent self-organization and tissue patterning. Importantly, different models of the mammalian conceptus have been developed using mouse and human stem cells¹³⁻²⁸. This emerging field aims to use stem cell cultures to create organized embryo-like structures (or embryoids), whose development and architecture bear significant similarities to their *in vivo* counterparts. Embryoids are distinct from organoids, as organoids are organized multicellular structures that mimic the development, regeneration and homeostasis of a single tissue or organ. In contrast, embryoids aim to model integrated development of the entire conceptus or a significant portion thereof. In general, embryoids have a more reproducible cellular organization and architecture than organoids, as bioengineering approaches are commonly deployed to guide their development and culture time is limited to a few days. In addition, the stem cells used in embryoids have been well established and their cultures are robust. This review discusses the developmental principles manifested in the development of embryoids and their applications for advancing human embryology (particularly at the post-implantation stages) and reproductive and regenerative medicine. Bioengineering tools used for controlling multicellular interactions and self-organization

critical for embryoid development are highlighted. We conclude with a discussion of important next steps to leverage advanced bioengineering controls of multicellular interactions to promote the continuous, progressive development of this exciting nascent field.

Mammalian development as a reference framework

The development of embryoids, like that of embryos, involves the emergence of organized multicellular structures, through coordinated cellular processes including pattern formation, morphogenesis, cell differentiation and growth. Here we first discuss the principles of early mouse and human development before turning to how these are manifest in embryoids.

During early development, both mouse and human embryos develop from a zygote and proceed through recognizable stages of morula, blastula, gastrula, neurula and organogenesis (Figure 1, Box 1, Box 4). The overall program of pre-implantation development from a zygote to a blastocyst is conserved between mice and humans, leading to the formation of the blastocyst, containing an outer trophectoderm (TE) layer surrounding a cavity (blastocoel) and an inner cell mass (ICM) on one side of the cavity^{29,30} (Figure 1, Box 4). As the blastocyst develops, the ICM becomes further segregated into two cell populations: the pluripotent epiblast (EPI) and primitive endoderm (PE; or hypoblast in human)^{29,30}.

The timing of blastocyst implantation differs between mice and humans (E5 in mouse and E7 in human). Furthermore, morphogenesis and lineage developments during early post-implantation mouse and human development show distinct features^{2,31} (Figure 1, Box 4). Mouse development from E5 - E6.5 leads to the formation of a cup-shaped EPI juxtaposed with TE-derived extraembryonic ectoderm (ExE), enclosing the pro-amniotic cavity. Concurrently, the PE forms the visceral endoderm (VE) that envelops both EPI and ExE. In contrast, soon after human blastocyst implantation, while the EPI undergoes epithelization and lumenogenesis to form the pro-amniotic cavity⁵⁻⁷, EPI cells closer to invading TE cells become specified into the amniotic ectoderm (AM)³², with remaining pluripotent EPI cells forming the embryonic disc. Thus, in pre-gastrulation human embryo, the pro-amniotic cavity is surrounded by a continuous epithelium with AM cells on one side and EPI cells on the other.

Studies of mouse gastrulation support the importance of extraembryonic tissues^{33,34} (Figure 1, Box 4). In particular, regional patterning of VE in pre-gastrulation mouse embryo leads to a gradient of WNT and NODAL signaling and the establishment of the anterior (A) - posterior (P) axis of the embryo^{35,36}. Importantly, developmental signals involving WNT, NODAL and BMP at the proximal, posterior end of the EPI instruct EPI cells to form the PS by E6.5 and ingress through the PS to acquire mesoderm and endoderm fates³⁷⁻³⁹. Human gastrulation initiates around E14. However, given limited access to post-implantation human tissues, gastrulation remains one of the most mysterious phases of human development.

During mouse gastrulation, primordial germ cells (PGCs), precursors of sperm and egg, emerge at the boundary between posterior EPI and ExE^{40,41}. Data on human PGC specification remain sparse⁴². Existing data from NHP monkey embryos⁴³ suggest that

primate PGCs may emerge in the nascent AM prior to the gastrulation. Additional studies are required to determine whether the same is true for human PGCs.

In mouse and human embryos, gastrulation transforms the EPI into a trilaminar structure consisting of definitive ectoderm, mesoderm and endoderm. The three germ layers undergo inductive interactions to pattern layers and specify new cell types, driving organ rudiment development (Box 4). Following gastrulation, the ectoderm undergoes neurulation, in which the neural plate is first patterned in the dorsal ectoderm before folding into the neural tube (NT)^{44,45} (Box 4). Cells in the NT continue to differentiate into different classes of neuronal progenitors^{46,47}. Concomitantly, mesodermal cells are organized into different regions to form the primordia of major organ systems including cardiovascular and lymphatic systems and skeletal muscle cells. Simultaneously, the endoderm will fold to form the primitive gut tube, which will produce the digestive and respiratory systems.

Embryonic and extraembryonic stem cells as building blocks

As a bottom-up approach, the development of embryoids uses embryonic and extraembryonic stem cells, including those derived from embryos, as building blocks to construct models to recapitulate embryonic development (Figure 2).

Mouse stem cells

The EPI cells of the mouse blastocyst are pluripotent, and their functional, epigenetic, and signaling properties have been extensively characterized. These studies reveal that pluripotency is dynamic and progressive. As the mouse embryo develops from blastula to gastrula, EPI cells transit from a naïve state, in which they do not respond to inductive signals, to a primed state in which they readily differentiate⁴⁸. The transition between naïve and primed states has been referred to as formative pluripotency during which EPI cells gain the capacity to make lineage decisions⁴⁹.

Mouse pluripotent stem cells (mPSCs) in culture display a similar continuum of states. Mouse embryonic stem cells (mESCs) with naïve pluripotency can be isolated directly from the ICM of the mouse blastocyst and maintained in culture^{50,51}. In contrast, mouse stem cells corresponding to the primed state, known as mouse epiblast stem cells (mEpiSCs), are derived from the post-implantation mouse EPI^{52,53}. mEpiSCs exhibit more advanced developmental features consistent with the early-gastrulation EPI⁵⁴. Mouse EPI-like cells (mEpiLCs) with formative pluripotency have been generated from mESCs *in vitro*, with a transcriptional profile consistent with early post-implantation mouse EPI⁵⁵.

Stem cell lines representative of extraembryonic lineages in the mouse blastocyst have also been established, including mouse trophoblast stem cells (mTSCs)⁵⁶ and extraembryonic endoderm (XEN) cells representing the stem cell population of the PE⁵⁷.

Human stem cells

Human ESCs (hESCs) have also been successfully derived from human blastocysts⁵⁸. However, hESCs have transcriptome and methylome different from the EPI of the human blastocyst^{59,60}, suggesting that the conditions in which hESCs are cultured fail to capture the

pre-implantation developmental program of the human embryo. Instead, hESCs are developmentally similar to the post-implantation, pre-gastrulation EPI in cynomolgus monkey embryos¹². Consistently, hESCs more closely resemble mEpiSCs than mESCs in terms of molecular properties, lineage potency, and culture conditions^{48,52,53}. However, there are still some notable differences in gene expression⁶¹ and in the propensity for PGC formation between hESCs and mEpiSCs, as hESCs can initiate PGC formation whereas mEpiSCs cannot^{62,63}. There are recent reports showing derivations of hESC lines from human blastocysts with naïve pluripotency features^{64,65}, and of chemical reprogramming cocktails capable of converting hESCs from primed to naïve pluripotency^{66,67}. However, functional validations of naïve pluripotency including chimera formation and germline transmission as well as tetraploid complementation, which have been used for mESCs, cannot be implemented with human cells for ethical reasons. To address this issue, there are ongoing discussions of a testable functional framework to assess naïve pluripotency in human cells³¹.

Somatic human cells can also be converted to a pluripotent state by cell fusion, somatic cell nuclear transfer, transcription factor-based reprogramming, and chemical reprogramming⁶⁸. Pluripotent stem cells generated by these reprogramming strategies are called induced pluripotent stem cells, or iPSCs. Human iPSCs (hiPSCs) are considered molecularly and functionally equivalent to hESCs⁶⁹.

Recently, through chemical screening, individual blastomeres isolated from eight-cell stage mouse morula were successfully cultured to establish mouse expanded potential stem cells (mEPSCs) that appear to possess developmental potency for all embryonic and extraembryonic lineages in blastocyst chimaera assays^{70,71}. Using similar approaches, human EPSCs (hEPSCs) were also derived from primed hESCs and hiPSCs, and hEPSCs are shown to have the potency to form trophoblast stem cells⁷².

In contrast to mouse extraembryonic stem cells, human extraembryonic stem cells have only emerged recently. Using chemical screening, human trophoblast stem cells (hTSCs) were first derived from human blastocysts and first-trimester placental tissues⁷³. Human hypoblast stem cells (hypoSCs) were also recently reported using chemically reset naïve hESC lines⁷⁴. Recent work showed that chemically reprogrammed naïve hESCs could give rise to hTSCs when cultured in appropriate conditions⁷⁵, and that both chemically reset and embryo-derived naïve hESCs could be used to derive hTSC lines⁷⁶. With the emergence of these human extraembryonic cell lines as well as hEPSCs, it becomes imperative for additional molecular and functional characterizations for authentication and establishing their developmental identities compared to their *in vivo* counterparts^{76,77}.

A rapidly growing toolbox of embryoids

Stem cells serve as building blocks for the development of embryoids that recapitulate different stages of mammalian development, from blastula through gastrula or early neurula and organogenesis (Figure 3). Development of embryoids use the same developmental principles that manifest in mammalian development. Importantly, embryoids have already generated new insights into early mammalian development.

Embryoid to model blastocyst development

The first embryoid to model blastocyst formation (or blastoid) was successfully developed by mixing mESCs with mTSCs at a defined ratio under appropriate culture conditions, leading to their self-assembly into a tissue organization reminiscent of the mouse blastocyst²². As *in vivo*, mouse blastoids possess an outer TE layer surrounding a compact ICM-like compartment, and their transcriptome is more similar to mouse blastocyst than is achieved by simply combining mESC and mTSC transcriptomes. Mouse blastoids have been used to dissect interactions between embryonic and extraembryonic compartments, revealing that NODAL and BMP signals from the ICM-like compartment are important for growth and morphogenesis of TE cells²². This insight has proven useful for improving culture conditions of mTSCs⁷⁸. In the initial blastoid protocol, further cell segregation and sorting of ICM-like cells into PE-like cells was inefficient. Optimization studies yielded improved conditions in which the relative proportions of the three cell lineages (EPI-, TE- and PE-like cells) more closely resemble that of mouse blastocyst⁷⁹. Remarkably, culture of mEPSCs in appropriate conditions yields self-organized blastoids consisting of EPI-, TE- and PE-like cells²⁶. Another recent work mixing mEPSCs with mTSCs also led to the formation of blastoids that showed developmental progression from the pre- to post-implantation egg cylinder morphology *in vitro*⁸⁰, similar to the mouse ETX embryoid described below. Although capable of implanting in the mouse uterus, all of the mouse blastoids fail to develop much further than the blastocyst stage either *in vitro* or *in vivo*. The reasons for this are not currently understood and may point to a requirement for greater organization than currently achieved in mouse blastoids. It remains to be determined whether human blastoids can be generated by using either naïve hPSCs or hEPSCs with or without hTSCs or hypoSCs under suitable culture environments.

Human amniotic sac embryoid

During early post-implantation human development, a patterned bipolar EPI-AM structure arises from the EPI. It was recently shown that culturing primed hPSCs on a soft culture surface together with native extracellular matrix (ECM) proteins (*i.e.*, Geltrex) diluted in culture medium leads to the formation of a spherical luminal hPSC colony²⁰. This observation is consistent with the intrinsic lumenogenic property associated with primed but not naïve hPSCs^{81,82}. Interesting, hPSCs in the colony lose pluripotency and differentiate into amniotic cells, even without exogenous inductive factors in the culture medium²⁰. If only one of these culture elements is present, either a soft substrate or diluted gel in the culture medium, primed hPSCs form luminal sacs but retain pluripotency, suggesting that amniotic differentiation of hPSCs is mechanosensitive²⁰. Amniotic differentiation of hPSCs requires endogenous BMP signaling, and its inhibition under amnion-differentiation conditions is sufficient for rescuing hPSC pluripotency²⁰.

A small fraction of luminal sacs, rather than differentiating entirely into squamous amniotic tissues, spontaneously break symmetry and form a bipolar structure with columnar pluripotent cells on one side and squamous amniotic cells on the other, mimicking EPI-AM patterning in the pre-gastrulation human embryo²⁰. This model is termed the post-implantation amniotic sac embryoid (PASE). Symmetry breaking in the PASE also depends on BMP activity, and active BMP signaling is only evident at the prospective AM-like

pole²⁰. Progressive development of the PASE results in EPI-like cells further differentiating into PS-like cells. This spontaneous symmetry breaking occurs in only 5 – 10% of luminal hPSC sacs. To increase efficiency of PASE formation, a microfluidic PASE model has recently been developed²⁷. This microfluidic device allows small clusters of primed hPSCs to be grown in small indentations with separate channels supplying culture medium to each side²⁷. Flowing BMP4 in only one of these channels leads reproducible patterning of amniotic cells only on the side exposed to BMP4²⁷. The opposite side remains pluripotent but soon goes on to differentiate into PS-like cells²⁷. The identity of PS derivatives can be modulated by stimulating this side of the cell clusters with additional ligands: WNT stimulation together with either BMP4 or ACTIVIN-A leads to posterior and anterior PS derivatives, respectively²⁷. Importantly, human PGC-like cells (hPGCLCs) emerge in the microfluidic PASE before it initiates gastrulation-like events²⁷, suggesting applications of the microfluidic PASE model for studying the origin and specification of hPGCs.

The PASE represents the first embryoid to model early post-implantation development of the EPI and AM compartments of the human embryo. It also suggests the inductive role of AM in triggering human gastrulation. Although the microfluidic PASE model significantly improves the controllability of EPI-AM patterning, further asymmetries are not demonstrated and the EPI-like compartment is either entirely anterior or posterior in character. Prolonged culture of the PASE is also limited by the confined space in the microfluidic device. Furthermore, disseminating cells from the PASE mimicking gastrulation would lead to its disassembly. Future efforts should be devoted to identifying a strategy to prolong the culture of the PASE and promote self-organization of gastrulating cells. It is possible that adding human extraembryonic stem cells including hypoSCs to the PASE will be helpful for these efforts.

2D models of gastrulation

Treatment of primed hPSCs confined to micropatterned colonies with BMP4 reproducibly leads to organized differentiation with putative TE-like cells on the colony outer edge, ectodermal cells in the colony center, and mesodermal and endodermal cells forming two layers in between^{18,83,84}. This multicellular pattern is consistent as in the gastrulating mammalian embryo. However, the fate territories in the 2D gastrulation model are adjacent on a 2D surface rather than layered one on top of the other as in *in vivo*. Although the 2D geometry is artificial and distinct from the 3D topology of mammalian embryos, the reproducibility and compatibility with live imaging of the 2D gastrulation model has allowed quantification of the self-organized signaling dynamics that drive these patterning events^{85–88}. These studies have revealed that rather than creating stable gradients, cells generate dynamic expanding fronts of endogenous WNT and NODAL signaling that are interpreted combinatorially to pattern different germ layers. These studies can serve as a template for investigating the mechanisms of patterning through signaling dynamics in other embryoids. Interestingly, in colonies treated with WNT rather than BMP4, similar cell fate patterns are observed but with a different mechanism, which involves a wave of EMT that sensitizes cells to the exogenous WNT signal⁸⁹. Mouse gastrulation has also been successfully modeled using the 2D gastrulation embryoid with mouse EpiLCs⁹⁰. Importantly, findings from this mouse embryoid have been compared to the mouse embryo,

providing a more direct validation, which for obvious reasons is not possible for human gastrulation embryoids. Micropatterning can be readily combined with other bioengineering approaches. For example, recent work has shown that overlaying gradients of exogenous ligands on micropatterned 2D gastrulation embryoids can bias the resulting fate pattern in a reproducible way⁹¹. Specifically, a gradient of BMP4 (and in some cases a counteracting gradient of BMP antagonist NOGGIN) generated in a microfluidic device induced axially organized patterning of the germ layers along the gradient, breaking the radial symmetry of 2D circular colonies⁹¹.

3D models of gastrulation

In addition to blastoids, mESCs and mTSCs can be cultured together in conditions that promote their self-organization to model post-implantation mouse development. In such models, rather than forming structures that morphologically resemble the mouse blastocyst, mESCs and mTSCs form separate compartments before fusing together. Each compartment undergoes lumenogenesis, with the resulting lumens merging together, resulting in a structure resembling the egg cylinder stage mouse embryo (referred to as the ETS embryoid)¹⁹. Remarkably, the ETS embryoid initiates developmental events mimicking both germ cell formation and PS development in an asymmetric manner¹⁹. This is surprising as VE, which is critical for A-P symmetry breaking *in vivo*, is not present in the ETS embryoid, although further studies show that adding XEN cells into the ETS embryoid improves this model (called the ETX embryoid)²³.

A 3D embryoid for modeling symmetry breaking of the EPI has also been developed starting from primed hPSCs²⁶. In this model, hPSCs grown in 3D and treated with a low dose of BMP4 form luminal sacs before polarizing into two opposing regions displaying gene expression patterns associated with ectoderm and mesoderm induction²⁶. This observation is similar to cell fate patterning that emerges from the 2D human gastrulation embryoid¹⁸. However, as the initial degree of symmetry is higher in 3D (sphere *vs.* disk), the development of the 3D human gastrulation embryoid involves spontaneous symmetry breaking while in 2D it does not.

Finally, an embryoid model beginning from only mESCs or primed hPSCs has been shown to be able to model the post-gastrulation development of the posterior portion of the mouse and human embryos, respectively^{17,21,28,92}. Growing these cells in aggregates of defined size and exposing them to a properly timed pulse of WNT activation leads to the formation of a tail-bud-like structure on one end of the aggregate that continues with axial organization, somitogenesis, PGC specification and even NT formation^{17,21,28,92}. These structures, known as gastruloids, recapitulate some essential features of A-P axial patterning of the post-gastrulation mouse and human embryos as revealed by HOX gene expression and somite formation^{21,28,92}. The mouse gastruloid can even be coaxed to generate a primitive beating heart following pathways similar to those of the mouse embryo⁹³. These mouse and human gastruloids all lack anterior structures, such as the forebrain, likely due to the posteriorizing effect of WNT signaling.

While the 3D embryoid models of gastrulation show remarkable emergence of patterning and morphogenesis, they lack the controllability and reproducibility of the 2D gastrulation

models. Bioengineering approaches, which can control cell-cell interactions and modulate symmetry breaking and patterning, as in the microfluidic PASE²⁷, will be useful for improving the controllability and reproducibility of the 3D gastrulation models.

Models of neurulation

The nervous system acquires its form and pattern during the neurulation stage. Several stem cell-based neurulation models have been developed, which focus on the ectodermal germ layer, the source of the nervous system. One of the earliest studies showed that 3D spherical luminal sacs composed of NE cells, reminiscent of the NT, could be grown from single mESCs under appropriate neural differentiation conditions¹⁵. These neural sacs were entirely dorsal in character but could be patterned by exogenous ventralizing or posteriorizing signals. Optimizing the gel matrix in which neural sacs were embedded also improved their dorsal (D)-ventral (V) patterning, showing the power of bioengineering approaches to optimize conditions for embryoid self-organization⁹⁴. D-V patterned neural sacs mimicking human NT development were recently demonstrated using primed hPSCs under a culture condition similar to that used for mESCs²⁷.

Recently, several 2D models have been reported to recapitulate patterning of a significant portion of the ectodermal germ layer. One study with primed hPSCs showed that regional patterning of neural crest and NE cells could be created on micropatterned surfaces, mimicking neural induction as the first step of the neurulation process, and that this emergent regional patterning was regulated by mechanical control of BMP-SMAD signaling²⁴. Other recent studies have recapitulated patterning of all four major fates within the ectoderm during neural induction – neural, neural crest, placode and epidermis^{25,95}. Modulating both BMP and WNT signaling enabled control over the fates that emerged at the border between neural and non-neural ectoderm⁹⁵, and one of these models was shown to be useful for modeling developmental effects of the mutations that cause Huntington's disease²⁵. In the future, models that recapitulate not only regional fate patterning, but also morphogenesis involved in neural plate folding and neural fold closure, could both lead to new fundamental knowledge of this stage of human development and provide essential systems for research into human NT closure defects.

Bioengineering tools to control embryoid development

Mammalian embryogenesis is a context-dependent process, involving interactions between multiple, co-emerging embryonic and extraembryonic cell lineages that are intricately organized in 3D. This 3D context provides spatial boundary conditions, as well as biochemical and biomechanical inputs and positional information that are often absent in conventional 2D culture vessels. Although mouse and human stem cells can be efficiently differentiated into specialized cell types under classical 2D cultures, poor control over initial seeding conditions and tissue growth lead to disorganized cell fate patterns and tissue shapes. Bioengineering tools such as microfluidics or microfabricated cell culture substrates have been proven highly effective to 'reconstruct', in a bottom-up fashion, the missing 3D physical and biochemical context of the early embryo (Figure 4). Indeed, recent advances in bioengineering and biomaterials not only promote the reproducibility of embryoids, *e.g.* to

facilitate the development of quantitative assays, but also enable systematic studies of how the complex array of extrinsic inputs influences embryonic development.

Micropatterning to control tissue shape

The simplest and perhaps most adopted approach to influence multicellular self-organization is based on cultures of cell colonies selectively on cell adhesive substrates that are designed to spatially control tissue size and shape. This can be readily achieved through micropatterning, a classical microfabrication technique widely used to study how cell or tissue shape affects cellular phenotypes⁹⁶. As illustrated in 2D embryoid models, 2D micropatterning provides significant advantages as an assay technology, given their scalability and reproducibility, coupled with the ability to manipulate culture conditions and the simplicity of live imaging. Importantly, 2D micropatterning can be integrated with microfluidics⁹¹ and cell mechanics tools (such as micropost force sensors or traction force microscopy⁹⁷) for dynamic, quantitative measurements and perturbations of soluble biochemical signals and insoluble biophysical cues. These unique features have not been fully exploited to date, but are important for future studies to examine the roles of tissue geometry and mechanical forces in influencing cell signaling and cell-cell communication to regulate patterning in 2D embryoids.

Using similar microfabrication approaches, micropatterning can be extended from 2D to 3D, such as to embed cells within microscale cavities in soft materials such as hydrogels or viscoelastic polymers. This approach has been demonstrated for tubular mammary epithelia, shedding light on mechanisms of branching morphogenesis⁹⁸. When single primed hPSCs are grown in microcavities overlaid with Matrigel, the cells self-organize and form a single central lumen with a defined geometry⁸¹. Such tissues might serve as precursors for the generation of new embryoids, *e.g.* for modeling NT patterning along the A-P and/or D-V axes.

Microwell arrays for controlling initial cell aggregation

Another simple yet useful microfabrication approach for improving the consistency of embryoid development is the microwell array. The microwell array can be used to trap cells in suspension to promote their initial aggregation into spheroids of controlled sizes and multicellular compositions. This approach, for example, has been exploited to reproducibly generate blastoids from mESC / mTSC aggregates in microwell arrays composed of agarose hydrogels fabricated by micromolding with PDMS stamps²². Along similar lines, the AggreWell™ plate, a commercial microwell array, was used to improve the reproducibility and efficiency in generating ETX embryoids and mEPSC-based blastoids^{23,26,80}.

For both micropatterning and microwell arrays, their impacts on embryoid development seem to derive from their influences on setting up the initial number of cells in each cell colony and colony geometrical boundaries. Colony geometry can directly influence cell signaling and cell-cell communication through regulatory mechanisms involving dynamic morphogenetic cues and diffusible signals^{24,84,85}. It remains elusive how the initial number of cells (or cell density) in each cell colony affects progressive embryoid development. Existing data suggest its effect on cell polarity, paracrine signaling, the actin cytoskeleton,

and mechanotransduction, which are known to regulate classic developmental signaling events.

Microfluidics for establishing signaling centers and gradients and stem cell niches

Whereas micropatterned substrates and microwell arrays offer an effective means to control cell colony shape and aggregate composition, the environment in which the cells are grown is typically isotropic and static, and thus poorly suited to recapitulate the spatiotemporal dynamics of morphogen signaling operating *in vivo*. Because of its ability to precisely manipulate tiny quantities of fluids and establish dynamic chemical gradients, microfluidics offers exciting opportunities to control morphogen signaling in space and time such as to establish artificial signaling centers to direct multicellular self-organization and patterning.

An example along this line reported the development of microfluidic devices to expose micropatterned 2D gastrulation embryoids to linear morphogen gradients generated via passive diffusion (*i.e.* a “source and sink” type gradient system)⁹¹. Beyond establishing controlled biomolecular gradients, microfluidics offers a powerful way to optimize and standardize advanced embryoid cultures, as demonstrated by the microfluidic PASE²⁷. This and similar microengineered 3D culture systems should be particularly useful for designing multicellular embryoid systems with the robustness and scalability needed in translational applications such as high-content screening.

It is worth noting that artificial signaling centers in multicellular self-organization and development can also be established using microbeads loaded with signaling molecules⁹⁹, optogenetics¹⁰⁰, or through co-culture with morphogen-secreting cells¹⁰¹. A recent work demonstrated optogenetic stimulation for local activation of WNT signals in both 2D and 3D human gastrulation models to drive mesendoderm differentiation¹⁰⁰. In another work, an inducible Shh-producing cell aggregate was embedded at one pole of an hPSC spheroid, mimicking a developmental organizer, to promote ordered self-organization along D-V and A-P axes in a forebrain organoid model¹⁰¹.

Advanced biomaterials to mimic ECM

Mammalian development involves not only cell-cell interactions, but also cell-ECM interactions that guide embryonic organization, cellular differentiation and morphogenesis. The ECM is synthesized and secreted by embryonic cells beginning at the earliest stages of development. Providing adhesive substrates in a 3D context, the ECM further defines tissue boundaries to guide cell migration and functions as a dynamic repository for growth factors to regulate morphogen signaling. Importantly, embryonic cells sense and respond to the 3D organization and physical properties of the ECM through mechanotransductive processes involving integrin-mediated adhesions and the intracellular actin cytoskeleton¹⁰². An exciting contemporary research question is how these mechanotransduction processes interact with growth-factor-mediated developmental signaling to regulate cellular differentiation and patterning¹⁰².

The importance of basement membrane-mediated integrin signaling in transforming amorphous EPI cells into an apico-basally polarized luminal EPI sac was first shown in the peri-implantation mouse embryo¹⁰³. Indeed, the use of 3D ECM cultures to promote the

development of mESCs and primed hPSCs into luminal EPI-like structures has been an important first step for the development of different embryoids^{19,20,23,26}. In view of these data, how can one then explain the remarkable level of self-organization and patterning seen in the mouse and human gastruloids grown in suspension, *i.e. without* a surrounding 3D matrix support?^{13,17,21} Two recent papers that report the exposure of mouse gastruloids to low percentage Matrigel at a later culture time point might shed light on the role of ECM in morphogenesis in the gastruloid. Intriguingly, instead of observing gene expression patterns in the absence of any visible morphogenesis, as in mouse gastruloids derived in suspension culture^{17,21}, the provision of Matrigel resulted in the development of somites and a NT^{28,92}, suggesting that fate patterns could arise even in morphologically rather disorganized tissues and elaborate morphogenesis and tissue formation might be dependent on physical contacts with ECM. However, whether Matrigel exerts its function in the mouse gastruloid through adhesive signaling or mechanical interactions or both remains to be elucidated.

All of the above examples have relied on native ECM isolated from animal tissues, in particular Matrigel and Geltrex, basement membrane extracts derived from mouse tumor tissues. The main limitations of these native ECM, *e.g.* its batch-to-batch variability and potential immunogenicity, are widely documented. However, despite sizeable efforts in biomaterial development over the last decade, it has not yet been possible to identify synthetic alternatives that can completely replace native ECM in 3D cultures. However, some progress has been reported in the use of Matrigel alternatives for embryoid cultures. For example, Poh and colleagues utilized fibrin matrices, a clinically approved biomaterial generated from fibrinogen, to coax mESC colonies to differentiate and form spatially organized germ layers¹⁶. Interestingly, the authors reported the roles of matrix dimensionality, stiffness, as well as cell-cell adhesion in promoting germ layer self-organization¹⁶.

An approach based on chemically defined, poly(ethylene glycol) (PEG)-based hydrogels was explored in the context of 3D NT models generated from mESCs^{15,94}. By systematically screening PEG matrices of variable stiffness, degradability, and ECM composition, the authors identified a parameter window in which apico-basally polarized NE sacs with proper D-V patterning robustly emerged, with an efficiency greater than achieved in Matrigel⁹⁴. More recently, synthetic hydrogels were applied to the 3D human gastrulation embryoid, by using two commercially available hydrogel systems (physically crosslinked PNIPAAm-PEG gel and an Fmoc-based supramolecular gel) that were admixed with Matrigel²⁶. Beyond assisting translational applications of embryoids, the exquisite modularity of such chemically and physically defined hydrogel systems will facilitate a systematic dissection of the independent roles of extrinsic ECM factors (including matrix stiffness, porosity, degradability, and ECM composition) in early development.

Conclusions and future directions

Understanding human development has been one of the central goals of modern biology. To circumvent the limited availability of human samples, conventional mammalian developmental biology studies have relied heavily on animal models, including NHP monkeys. In all of these models, the need for *in utero* development prevents precise

manipulations and high-resolution observation. As a matter of fact, there will never be sufficient embryonic materials - from humans, NHP monkeys, or other mammalian species - available for quantitative assays with a level of resolution offered by synthetic *in vitro* embryoid systems. As a bottom-up approach using stem cells to model embryonic development without using intact embryos, embryoids are quickly becoming an essential experimental tool for advancing mammalian embryology. In particular, human embryoids are the only method available to study human embryological events between the onset of gastrulation and 4 – 5 weeks post-fertilization when the earliest fetal tissues from elective terminations are available. By this time point, the primordia of most of the major organ systems have formed in the recognizable fetal body, so it is imperative to develop alternative models to understand the origins of the human body plan.

The development of embryoids integrates knowledge and methodologies from stem cell biology, developmental biology, synthetic biology, tissue engineering, and bioengineering. Coupled with the ease of genetically modifying stem cell lines, the ability to manipulate culture conditions and the simplicity of live imaging, embryoids are becoming robust and attractive systems to disentangle cellular behaviors and signaling interactions that drive mammalian embryogenesis. Using lineage and signaling reporter lines, embryoids offer exciting trackable systems to study pattern formation, morphogenesis, cell differentiation, and growth and how these developmental processes are dynamically regulated and coordinated during embryonic development. Embryoids are also useful for elucidating intracellular signaling dynamics and gene regulatory networks and their cross-regulation with cell mechanics and morphogenetic signals during embryonic development and for studying classic developmental biology questions, such as symmetry breaking, scaling and induction.

Directed differentiations of hPSCs towards clinically relevant cell lineages using conventional 2D cultures have made significant progress over the last two decades and are largely based on developmental biology knowledge generated from model organisms to optimize growth and differentiation factors to modulate relevant developmental signaling pathways. However, intricate cell-cell interactions involved in embryonic development, which are important for lineage specification and functional maturation, are often missing and difficult to recapitulate in conventional 2D cultures. Thus, it is possible that continuous development of human embryoids can lead to advanced 3D cultures in which human stem cells can undergo successive developmental stages to produce tissue progenitors and fully differentiated cells with better fidelity to their *in vivo* counterparts in terms of gene expression, epigenetics, and function.

Nonetheless, the widespread utility of embryoids and their broad impact on human embryology and reproductive medicine will depend upon continuous improvements of their controllability, scalability, reproducibility and standardization and the commercial availability of culture platforms used for embryoid development. More sophisticated embryoid platforms, such as the microfluidic PASE, will require additional collaborative efforts between bioengineers and stem cell and developmental biologists for their dissemination to the broad research community. In principle, embryoids can be integrated

with multi-well plate formats to achieve highly parallelized assays compatible with existing automation workflows and screening infrastructure.

Mouse embryoids, which contain all embryonic and extraembryonic lineages and their correct organizations to mimic mouse development from pre-implantation to early gastrulation, have been successfully developed^{19,22,23,26}. Since culture protocols are available to enable whole mouse embryos to develop *in vitro* from the pre-gastrula to the early organogenesis stages¹⁰⁴, it is conceivable that mouse embryoids will eventually be able to mimic whole mouse embryonic development to the organogenesis stages. Compared to mouse embryoids, human embryoids developed so far have only used primed hPSCs to model post-implantation developmental events associated with the EPI lineage. It is foreseeable that as hTSCs and human hypoblast stem cell lines become available and further authenticated, these extraembryonic cells will be integrated into existing human embryoids (as in the mouse blastoids and ETS and ETX embryoids^{19,22,23}), allowing their prolonged and organized development and studies of the roles of embryonic-extraembryonic interactions in guiding implantation, placentation, embryonic patterning and gastrulation. Another future direction will be to leverage hPSCs possessing developmental potency for both embryonic and extraembryonic cell lineages (such as naïve hPSCs and hEPSCs) and identify suitable culture conditions to guide their development into embryoids that contain organized embryonic and extraembryonic structures²⁶. Architecturally and functionally competent endometrial culture systems are available using both human primary cultures and established cell lines¹⁰⁵. In the future, it will be important to use human embryoids containing TE-like cells coupled with endometrial culture systems to model human implantation and placentation, in the hope of understanding the interrelationship between embryonic and placental development.

We envision that continuous developments of mouse and human embryoids in the next 5 – 10 years will incorporate new advances of developmental biology, stem cell biology and bioengineering and will lead to new understanding of intracellular signaling and cell fate dynamics at single-cell resolution, extracellular movement of developmental signals at cellular and tissue scales, and embryonic-extraembryonic interactions and their critical roles in guiding embryonic development. It is foreseeable that advanced approaches integrating different bioengineering tools will further promote the controllability and reproducibility of different embryoids. Bioengineering tools to dynamically control the cellular environment, such as by modulating morphogen gradients, symmetry breaking and local signaling centers, will be essential for improving embryoids and gaining new insights in their developments. Next-generation 3D human embryoids (such as human ETX embryoids) or even new human embryoid systems mimicking organogenesis (including the brain, heart, blood and gut) will likely emerge. We also envision that in the next 5 – 10 years initial translational applications of human embryoids will allow studying genetic and environmental causes of recurrent implantation failure (by combining human embryoids with endometrial culture systems) and early birth structural defects such as NT defects (NTDs) and congenital heart defects. Embryoids also have the potential to replace *in vivo* teratoma assays commonly used for establishing stem cell pluripotency. ‘Organism-level’, high-throughput, embryoid-based screening pipelines will likely emerge in the near future. We also envision that an important next technological milestone is to achieve a human embryoid that can recapitulate the entire

gastrulation process and the development of the trilaminar germ disc containing the three organized definitive germ layers.

Validation of findings from embryoids using *in vivo* controls will be important to evaluate their developmental relevance⁹⁰. However, this is challenging for human embryoids that aim to recapitulate post-gastrulation human development, given the scarcity of relevant human embryo data¹⁰⁶. This challenge will be partially addressed by the recent progress of NHP monkey embryo studies^{10–12}, which provide quantitative transcriptomic and epigenomic profiles of monkey cells at post-gastrulation developmental stages. Nonetheless, it becomes imperative to establish a molecular and cellular standard to assess the authenticity and equivalency and establish developmental identities of human embryoids compared to their *in vivo* counterparts. This might require the current 14-day rule governing the *in vitro* human embryo research to be extended to a post-gastrulation developmental stage⁹.

Accompanying the emergence of different human embryoids, there are ongoing discussions and recommendations from the bioethics community on their regulation^{107,108}. Even though the existing human embryoids are far from being equivalent to human embryos, as we continue to improve and generate new human embryoids, they are expected to more closely mimic human embryos, in terms of cell organization, morphogenesis and gene expression. The continuous development and progression of this nascent field will inevitably lead to important bioethical questions: What should the ethical status of human embryoids be and how should they be regulated? What does determine the developmental potential of human embryoids in culture and equally as important, can it be functionally assessed? Discussions about these questions are clearly out of the scope of this review, and the readers are referred to recent commentaries elsewhere^{107,108}. Currently there is little explicit regulation of human embryoid research. However, a consensus among researchers working in this field (including the authors) has urged regulators to prohibit implantation of human embryoids into mammalian uterus and ban the use of human embryoids for reproductive purposes^{107,108}. As this nascent 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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Box 1.**Glossary**

- **Conceptus**
The products of conception at all stages of development from zygote to birth. These include the embryo proper, the fetus, the placenta, and all extraembryonic membranes. The term “embryo proper” refers to those parts of the conceptus that will form the new body and excludes extraembryonic tissues. Often, the terms “embryo” and “conceptus” are used interchangeably.
- **Pre-implantation development**
The first few days of development, from fertilization to implantation, during which the conceptus travels down the oviduct toward the uterus. It encompasses the first 7 – 9 days after fertilization in humans.
- **Morula**
The very early stage in a conceptus when cleavage has resulted in a solid ball of cells.
- **Implantation**
The process of attachment and invasion of the conceptus to the uterine tissues that occurs around day 7–9 after fertilization in humans. Implantation establishes the fetal-maternal interface leading to later placental development.
- **Blastula and blastocyst**
The stage of the conceptus prior to implantation is termed blastula. At this stage, the conceptus is called a blastocyst.
- **Peri-implantation development**
The development of the conceptus in the uterine tissues prior to gastrulation.
- **Gastrula and gastrulation**
Gastrulation describes the process by which the three definitive germ layers of the embryonic compartment of the conceptus are formed. Gastrulation begins around day 14 in humans. The gastrulation stage conceptus is termed gastrula.
- **Primitive streak**
The embryonic structure that breaks radially symmetry by establishing the anterior-posterior axis and establishes bilateral symmetry (alignment of equivalent structures on both sides of the anterior-posterior axis), the site of gastrulation, and the formation of the germ layers. In the human embryo, the primitive streak appears around day 14 after fertilization.
- **Neurula and neurulation**

Neurulation describes the process by which the neural tube is formed from the ectodermal neural plate. The neural tube will give rise to the brain and spinal cord. The neurulation stage conceptus is termed neurula.

- Organogenesis

The development of specific organs in the embryo such as the brain and heart. Organogenesis starts soon after gastrulation. In humans, organogenesis commences during the 4th week after fertilization.

- Embryonic and fetal stages

The embryonic stage begins with the division of the zygote and encompasses the development of the body plan and formation of the organs. This is followed by the fetal stage, during which growth and maturation of tissues and organs occurs. In humans, the fetal stage begins during the 9th week after fertilization and continues to birth.

Box 2.**Classic developmental concepts and principles**

- **Developmental potency**
Developmental potency describes the ability of a cell in the embryo to differentiate into other cell types. There is a continuum of developmental potency following progressive development of the embryo, from totipotency, pluripotency, multipotency, oligopotency, and finally unipotency.
- **Pattern formation**
Pattern formation is the process by which initially equivalent cells in a developing tissue acquire identities that lead to a well-ordered spatial pattern of cell activities. Pattern formation can involve positional information or cell sorting (see below).
- **Positional information**
The concept of positional information proposes that cells in a developing tissue acquire positional values as in a coordinate system, which they interpret by developing in particular ways to give rise to spatial patterns. Positional information often involves diffusion of morphogens, leading to signaling gradients and differential gene expression in a morphogen concentration-dependent manner. A key feature of positional information being the basis for pattern formation is that there is no pre-pattern in the developing tissue.
- **Cell sorting**
Pattern formation in a developing tissue can initiate from the specification of different cell types in the tissue in a salt-and-pepper fashion, which is followed by sorting of different cell types into distinct domains from where different tissues are formed. Cell sorting involves a morphogenetic process during which individual cells exchange neighbors, increasing the number of homotypic contacts and decreasing the number of heterotypic contacts.
- **Symmetry breaking**
Symmetry breaking is the process by which an initially homogeneous system acquires an asymmetry along an axis. While external cues can induce or assist symmetry breaking, asymmetries can emerge spontaneously without such input, guided by self-organization (see below).
- **Self-organization**
Self-organization, as a non-equilibrium process, can be defined as the formation of complex patterned structures from units of less complexity by collective, non-linear interactions, without referring to an external blueprint or template. These local internal interactions typically form feedback loops, thereby conferring robustness to the system. Other common features found in

self-organizing systems are non-linearity, symmetry breaking, and the emergence of patterns through amplifications of stochastic fluctuations.

- Embryonic induction

Embryonic induction is an interaction between one (inducing) tissue and another (responding) tissue, as a result of which the responding tissue undergoes a change in its direction of differentiation.

- Signaling center

A localized region of the embryo that exerts a special influence on surrounding cells, usually by means of secreted signaling molecules, and thus instructs how those cells develop.

- Organizer

A signaling center that directs the development of the whole embryo or of part of the embryo.

- Community effect

Community effect describes cell-cell communication among a group of nearby cells in a developing tissue, which is necessary for them to maintain coordinated behaviors.

- Morphogenetic regulation

Tissue-scale morphogenetic changes, including changes in cell shape, number, position and force, can work in concert with classic developmental signaling events mediated by diffusible signals to mediate gene expression and cell fate specification.

Box 3.**Clinical benefits of human embryology**

Advancing fundamental understanding of human embryogenesis can provide a scientific foundation for improving assisted human reproduction and prevention of pregnancy loss, birth defects and teratogenesis. It will also advance the biology of germ cells and treatment of infertility. Advancing understanding of human implantation will help develop effective contraception technologies and treatments of recurrent implantation failure. Detailed understanding of the widespread epigenetic programming during human embryonic development can provide important insights for disease progression in later life. Studying human development is critical for improving stem cell differentiation protocols to mimic embryogenesis, in order to achieve desired cell functions for research and therapy.

Box 4.**Early mouse and human development****Pre-implantation development**

Pre-implantation mouse and human development displays intricate self-organization and autonomy. After fertilization, the one-cell zygote undergoes cleavage cell divisions to form a solid ball of cells resembling a mulberry (and hence the name morula). Cells of the morula begin to differentiate, leading to blastocyst formation. In the blastocyst, the trophectoderm (TE) surrounds a fluid-filled cavity (blastocoel) with an inner cell mass (ICM) on one side. The TE is an extraembryonic tissue and will give rise to the placenta. As the blastocyst develops, the ICM becomes segregated into two distinct cell populations: the embryonic epiblast (EPI), which will give rise to the embryo proper, and a second extraembryonic tissue known as the primitive endoderm (PE; or hypoblast in human). Pre-implantation development has been extensively studied using the mouse embryo, revealing important cellular and morphogenetic events including position-dependent TE / ICM patterning, the blastocoelic cavity formation, and lineage segregation and sorting of EPI and PE in the ICM. The readers are referred to some excellent reviews on the mouse blastocyst development^{29,30}. Human blastocyst formation remains incompletely understood^{3,109}. Existing data suggests that there are differences in timing, in gene expression and potentially in mechanisms of lineage development and function between mice and humans during pre-implantation development².

Peri-implantation development

Successful implantation involves a bilateral interaction between a competent blastocyst and a receptive uterus. Implantation of the blastocyst (E5 in mouse and E7 in human) triggers major morphological reorganization and lineage developments. Upon implantation of the mouse blastocyst, the TE adjacent to the EPI (polar TE) forms the extraembryonic ectoderm (ExE) and ectoplacental cone. Concomitantly, the EPI and ExE each undergo lumenogenesis so that separate apical lumens are formed in each compartment^{82,103}. The two luminal cavities soon fuse to establish the pro-amniotic cavity, leading to the formation of a cup-shaped EPI juxtaposed with the ExE at E6 (the egg cylinder). From E5 to E6, the PE forms the parietal endoderm and visceral endoderm (VE). By E6, the VE envelops both the EPI and ExE, setting the stage for gastrulation.

Morphogenesis and lineage development in the peri-implantation human embryo show distinct features compared with mice³². Upon implantation, the EPI undergoes lumenogenesis to form the pro-amniotic cavity⁵⁻⁷, similar to the mouse EPI. Distinctly, the luminal EPI soon breaks symmetry and resolves into the bipolar patterned EPI-amnion sac^{7,31}. Specifically, EPI cells adjacent to invading polar TE cells differentiate into the amniotic ectoderm (AM)^{7,31}, an extraembryonic tissue involved in future fetal membrane development. The EPI cells at the opposite pole adjacent to the hypoblast remain pluripotent and become thickened and more columnar, forming the embryonic disc. Thus, the pre-gastrulation EPI displays distinct topologies between humans and mice: discoid in human and cup-shaped in mouse. The mouse embryo does not develop

the bipolar EPI-amnion structure. In mice, the AM emerges as amniotic folds at the junction of the EPI and ExE during gastrulation^{110,111}.

Gastrulation

Prior to mouse gastrulation, reciprocal interactions between EPI, ExE and VE lead to regionalized patterning in these tissues^{33,34}. Regionalization of VE is particularly important, leading to the formation of the anterior VE or AVE at the prospective anterior side of the embryo^{35,36}. The AVE secretes antagonists to shield overlying EPI cells from differentiation. Development of the AVE thus breaks radial symmetry and marks anterior-posterior (A-P) axis formation in the mouse embryo. Soon after, gastrulation is initiated at the proximal, posterior end of the EPI by a convergence of BMP-WNT-NODAL signaling interactions between EPI, ExE and VE³⁷⁻³⁹. The antagonists secreted by the AVE block signaling and impart neuroectoderm characters at the anterior pole of the EPI³⁶, whereas signals at the proximal, posterior end of the EPI instruct cells to undergo an epithelial-mesenchymal transition (EMT) and ingress through the PS to acquire mesendodermal fates³⁷. During mouse gastrulation, primordial germ cells (PGCs) emerge at the proximal, posterior end of the EPI^{40,41}. Experimental evidence supports that prospective PGCs are selected from somatic, gastrulating EPI cells by dose-dependent BMP signals that originate from the ExE¹¹².

Gastrulation remains one of the most mysterious phases of human development. Recent studies of NHP monkey embryos suggest conserved mechanisms are likely in play for A-P patterning during human gastrulation^{12,43}. Limited data from NHP monkey⁴³ and *in vitro* cultured human⁴² embryos suggest that PGCs may emerge in the dorsal nascent AM soon after implantation. This unexpected finding will require additional confirmation.

After gastrulation, the EPI in mouse and human embryos transforms into a trilaminar structure consisting of ectoderm, mesoderm and endoderm. As gastrulation proceeds, it brings subpopulations of cells in the three germ layer lineages into proximity so that they can undergo inductive interactions to pattern layers and specify new cell types, driving the development of organ rudiments.

Neurulation

Gastrulation is followed by neurulation. During neurulation, the ectoderm is first patterned into the neuroectoderm (neural plate) in the medial portion of the embryo flanked by future epidermis. At the border between these regions, the neural crest and placodes form in response to BMP and WNT signals emanated from surrounding tissues^{113,114}. The neural plate soon folds into the neural tube (NT)^{44,45}, with its anterior and posterior regions giving rise to the brain and spinal cord, respectively. Cells in the NT continue to differentiate under the influence of inductive factors emanating from surrounding tissues. Sonic hedgehog (Shh)-mediated transcriptional networks that control ventral patterning of the mouse spinal cord have been well elucidated^{46,47}.

Human neurulation remains challenging to study, even though NT closure defects remain one of the most common birth defects^{44,45}. Recent studies suggest that late-manifesting neurodegenerative disorders, such as Huntington's disease, may have a

neurodevelopmental component^{25,115}. The role of early nervous system development in late-onset neurodegenerative disorders remains a debated topic.

While the ectoderm undergoes neurulation, the mesoderm and endoderm also become further specified. Specifically, mesodermal cells are organized into cardiogenic mesoderm, axial mesoderm of the prechordal plate and notochord, paraxial mesoderm, intermediate mesoderm and lateral plate mesoderm. Each of these mesodermal regions undergoes some form of segmentation. The most evident and complete segmentation occurs in the paraxial trunk mesoderm, where each segment becomes an entirely separate somite. Much of the other mesodermal regions develop into embryonic connective tissues, cardiovascular and lymphatic systems, skeletal muscle cells, most of the urogenital system, and the lining of the pericardial, pleural and peritoneal cavities. Following gastrulation, the endoderm folds to form the primitive gut tube consisting of three subdivisions: foregut, midgut, and hindgut, which subsequently give rise to the epithelial lining of the digestive and respiratory systems.

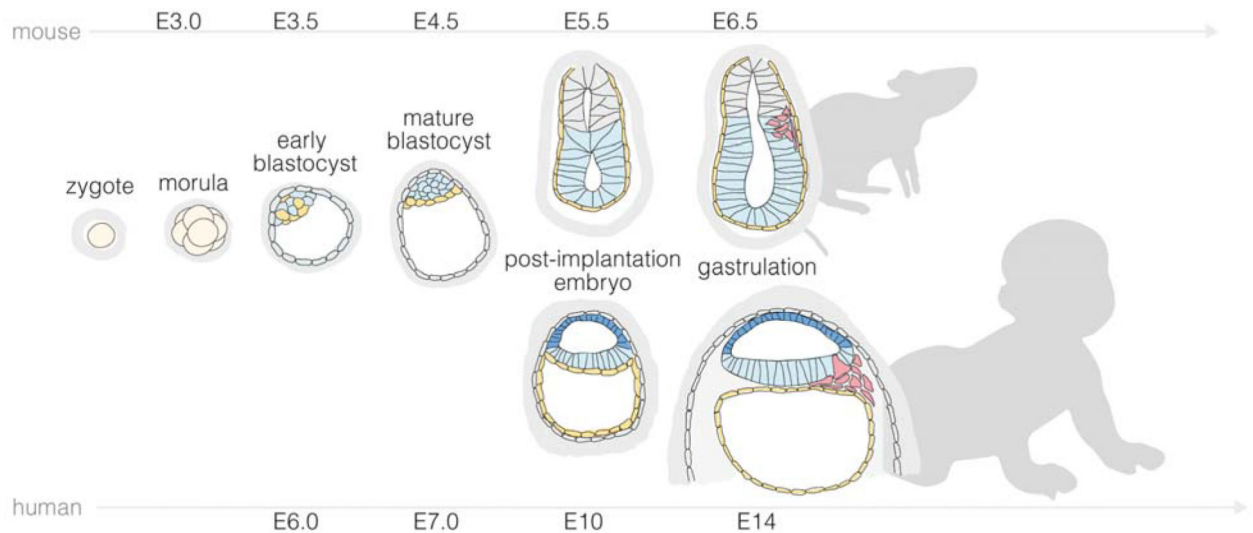


Figure 1. Overview of mouse and human development from pre-implantation to the onset of gastrulation.

Prior to implantation, both mouse and human embryos undergo cell divisions culminating in the development of a blastocyst, comprising an outer trophectoderm (TE) layer and an inner cell mass (ICM) that further segregates into epiblast (EPI) and primitive endoderm (PE; hypoblast in humans). The timing of blastocyst implantation differs between mice and humans (E5 in mouse and E7 in human). Furthermore, morphogenesis and lineage developments during peri-implantation mouse and human development show distinct features. Mouse development from E5 - E6.5 leads to the formation of a cup-shaped EPI juxtaposed with TE-derived extraembryonic ectoderm (ExE), enclosing the pro-amniotic cavity. Concurrently, the PE forms the visceral endoderm (VE) that envelops both EPI and ExE. In contrast, soon after human blastocyst implantation, while the EPI undergoes lumenogenesis to form the pro-amniotic cavity, EPI cells adjacent to polar TE cells become specified into the amniotic ectoderm (AM), with remaining pluripotent EPI cells forming the embryonic disc. By E6.5 for mice and E14 for humans, gastrulation is initiated in the posterior EPI compartment. Mouse primordial germ cells (PGCs) emerge at the boundary between posterior EPI and ExE at the onset of gastrulation. Data on primate PGC specification remain sparse. Existing data suggest that human PGCs may emerge in the nascent AM prior to the gastrulation. Human PGC specification requires additional studies for clarification. For peri-implantation mouse and human embryos, only their embryonic regions are shown.

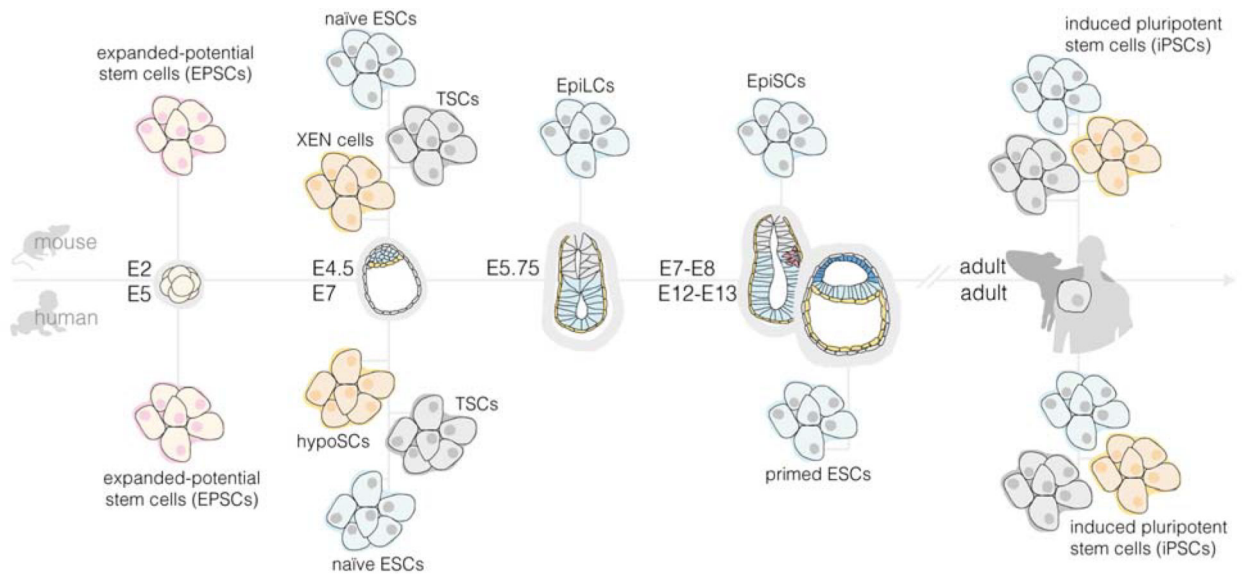


Figure 2. Mouse and human embryonic and extraembryonic stem cells and their corresponding developmental potencies.

Expanded potential stem cells (EPSCs) are established by isolating individual cells (or blastomeres) from eight-cell stage mouse and human embryos (or morula). By isolating cells from mouse blastocysts, mouse embryonic stem cells (mESCs) with naïve pluripotency, trophoblast stem cells (mTSCs), and extraembryonic endoderm (mXEN) cells representing the stem cell population of the primitive endoderm (PE) have been established. Similarly, human trophoblast stem cells (hTSCs) have been derived from human blastocyst. Primed mouse ESCs, known as mouse epiblast stem cells (mEpiSCs), are derived from the late post-implantation, pre-gastrulation mouse epiblast (EPI). Mouse EPI-like cells (EpiLCs) with an intermediate or formative state between naïve and primed pluripotency have been generated from mESCs *in vitro*, with a transcriptional profile similar to the early post-implantation mouse EPI. Human ESCs (hESCs) with primed pluripotency have also been derived from pre-implantation human blastocysts. Using strategies such as reprogramming, differentiated somatic mouse and human cells can be converted to a pluripotent state to establish induced pluripotent stem cells, or iPSCs. Using chemical cocktails, primed hESCs can be reverted into a naïve-like pluripotent state. Human hypoblast stem cells (hypoSCs) can be generated using these chemically reset naïve hESC lines.

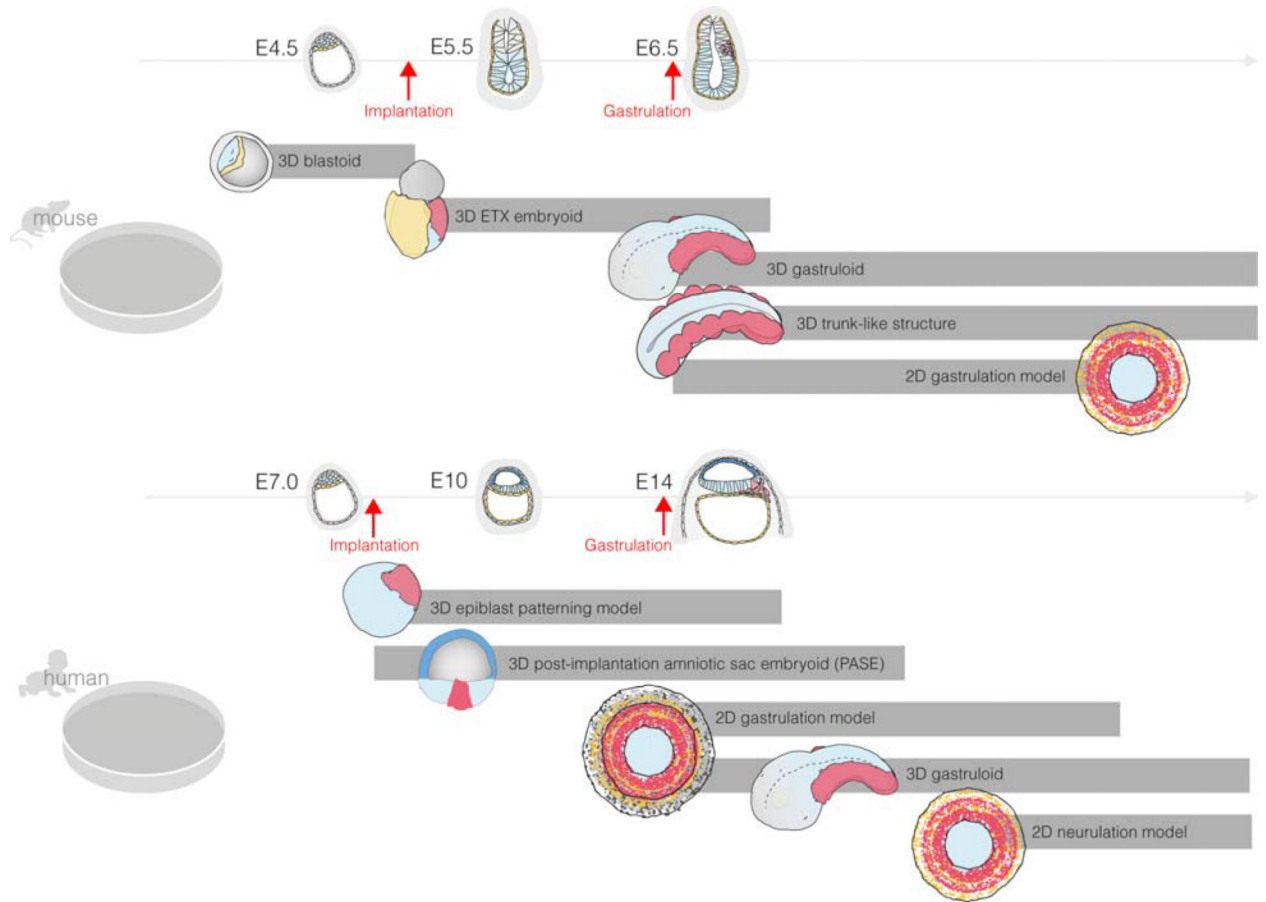


Figure 3. Existing embryoids that recapitulate different stages of mouse (top) and human development (bottom), from pre-implantation through gastrulation or early neurulation and organogenesis.

3D blastoid: Embryoid to model pre-implantation blastocyst development. 3D ETX embryo: Embryoid to model post-implantation embryo development up to early gastrulation. 3D gastruloid and trunk-like structure: Embryoid to model post-gastrulation development of the posterior portion of the embryo. 2D gastrulation model: Embryoid to model germ layer patterning during gastrulation. 3D epiblast patterning model: Embryoid to model epiblast morphogenesis and patterning during early post-implantation development. 3D post-implantation amniotic sac embryo (PASE): Embryoid to model post-implantation human development up to early gastrulation. 2D neurulation model: Embryoid to model the neurulation process, leading to neural tube development and patterning.

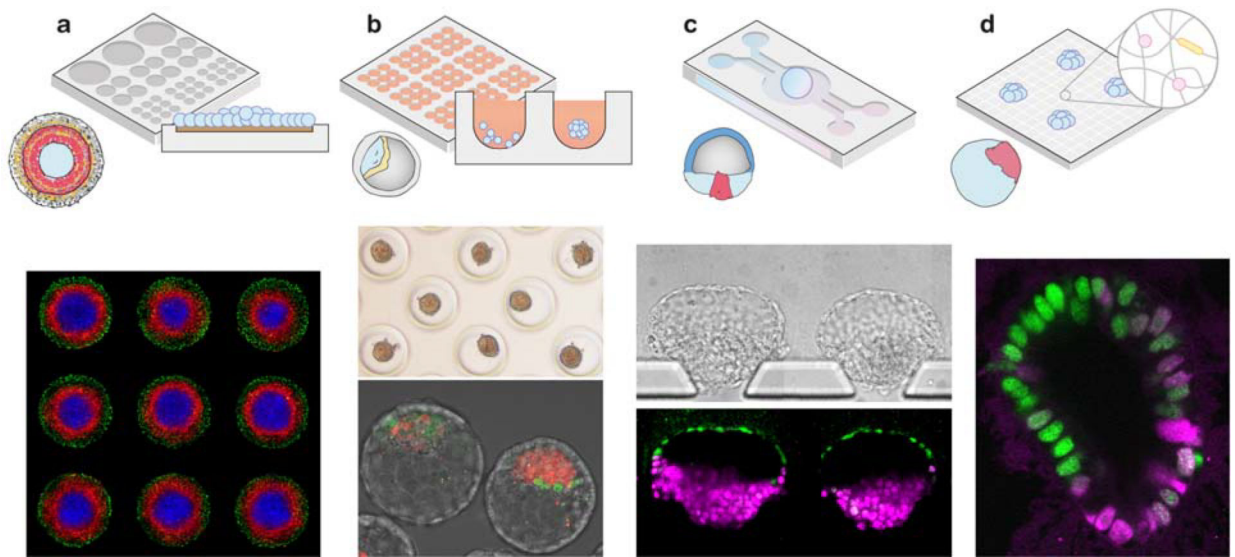


Figure 4. Bioengineering tools to promote multicellular interaction and self-organization in embryoid development.

(a) Micropatterning to generate 2D circular colonies of hPSCs to model germ layer patterning during gastrulation. Immunofluorescence image shows emergence of concentric gene expression regions, mimicking development of the germ layers (SOX2+ ectoderm, *blue*; TBXT+ mesendoderm, *red*) as well as a GATA3+ extraembryonic layer (*green*). Image from A. Yoney and E.D. Siggia. (b) Microwell array to promote cell aggregation and development of mouse blastoids. Top: Microwell arrays composed of agarose hydrogels to promote aggregation of mESCs and mTSCs. Bottom: Merged image showing two blastoids, with a layer of mTSCs surrounding a cavity and a cluster of mESCs mimicking the inner cell mass. Immunostaining: NANOG (*red*) and GATA6 (*green*). Images from N. Rivron. (c) Microfluidics to control spatiotemporal morphogen signaling and tissue patterning. Bright-field (top) and immunofluorescence (bottom) images of an array of post-implantation amniotic sac embryoids (PASEs), showing molecular asymmetry and tissue patterning, with TFAP2A+ amniotic cells on one pole (*green*) and TBXT+ gastrulating cells (*magenta*) on the opposite pole. Images from Y. Zheng. (d) Chemically and physically defined hydrogels for 3D embryoid development. Immunofluorescence image of a 3D human gastrulation embryoid for modeling epiblast morphogenesis and patterning (SOX2, *green*; TBXT, *magenta*). Image from M. Simunovic.