Stem cell-based models of early mammalian development

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ABSTRACT

The complex process by which a single-celled zygote develops into a viable embryo is nothing short of a miraculous wonder of the natural world. Elucidating how this process is orchestrated in humans has long eluded the grasp of scientists due to ethical and practical limitations. Thankfully, pluripotent stem cells that resemble early developmental cell types possess the ability to mimic specific embryonic events. As such, murine and human stem cells have been leveraged by scientists to create in vitro models that aim to recapitulate different stages of early mammalian development. Here, we examine the wide variety of stem cell-based embryo models that have been developed to recapitulate and study embryonic events, from pre-implantation development through to early organogenesis. We discuss the applications of these models, key considerations regarding their importance within the field, and how such models are expected to grow and evolve to achieve exciting new milestones in the future.

KEY WORDS: Blastoid, Embryogenesis, Embryoid, Gastruloid, Organogenesis, Stem cell

Introduction

Over the past few decades, there have been few advancements in the fields of developmental biology and stem cell biology that have been more exciting than studies of human pluripotent stem cells (hPSCs). Through these studies, it has become appreciated that the availability of hPSCs opens up previously inaccessible phases of early human development to experimental studies. Understanding human development has been historically challenging primarily due to ethical limitations in studying human embryonic tissues, but also due to differences in the developmental dynamics between humans and typically used model species such as mice. As such, the availability of hPSCs has ignited the field, providing the means to develop in vitro models of human development that offer experimental controls while preserving human relevancy. Using such stem cell-based embryo models (‘embryoids’), researchers now have convenient and powerful experimental tools that can be used to uncover the complex symphony of molecular and cellular events that occur during human development.

In this Review, we first detail the various types of stem cells that have been used in the development of different embryos. We then highlight how these models have been applied to study various stages of early mammalian development, from pre-implantation blastocyst formation and gastrulation through to the early stages of organogenesis, and their limitations. Finally, we highlight some of the challenges and future directions for the field.

Varieties of stem cells

As the field of stem cell research has grown over the years, scientists have developed a variety of stem cell types from embryos at pre- and post-implantation stages of development (Fig. 1), and are continuing to generate new ones. Human embryonic stem cells (hESCs) were first derived from human blastocysts (Thomson et al., 1998) and, less than a decade later, it was discovered that human somatic cells could be reprogrammed into a pluripotent phenotype to generate human induced pluripotent stem cells (hiPSCs) (Junying et al., 2007; Takahashi et al., 2007). Conventional hESCs and hiPSCs, together termed human pluripotent stem cells (hPSCs), are developmentally similar to pre-gastrulation stage epiblast (EPI) cells of primate monkey and human embryos (Nakamura et al., 2016). As these pre-gastrulation EPI cells are primed for germ layer specification (Rossant and Tam, 2017), conventional hPSCs are considered to exist in a ‘primed’ pluripotency state. After intensive studies, hPSCs that model earlier stage ‘naïve’ pluripotent cells of the inner cell mass (ICM) of the blastocyst were developed (Takashima et al., 2014; Theunissen et al., 2014; Guo et al., 2016). It should be noted, however, that mouse ESCs (mESCs) derived from mouse blastocysts are analogous to naïve hPSCs in that they mimic pre-implantation pluripotent ICM cells (Boroviak et al., 2014; Ying et al., 2008), whereas mouse EPI-like stem cells (Episcs) are derived from post-implantation epiblast tissues and are developmentally similar to primed hPSCs (Tesar et al., 2007; Brons et al., 2007; Kojima et al., 2014). A new population of PSCs, referred to as expanded/extended pluripotent stem cells (EPSCs), has also been developed and is capable of differentiating into cell types reminiscent of embryonic and extraembryonic lineages in mice (Yang et al., 2017a,b) and in humans (Guo et al., 2016). More recently, researchers have developed mouse totipotent stem cells (TotiSCs), which exhibit molecular similarities to two- and four-cell stage blastomeres (Shen et al., 2021; Hu et al., 2022). Similarly, cells resembling the eight-cell (8C) stage human blastomeres, coined 8C-like cells (8CLCs), have recently been derived from hPSCs (Mazid et al., 2022).

In addition to developing human ICM lineage-related stem cells, there are intensive ongoing efforts to generate human extraembryonic stem cell lines that resemble the trophoderm (TE) and hypoblast/primitive endoderm (PE) lineages. For example, human trophoblast stem cells (hTSCs) have been derived from human blastocysts and early placentas (Okae et al., 2018). Additionally, naïve hPSCs (Cinkornpumin et al., 2020; Lo et al., 2021), primed hPSCs (Viukov et al., 2022 preprint) and 8CLCs (Mazid et al., 2022) have recently been used to generate stem cell lines with transcriptomic similarities to human TE. Although bona fide human hypoblast stem cells currently do not exist, naïve hPSCs have recently been used to generate expandable naive extraembryonic endoderm (nEnd) cells that display transcriptomic similarities to human blastocyst-derived hypoblast cells (Linneberg-Agerholm et al., 2021).
et al., 2019). As new stem cell types continue to emerge and be characterized and authenticated (reviewed by Posfai et al., 2021b), they will no doubt add to the toolbox for generating in vitro models of human development.

**Modeling pre-implantation blastocyst development**

Although there are considerable differences in embryonic development between mice and humans, some aspects of pre-implantation development are shared between the two species, with both forming a structure known as a blastocyst, which contains an outer TE layer surrounding a cavity (blastocoel) with the ICM on one side of this cavity (Fig. 1). Significant progress has been made in generating stem cell-based models of pre-implantation development, specifically in developing blastocyst models, or ‘blastoids’, that contain all three lineages (EPI, TE and PE) found in blastocysts. Ever since the first demonstrations of blastoid formation using mouse stem cells (Rivron et al., 2018), there has been excitement about the prospect of generating human blastoids (reviewed by Fu et al., 2021). Indeed, the recent successful development of human blastoids now paves the way for using these controllable experimental tools for studying classic developmental concepts in human blastocyst formation, including developmental potency, lineage diversification, patterning formation, cell sorting and embryonic induction. Moreover, because the dynamics of human implantation and peri-implantation development are difficult to study, human blastoids are becoming an attractive tool for advancing our understanding of these stages of human development.

**Mouse blastoids**

Various techniques have been used for generating mouse blastoids, yielding a diverse array of blastoids with different features (Fig. 2A). As mESCs are incapable of differentiating into extraembryonic lineages, the first demonstration of mouse blastoids was achieved by seeding mTSCs on top of mESC aggregates formed inside a microwell (Rivron et al., 2018), offering insights into the embryonic inductions that direct trophoblast development (Rivron et al., 2018). The blastoid generation protocol was then made more efficient via the inclusion of mTSCs with a gene expression profile reminiscent of polar tropheoblasts (Frias-Aldeguer et al., 2019 preprint). Another blastoid model was created by seeding mTSCs onto mEPSC aggregates formed in a microwell (coined EPS-blastoids) (Sozen et al., 2019). Both types of blastoids demonstrated that the presence of mTSCs could spontaneously induce the formation of an embryonic-abembryonic axis within blastoids (Frias-Aldeguer et al., 2019 preprint; Sozen et al., 2019). These blastoids even seem to enable the formation of primitive endoderm-like (PE-like) cells (Rivron et al., 2018; Frias-Aldeguer et al., 2019 preprint; Sozen et al., 2019). The EPS-blastoid further shows that its PE-like epithelium can give rise to cells that are transcriptomically similar to parietal endoderm and visceral endoderm, both of which are derivatives of the PE (Sozen et al., 2019). Another recently developed in vitro 3D protocol for differentiating mEPSCs into EPI-, TE- and PE-like cells resulted in the formation of mouse blastoids with similar morphology and lineage allocation to mouse blastocysts (Li et al., 2019). An alternative mouse blastoid model has also been generated without the use of extraembryonic stem cells by reprogramming mouse pluripotent stem cells (mPSCs) into induced blastocyst-like precursor cells (iBLC-PCs) that self-organize into induced blastocyst-like cysts (iBLCs) (Kime et al., 2019). More recently, two different mouse blastoids, coined totipotent blastomere-like cell blastoids (TBLC-blastoids) (Zhang et al., 2022 preprint) and totipotent stem-cell blastoids (TPS-blastoids) (Xu et al., 2022), have been generated from mouse cells resembling two- and four-cell blastomeres. The creation of mouse blastoids from cells that resemble early blastomeres provides a new capacity for researchers to model the early stages of blastocyst development in vitro in a way that cannot currently be achieved using human cells.

**Human blastoids**

Human blastoids offer the first complete model of a human embryo, and much of their success is owed to the strategies implemented to create mouse blastoids. Currently, two main techniques have been used to generate human blastoids: manipulation of hPSCs and direct reprogramming of adult human cells (Fig. 2B). Given the developmental potency of naïve hPSCs and hEPSCs to give rise to both embryonic- and extraembryonic-like cells (Guo et al., 2021; Yang et al., 2017a,b; Gao et al., 2019), it is not surprising that these cells have been used successfully for developing human blastoids (Kagawa et al., 2022; Yu et al., 2021; Yanagida et al., 2021; Fan et al., 2021; Sozen et al., 2021). Blastoids generated from
centrifuging naïve hPSCs down into rounded microwells to facilitate self-organization yielded interesting findings concerning the coordination of morphogenesis and lineage segregation events (Yanagida et al., 2021). Similar blastoids were generated by seeding naïve hPSCs into a microwell array and placing the wells into a hypoxic chamber (Kagawa et al., 2022), whereas other groups aggregated naïve hPSCs in pyramid wells and generated distinct waves of hypoblast and trophoblast differentiation to form blastoids (Yu et al., 2021). Alternatively, aggregating hEPSCs into a pyramid well has proven to be a successful strategy for generating blastoids, even though co-culture with TSCs in this method was shown to be unsuccessful in generating a faithful TE-like epithelium (Sozen et al., 2021). Another blastoid generation method involves mixing hEPSCs and EPS-derived TE-like cells at a 1:4-1:5 ratio, yielding structures with a blastocyst-like morphology, albeit at a low efficiency (Fan et al., 2021). These human blastoids exhibit transcriptomic and morphological similarities to human blastocysts, and some of them also appear to possess the ability to initiate implantation-like events in the presence of endometrial cells. Similarly, blastoids have been derived using 8CLCs, which also demonstrate morphological and transcriptomic similarities to human blastocysts (Mazid et al., 2022). These 8CLC-derived blastoids are uniquely poised to offer insights into the formation of extraembryonic lineages during the progressive development from the blastula to the blastocyst.

Another recent study claimed that, under appropriate chemical-physical environments, primed hPSCs can form trophoblast-like cells, and this was leveraged to generate what appear to be 3D structures possessing cellular distributions reminiscent of a human blastocyst (Imamura et al., 2022 preprint). This particular model remains to be validated; nonetheless, it challenges previously understood limitations of primed hPSCs and suggests that hPSCs may be more plastic than once thought. An alternative method for generating human blastoids is through direct reprogramming of human somatic cells. The blastoids generated via this reprogramming-based method have been termed ‘iBlastoids’ (Liu et al., 2021). It should be noted, however, that careful examination of transcriptomic data suggests that alleged TE-like cells reported to be present in iBlastoids are, in fact, amnion-like cells (Zhao et al., 2021 preprint; Zheng et al., 2022).

**Challenges and opportunities in blastoid research**

When evaluating blastoids, an important consideration is their stability and how well they can replicate both blastocyst...
development and the transition into peri-/post-implantation embryonic stages when compared with natural embryos. Studying human blastocyst implantation in vivo obviously faces many practical and ethical limitations, and replicating an in vivo-like environment for faithful blastoid culture can be difficult when precise endpoints are difficult to pin down. As such, studying the implantation of mouse blastoids into a pseudopregnant mouse uterus typically serves as an essential baseline upon which mouse and human blastoid implantation studies can be based.

Studies that transplanted mouse blastoids into a pseudopregnant mouse uterus between E2.5 and E3.5 demonstrated a recapitulation of natural implantation events such as discrete formation of a patterned decidua, formation of trophoblast giant cells and induction of uterine vascular permeability (Sozen et al., 2019; Rivron et al., 2018; Li et al., 2019; Kime et al., 2019). However, many of these implantation events occurred at a low efficiency, and the degradation and altered morphology of some of the implanted blastoids suggests a degree of blastoid resorption into the uterine tissue (Sozen et al., 2019; Kime et al., 2019). The mechanisms of natural embryo resorption are various and complex, and include placental dysfunction (Reynolds et al., 2006) and problems with immune tolerance between fetal and maternal tissues (Zenclussen et al., 2005). Indeed, careful examination of how blastoids may induce placental dysfunction after implantation or how effectively blastoids can establish immune tolerance with maternal tissues may hold the key to overcoming this obstacle and developing more stable implantation experiments.

Human blastoids also exhibit evidence of implantation competency in in vitro implantation studies, e.g. when using in vitro cultures of human endometrial cells (Kagawa et al., 2022) or engineered in vitro culture systems that model implantation in the absence of maternal tissues (Fan et al., 2021; Liu et al., 2021; Shahbazi et al., 2016; Deglincerti et al., 2016). To this end, hPSC-based endometrial organoids might provide faithful models of the human uterus for future blastoid implantation studies (reviewed by Hibaoui and Feki, 2020). Interestingly, some human blastoids do not implement implantation but still undergo morphogenetic events and lineage transitions resembling those occurring during peri-implantation development, such as segregated outgrowths and amniotic cavity-like structure formation (Yu et al., 2021) or the formation of a radially organized EPI-like structure around a central lumen with a surrounding extraembryonic structure containing hypoblast-like cells (Sozen et al., 2021). However, such morphogenetic events occur at a low efficiency, and continuous development of human blastoids has not shown faithful formation of amnion-like tissue or a primitive streak-like structure – two important early post-implantation developmental hallmarks.

Adapting recent advances in prolonged ex vivo cultures of natural embryos into blastoid cultures might promote their continuous development (Aguilera-Castrejon et al., 2021; Ichikawa et al., 2022; Govindasamy et al., 2021). Indeed, existing protocols for the ex utero culture of natural mouse embryos have recently been adapted for the prolonged culture of embryo models such as naïve mESC-derived synthetic embryos (sEmbryos) (Tarazi et al., 2022) and ETIX embryos comprising mESCs, TSCs and Gata4-expressing mESCs, referred to as induced XEN (iXEN) cells (Amadei et al., 2022). Both of these embryooids can be grown in ex utero roller culture systems beyond the E5.5 stage and into post-gastrulation stages. Looking forward, the integration of faithful human uterine models with robust ex vivo culture systems is expected to be an upcoming milestone that will not only further validate existing embryoids, but usher in a new age of understanding human implantation dynamics.

**Modeling peri-implantation development**

While efforts to develop blastoids for modeling pre-implantation development are ongoing, embryooids that recapitulate peri-implantation and pre-gastrulation developmental events, centering on the EPI lineage, have also been developed. There has also been significant progress in developing models that recapitulate TE lineage diversification and development during the early placentaent process.

**Mouse peri-implantation models**

Innovations in the assembly of different stem cells have yielded a variety of powerful mouse peri-implantation models (Fig. 3A). The first was the ETS embryo, which combines mouse ESCs and TSCs into a structure reminiscent of an E6.5 mouse embryo (Harrison et al., 2017). A similar model known as the ETX embryo was later developed and employs the spontaneous assembly of extraembryonic endoderm (XEN) stem cells with ESCs and TSCs to generate a model of the compartmentalized mouse embryo (Sozen et al., 2018; Zhang et al., 2019). The more recently developed ETIX embryo initially resembles a peri-implantation mouse embryo and can develop into later post-implantation stages, and even demonstrates the formation of beating cardiac tissue in addition to trunk-like structures, gut tube-like structures, primordial germ cell-like cells and what appear to be VEG-derived yolk sac structures with blood islands (Amadei et al., 2022).

**Human peri-implantation models**

The topology of the pre-gastrulation human embryo differs considerably from that of the mouse egg cylinder before gastrulation. Another distinction between human and mouse pre-gastrulation development is with respect to the formation of the amnion. Specification of the amnion from the EPI is a key feature of pre-gastrulation human embryos, whereas mouse embryos do not possess an amnion before gastrulation (Yang et al., 2021b). Recent investigations have revealed that clusters of primed hPSCs can be coaxed into forming luminal cysts of squamous amnion-like cells when cultured in a 3D gel culture in which mechanical signals of the extracellular matrix overlay are modulated (Shao et al., 2017a). This amniotic differentiation of primed hPSCs can also be regulated via asymmetric BMP4 activity in order to generate asymmetric cysts, termed the post-implantation amniotic sac embryoid (PASE). This structure morphologically and transcriptomically resembles the natural amniotic sac and can even initiate events resembling the development of primordial germ cells (PGCs) and posterior definitive mesoderm (Fig. 3B) (Shao et al., 2017b; Zheng et al., 2019). Using fit-to-purpose microfluidics, multiple PASE models have been generated to facilitate the induction of definitive mesoderm and endoderm for extended observation and perturbation (Zheng et al., 2019).

Continuous development of the PASE, however, is limited as it disintegrates as gastrulating cells delaminate from the PASE structure. One possible approach to promote extended PASE development is to incorporate extraembryonic lineages into the PASE. This would provide additional embryonic-extraembryonic interactions and associated physical boundaries that might promote patterning of the pluripotent pole and definitive germ layer organization. Recent work suggests that amnion specification in non-human primate embryos occurs in two distinct waves and, following on from this, leveraged naïve hPSCs to replicate the
timing and morphogenesis of these reported waves (Rostovskaya et al., 2022). Future directions could include a closer examination of the timing and morphogenesis events of amniogenesis in peri-implantation non-human primate and human embryos, as well as in human embryoids developed using naïve and primed hPSCs.

**Placentation models**

While the EPI lineage inside the blastocyst undergoes lineage diversification and organization at the peri-implantation stage, so too does the extraembryonic TE. Upon implantation, the TE segregates into multiple different trophoblast lineages, including syncytiotrophoblast, extravillous trophoblast and cytotrophoblast, to facilitate invasion into the maternal tissue and formation of the placenta. The recent development of trophoblast organoids offers new insights into placental development (reviewed by Zhou et al., 2021). These trophoblast organoids can be developed from hPSCs (Karvas et al., 2020; Telugu et al., 2013; Roberts et al., 2018; Sheridan et al., 2019) or hTSCs (Turco et al., 2018; Haider et al.,

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**Fig. 3. Overview of mouse peri-/post-implantation embryo models.** (A) Schematic of the different processes that have been implemented to generate 2D and 3D models of gastrulation or to model peri-gastrulation events in vitro using mouse pluripotent stem cells (mPSCs). Pluripotent mPSCs can be micropatterned and subjected to different signaling events to generate 2D structures that recapitulate different aspects of gastrulation, such as primitive streak formation. Alternatively, mPSCs can be cultured into 3D aggregates that can then be manipulated to recapitulate different aspects of gastrulation depending on the protocol. (B) Schematic of the different processes implemented to generate 3D models of gastrulation using mPSCs in combination with extraembryonic/extraembryonic-like cells [TSCs, trophoblast stem cells; XEN cells or induced XEN (iXEN) cells; EPILCs, epiblast-like cells; ESCs, embryonic stem cells; XEGs, XEN-enhanced gastruloids; ETS embryo, ESC- TSC-derived embryo; ETX/ETiX embryo, ESC- TSC- and XEN/iXEN-derived embryo; sEmbryo, synthetic embryo]. Different cell types can be combined by generating distinct 3D aggregates and combining them in co-cultures or by co-culturing the different cell types and allowing them to self-organize into 3D structures.
Future efforts in modeling placentation might integrate embryoids, including blastoids, and trophoblast organoids/hPSC-derived trophectoderm with natural uterine tissues/hPSC-derived uterine organoids to model trophoblast-uterine interactions during placentaion. Additionally, the use of patient-specific hiPSC-derived uterine organoids could be used to investigate how different disease states or genetic factors may affect implantation.

Modeling gastrulation

A favorite adage among developmental biologists is the timeless quote by Lewis Wolpert that states ‘it is not birth, marriage, or death, but gastrulation which is truly the most important time in your life.’ During gastrulation, the major body axes are formed, the three definitive germ layers (ectoderm, mesoderm and endoderm) are established and cell migration commences in order to organize the newly formed tissues. Recent advances using mouse and human stem cells, in both 2D and 3D cultures, have led to the generation of promising models of gastrulation that can now allow us to gain a detailed mechanistic understanding of gastrulation-related events.

Mouse gastrulation models

The development of in vitro mouse gastrulation models has been aided by the abundance of in vivo mouse data available for their authentication and validation. Given their consistency and compatibility with live imaging, 2D mouse gastrulation models are particularly well-equipped to leverage in vivo mouse data for comparison and validation in ways that are currently not possible with human models. Validations for 2D mouse gastrulation models have evolved as new data continue to emerge to reveal insights into dynamic mouse embryonic events. For example, one 2D mouse gastrulation model converted micropatterned mESCs into EPI-like cells, examined the effects of different signaling activities on the specifications of different regional identities and compared the data with in vivo mouse data (Morgani et al., 2018).

In addition to 2D mouse gastrulation models, recent years have seen rapid progress in generating 3D mouse gastrulation models by controlling culture environments and chemical stimulations of aggregates of either mESCs (van den Brink et al., 2014; Girgin et al., 2021a; Veenvliet et al., 2020; Turner et al., 2017; Beccari et al., 2018; Rossi et al., 2020, 2021 preprint; Xu et al., 2021; Anlaş et al., 2021a,b preprint) (Fig. 3A) or mESCs and various mouse extraembryonic stem cells (Harrison et al., 2017; Girgin et al., 2021b; Bérenger-Currias et al., 2022; Sozen et al., 2018; Amadei et al., 2021, 2022) (Fig. 3B). The term ‘gastruloid’ was originally coined in a 2014 publication that cultured 3D aggregates of mESCs in U-bottomed microwells and subjected them to N2B27 medium with pulses of WNT signaling such that the cells formed aggregates that recapitulate some hallmarks of gastrulation, such as symmetry breaking, axial elongation and germ layer specification (van den Brink et al., 2014). More recent efforts have expanded upon the original mouse gastruloid protocol to probe the mechanical contributions of 3D culture environments to create new biomimetic niches that enable symmetry breaking and elongation. Protocols have been developed that leverage hydrogel niches and use WNT inhibition instead of WNT activation to achieve gastruloids with anterior neural tissues (Girgin et al., 2021a).

Other protocols have used similar niches to instead initiate the formation of trunk-like structures (Veenvliet et al., 2020), and there are even some non-adherent niches that promote axial polarization and spatially localized signaling similar to that observed in mouse embryos (Turner et al., 2017; Beccari et al., 2018). A unified mouse gastruloid protocol that aims to produce consistent gastruloids across a variety of different cell lines and is compatible with live imaging has also been recently reported (Anlaş et al., 2020, 2021a,b). Alternative methods have merged mESC aggregates in the gastruloid culture with cell-based signaling centers for signaling control and axial organization, and these advanced gastruloids have demonstrated the formation of primitive organ systems similar to those found in neurula-stage mouse embryos, including the neural tube, beating cardiac tissue and a primitive gut tube (Xu et al., 2021).

The importance of understanding the contributions of extraembryonic tissues to gastrulation has motivated the creation of mouse gastrulation models from multiple types of stem cells (Fig. 3B). For example, it is known that signaling interactions between the EPI and ExE are responsible for the formation of the distal visceral endoderm, and that the anterior visceral endoderm plays a crucial role in patterning of the EPI required for primitive streak development and the initiation of gastrulation (reviewed by Tam and Loebel, 2007). Mouse gastruloid models generated by assembling mTSCs and mESCs undergo events that closely resemble those seen during the development of in vitro cultured mouse embryos, not only with respect to the morphologies of the mTSC and mESC compartments, but also with regard to the induction of definitive mesoderm and PGC-like cells (PGCLCs) (Harrison et al., 2017). Incorporating mTSCs into the mouse gastruloid protocol has also been explored to examine the development of anterior brain-like regions in mouse gastruloids (Girgin et al., 2021b). Interestingly, researchers claim that the development of neuroepithelial structures can also been seen in mouse gastruloids generated by assembling mESCs and XEN cells, coined XEN-enhanced gastruloids (XEGs) (Bérenger-Currias et al., 2022). Indeed, prolonged culture of E16.5/17.5 embryos has been shown to generate models with transcriptomic similarities to those of natural gastrulating mouse embryos (Sozen et al., 2018; Amadei et al., 2021, 2022). In addition, and as mentioned previously, the electronically controlled ex utero culture of co-aggregated naïve mESCs, naïve mESC-derived TE-like cells, and naïve mESC-derived XEN-like cells has yielded sEmbryos that can grow into post-gastrulation, early organogenesis stage embryos (Tarazi et al., 2022).

Human gastrulation models

Studies of gastrulation using natural mouse embryos and in vitro mouse gastrulation models have provided clues into the gastrulation process in humans. However, distinct differences regarding morphology and both signaling and genetic mechanisms exist between murine and human gastrula. For example, it is unknown whether a signaling center similar to the anterior visceral endoderm exists in the pre-gastrulation human embryo. In addition, morphological differences between human and mouse peri-gastrulation embryos result in different mechanical and paracrine signaling cues for the EPI before gastrulation (reviewed by Molè et al., 2020). Knowledge of these differences has motivated the development of human gastrulation models using primed hPSCs (Fig. 4).

With geometric constraints and supplemented morphogens, 2D colonies of primed hPSCs have been shown to develop a thickened primitive streak-like structure together with concentric rings of ectodermal, mesodermal, endodermal and extraembryonic domains (Warmflash et al., 2014; Minn et al., 2020). Several mechanisms have been explored to explain this gastrulation-like tissue patterning, including spatiotemporal dynamics of BMP, WNT and NODAL activities (Chhabra et al., 2019; Martyn et al., 2019b; Tewary et al., 2017), diffusion of endogenous inhibitors (Martyn et al., 2019a; Tewary et al., 2017; Etoc et al., 2016), and spatial
distribution of signaling receptors (Etoc et al., 2016). The migration of gastrulating cells (Martyn et al., 2019b), the induction of organizers (Martyn et al., 2018) and the depletion of aneuploid cells during gastrulation (Yang et al., 2021a) have also been investigated using 2D human gastrulation models. For example, cell tracking techniques have revealed fate-dependent cell migration in the 2D human gastrulation model that resembles the migration observed in natural mouse embryos (Martyn et al., 2019b). Xenografts of organizer-like regions derived from 2D human gastrulation models into chick embryos has demonstrated the formation of a secondary axis reminiscent of natural axis self-organization in chicks (Martyn et al., 2018). Furthermore, single cell data from the 2D human gastrulation model has revealed a mechanism for aneuploidy elimination that resembles the mechanism implied from available human embryo data (Yang et al., 2021a). The 2D human gastrulation model can also be combined with bioengineering tools to further engineer and perturb the system. For example, when the cells are cultured on a soft matrix, gastrulation-like nodes form instead of gastrulation-like rings (Muncie et al., 2020). In addition, exogenous chemical gradients achieved by microfluidic devices have been shown to establish axial germ-layer domains in the 2D gastrulation model (Manfrin et al., 2019), which more accurately resemble the morphology of germ layer organization during gastrulation.

3D human gastrulation models have also been developed. When supplemented with exogeneous morphogens, hPSCs embedded in soft gel matrix form 3D aggregates and spontaneously exhibit symmetry breaking with patterned gene expression (Simunovic et al., 2019). This model has since been expanded upon to include primed hPSC-derived cells with an extraembryonic transcriptional signature; in the absence of exogenous morphogens, the resulting structures reveal the development of cell types resembling those of early gastrulation embryos (Simunovic et al., 2022). Using a 3D culture protocol similar to that used for mouse gastruloid cultures, 3D aggregates of primed hPSCs break symmetry and form an anterior-posterior (A-P) axis; such human gastruloids undergo elongation along this axis with spatial organization of what appears to be three germ layers (Moris et al., 2020; Olmsted and Paluh, 2021). Furthermore, using shaking cultures, human gastruloids exhibit the formation of more-organized tissue structures, such as a primitive gut tube-like structure and a spinal cord-like structure (Olmsted and Paluh, 2021).

Challenges in developing gastrulation models
Compared with 2D gastrulation models, 3D gastrulation models bear higher resemblance to in vivo embryo morphology (Moris et al., 2020; Olmsted and Paluh, 2021; Harrison et al., 2017; Sozen et al., 2018). Furthermore, 3D gastrulation models exhibit the exciting potential of mimicking early organogenesis under prolonged culture conditions (Veenvliet et al., 2020; Rossi et al., 2020, 2021 preprint). The efficiency, controllability and reproducibility of the 3D gastrulation models, however, remain suboptimal. It should also be noted that 3D gastruloids do not contain a structure analogous to the primitive streak, whereas the highly controllable 2D gastrulation models possess a localized region of primitive streak-like formation. Indeed, 3D mouse gastruloids typically resemble post-gastrulation E8.5 mouse embryos compared with 2D mouse gastrulation models, which usually resemble E7 mouse embryos instead. Furthermore, it is far more difficult to closely examine the dynamic formation of a 3D gastruloid. As such, it can be difficult to pinpoint and address the causes of variability between different models and even between different gastruloids generated by the same protocol. Gastrulation is a
complex process, requiring precise spatiotemporal physical and chemical controls. In most current 3D gastrulation approaches, very few external controls are applied to the culture system, suggesting that the development of 3D gastrulation models is highly dependent on the boundary conditions of the culture and the stochastic behaviors and self-organizing properties of the initial cell populations.

The development of an in vitro human gastrulation model that exhibits high fidelity and integrity is therefore still a long-term pursuit for human embryoid research. Although extraembryonic structures have often been overlooked, likely due to both ethical concerns and technical difficulties, such extraembryonic structures are crucial for embryo development as they provide protection, nutrition, physical confinement and chemical signaling. In the case of human gastrulation, it is believed that the amnion provides an inductive role in triggering mesoderm induction (Zheng et al., 2019; Yang et al., 2021b), and the hypoblast plays a functional role in establishing the A-P axis (Amadei et al., 2021). Additionally, the yolk sac may function via nutritional supplementation and hematopoiesis initialization (Ross and Boroviak, 2020). At this time, there are only a handful of murine gastrulation models with integrated extraembryonic structures (Harrison et al., 2017; Sozen et al., 2018; Amadei et al., 2021, 2022). Nonetheless, these murine gastrulation models raise the possibility of achieving more advanced organogenesis and suggest potential strategies for more advanced human gastrulation models.

**Modeling early organogenesis**

Although modeling late organogenesis in embryoids is still out of reach, current 3D gastrulation models are taking steps towards faithful modeling of early organogenesis events in vitro. In particular, as we have already highlighted, there are mouse gastrulation models in which early organogenesis events have unfolded. These studies signify the promising future applications of 3D embryoids for generating functional organs, which could potentially be used for disease modeling and drug screens or even for therapeutic transplantation – the holy grail of regenerative medicine.

**Neural development**

Considerable efforts have been made in using hPSCs to develop in vitro models of early neural development (Fig. 5). The neural tube (NT) serves as the embryonic precursor to the central nervous system. Not surprisingly, therefore, most efforts in modeling early neural development have so far centered on recapitulating the NT formation process (or the neurulation process) and NT patterning along the A-P and dorsal-ventral (D-V) axes.

The first step in the neurulation process, neural conversion of the ectoderm, has been modeled successfully by seeding primed hPSCs in micropatterned colonies (Xue et al., 2018). Under a uniform neural induction chemical environment, these hPSC colonies spontaneously pattern into central neural plate (NP)-like tissue and peripheral neural plate border (NPB)-like tissue (Fig. 5). Although this neuroectoderm patterning model lacks non-neural ectoderm (NNE), which abuts the NPB in vivo, emergence of NNE tissue was achieved by supplementing exogeneous BMP4 into the neuroectoderm patterning model, and cells expressing neural crest markers were observed between the neural and non-neural regions (Britton et al., 2019). A similar micropatterning strategy to model the neurulation process in 3D showed that micropatterned hPSC

![Fig. 5. Overview of neural development models. Examples of stem cell-based models used to study neural induction and patterning events in vitro. Both 2D and 3D models have been used to examine ectoderm differentiation and patterning of the neuroepithelium along the body axes. In these models, human pluripotent stem cells (hPSCs) are coaxed into replicating aspects of neural patterning depending on the different protocol employed. Patterning of the neural plate, the neural plate border and non-neural ectoderm can be achieved using 2D circular micropatterns. AP patterning can be replicated via exposure of elongated 2D patterns to signaling gradients, while DV patterning of neuroepithelial cysts can be modeled using hPSCs in a 3D culture environment. Neurulation, or folding of the neural tube, can also be modeled in vitro by generating hollow 3D tubes from micropatterned hPSCs and exposing the system to neural differentiation medium. The cells attached to the substrate model the neural plate and self-organize in a process that closely resembles neurulation; such a model even produces cells resembling neural crest cells.](image-url)
colonies develop a lumenal neural cyst at the center of the colonies (Haremaki et al., 2019). Reminiscent of the NT, the central neural cyst is surrounded by NPB and NNE derivatives. Models that demonstrate events reminiscent of NP folding have also been developed (Fig. 5). Such models imitate different aspects of the NP folding process, leading to the formation of a closed NT-like tissue (Sahni et al., 2021; Karzbrun et al., 2021; Lee et al., 2022).

D-V patterning of the spinal cord has also been imitated using stem cell-derived neural development models (Fig. 5). Floating aggregates of mESCs or hPSCs exhibit concentric or local D-V patterning under posteriorizing and ventralizing neural induction conditions (Duval et al., 2019; Ogura et al., 2018). Under similar chemical environments, other models have used mESCs or hPSCs embedded in extracellular matrices (Meinhardt et al., 2014; Ranga et al., 2016; Zheng et al., 2019; Abdel Fattah et al., 2021), showing that the embedded cells form lumenal neural cysts that exhibit proper D-V patterning.

Neuroectoderm patterning and neurulation happen shortly after gastrulation. Thus, some gastrulation models that are more advanced on the developmental timeline exhibit development of neural tissues (Beccari et al., 2018; Girgin et al., 2021b). Embedding mouse gastruloids in Matrigel has been used to further refine gastruloid morphology to a trunk-like structure, featuring tissues resembling the NT, somites and neuroepithelial progenitors (NMPs) (Veenenvliet et al., 2020). Similar trunk-like structures have also been developed using primed hPSCs (Yaman et al., 2022 preprint). As mentioned above, mouse gastruloids leveraging cell-based signaling centers exhibit a neurulation-like process and the formation of a NT-like structure that exhibits A-P and D-V patterning (Xu et al., 2021). Similarly, ETiX and sEmbryo embryoids possess what appear to be forebrain and midbrain regions as well as a NT-like structure (Amadei et al., 2022; Tarazi et al., 2022). Development of the peripheral nervous system has also been modeled in hPSC-derived elongating multi-lineage organized (EMLO) gut rudiments (Olmsted and Paluh, 2021), which demonstrate the interplay between the development of important organ structures (such as the primitive gut tube) and the co-development of the central and peripheral nervous system.

**Somitogenesis**

A number of 3D embryoid systems have been shown to model the development of other early developmental structures essential for organ formation (Fig. 6). The development of somite-like structures from mesodermic mesoderm, for example, often features in mouse gastruloids and has also been reported in the trunk-like structures of ETiX embryoids (van den Brink et al., 2020; Xu et al., 2021; Amadei et al., 2022). The sEmbryo model is also claimed to develop at least four pairs of somite-like structures after prolonged culture (Tarazi et al., 2022). Specifically, trunk-like structures derived from mESC and hPSC aggregates contain somite-like structures surrounding a NT-like structure (Veenenvliet et al., 2020; Yaman et al., 2022 preprint). Recent work has also generated ‘somitoids’, which are hPSC-derived structures that model the formation of A-P-patterned somite-like epithelial structures (Miao et al., 2022 preprint; Sanaki-Matsumiya et al., 2022). Some of these models develop NMPs at their posterior end, which further bifurcate into somite-related or neural-related cells in response to different levels of exogeneous WNT signaling (Sanaki-Matsumiya et al., 2022; Yaman et al., 2022 preprint).

**Gut tube formation**

Researchers have been able to create mouse gastruloids that contain a gastruloid-spanning primordium resembling patterned anterior foregut, midgut and hindgut (Vianello and Lutolf, 2020 preprint). It has also been shown that mouse gastruloids develop an A-P and D-V patterned primitive gut tube alongside neural and cardiac structures (Xu et al., 2021). Additionally, mESC-derived trunk-like structures possess what appear to be gut-like epithelial structures (Veenenvliet et al., 2020), while hPSC-derived EMLO gastruloids seem to develop primitive gut tube-like structures alongside central and peripheral neurons (Olmsted and Paluh, 2021). The ETiX and sEmbryo embryoids have also been reported to demonstrate the development of the definitive endoderm into a gut tube-like structure (Amadei et al., 2022; Tarazi et al., 2022).

**Cardiogenesis**

Finally, one additional realm of early organogenesis that has seen exciting progress in embryoid research is cardiogenesis. By adding cardiogenic factors to gastruloid culture protocols, researchers have shown that mouse gastruloids develop beating cardiac tissues and associated vasculature (Rossi et al., 2020, 2021 preprint; van den Brink et al., 2014). These gastruloid models were recently expanded to allow the development of hematopoietic precursor-like cells and erythroid-like cells that are spatially localized to a vascular-like structure in a manner reminiscent of the development of blood cells in vivo (Rossi et al., 2020, 2021 preprint). Other mouse models of peri-/post-implantation have also been shown to model cardiogenesis alongside other organogenesis events (Xu et al., 2021; Olmsted and Paluh, 2022; Amadei et al., 2022; Tarazi et al., 2022). This includes the expansion of EMLO gastruloids such that they include a cardiac-like region (in structures termed ‘EMLOCs’) alongside the development of neural tissues, thus mimicking an innervated human heart (Olmsted and Paluh, 2022).

Although the presence of different tissues and organs in different embryoids is still in a continuous process of validation, it is becoming clear that embryoids provide an embryonic-like environment that is conducive for initiating organ development from the three germ layers; this advantageous feature might enable researchers to reliably generate structures that more closely resemble functional organs. In addition, embryoids may provide the possibility to model interorgan communication during development, allowing the coordinated development and growth of multiple tissues and organs. Such 3D embryoid systems with features resembling early organogenesis are promising for providing new insights into how different tissues form in relation to one another.

**Conclusions and future directions**

In this Review, we have reflected on recent progress in generating stem cell-based models of early mammalian development. Although this progress takes us closer to generating embryos in vitro that truly mimic their in vivo counterparts, it also raises inevitable bioethical questions, especially as human embryoid protocols become optimized. For discussions of such important bioethical issues in embryoid research, we refer readers to other recent excellent reviews (Fu et al., 2021; Rivron and Fu, 2021; Rossant and Tam, 2021; Posfai et al., 2021a; Clark et al., 2021; Weatherbee et al., 2021).

Despite the incredible progress, it is clear that further improvements are needed to achieve greater efficiency and controllability in embryoid development. In our view, such improvements can best be achieved through integrative approaches in which molecular and cellular bioengineering tools and biomaterials systems are incorporated to precisely modulate spatiotemporal biochemical and biomechanical signals in embryoid cultures (Xu et al., 2021; Manfrin et al., 2019; Zheng et al., 2019; reviewed by Shao and Fu, 2022). The importance of proper validation of human
embryoids as faithful models of human development can also not be overstated. Most current human embryoid studies rely on snapshots of tissue morphology, lineage marker expression, and transcriptome data for authentication, with limited functional validations to ascertain progressive lineage development and tissue organization. Making matters worse, ethical and technical limitations on human embryo studies make it very challenging to obtain information about human post-implantation embryonic development. Nonetheless, studies of non-human primate embryos that more closely resemble human embryos are making rapid progress, including prolonged in vitro culture up to the early organogenesis stages (Niu et al., 2019; Ma et al., 2019; Nakamura et al., 2016; Boroviak et al., 2018; Bergmann et al., 2022). We expect that in vivo data from non-human primates, particularly for post-implantation development stages, will become more widely available. Such information will be valuable for the authentication and validation of human embryoids. Given the availability of in vivo non-human primate embryo data, we also expect to see new embryoids being generated from non-human primate stem cells in the near future.

It should also be noted that in most human embryoids, extraembryonic tissues from the TE or hypoblast lineages are missing. This is partially due to the fact that human extraembryonic stem cells related to these lineages remain less established compared with their mouse counterparts. Intensive efforts are therefore currently being directed to establish and characterize new human extraembryonic stem cells (Linneberg-Agerholm et al., 2019; Guo et al., 2021; Dong et al., 2020; Cinkornpumin et al., 2020; Gao et al., 2019). A human embryoid containing both embryonic and extraembryonic compartments will no doubt further our understanding of embryonic-extraembryonic interactions in human development. Moreover, the inclusion of extraembryonic tissues in human embryoids will also likely promote their progressive development by providing not only structural integrity and support, but also endogenous tissue communication signals, which are difficult to fully recapitulate through artificial controls.

Although modeling late organogenesis in embryoids is still out of reach, embryoids present promising models for studying...
organogenesis beyond the ‘single-organ’ level. Interorgan communication in development has a significant impact on the spatial patterning and mesoscale morphology of organs. Such interactions exist not only between the organs developed from one specific germ layer, but also between organs derived from different germ layers. Currently, there are only a few embryoid studies in which ‘multi-organ’ co-development has been reported. These include mouse and human trunk-like structures exhibiting the co-development of NT- and somite-like structures (Sanaki-Matsumiya et al., 2022; Yaman et al., 2022 preprint). Another human trunk model reported what appears to be spinal cord neurons and skeletal muscle cells (Faustino Martins et al., 2020), and a heart-forming model has also been developed to recapitulate early heart and foregut co-development (Dra Khalis et al., 2021). These new embryoids with ‘multi-organ’ development signify promising approaches for studying multidirectional interactions between developing organs in mammalian organisms.

In addition to the continued development and evolution of these models, it is also important to look ahead to examine how these models could potentially be used in the future to study and perhaps even solve health problems that other methods are unable to tackle. As previously mentioned, implantation studies leveraging blastoids present a promising avenue for studying the mechanisms of implantation failure associated with infertility, and the resulting insights can also be used to develop new forms of contraception that are safer and more effective than those currently available. Similarly, peri-implantation models of human embryos can facilitate studies of amnion formation in humans that can lead to exciting breakthroughs in our understanding of the amniotic membrane; such breakthroughs could lead to new preventative treatments for abnormalities such as preterm premature rupture of the membrane. Post-implantation embryoids that model the mechanisms of early organ development open the door up for targeted studies of birth defects, many of which remain poorly understood (Feldkamp et al., 2017). An augmented understanding of human development could inform the creation of new preventative and therapeutic measures of these birth defects. Finally, one of the most exciting directions for embryoid research is the development of new toxicological testing assays that could inform the development of new chemicals and medicines that can greatly improve the state of maternal and fetal health in our society.

In summary, there has been immense progress within the embryoid field over the past few years. Embryoids are becoming powerful experimental tools for studying mammalian development at the tissue and organ levels, and particularly in the context of primate development. There are several new exciting opportunities for embryoid research, including using bioengineering tools to improve the efficiency, reproducibility, and controllability of embryoid formation, using embryoids to study implantation and placentation, and last, but not least, using embryoids to study organogenesis beyond the ‘single-organ’ level. Only by reflecting on the past and analyzing the present can we recognize the doorways that have been opened by these embryoid studies. There is plenty room for embryoids to develop and grow!

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