# Stem cell-based models of early mammalian development

Aidan H. Terhune[1], Jeyoon Bok[1], Shiyu Sun[1], and Jianping Fu[1,2,3]

[1]Department of Mechanical Engineering, University of Michigan, Ann Arbor, Michigan 48109, USA; [2]Department of Biomedical Engineering, University of Michigan, Ann Arbor, Michigan 48109, USA; [3]Department of Cell & Developmental Biology, University of Michigan Medical School, Ann Arbor, MI 48109, USA.

Correspondence should be addressed to: J. Fu (jpfu@umich.edu)

## 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 certain embryonic development 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 embryo 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.

## 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 or zebrafish. 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 during human development.

In this Review, we first detail the various types of stem cells that have been used in the development of different embryoids. 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 human stem cell research has grown over the years, scientists have developed a variety of stem cell types 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]. Since these pre-gastrulation EPI cells are primed for germ layer specification [Rossant & Tam 2017], conventional hPSCs are considered to exist in a 'primed' pluripotency state. After intensive studies, hPSCs that model the '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][Kojima et al. 2014]. A new kind 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 the extraembryonic lineages in mice [Yang et al. 2017a][Yang et al. 2017b] and in humans [Gao et al. 2019]. More recently, researchers have developed mouse totipotent stem cells (TotiSCs), which exhibit molecular similarities to 2- and 4-cell stage blastomeres [Shen et al. 2021][Hu et al 2022]. Similarly, cells resembling the 8-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 trophectoderm (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][Io et al. 2021], primed hPSCs [Viukov et al. 2022] and 8CLCs [Mazid et al. 2022] have recently been used to generate stem cell lines with transcriptomic similarities to human TE. While there currently do not exist *bona fide* human hypoblast stem cells, naïve hPSCs have recently been used to generate expandable naïve extraembryonic endoderm (nEnd) cells that display transcriptomic similarities to human blastocyst-derived hypoblast cells [Linneberg-Agerholm et al. 2019]. As new stem cell types continue to emerge and be characterized and authenticated [Reviewed in Posfai et al. 2021b], they will no doubt add to the toolbox for generating *in vitro* models of the many facets of human development.

**Modeling pre-implantation blastocyst development**

While there are considerable differences in embryonic development between mice and humans, some aspects of pre-implantation development are shared between the two species, leading to the formation of the blastocyst, containing an outer TE layer surrounding a cavity (blastocoel) and the ICM on one side of the cavity (**Figure 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 in 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, pattering formation, cell sorting, and embryonic induction. Moreover, because the dynamics of implantation and peri-implantation development of human blastocysts are difficult to study, human blastoids are becoming an attractive tool to advance our understanding of human implantation and peri-implantation development.

*Mouse blastoids*

Various techniques have been used for generating mouse blastoids, yielding a diverse array of blastoids with different features (**Figure 2a**). Since mESCs are incapable of differentiating into extraembryonic lineages like naïve hPSCs can, the first demonstration of mouse blastoids was achieved by seeding mTSCs on top of mESC aggregates formed inside of a microwell [Rivron et al. 2018]. The first demonstration of mouse blastoids had already offered insights into embryonic inductions that direct trophoblast development [Rivron et al. 2018]. The blastoid generation protocol was made more efficient via the inclusion of mTSCs with a gene expression profile reminiscent of polar trophoblasts [Frias-Aldeguer et al. 2019]. Another blastoid was created by seeding mTSCs onto mEPSC aggregates formed in a microwell (coined EPS-blastoids) [Sozen et al. 2019]. Both kinds of blastoids demonstrated that the presence of mTSCs could spontaneously induce the formation of an embryonic-abembryonic axis within blastoids [Frias-Aledeguer et al. 2019][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][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, 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 results 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 mPSCs into induced blastocyst-like precursors (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] and totipotent stem-cell blastoids (TPS-blastoids) [Xu et al. 2022], have been generated from mouse cells resembling 2- and 4-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 (**Figure 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][Yang et al. 2017b][Gao et al. 2019], it is not surprising that these cells are used 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 a. 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 followed by distinct waves of hypoblast and trophoblast differentiation to form blastoids [Yu et al. 2021]. Alternatively, aggregating hEPSC 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 by mixing hEPSCs and EPS-derived TE-like cells at a 1:4-1:5 ratio yielded structures with a blastocyst-like morphology, though 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 also 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 work claimed that under appropriate chemophysical environments primed hPSCs could 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]. 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 instead [Zhao et al. 2021]. Indeed, a major challenge regarding the evaluation human blastoids is a relative lack of natural human and non-human primate embryo data for validation and authentication. There is also an increasing recognition of current confusion about how to distinguish TE-like cells from amnion-like cells in human embryoids [Zhao et al. 2021][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 blastocyst development into peri-/post-implantation embryonic stages. Studying human blastocyst implantation *in vivo* faces many practical and ethical limitations, so replicating an *in vivo*-like environment for faithful blastoid culture can be difficult when precise endpoints are often difficult to pin down. As such, *in vivo* studies of blastoid implantation in mice serve as an essential baseline upon which human blastoid implantation studies can be based. By transplanting blastoids into a pseudopregnant mouse uterus between E2.5 and E3.5, evidence was found suggesting 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, among which are placental dysfunction [Reynolds at 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 using models of the human uterus such as *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 in Hibaoui &Feki, 2020]. Interestingly, some of the human blastoids do not implement implantation models but still undergo morphogenetic events and lineage development that resemble peri-implantation development such as segregated outgrowths and amniotic cavity-like structure formation [Yu et al. 2021] or 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 the amnion-like tissue nor a primitive streak-like structure, two important early post-implantation human developmental hallmarks.

Adapting recent advances in prolonged *ex vivo* cultures of natural embryos into blastoid cultures might promote their continuous development into post-implantation developmental stages [Aguilera-Castrejon et al. 2021][Ichikawa et al. 2021][Govindasamy et al. 2021]. Indeed, existing protocols for *ex utero* culture of natural mouse embryos have recently been adapted to accommodate cutting-edge embryoids such as naïve mESC-derived synthetic embryos (sEmbryos) [Tarazi et al. 2022] and ETiX embryoids comprised of mESCs, TSCs, and Gata4-expressing mESCs referred to as induced XEN (iXEN) cells [Amadei et al. 2022]. Both of these

embryoids can be grown in an *ex utero* roller culture system past the E5.5 stage and into post-gastrulation organogenesis stages. Looking forward, the integration of faithful human uterine models with robust *ex vivo* culture systems for embryoid implantation studies 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, embryoids that recapitulate peri-implantation and pre-gastrulation developmental events centering on the EPI lineage have also been developed using primed hPSCs. There is also significant progress in the development of placenta models that recapitulate TE lineage diversification and development during the early placentation process.

*Mouse peri-implantation models*

Currently, there exist few mouse models that recapitulate peri-implantation development, but innovations in the assembly of different mouse stem cells have yielded a variety of powerful family of mouse models (**Figure 3a)**. The first is the ETS embryo which combined mouse ESCs and TSCs into a structure reminiscent of E6.5 mouse embryos [Harrison et al. 2017]. A similar model was later developed that employs the spontaneous assembly of extraembryonic endoderm (XEN) stem cells with ESCs and TSCs to generate a model of the compartmentalized mouse embryo known as the ETX embryoid [Sozen et al. 2018][Zhang et al. 2019]. The more recently developed ETiX embryoid 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 VE-derived yolk sac structures with blood islands [Amadei et al. 2022].

*Human peri-implantation models*

The topology of pre-gastrulation human embryos differ considerably from 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, which is in stark contrast to mouse embryos which do not possess an amnion prior to gastrulation [Yang et al. 2021b]. Recent investigations have revealed that clusters of primed hPSCs can be coaxed into forming lumenal 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 so as to generate asymmetric cysts, termed the post-implantation amniotic sac embryoid (PASE), which morphologically and transcriptomically resembles the natural amniotic sac and can even initiate events resembling the induction of primordial germ cells (PGCs) in the amniotic pole and posterior definitive mesoderm induction in the pluripotent pole (**Figure 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 since 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 in the PASE. Recent work suggests that amnion specification in NHP embryos occurs in two distinct waves, and this study 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 NHP 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 in Zhou et al. 2021]. These trophoblast organoids are 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. 2018]. Future efforts in modeling the 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 the placentation. One such example could be the use of patient specific hiPSC-derived uterine organoids to investigate how different disease states or genetic factors may affect implantation mechanics.

**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, 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 useful for gaining 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 embryogenic events. For example, one 2D mouse gastrulation model converted micropatterned mESCs into EPI-like cells, examined the effects of different signalling 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 either aggregates of 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][Xu et al. 2021][Anlaş et al. 2020][Anlaş et al. 2021] (**Figure 3a**) or aggregates of mESCs and mouse extraembryonic stem cells [Harrison et al. 2017][Girgin et al. 2021b][ Bérenger-Currias et al. 2020][Sozen et al. 2018][Amadei et al. 2021][Amadei et al. 2022] (**Figure 3b**). Specifically, 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 signalling such that the aggregates 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 utilize 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][Anlaş et al. 2021]. 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 neurula-stage mouse embryos, including the neural tube, beating cardiac tissue, and primitive gut tube [Xu et al. 2021]. Modeling organogenesis via gastruloids will be discussed at length in a later section.

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 (**Figure 3b**). For instance, it is known that signaling interactions between the EPI and ExE are responsible for the formation of the distal VE, and that the anterior VE plays a critical role in patterning of the EPI required for primitive streak development and the initiation of gastrulation [Reviewed in Tam & Loebel 2007]. Mouse gastrulation 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. 2020]. Indeed, prolonged culture of ETX/ETiX embryoids has shown to generate models with transcriptomic similarities to natural gastrulating mouse embryos [Sozen et al. 2018][Amadei et al. 2021][Amadei et al. 2022]. As previously mentioned, electronically-controlled *ex utero* culturing 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 early organogenesis stages [Tarazi et al. 2022].

*Human gastrulation models*

Studies of mouse gastrulation using natural mouse embryos and *in vitro* gastrulation models have provided clues into the gastrulation process in humans. However, distinct differences in morphologies and signaling and genetic mechanisms between murine and human gastrula exist. For example, it is unknown if a signaling center similar to the anterior VE exists in the pre-gastrulation human embryo, and the morphological differences between human and mouse peri-gastrulation embryos result in different mechanical and paracrine signaling cues for the EPI prior to gastrulation [Reviewed in Molè et al 2020]. Knowledge of these differences has motivated the development of human gastrulation models using primed hPSCs (**Figure 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 / 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], induction of organizers [Martyn et al. 2018], and depletion of aneuploid cells during gastrulation

[Yang et al. 2021a] have also been investigated using the 2D human gastrulation model. Cell tracking techniques reveal fate-dependent cell migration in the 2D human gastrulation model that resembles migration in natural mouse embryos [Martyn et al. 2019b]. Xenografts of organizer-like regions derived from the 2D human gastrulation model into chick embryos demonstrate the formation of a secondary axis reminiscent of natural axis self-organization in chicks [Martyn et al 2018]. Single cell data from the 2D human gastrulation model revealed a mechanism for aneuploidy elimination that resembles 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 2D human gastrulation model is 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. 2018], which more accurately resemble the morphology of germ layer organization during gastrulation.

3D human gastrulation models have also been developed. With proper exogeneous morphogens supplemented, 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, and in the absence of exogenous morphogens the resulting structures reveal the development of cell types resembling those of early gastrulation [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 the A-P axis with spatial organization of what appear to be three germ layers [Moris et al. 2020][Olmsted & Paluh 2021]. Furthermore, using shaking cultures, human gastruloids show more organized tissue development, such as a primitive gut tube-like structure and a spinal cord-like structure [Olmsted & Paluh 2021].

*Challenges in developing gastrulation models*

Compared to 2D gastrulation models, 3D gastrulation models have higher fidelity to *in vivo* embryo morphology [Moris et al. 2020][Olmsted & Paluh 2021][Harrison et al. 2017][Sozen et al. 2018]. Furthermore, 3D gastrulation models exhibit the exciting potential of early organogenesis under prolonged culture conditions [Veenvliet et al. 2020][Rossi et al. 2021]. 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 to 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. For current 3D gastrulation models, very few external controls are applied in their cultures, suggesting the development of current 3D gastrulation models is highly dependent on the boundary conditions of the culture and 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 still therefore a long-term pursuit for human embryoid research. While extraembryonic structures have often been overlooked, likely due to both ethical concerns and technical difficulties, such extraembryonic structures are critical 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 by nutrition supplementation and hematopoiesis initialization [Ross & 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][Amadei et al. 2022]. Nonetheless, these murine gastrulation models light up the possibility of achieving more advanced organogenesis and point out potential strategies for more advanced human gastrulation models.

**Modeling early organogenesis**

While 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, and 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 for disease modeling and drug screens or even for therapeutic transplantation, the holy grail for regenerative medicine.

*Neural development*

Considerable efforts have been made in using hPSCs to develop *in vitro* models of early neural development. 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 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, hPSC colonies spontaneously pattern into central neural plate (NP)-like tissue and peripheral neural plate border (NPB)-like tissue. While 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] (**Figure 5a**). A similar micropatterning strategy to model the neurulation process in 3D showed that micropatterned hPSC 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 (**Figure 5b**). 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 (**Figure 5a**). Floating aggregates of mESCs or hPSCs exhibit concentric or local DV 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 neuromesodermal progenitors (NMPs) [Veenvliet et al. 2020]. Similar trunk-like structures have also been developed using primed hPSCs [Yaman et al. 2022]. 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, the ETiX and sEmbryo embryoids possess what appear to be the forebrain and midbrain regions and 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) gastruloids [Olmsted & 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 (**Figure 6**). Development of somite-like structures from presomitic mesoderm, for example, is often featured in mouse gastruloids and has also been reported in the trunk-like structures and in the development of the ETiX embryo [van den Brink et al. 2020][Xu et al. 2021][Amadei et al. 2022]. The sEmbryo model is also claimed to develop at least 4 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 [Veenvliet et al. 2020][Yaman et al. 2022]. Recent work has also generated somitoids - hPSC-derived structures that model the formation of AP-patterned somite-like epithelial structures [Miao et al. 2022][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].

*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 & Lutolf 2021]. 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 [Veenvliet et al. 2020], while hPSC-derived EMLO gastruloids seem to develop primitive gut tube-like structures alongside central and peripheral neurons [Olmsted & Paluh 2021]. The ETiX and sEmbryo embryoids have also been reported to include the development of definitive endoderm into a gut tube-like structure [Amadei et al. 2022][Tarazi et al. 2022].

*Cardiogenesis*

Lastly, one additional realm of early organogenesis that has seen exciting progress in embryoid research is cardiogenesis. By adding cardiogenic factors to gastruloid culture protocols, recent mouse gastruloids develop beating cardiac tissues and associated vasculature [Rossi et al. 2020][van den Brink et al. 2014]. These gastruloid models were recently expanded to allow for 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. 2021]. Other mouse models of peri-/post-implantation have also been shown to model cardiogenesis alongside other organogenesis events [Xu et al. 2021][Olmsted & Paluh 2022][Amadei et al. 2022][Tarazi et al. 2022]. This includes the expansion of EMLO gastruloids with the development of a cardiac-like region (EMLOCs) alongside development of neural tissues, thus mimicking an innervated human heart [Olmsted & 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 layer lineages; this advantageous feature might enable researchers to reliably generate structures that more closely resemble functional organs. In addition, embryoids may provide a possibility to model interorgan communication during development, which leads to coordinated development and growth of multiple tissues and organs. Many 3D embryoid systems with features resembling early organogenesis are indeed 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. While 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 & Fu 2021][Rossant & 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. 2018][Zheng et al. 2019][Reviewed by Shao & 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 in the embryoids. 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 NHP embryos that are more closely related to humans are making rapid progress, including their 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 *in vivo* data from NHPs, particularly for the 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* NHP embryo data, we also expect to see new embryoids being generated from NHP 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 (**Figure 4**). This is partially due to the fact that human

563 extraembryonic stem cells related to these lineages remain less established compared to their
564 mouse counterparts. Intensive efforts are therefore currently being directed to establish and
565 characterize new human extraembryonic stem cells [Linneberg-Agerholm et al. 2019][Guo et al.
566 2021][Dong et al. 2020][Cinkornpumin et al. 2020][Gao et al. 2019]. A human embryoid
567 containing both embryonic and extraembryonic compartments will no doubt promote our
568 understanding of embryonic-extraembryonic interactions in human development. Moreover, the
569 inclusion of extraembryonic tissues in human embryoids will also likely improve their
570 progressive development by providing not only structural integrity and support, but also
571 endogenous tissue communication signals, which are difficult to fully recapitulate through
572 artificial controls.
573 While modeling late organogenesis in embryoids is still out of reach, embryoids present
574 promising models to study organogenesis beyond the "single-organ" level. Interorgan
575 communication in development has a significant impact on the spatial patterning and mesoscale
576 morphology of organs. Such interactions exist not only between the organs developed from one
577 specific germ layer, but also between organs derived from different germ layers. Currently, there
578 are only a few embryoid studies in which "multi-organ" co-development has been reported
579 (**Figure 6**), including mouse and human trunk-like structures exhibiting the co-development of
580 NT- and somite-like structures [Sanaki-Matsumiya et al. 2022][Yaman et al. 2022]. Another
581 human trunk model reported what appear to be spinal cord neurons and skeletal muscle cells
582 [Faustino Martins et al. 2020], and a heart-forming model has also been developed to recapitulate
583 early heart and foregut co-development [Drakhlis et al. 2021]. These new embryoids with
584 "multi-organ" development signify promising approaches for studying multidirectional
585 interactions between developing organs in mammalian organisms.
586 In addition to the continued development and evolution of these models, it is also
587 important to look ahead to examine how these models may be used to study and perhaps even
588 solve health problems that other methods are unable to tackle. As previously mentioned,
589 implantation studies leveraging blastoids present a promising avenue for studying the
590 mechanisms of implantation failure associated with infertility, and the resulting insights can also
591 be used to develop new forms of contraception that are safer and more effective than those
592 currently available. Similarly, peri-implantation models of human embryos can facilitate studies
593 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 opens the door for targeted studies of birth defects. The majority of lethal birth defects are congenital organ defects, and the etiology of such lethal defects remains poorly understood [Feldkamp et al. 2017]. An augmented understanding of human development will inform the creation of new preventative and therapeutic measures of these birth defects. Lastly, one of the most exciting directions for embryoid research is the development of new toxicological testing assays that can 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 to study 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 their efficiency and controllability, 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 up by these embryoid studies. There is plenty room for embryoids to develop and grow!

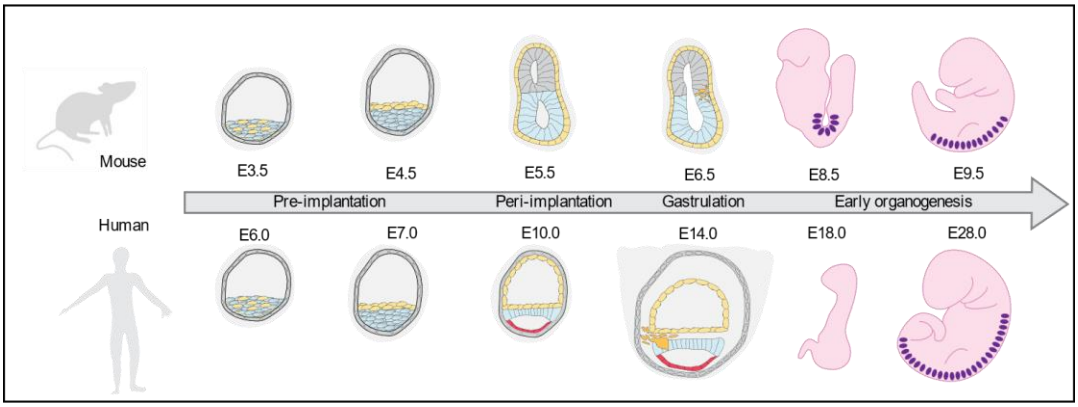


**Figure 1. Overview of early mammalian embryogenesis**

Humans and mice exhibit very different developmental timelines, particularly in late embryogenesis. However, pre-implantation stages of development are more similar between the two. Both species form blastocysts prior to implantation that consist of an outer layer of trophectoderm (dark grey) that houses an inner cell mass (ICM) that separates into pluripotent epiblast cells (blue) and hypoblast cells (yellow; primitive endoderm or extraembryonic endoderm for mice). In humans, peri-implantation development leads to formation of the amnion (red), which is believed to play a role in the formation of the primitive streak (orange) during gastrulation. Following gastrulation, embryos of both species begin the formation of key organ progenitors, including the somites (purple).

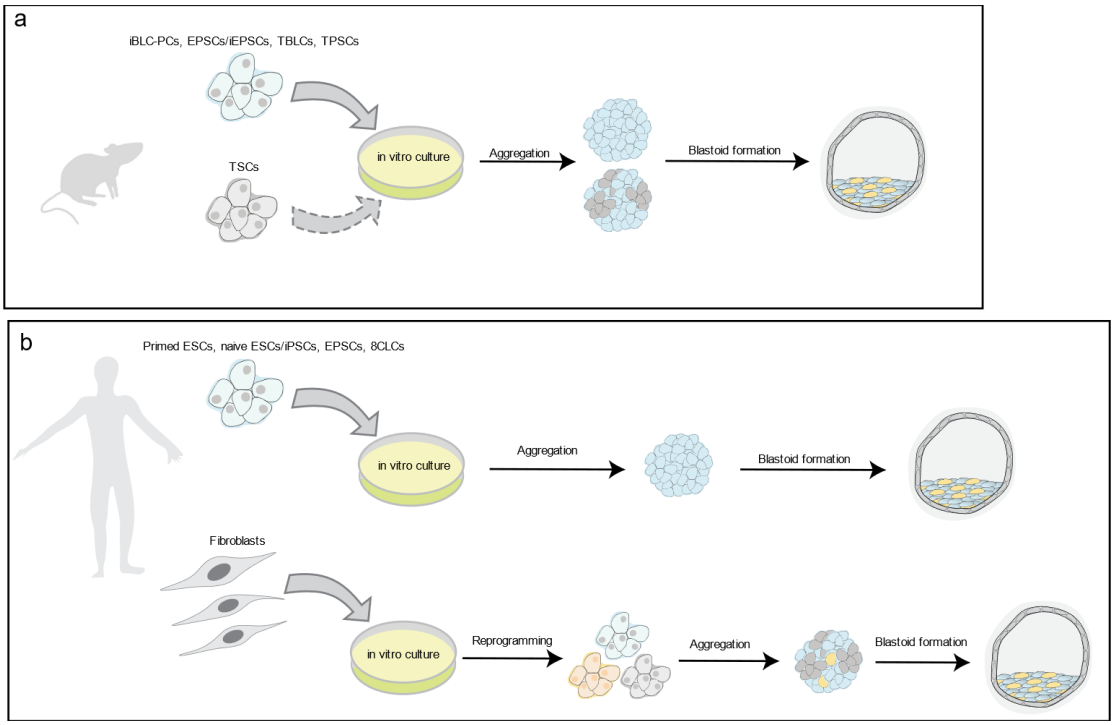


**Figure 2. Overview of blastoid formation procedures**

a) Schematic of the process for generating blastoids from various types of murine stem cells. Different pluripotent cell types (iBLC-PCs, EPSCs/iEPSCs, TBLCs, TPSCs) have been used to create blastoids, many of which can be used in conjunction with trophoblast stem cells (TSCs) to facilitate the formation of a trophectoderm-like compartment. Other models rely on the differentiation potential of pluripotent stem cells to form this compartment in addition to extraembryonic endoderm-like cells. b) Schematic of the different processes for generating human blastoids. Multiple pluripotent stem cell types (primed ESCs, naïve ESCs/iPSCs, EPSCs, 8CLCs) have been used to generate human blastoids with extraembryonic-like compartments. Additionally, the reprogramming adult fibroblasts into the three founding lineages for subsequent blastoid formation has also been attempted.

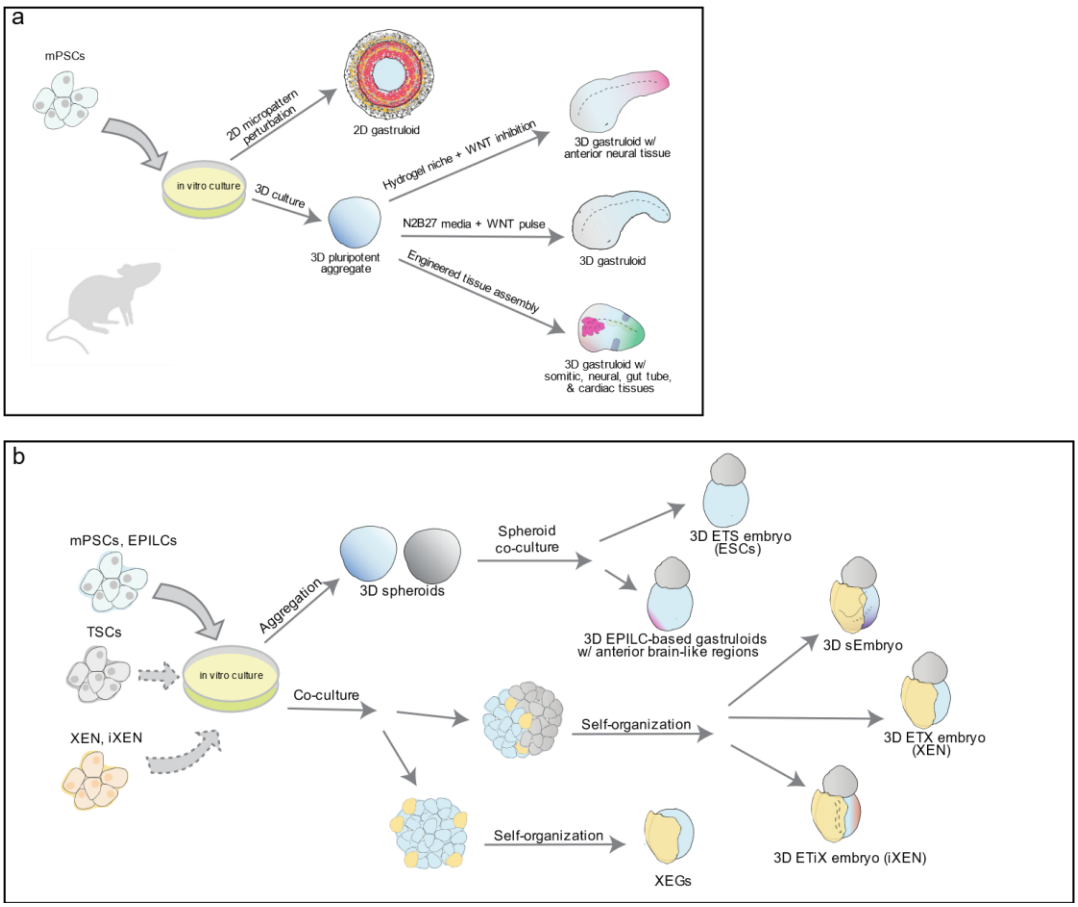


**Figure 3. Overview of mouse peri-/post-implantation embryo models**

a) Schematic of the different processes that have been implemented to generate 2D and 3D models of gastrulation or model peri-gastrulation events in vitro using mPSCs alone. Pluripotent mPSCs can be micropatterned and subjected to different signaling events to generate 2D patterns that recapitulate different aspects of gastrulation such as primitive streak formation. Alternatively, mPSCs and can be cultured into 3D aggregates which can be manipulated into recapitulating different aspects of gastrulation depending on the protocol. b) Schematic of the different processes implemented to generate 3D models of gastrulation using mPSCs in addition to extraembryonic/extraembryonic-like cells (TSCs, XEN cells, or iXEN cells). Different cell types can be combined by generating distinct 3D aggregates and combining them in a co-culture or by co-culturing the different cell types and allowing them to self-organize into 3D structures.

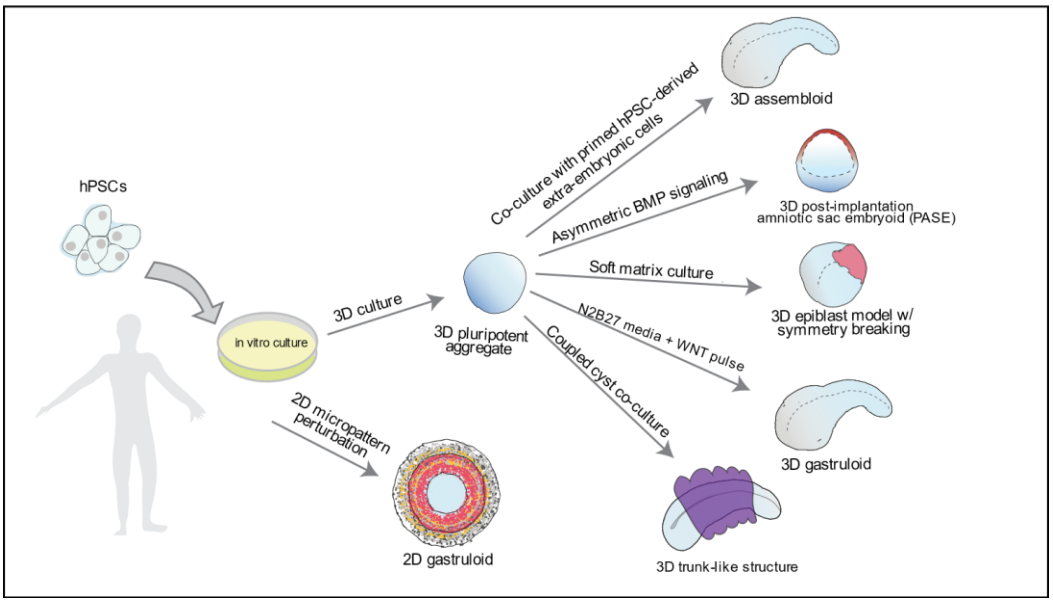


**Figure 4. Overview of human peri-/post-implantation embryo models**

General schematic of the different processes that have been implemented to generate models of gastrulation or peri-gastrulation events in vitro using hPSCs. Pluripotent mPSCs can be micropatterned and subjected to different signaling events to generate 2D patterns that recapitulate different aspects of gastrulation such as primitive streak formation. Various 3D models of peri-/post-implantation human embryos have been generated by culturing 3D aggregates of hPSCs and subjecting them to different culture protocols to model different aspects of peri-/post-implantation development.

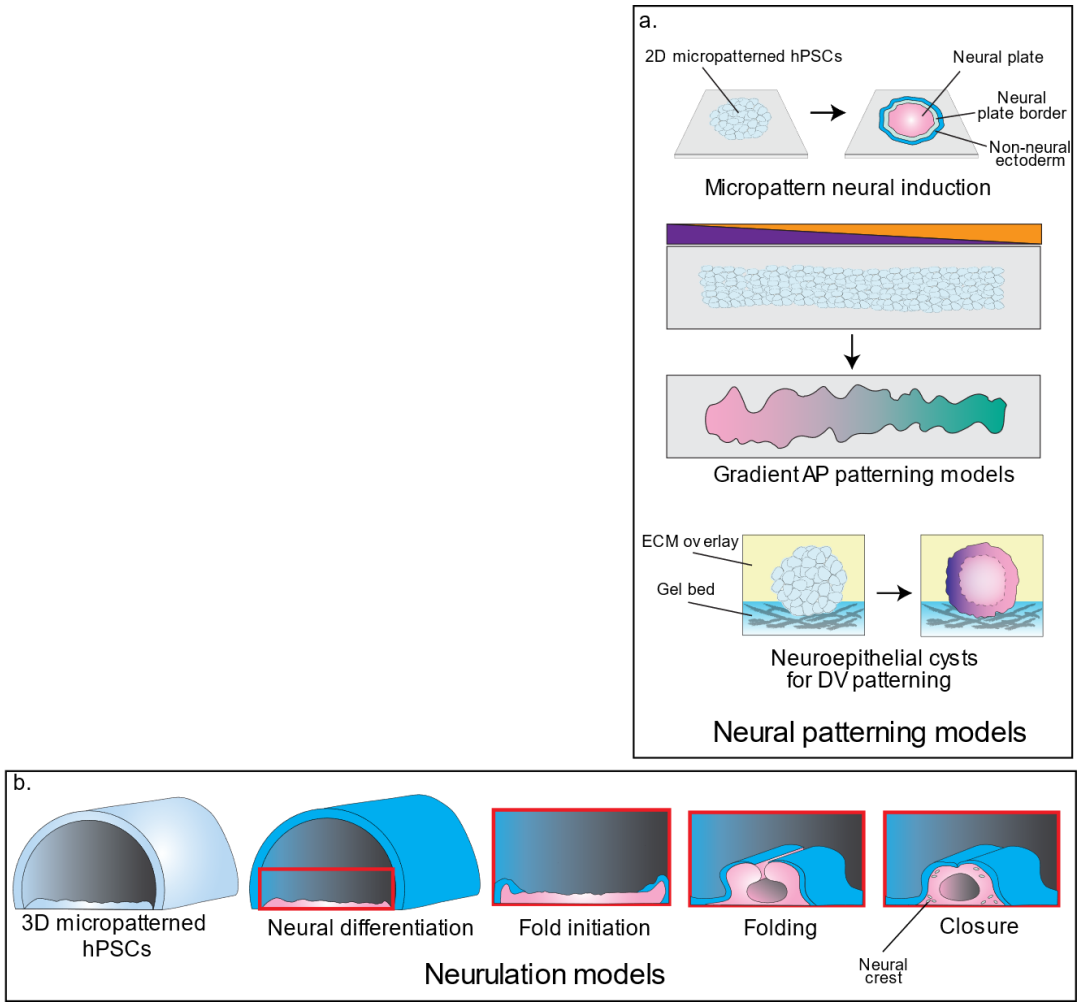


**Figure 5. Overview of neural development models**

A) Examples of the stem cell-based models used to study neural induction and patterning events in vitro. Both 2D and 3D models are used to examine ectoderm differentiation and patterning of the neuroepithelium along the body axes. In these models, hPSCs have been coaxed into replicating modeling aspects of neural patterning depending on the different protocol employed. Patterning of neural plate, 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, and DV patterning of neuroepithelial cysts can be modeled using hPSCs in a 3D culture environment. B) Neurulation, or folding of the neural tube, can 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 will model the neural plate and self-organize in a process that closely resembles neuralation and even produces cells resembling neural crest cells.

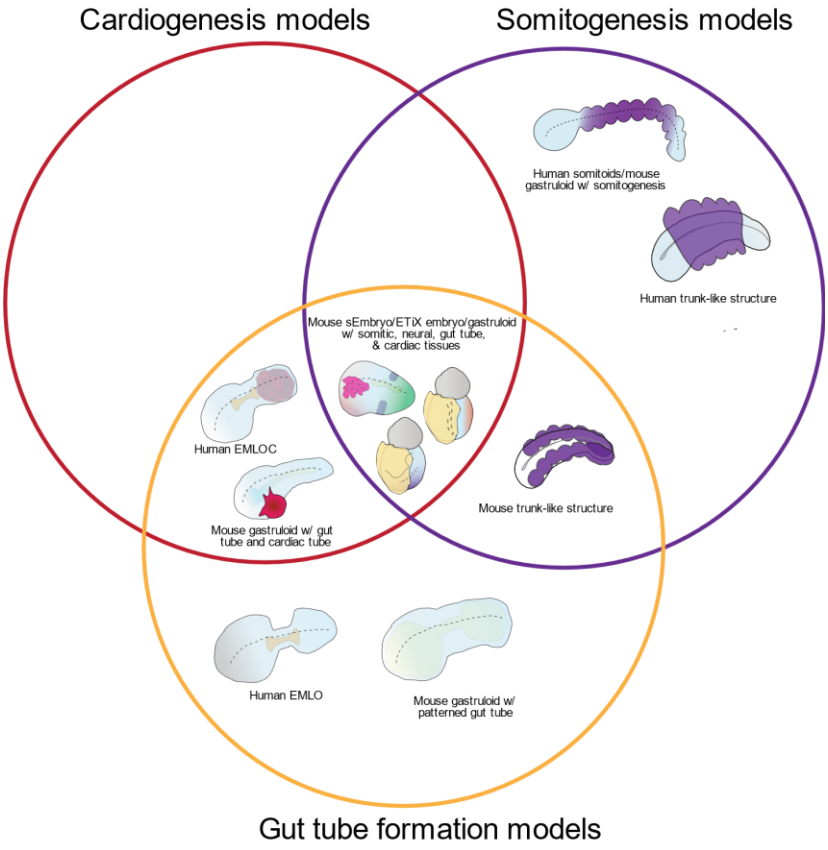


**Figure 6. Overview of early organogenesis in in vitro models**

The various mouse and human gastruloid models that feature events resembling early organogenesis are shown. Note that some models recapitulate several aspects of organogenesis.

References


1. Abdel Fattah, A.R., et al., Actuation enhances patterning in human neural tube organoids. Nature Communications, 2021. 12(1).
2. Aguilera-Castrejon, A., et al., Ex utero mouse embryogenesis from pre-gastrulation to late organogenesis. Nature, 2021. **593**(7857): p. 119-124.
3. Amadei, G., et al., Inducible Stem-Cell-Derived Embryos Capture Mouse Morphogenetic Events In Vitro. Developmental Cell, 2021. 56(3): p. 366-382.e9.
4. Amadei, G., et al., Stem cell-derived mouse embryos develop within an extra-embryonic yolk sac to form anterior brain regions and a beating heart. 2022, Cold Spring Harbor Laboratory.
5. Anlaş, K., et al., Gastruloids: Embryonic Organoids from Mouse Embryonic Stem Cells to Study Patterning and Development in Early Mammalian Embryos. 2021, Springer US. p. 131-147.
6. Anlaş, K., et al., Dynamics of anteroposterior axis establishment in a mammalian embryo-like system. 2021, Cold Spring Harbor Laboratory.
7. Beccari, L., et al., Multi-axial self-organization properties of mouse embryonic stem cells into gastruloids. Nature, 2018. 562(7726): p. 272-276.
8. Bérenger-Currias, N.M.L.P., et al., Extraembryonic endoderm cells induce neuroepithelial tissue in gastruloids. 2020, Cold Spring Harbor Laboratory.
9. Bergmann, S., et al., Spatial profiling of early primate gastrulation in utero. Nature, 2022.
10. Boroviak, T., et al., Single cell transcriptome analysis of human, marmoset and mouse embryos reveals common and divergent features of preimplantation development. Development, 2018. **145**(21): p. dev167833.
11. Boroviak, T. et al. The ability of inner-cell-mass cells to self-renew as embryonic stem cells is acquired following epiblast specification. Nat. Cell Biol. 16, 513–525 (2014).
12. Britton, G., et al., A novel self-organizing embryonic stem cell system reveals signaling logic underlying the patterning of human ectoderm. Development, 2019. 146(20): p. dev179093.

13. Chhabra, S., et al., Dissecting the dynamics of signaling events in the BMP, WNT, and NODAL cascade during self-organized fate patterning in human gastruloids. PLOS Biology, 2019. **17**(10): p. e3000498.
14. Cinkornpumin, J.K., et al., Naive Human Embryonic Stem Cells Can Give Rise to Cells with a Trophoblast-like Transcriptome and Methylome. Stem Cell Reports, 2020. 15(1): p. 198-213.
15. Clark, A.T., et al., Human embryo research, stem cell-derived embryo models and in vitro gametogenesis: Considerations leading to the revised ISSCR guidelines. Stem Cell Reports, 2021. **16**(6): p. 1416-14214.
16. Demers, C.J., et al., Development-on-chip: in vitro neural tube patterning with a microfluidic device. Development, 2016. 143(11): p. 1884-1892.
17. Deglincerti, A., et al., Self-organization of the in vitro attached human embryo. Nature, 2016. **533**(7602): p. 251-254.
18. Dong, C., et al., Derivation of trophoblast stem cells from naïve human pluripotent stem cells. eLife, 2020. **9**.
19. Drakhlis, L., et al., Human heart-forming organoids recapitulate early heart and foregut development. Nature Biotechnology, 2021. **39**(6): p. 737-746.
20. Duval, N., et al., BMP4 patterns Smad activity and generates stereotyped cell fate organisation in spinal organoids. Development, 2019. 146(14): p. dev175430.
21. Etoc, F., et al., A Balance between Secreted Inhibitors and Edge Sensing Controls Gastruloid Self-Organization. Developmental Cell, 2016. **39**(3): p. 302-315.
22. Fan, Y., et al., Generation of human blastocyst-like structures from pluripotent stem cells. Cell Discovery, 2021. 7(1).
23. Faustino Martins, J.-M., et al., Self-Organizing 3D Human Trunk Neuromuscular Organoids. Cell Stem Cell, 2020. 26(2): p. 172-186.e6.
24. Feldkamp, M.L., et al., Etiology and clinical presentation of birth defects: population based study. BMJ, 2017: p. j2249.
25. Frias-Aldeguer, J., et al., Embryonic signals perpetuate polar-like trophoblast stem cells and pattern the blastocyst axis. 2019, Cold Spring Harbor Laboratory.
26. Fu, J., A. Warmflash, and M.P. Lutolf, Stem-cell-based embryo models for fundamental research and translation. Nature Materials, 2021. 20(2): p. 132-144.

27. Gao, X., et al., Establishment of porcine and human expanded potential stem cells. Nature Cell Biology, 2019. 21(6): p. 687-699.
28. 26. Girgin, M.U., et al., Bioengineered embryoids mimic post-implantation development in vitro. Nature Communications, 2021. 12(1).
29. Girgin, M.U., et al., Gastruloids generated without exogenous Wnt activation develop anterior neural tissues. Stem Cell Reports, 2021. 16(5): p. 1143-1155.
30. Govindasamy, N., et al., 3D biomimetic platform reveals the first interactions of the embryo and the maternal blood vessels. Developmental Cell, 2021. **56**(23): p. 3276-3287.e8.
31. Guo, G., et al., Human naive epiblast cells possess unrestricted lineage potential. Cell Stem Cell, 2021. 28(6): p. 1040-1056.e6.
32. Guo, G. et al. Naive pluripotent stem cells derived directly from isolated cells of the human inner cell mass. Stem Cell Rep. 6, 437–446 (2016).
33. Haider, S., et al., Self-Renewing Trophoblast Organoids Recapitulate the Developmental Program of the Early Human Placenta. Stem Cell Reports, 2018. **11**(2): p. 537-551.
34. Haremaki, T., et al., Self-organizing neuruloids model developmental aspects of Huntington's disease in the ectodermal compartment. Nature Biotechnology, 2019. 37(10): p. 1198-1208.
35. Harrison, S.E., et al., Assembly of embryonic and extraembryonic stem cells to mimic embryogenesis in vitro. Science (American Association for the Advancement of Science), 2017. 356(6334).
36. Hibaoui, Y. and A. Feki, Organoid Models of Human Endometrial Development and Disease. Frontiers in cell and developmental biology, 2020. **8**.
37. Hu, Y., et al., Induction of mouse totipotent stem cells by a defined chemical cocktail. Nature, 2022.
38. Ichikawa, T., et al., An ex vivo system to study cellular dynamics underlying mouse peri-implantation development. Developmental Cell, 2022. 57(3): p. 373-386.e9.
39. Imamura, S., et al., Human blastoid from primed human embryonic stem cells. 2022, Cold Spring Harbor Laboratory.

40. Io, S., et al., Capturing human trophoblast development with naive pluripotent stem cells in vitro. Cell Stem Cell, 2021. 28(6): p. 1023-1039.e13.
41. Junying, Y.U., et al., Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells. Science, 2007. 318(5858).
42. Kagawa, H., et al., Human blastoids model blastocyst development and implantation. Nature, 2022. 601(7894): p. 600-605.
43. Karvas, R.M., et al., Use of a human embryonic stem cell model to discover GABRP, WFDC2, VTCN1 and ACTC1 as markers of early first trimester human trophoblast. Molecular Human Reproduction, 2020. **26**(6): p. 425-440.
44. Karzbrun, E., et al., Human neural tube morphogenesis in vitro by geometric constraints. Nature, 2021. 599(7884): p. 268-272.
45. Kime, C., et al., Induced 2C Expression and Implantation-Competent Blastocyst-like Cysts from Primed Pluripotent Stem Cells. Stem Cell Reports, 2019. 13(3): p. 485-498.
46. Kojima, Y., et al., The Transcriptional and Functional Properties of Mouse Epiblast Stem Cells Resemble the Anterior Primitive Streak. Cell Stem Cell, 2014. 14(1): p. 107-120.
47. Lee, J.-H., et al., Production of human spinal-cord organoids recapitulating neural-tube morphogenesis. Nature Biomedical Engineering, 2022. 6(4): p. 435-448.
48. Li, R., et al., Generation of Blastocyst-like Structures from Mouse Embryonic and Adult Cell Cultures. Cell, 2019. 179(3): p. 687-702.e18.
49. Linneberg-Agerholm, M., et al., Naïve human pluripotent stem cells respond to Wnt, Nodal, and LIF signalling to produce expandable naïve extra-embryonic endoderm. Development, 2019. 146(24): p. dev180620.
50. Liu, X., et al., Modelling human blastocysts by reprogramming fibroblasts into iBlastoids. Nature, 2021. 591(7851): p. 627-632.
51. Ma, H., et al., In vitro culture of cynomolgus monkey embryos beyond early gastrulation.Science, 2019. 366(6467).
52. Manfrin, A., et al., Engineered signaling centers for the spatially controlled patterning of human pluripotent stem cells. Nature Methods, 2019. 16(7): p. 640-648.

53. Martyn, I., A.H. Brivanlou, and E.D. Siggia, A wave of WNT signalling balanced by secreted inhibitors controls primitive streak formation in micropattern colonies of human embryonic stem cells. Development, 2019. 146(6): p. dev172791.
54. Martyn, I., et al., Self-organization of a human organizer by combined Wnt and Nodal signalling. Nature, 2018. 558(7708): p. 132-135.
55. Martyn, I., E.D. Siggia, and A.H. Brivanlou, Mapping cell migrations and fates in a gastruloid model to the human primitive streak. Development, 2019. 146(17): p. dev179564.
56. Mazid, M.A., et al., Rolling back human pluripotent stem cells to an eight-cell embryo-like stage. Nature, 2022. **605**(7909): p. 315-324.
57. Meinhardt, A., et al., 3D Reconstitution of the Patterned Neural Tube from Embryonic Stem Cells. Stem Cell Reports, 2014. **3**(6): p. 987-999.
58. Miao, Y., et al., Reconstruction and deconstruction of human somitogenesis in vitro. 2022, Cold Spring Harbor Laboratory.
59. Minn, K.T., et al., High-resolution transcriptional and morphogenetic profiling of cells from micropatterned human ESC gastruloid cultures. eLife, 2020. **9**.
60. Molè, M.A., A. Weberling, and M. Zernicka-Goetz, Comparative analysis of human and mouse development: From zygote to pre-gastrulation. Current topics in developmental biology, 2020. **136**.
61. Morgani, S.M., et al., Micropattern differentiation of mouse pluripotent stem cells recapitulates embryo regionalized cell fate patterning. eLife, 2018. 7.
62. Moris, N., et al., An in vitro model of early anteroposterior organization during human development. Nature, 2020. 582(7812): p. 410-415.
63. Muncie, J.M., et al., Mechanical Tension Promotes Formation of Gastrulation-like Nodes and Patterns Mesoderm Specification in Human Embryonic Stem Cells. Developmental Cell, 2020. **55**(6): p. 679-694.e11.
64. Nakamura, T., et al., A developmental coordinate of pluripotency among mice, monkeys and humans. Nature, 2016. **537**(7618): p. 57-62.54.
65. Niu, Y., et al., Dissecting primate early post-implantation development using long-term in vitro embryo culture. Science, 2019 366(6467).

66. Ogura, T., et al., Three-dimensional induction of dorsal, intermediate and ventral spinal cord tissues from human pluripotent stem cells. Development, 2018. 145(16): p. dev162214.
67. Okae, H., et al., Derivation of Human Trophoblast Stem Cells. Cell Stem Cell, 2018. 22(1): p. 50-63.e6.
68. Olmsted, Z.T. and J.L. Paluh, Co-development of central and peripheral neurons with trunk mesendoderm in human elongating multi-lineage organized gastruloids. Nature Communications, 2021. 12(1).
69. Olmsted, Z.T. and J.L. Paluh, A Combined Human Gastruloid Model of Cardiogenesis and Neurogenesis. 2022, Cold Spring Harbor Laboratory.
70. Posfai, E., et al., All models are wrong, but some are useful: Establishing standards for stem cell-based embryo models. Stem Cell Reports, 2021. **16**(5): p. 1117-1141.
71. Posfai, E., et al., Evaluating totipotency using criteria of increasing stringency. Nature Cell Biology, 2021. 23(1): p. 49-60.
72. Ranga, A., et al., Neural tube morphogenesis in synthetic 3D microenvironments. Proceedings of the National Academy of Sciences, 2016. **113**(44): p. E6831-E6839.
73. Reynolds, L.P., et al., Evidence for altered placental blood flow and vascularity in compromised pregnancies. JPhysiol 2006, 572(1):51–58.
74. Rivron, N.C., et al., Blastocyst-like structures generated solely from stem cells. Nature, 2018. 557(7703): p. 106-111.
75. Rivron, N.C. and J. Fu, It takes a village to form embryo models. Stem Cell Reports, 2021. **16**(5): p. 1011-1013.
76. Roberts, R.M., et al., Specification of trophoblast from embryonic stem cells exposed to BMP4. Biology of Reproduction, 2018. **99**(1): p. 212-224.
77. Ross, C. and T.E. Boroviak, Origin and function of the yolk sac in primate embryogenesis. Nature Communications, 2020. **11**(1).
78. Rossant, J. and P.P.L. Tam, New Insights into Early Human Development: Lessons for Stem Cell Derivation and Differentiation. Cell Stem Cell, 2017. **20**(1): p. 18-28.
79. Rossant, J. and P.P.L. Tam, Opportunities and challenges with stem cell-based embryo models. Stem Cell Reports, 2021. **16**(5): p. 1031-1038.

80. Rossi, G., et al., Capturing Cardiogenesis in Gastruloids. Cell Stem Cell, 2021. 28(2): p. 230-240.e6.
81. Rossi, G., et al., Gastruloids asin vitromodels of embryonic blood development with spatial and temporal resolution. 2021, Cold Spring Harbor Laboratory.
82. Rostovskaya, M., et al., Amniogenesis occurs in two independent waves in primates. Cell Stem Cell, 2022. **29**(5): p. 744-759.e6.
83. Sanaki-Matsumiya, M., et al., Periodic formation of epithelial somites from human pluripotent stem cells. Nature Communications, 2022. **13**(1).
84. Sahni, G., et al., A Micropatterned Human-Specific Neuroepithelial Tissue for Modeling Gene and Drug-Induced Neurodevelopmental Defects. Advanced Science, 2021. **8**(5): p. 2001100.
85. Shahbazi, M.N., et al., Self-organization of the human embryo in the absence of maternal tissues. Nature Cell Biology, 2016. 18(6): p. 700-708.
86. Shao, Y. and J. Fu, Engineering multiscale structural orders for high-fidelity embryoids and organoids. Cell stem cell, 2022. **29**(5).
87. Shao, Y., et al., Self-organized amniogenesis by human pluripotent stem cells in a biomimetic implantation-like niche. Nature Materials, 2017. 16(4): p. 419-425.
88. Shao, Y., et al., A pluripotent stem cell-based model for post-implantation human amniotic sac development. Nature Communications, 2017. 8(1).
89. Shen, H., et al., Mouse totipotent stem cells captured and maintained through spliceosomal repression. Cell, 2021. 184(11): p. 2843-2859.e20.
90. Sheridan, M.A., et al., Early onset preeclampsia in a model for human placental trophoblast. Proceedings of the National Academy of Sciences, 2019. **116**(10): p. 4336-4345.
91. Simunovic, M., et al., A 3D model of a human epiblast reveals BMP4-driven symmetry breaking. Nature Cell Biology, 2019. 21(7): p. 900-910.
92. Simunovic, M., E.D. Siggia, and A.H. Brivanlou, In vitro attachment and symmetry breaking of a human embryo model assembled from primed embryonic stem cells. Cell stem cell. **29**(6).

93. Sozen, B., et al., Self-assembly of embryonic and two extra-embryonic stem cell types into gastrulating embryo-like structures. Nature Cell Biology, 2018. 20(8): p. 979-989.
94. Sozen, B., et al., Self-Organization of Mouse Stem Cells into an Extended Potential Blastoid. Developmental Cell, 2019. 51(6): p. 698-712.e8.
95. Sozen, B., et al., Reconstructing aspects of human embryogenesis with pluripotent stem cells. Nature Communications, 2021. 12(1).
96. Takahashi, K., et al., Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell, 2007. 131(5): p. 861-872.
97. Takashima, Y., et al., Resetting Transcription Factor Control Circuitry toward Ground State Pluripotency in Human. Cell, 2014. **158**(6): p. 1254-1269.79.
98. Tewary, M., et al., A stepwise model of Reaction-Diffusion and Positional-Information governs self-organized human peri-gastrulation-like patterning. Development, 2017. 144(23): p. 4298-4312.
99. Tam, P.P.L. and D.A.F. Loebel, Gene function in mouse embryogenesis: get set for gastrulation. Nature Reviews Genetics, 2007. **8**(5): p. 368-381.
100. Tarazi, S., et al., Post-Gastrulation Synthetic Embryos Generated Ex Utero from Mouse Naïve ESCs. Cell, in press, 2022.
101. Telugu, B.P., et al., Comparison of extravillous trophoblast cells derived from human embryonic stem cells and from first trimester human placentas. Placenta, 2013. **34**(7): p. 536-543.
102. Tesar, P.J., et al., New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature, 2007. **448**(7150): p. 196-199.
103. Theunissen, T. W. et al. Systematic identification of culture conditions for induction and maintenance of naive human pluripotency. Cell Stem Cell 15, 471–487 (2014).
104. Thomson, J.A., et al., Embryonic Stem Cell Lines Derived from Human Blastocysts. Science (American Association for the Advancement of Science). 282(5391).
105. Turco, M.Y., et al., Trophoblast organoids as a model for maternal–fetal interactions during human placentation. Nature, 2018. **564**(7735): p. 263-267.

106. Turner, D.A., et al., Anteroposterior polarity and elongation in the absence of extraembryonic tissues and spatially localised signalling in Gastruloids, mammalian embryonic organoids. Development, 2017. 144(21): p. 3894-3906.
107. Van Den Brink, S.C., et al., Single-cell and spatial transcriptomics reveal somitogenesis in gastruloids. Nature, 2020. 582(7812): p. 405-409.
108. Van Den Brink, S.C., et al., Symmetry breaking, germ layer specification and axial organisation in aggregates of mouse embryonic stem cells. Development, 2014. 141(22): p. 4231-4242.
109. Veenvliet, J.V., et al., Mouse embryonic stem cells self-organize into trunk-like structures with neural tube and somites. Science (American Association for the Advancement of Science), 2020. 370(6522).
110. Vianello, S. and M.P. Lutolf, In vitro endoderm emergence and self-organisation in the absence of extraembryonic tissues and embryonic architecture. 2020, Cold Spring Harbor Laboratory.
111. Viukov, S., et al., Human primed and naïve PSCs are both competent in differentiating into bona fide trophoblast stem cells. 2022, Cold Spring Harbor Laboratory.
112. Warmflash, A., et al., A method to recapitulate early embryonic spatial patterning in human embryonic stem cells. Nature Methods, 2014. 11(8): p. 847-854.
113. Weatherbee, B.A.T., T. Cui, and M. Zernicka-Goetz, Modeling human embryo development with embryonic and extra-embryonic stem cells. Developmental Biology, 2021. 474: p. 91-99.
114. Xu, P.-F., et al., Construction of a mammalian embryo model from stem cells organized by a morphogen signalling centre. Nature Communications, 2021. 12(1).
115. Xu, Y., et al., Derivation of totipotent-like stem cells with blastocyst-like structure forming potential. Cell Research, 2022.
116. Xue, X., et al., Mechanics-guided embryonic patterning of neuroectoderm tissue from human pluripotent stem cells. Nature Materials, 2018. **17**(7): p. 633-641.
117. Yaman, Y.I., R. Huang, and S. Ramanathan, Coupled organoids reveal that signaling gradients drive traveling segmentation clock waves during human axial morphogenesis. 2022, Cold Spring Harbor Laboratory.

118. Yanagida, A., et al., Naive stem cell blastocyst model captures human embryo lineage segregation. Cell Stem Cell, 2021. 28(6): p. 1016-1022.e4.
119. Yang, J., et al., Establishment of mouse expanded potential stem cells. Nature, 2017. 550(7676): p. 393-397.
120. Yang, M., et al., Depletion of aneuploid cells in human embryos and gastruloids. Nature Cell Biology, 2021. **23**(4): p. 314-321.
121. Yang, R., et al., Amnion signals are essential for mesoderm formation in primates. Nature Communications, 2021. **12**(1).
122. Yang, Y., et al., Derivation of Pluripotent Stem Cells with In Vivo Embryonic and Extraembryonic Potency. Cell, 2017. 169(2): p. 243-257.e25.
123. Ying, Q.-L. et al. The ground state of embryonic stem cell self-renewal. Nature 453, 519–523 (2008).
124. Yu, L., et al., Blastocyst-like structures generated from human pluripotent stem cells. Nature, 2021. 591(7851): p. 620-626.
125. Zenclussen, A.C., et al., Abnormal T-cell reactivity against paternal antigens in spontaneous abortion - Adoptive transfer of pregnancyinduced CD4 (+) CD25 (+) T regulatory cells prevents fetal rejection in a murine abortion model. Am J Pathol 2005, 166(3):811–822.
126. Zhang, P., et al., Highly efficient generation of blastocyst-like structures from mouse totipotent blastomere-like cells. 2022, Cold Spring Harbor Laboratory.
127. Zhang, S., et al., Implantation initiation of self-assembled embryo-like structures generated using three types of mouse blastocyst-derived stem cells. Nature Communications, 2019. 10(1).
128. Zhao, C., et al., Reprogrammed blastoids contain amnion-like cells but not trophectoderm. 2021, Cold Spring Harbor Laboratory.
129. Zheng, Y., et al., Controlled modelling of human epiblast and amnion development using stem cells. Nature, 2019. 573(7774): p. 421-425.
130. Zheng, Y., et al., Single-cell analysis of embryoids reveals lineage diversification roadmaps of early human development. Cell Stem Cell, in press, 2022.
131. Zhou, J., et al., Modeling human peri-implantation placental development and function. Biology of Reproduction, 2021. 105(1): p. 40-51.