Towards Developing Human Organs *via* Embryo Models and Chimeras

Jun Wu[1, 2, 3] and Jianping Fu[4, 5, 6]

[1] Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA;
[2] Hamon Center for Regenerative Science and Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA;
[3] Cecil H. and Ida Green Center for Reproductive Biology Sciences, University of Texas Southwestern Medical Center, Dallas, TX, USA;
[4] Department of Mechanical Engineering, University of Michigan, Ann Arbor, Michigan 48109, USA;
[5] Department of Biomedical Engineering, University of Michigan, Ann Arbor, Michigan 48109, USA;
[6] Department of Cell & Developmental Biology, University of Michigan Medical School, Ann Arbor, MI 48109, USA.

*Correspondence should be addressed to J.W. (email: jun2.wu@utsouthwestern.edu) and J.F. (email: jpfu@umich.edu)

Abstract

Developing functional organs from stem cells remains a challenging goal in regenerative medicine. Existing methodologies, such as tissue engineering, bioprinting and organoids, only offer partial solutions. This Perspective focuses on two emerging approaches promising for engineering human organs from stem cells: stem cell-based embryo models and interspecies organogenesis. Both approaches exploit the premise of guiding stem cells to mimic natural development. We begin by summarizing what is known about early human development, as a blueprint for recapitulating organogenesis in both embryo models and interspecies chimeras. The latest advances in both fields are discussed, before highlighting the technological and knowledge gaps to be addressed before the goal of developing human organs could be achieved using the two approaches. We conclude by discussing challenges facing embryo modeling and interspecies organogenesis and outline future prospects for advancing both fields towards the generation of human tissues and organs for basic research and translational applications.

Introduction

For a wide range of life-threatening diseases such as end-stage kidney, liver, heart and lung failure, whole organ transplantation often stands as the only viable treatment option. However, a global shortage of donor organs has exacerbated the issue. Notably, in the United States alone, more than 100,000 individuals find themselves on the national transplant waiting list at any given time, with 17 people dying daily as they wait for an organ transplant (https://www.organdonor.gov/learn/organ-donation-statistics). The scarcity of donor organs has prompted physicians and scientists to look for alternative solutions, including xenotransplantation of animal organs with close anatomical / physiological similartities to human ones. However, even though there is notable progress in using genetically modified organs from animals, such as those of pigs, for xenotransplantation therapies,[1] it remains uncertain whether animal organs are suitable for long-term human transplants.

In addition to xenotransplantation, there are other strategies available for organ engineering based on cultured human cells, including tissue engineering, bioprinting, and organoid technologies. Decades of tissue engineering studies have led to successful applications of various engineered tissue constructs, including most recent ones for skin and corneal tissue grafting to treat skin disease and restore vision, respectively.[2,3] Another emerging tissue engineering approach utilizes decellularized extracellular matrix (ECM) from different organs, such as the heart, liver, kidney and lung, to provide biomimetic scaffolds that support the generation of bioartificial organs.[4] Bioprinting allows precise deposition of bioinks (often containing cells) and support structures to create three-dimensional (3D) tissue architectures with unparalleled topological complexities.[5,6] The advent of organoids, 3D structures derived from self-organizing tissue-specific stem cells and progenitors, has shown considerable promise in modeling human organ development and disease.[7,8] There are notable recent progresses in enhancing the complexity of organoids, including incorporating vascular networks within organoids and assembling multi-tissue organoids to study intra- and inter-organ communication.[9] Although organ engineering studies based on tissue engineering, bioprinting and organoid technologies are becoming increasingly sophisticated, they still offer imperfect solutions. For instance they fall short in their ability to recapitulate essential functional elements, including vasculature, innervation, lymphatics, and the accurate number, diversity and organization of functional and supporting cell types from different germ layer lineages within solid organs.[10]

The focus of this Perspective is two cutting-edge strategies based on human stem cells that are promising for addressing the challenges of organ engineering: stem cell-based embryo models and interspecies organogenesis (**Figure 1**). Both approaches are grounded in a unified conceptual framework that emphasizes the replication of natural processes of germ layer lineage development and organization and microenvironments essential for organ formation. Mimicking the trajectory of natural embryonic development, formation of human organ primordia using both approaches would follow the canonical developmental blueprint, progressing from gastrulation to organogenesis. *In vivo*, the foundational cells for gastrulation and organogenesis are the pluripotent epiblast (EPI) cells. They differentiate and self-organize into patterned embryonic germ layers during gastrulation, setting the stage for subsequent tissue development and interaction. This, combined with tissue-level morphogenetic processes, leads to the formation of early organ structures.

Contrary to tissue engineering and bioprinting approaches that add layers of complexity in increments to primitive tissues composed of scaffolds and cultured cells, embryo models and interspecies organogenesis initiate organ development with inherent structural complexity through self-organization and -construction of embryonic germ layers guided by genetic programs. Both approaches, in theory, could enable human organs to develop in an environment that closely mimic their natural growth conditions, whether provided by embryo models themselves or in an interspecies host, as opposed to taking organ development out of its natural context as in tissue engineering or bioprinting. Similarly, despite their promise for modeling organ development, organoids are often generated in culture conditions distintly different from natural embryonic environments, such as missing supporting cell types from different embryonic germ layers. As such, organoids tend to lack the complex structural organization and tissue architecture of different germ layer lineages found in native organs that are essential for their physiological functions.

The rapid emergence of stem cell-based embryo models takes advantage of the recent knowledge that pluripotent stem cells (PSCs) largely follow the natural developmental programs of the epiblast cells when differentiated *in vitro* and their progenies possess remarkable self-organizing properties, giving rise to organized multicellular structures that mimic embryonic tissues.[7,11] In addition, the ever increasing knowledge of early post-implantation human development, leading to organogenesis, derived from studies on cultured and aborted human embryos,[12-19] provide critical information for guilding human embryo model development as well

as for their authentication and benchmarking. The exciting prospect of using embryo models for organ engineering has been further elevated by recent advancements of mouse embryo models, which recapitulate certain aspects of the germ layer lineage diversification and organization during gastrulation and early organogenesis, albeit with low efficiencies and developed organ primordia showing notable defects.[20-22] Similar progress in human embryo models, although currently lagging, would, in principle, lead to useful experimental systems for dissecting the molecular and cellular events driving human gastrulation and early organogenesis. Furthermore, such advanced human embryo models would contain most, if not all, foundational embryonic cell types essential for complex solid organ formation. We speculate that with proper spatial organization and interactions between embryonic germ layer lineages, like inherent programmed organogenesis *in vivo*, these embryo models would have the self-organizing potential to form different organ primordia, thereby opening up exciting new frontiers for organ engineering and related applications.

Human-animal chimeras and blastocyst complementation represent another promising route for generating transplantable human tissues and organs. Nature's intricate system for embryonic development creates functional tissues and organs through a dynamic interplay between genetic programming and extrinsic developmental niche. This guides embryonic cells in their differentiation and complex tissue formation. Advances in gene-targeting technologies and PSCs have greatly enhanced our understanding of how genetic and epigenetic factors drive embryonic development. Disruptions in these factors can lead to developmental defects, including missing cell lineages or organs, in embryos. This creates "empty" developmental niches that may be filled using donor PSCs, a strategy known as blastocyst complementation.[23] Initially demonstrated with mouse embryonic stem cells (ESCs) in 1993,[24] this technique was later adapted to interspecies contexts, successfully producing functional rat pancreas in mice.[25] These groundbreaking studies have continuously motivated interspecies organogenesis research towards the goal of growing human tissues and organs in other species.

Though numerous challenges remain, it is now techanically conceivable for the formation of human organ primordia in stem cell-derived embryo models or within animal hosts. It is the belief of the authors of this Perspective that with further optimizations, organ rudiments in embryo models or animal hosts could, in principle, develop and grow into fully functional organs, supported by *in vivo*-like developmental niches and nurtured by blood circulation systems

provided by embryo models or through advanced culture systems, such as artificial placentas, or animal hosts.

Even though functional human organs have yet to be generated through embryo modeling and interspecies organogenesis, this Perspective aims to encapsulate recent advances in both fields and speculate about their promise for regenerative medicine. We start by briefly summarizing human pluripotent and extraembryonic stem cells, which constitute the starting cell populations for both approaches. We then discuss the natural development program up to early organogenesis, side by side with the latest advancements in embryo modeling and interspecies organogenesis. We emphasize the importance of closely mimicking natural developmental processes to ensure proper germ layer diversification, interactions and organization, which are fundamental for tissue lineage specification and morphogenesis, ultimately leading to organ formation. We then elaborate on the challenges and expectations and conclude by addressing the future prospects and ethical considerations in embryo modeling and interspecies organogenesis. Given the numerous technical and ethical hurdles facing the two fields, it is our hope that this Perspective will provide a useful framework for guiding both fields towards one of the main goals of regenerative medicine: the generation of functional human tissues and organs for fundamental and translational applications.

Embryonic and Extra-embryonic Stem Cells

Different types of human PSCs (hPSCs) and/or extraembryonic stem cells have been utilized as starting cell populations in embryo modeling (**Table 1**) and interspecies organogenesis (**Table 2**). Stem cells of the three foundational lineages of early mouse embryos - epiblast, trophectoderm, and primitive endoderm - have all been well established *in vitro* as ESCs, trophoblast stem cells (TSCs), and extraembryonic endoderm stem cells (XENs), respectively.[26-29] Human TSCs (hTSCs) have only been derived recently from cytotrophoblasts, blastocysts or naïve hPSCs.[30-33] Although human stem cells analogous to mouse XENs have not yet been fully established, XEN-like cells have been reported through differentiation from naïve or intermediate hPSCs.[34-36]

The *in vivo* human pluripotency continuum has been recapitulated *in vitro* with various types of hPSCs representing distinct pluripotency states.[37] These hPSCs in different pluripotency states are believed to be suitable for modeling the behaviors of pluripotent epiblast cells at different stages of early human development. Conventional hPSCs represent post-implantation (or embryonic day or E10-12) rather than blastocyst stage (E6-7) human epiblast cells and reside in

the primed pluripotency state that show limited potential in differentiation towards extraembryonic cell lineages.[38] The unrestricted developmental potential of naïve mouse PSCs has inspired intensive efforts to derive naïve hPSCs. Assessment of naïve pluripotency in hPSCs, due to ethical challenges associated with stringent functional tests like germline transmission in chimeras and tetraploid complementation,[39] relies exclusively on molecular benchmarking. hPSCs cultured under various conditions, such as 5i/L/A,[40] t2iLGöY,[41] 4CL[42] and HENSM,[43] have met most, if not all, of the established molecular criteria. These naïve hPSCs exhibit transcriptomic profiles similar to pre-implantation E6-7 human epiblast cells, a result that supports multiple culture conditions to stabilize naïve pluripotency in humans. In contrast, some hPSCs initially thought of as naïve or naïve-like display transcriptomic features more similar to post-implantation E8-9 human epiblast cells, suggesting that these cells reside in intermediate states between naïve and primed pluripotency.[44-46] Formative pluripotency, one such intermediate state, has recently garnered some attention.[47] This state represents a developmental window when naïve pluripotency is reconfigured to prepare for multilineage competency, including germ cell specification. Although hPSCs with formative pluripotency have recently been reported,[38,48] currently there is a lack of well accepted criteria for authenticating the human formative pluripotency state.

Recent studies have identified mouse PSCs that display some characteristics consistent with totipotency; herein, these cells are collectively referred to as totipotent-like pluripotent stem cells (TPSCs). So far, reported mouse TPSCs include extended and expanded potential stem cells (EPSCs),[49,50] totipotent blastomere-like cells (TBLCs),[51] chemically induced totipotent stem cells (ciTotiSCs),[52] totipotent-like stem cells (TLSCs),[53] and totipotent potential stem (TPS) cells.[54] Although the establishment of stable human TPSCs remains elusive, several recent studies have successfully identified metastable human eight cell-like cells (8CLCs) under naïve hPSC cultures that activate a range of zygotic genome activation (ZGA) genes.[42,55,56] These putative human totipotent-like cells, whether transient or stable, provide a starting cell population likely useful for modeling pre-implantation human developmental events, ranging from early blastomere development to blastocyst formation. They also hold potential for applications in interspecies organogenesis.

Recapitulating Embryonic Development Leading to Organogenesis

Embryonic development serves as a blueprint for embryo modeling and interspecies organogenesis to form functional organs through spatiotemporally dynamic intercellular interactions and organizations. Organ formation *in vivo* necessitates stereotypical developmental progression, from the implantation, gastrulation to organogenesis (**Figure 2A**). Pre-implantation human development has been well characterized, thanks to *in vitro* culture conditions developed for fertilized embryos used in assisted human reproduction.[57] Human development from implantation to early organogenesis, however, is much less clear, due to both technical and ethical difficulties associated with intrauterine development after implantation. Our knowledge of post-implantation human development primarily comes from descriptive analyses of historical human embryo collections,[58] recent research on primary human embryonic samples,[17,18] and studies on cultured human embryos.[12-14,16,59,60] In this section, we discuss the current understanding of early human development, focusing on lineage development, morphogenetic events and dynamic tissue organizations that culminate in the formation of organ primordia. Alongside the exploration into the current understanding of early human development, we discuss what has been achieved in embryo modeling and interspecies organogenesis. For detailed discussions on human development from blastocyst formation to gastrulation, we direct readers to some recent comprehensive reviews.[61,62] It is important, however, to acknowledge that our discussion, especially regarding peri- and post-implantation development, is based on knowledge that may be incomplete or subject to revision by future research. Current understanding of the molecular and cellular mechanisms of human development should be best considered as evolving hypotheses rather than established facts. To serve this goal, we try to highlight issues of particular uncertainty or controversy and to indicate the limits of our knowledge.

Pre-implantation development

Pre-implantation human development displays notable autonomy and self-organization. Human embryos, from fertilization to implantation, progress through an ordered series of cell-fate decisions and symmetry-breaking events. This developmental sequence results in the formation of a blastocyst, composed oftrophectodermsurrounding the blastocoel and an inner cell mass (ICM) (**Figure 2A[i]**). Post-implantation, thetrophectodermcontributes to placental development. Within the blastocyst, the ICM differentiates into two distinct cell lineages: epiblast, forming the embryo, and hypoblast (HYP), or called primititve endodermin mice. Before implantation the epiblast and

hypoblast compartments are separated by a basal lamina, with hypoblast cells forming a polarized cuboidal epithelium lining the blastocoelic cavity (**Figure 2A[i]**).

Human blastocyst implantation initiates notable tissue reorganization and lineage development.[61-64] However, knowledge about dynamic cell lineage specification and differentiation, fate patterning, morphogenetic tissue organization, and underlying molecular and cellular mechanisms during early post-implantation human development remains limited. Histochemical analyses of early post-implantation human embryos reveal that invasive trophoblast cells at the embryonic pole of implanting blastocysts proliferate and establish connections with the maternal uterine tissue. Some of these cells lose plasma membranes, forming syncytiotrophoblasts, which grow and enclose the implanting blastocyst. Remaining trophoblast cells along the blastocyst wall maintain their membranes and constitute the cytotrophoblast.

Modeling blastocyst development

Recent years have witnessed significant advancements in the development of human blastocyst models, known as 'blastoids'[65-72] (**Table 1**). These models encompass all the founding cell lineages of the fetus and its supporting tissues and as such are considered as integrated embryo models (**Figure 2B[i]**). The generation of human blastoids was inspired by initial success in mouse blastoid formation.[73-75] Mouse blastoids are created by combining mTSCs with mESCs[73] or mEPSCs[74] in confining microwells, or by differentiating mEPSCs into EPI-, TE-, and PE-like cells in microwells[75] (**Table 1**). Additionally, mouse blastoids have been developed through chemical reprogramming of primed mouse epiblast stem cells (mEpiSCs) to form induced blastocyst-like precursors that subsequently self-organize into blastoids[76] (**Table 1**). Recently, mouse blastoids have also been derived from mouse TPSCs[53,54,77] (**Table 1**).

Providing mouse stem cells with geometric confinements is a critical step to promote cell-cell interactions and self-organization. This yields mouse blastoids with morphological features, lineage compositions and organization, and gene expression patterns showing different levels of similarities to mouse blastocysts.[53,54,73-77] Even though mouse blastoids transferred into the uteri of pseudopregnant mice could initiate an implantation-like process and induce decidualization, they exhibit very limited growth or development before resorption.[73-76] This suggests that mouse blastoids do not have the same developmental potential as mouse blastocysts. Optimizing mouse blastoids to progress through implantation, gastrulation and organogenesis, ultimately leading to

the formation of functional organs in surrogate mouse uteri or *in vitro*, remains an unrealized goal in embryo modeling. If achieved, this would represent a significant milestone in the use of mammalian embryo models for organ engineering.

Current methods for generating human blastoids are similar to those used for mouse blastoids generated from a single starting cell type.[53,54,75,77] Given their developmental potential for both embryonic- and extraembryonic-like cells,[31,32,34,49,78] naïve hPSCs and hEPSCs have been the cells of choice to create human blastoids (**Table 1**). These cells, sometimes with their derivatives, are placed in microwells and subjected to chemical inductions, promoting differentiation and self-organization into segregated EPI-, TE-, and HYP-like compartments. This process results in blastoids with different levels of similarities in morphology, global gene expression, and lineage composition to human blastocysts.[65-72] Human blastoids have also been generated with chemically reprogrammed hPSCs that resemble eight-cell stage blastomeres (*i.e.* 8CLCs)[42,55] (**Table 1**). It remains unclear whether human 8CLCs have the intrinsic capacity to differentiate and self-organize into blastoids without the influence of external factors. Alternatively, human blastoids have been generated from transitioning intermediates of somatic cell reprograming (iBlastoids)[79] and primed-to-naïve conversion[80] (**Table 1**). However, comparative transcriptome studies suggest that TE-like cells in iBlastoids may actually represent post-implantation amniotic ectoderm cells.[81,82] As an integrated embryo model, human blastoid research warrents careful scientific and ethical oversight processes.[83] Ethical constraints prohibit *in vivo* implantation studies of human blastoids.[83]

Besides mouse and human blastoids, researchers have recently created monkey[84] and bovine blastoids[85] using naïve-like monkey ESCs and through assembling bovine TSCs[86] and EPSCs,[87] respectively. Prolonged culture of monkey blastoids shows cellular features and molecular markers consistent with peri-gastrulation primate development.[84] Monkey and bovine blastoids, when transferred into surrogate uteruses, appear capable of establishing early pregnancy based on ultrasound observations and/or hormone level detections.[84,85] These *in vivo* transplantation assays provide the most stringent test of blastoid developmental potential. However, it is yet to be shown whether implanted monkey or bovine blastoids can exhibit stereotypical tissue organization and lineage diversification consistent with post-implantation development.

Interspecies chimeric contributions to blastocyst formation

Generation of human organs in animals *via* blastocyst complementation requires donor hPSCs to effectively contribute to the ICM of host blastocysts. Studies in mice reveal that ICM incorporation and chimera competency positively correlates with the developmental potential of donor PSCs: mouse TPSCs exhibit the highest capacity to form chimeras in embryonic tissues,[49,51,52] followed by naïve and intermediate/formative mouse PSCs, while primed mouse EpiSCs rarely contribute to mouse blastocyst ICMs.[39,88] Likewise, 8CLCs, naïve, naïve-like and intermediate hPSCs have shown robust colonization into ICMs of mouse, pig, rabbit and monkey blastocysts[42-45,89-93] (**Figure 2C[i]** and **Table 2**). In contrast, primed hPSCs were inefficiently incorporated into mouse, rabbit, cow or pig ICMs, and the cells undergo apoptosis following blastocyst injection[90,94,95] (**Table 2**).

Peri-implantation development

During peri-implantation human development, hypoblast proliferates, extending beyond the epiblast, differentiating into visceral and parietal endoderm. The visceral endoderm lies beneath the epiblast, forming a continuous, polarized cuboidal epithelium, while peripheral hypoblast cells transform into spindle-shaped parietal endoderm, creating the inner lining for the cytotrophoblast. The parietal endoderm expands gradually to line the entire inner cavity of the cytotrophoblast, leading to primary yolk sac formation (**Figure 2A[ii]**).

During primary yolk sac formation, extraembryonic mesoderm arises as spindle-shaped cells situated between parietal endoderm and cytotrophoblast. The origin of early extraembryonic mesoderm in humans is a subject of debate. One hypothesis suggests it may derive from either visceral or parietal endoderm. Another theory proposes that it could originate from peri-implantation epiblast cells while they form the amniotic cavity and undergo symmetry breaking to generate the amniotic ectoderm.[96,97] As human peri-implantation development progresses, extraembryonic mesoderm expands, enveloping the epiblast compartment and primary yolk sac, thereby separating them from the cytotrophoblast (**Figure 2A[ii]**).

During human peri-implantation development, the epiblast compartment forms the amniotic cavity through lumenogenesis[59] (**Figure 2A[ii]**). This lumenal epiblast sac gradually resolves into a bipolar structure, with epiblast cells neighboring invading cytotrophoblast cells becoming squamous amniotic ectoderm and remaining epiblast cells on the opposite pole maintaining pluripotency and forming a discoid embryonic disc. At this stage, the epiblast and

visceral endoderm form the bilaminar embryonic disc, positioned between the amniotic cavity (dorsally) and the primary yolk sac cavity (ventrally) (**Figure 2A[ii]**). Prior to gastrulation, a chorionic cavity forms in the extraembryonic mesoderm by dividing it into two layers. At this stage, the yolk sac structure beneath the bilaminar embryonic disc transitions from the primary to definitive yolk sac. How the definitive yolk sac forms to replace the primary yolk sac remains elusive. One theory suggests that the definitive yolk sac takes shape by visceral endoderm expansion, giving rise to a new membrane that pushes the primary yolk sac forward. It eventually pinches off from the primary yolk sac, with the primary yolk sac tissue degenerating into vesicles at the abembryonic end of the chorionic cavity. Simultaneously, the chorionic cavity expands, separating the human embryo with its attached amnion and yolk sac from the blastocyst's outer wall (now called chorion), suspended solely by a thick stalk of tissue, the connecting stalk. The cellular composition of human connecting stalk remains to be fully characterized and likely contains mainly extraembryonic mesoderm.

There are many fundamental questions unanswered about peri-implantation human development. Compared to mice, human amniotic ectoderm and extraembryonic mesoderm emerge earlier. Even though differentiations of amniotic ectoderm- and extraembryonic mesoderm-like cells from cultured hPSCs have been demonstrated,[98,99] the origins of these two lineages, molecular mechanisms underlying their specifications, and their roles in human peri-implantation development remain to be elucidated. Recent studies using cultured human embryos support the role of ECM signaling in the lumenogenesis and formation of the amniotic cavity in the epiblast during peri-implantation human development.[13,59] Another *in vitro* study suggests a role of ECM rigidity-dependent BMP signaling in regulating amniotic differentiation of primed hPSCs.[98] How ECM and developmental signaling, tissue mechanics and morphogenetic events, and lineage fate decisions are interconnected during peri-implantation human development remains elusive. It also remains to be clarified the molecular and cellular mechanisms underlying the primary and definitive yolk sac formation in humans. During human peri-implantation development, thetrophectodermderivatives become physically separated from the bilaminar embryonic disc by the amnion, a distinction from the pre-gastrulation structure of the mouse egg cylinder (**Figure 2A[ii]**).

Modeling peri-implantation development

Notable differences exist between mouse and human peri-implantation development.[61-64,100] Mouse models of peri-implantation development, made using embryonic and extraembryonic stem cells, have successfully mimicked tissue organization and lineage segregation seen in early post-implantation mouse embryos (**Table 1**). More recently, improvements of a rotating bottle culture system initially pioneered by Dennis New[101] have allowed for prolonged *ex utero* mouse embryo culture.[102] Importantly, this rotating bottle culture system has also enabled stem cell-derived mouse peri-implantation embryo models to progress beyond gastrulation, initiating early organogenesis, albeit with a very low efficiency and organ primordia exhibiting notable defects (**Table 1**).[20-22] Specifically, these mouse embryo models develop structures mimicking headfolds with brain subdivisions, a heart, a trunk structure with a neural tube and somites, a tail bud containing neuromesodermal progenitors (NMPs), and a gut tube.[20-22] These mouse studies showcase the exciting promise of stem cell-based embryo models for generating organ primordia through progressive development from the gastrulation to early organogenesis.

In extended 3D cultures, human blastoids show features of early post-implantation development, including amniotic cavity and primary yolk sac formation, growth and differentiation of thetrophectodermlineage, and the emergence of gastrulating cells.[71,80] However, the low efficiency of human blastoids exhibiting these developmental events limits their applications for studying peri- and post-implantation human development. Advancements in this area may be facilitated by ongoing research aimed at improving prolonged human blastocyst cultures *in vitro*, along with efforts in developing models of implantation and placentation using human endometrial cells.[69,70]

Besides prolonged 3D cultures of human blastoids,[71,80] there are other embryo models developed for studying human peri-implantation development. Early studies showed lumen formation as an intrinsic property of primed hPSCs, supporting their use for modeling amniotic cavity formation.[103] Additional studies revealed that naïve hPSCs could not readily form lumens, and the epiblast compartment of *in vitro* cultured human blastocysts only forms the amniotic cavity after epiblast cells exit the naïve pluripotency.[59] Recently, a study demonstrated that clusters of primed hPSCs in a 3D culture underwent lumenogenesis before evolving into a bipolar structure mimicking post-implantation amnion-EPI patterning (**Table 1**).[104] Progressive development of this structure showed delamination of gastrulating cells from the EPI-like pole, a feature consistent with the onset of gastrulation. More recently, a microfluidic amniotic sac model was developed,

allowing for controlled formation of primed hPSC clusters. This was followed by asymmetrical chemical stimulations of hPSC clusters in the microfluidic device, which improved the efficiency and controllability of the amniotic sac model (**Table 1**).[105] This model also demonstrated features consistent with induction of human primordial germ cells (PGCs) during peri-gastrulation human development.[105] This microfluidic amniotic sac model highlights the promising applications of bioengineering tools in controlling tissue geometry, as well as biochemical and biophysical conditions, for embryo modeling to boost their efficiency and controllability.

Another 3D peri-implantation human development model was also developed using primed hPSCs to model anterior (A)-posterior (P) symmetry breaking of the epiblast at the onset of gastrulation (**Table 1**).[106] A follow-up study utilized an assembloid approach to combine primed hPSCs and extraembryonic-like cells to examine the role of embryonic-extraembryonic interactions during the same developmental event (**Table 1**).[107]

Very recently, several new human embryo models have been reported, utilizing either naïve hPSCs or hEPSCs, and sometimes with their derivatives, to simulate human peri-implantation development up to the gastrulation[60,108-112] or early organogenesis[113] (**Figure 2B[ii]** and **Table 1**). Some of these embryo models exhibit complex cellular developments and organizations consistent with the development of nearly all known lineages and structures of peri-implantation human embryos. These structures include bilaminar disc formation, epiblast lumenogenesis for amniotic cavity formation, patterned amniogenesis, A-P symmetry breaking in the epiblast, human PGC specification, primary yolk sac formation, extraembryonic mesoderm and chorionic cavity development, and atrophectodermlineage-surrounding compartment. Although one such model reports signs of a trilaminar disc-like structure and primary neurulation,[113] it remains to be fully validated whether these most recent peri-implantation human development models can faithfully emulate the multifaceted human gastrulation process and even reach the early organogenesis stage.[60,108-112] Since the development of these embryo models relies on spontaneous aggregation and differentiation of naïve hPSCs or hEPSCs, they often exhibit suboptimal efficiency and/or disorganized cellular structures. Even though it remains to be seen how these models will be utilized as experimental tools to advance fundamental knowledge of peri-implantation human development, they represent the most recent advances of human embryo modeling.

Interspecies chimeric contributions to peri-implantation development

Despite exhibiting robust colonization of host blastocyst ICM, naïve hPSCs, such as those cultured in 5i/L/A and PXGL conditions, surprisingly show limited chimeric contribution in mouse, pig, and monkey peri-implantation or early post-implantation embryos[90-92] (**Table 2**). In comparison, hPSCs in intermediate states show improved contributions in early post-implantation chimera formation in pig (E20-E28),[90] mouse (E9.5-10.5)[43-45,89] and monkey (E15)[114] embryos (**Figure 2C[iii]** and **Table 1**). These findings indicate that intermediate or naïve-like hPSCs might be more effective as donor cells for interspecies blastocyst complementation, or that the culture condtions for naïve hPSCs need refinement for optimal use in interspecies chimera applications. Supporting this idea, naïve hPSCs cultured under 5i/L/A and PXGL conditions have been found to exhibit genomic instabilities and a loss of DNA methylation at primary imprints.[115,116]

Studies of mouse TPSCs suggest that, if successfully developed, human TPSCs could be valuable for interspecies chimera formation and blastocyst complementation. Several recent studies support this hypothesis (**Table 2**), with human EPSCs demonstrating increased chimera competency in both mouse and monkey embryos.[49,114,117] In addition, human cells were readily detected in E10.5 mouse embryos following blastocyst injection of human 8CLCs.[42]

Although primed hPSCs undergo apoptosis and cannot contribute to chimera formation following blastocyst injection, they can effectively engraft the posterior epiblast compartment in gastrula-stage mouse embryos and differentiate into cell lineages from all the three germ layers[118,119] (**Figure 2C[ii]**). Thus, utilizing primed hPSCs for interspecies organogenesis *via* an "EPI complementation" in gastrula-stage mouse embryos appears as an attractive alternative strategy.[88] To achieve this goal, a prolonged *ex utero* embryo culture system, like the one recently reported,[102] will be needed for prolonged culture of mouse gastrula, due to a lack of effective methods for transferring gastrula-stage embryos into a surrogate uterus. By grafting primed hPSCs into the posterior epiblast of an organogenesis-disabled, pre-gastrulation mouse embryo, it might be possible to generate human organ primordia through prolonged *ex utero* culture of these chimeric embryos.

Gastrulation and organogenesis

Gastrulation *in vivo* involves the formation of the primitive streak in the epiblast and differentiating epiblast cells moving through the PS, intercalating with underlying visceral endoderm cells, and

eventually replacing them with embryonic endoderm cells. The transition of visceral endoderm to definitive endoderm and the role of definitive endoderm in definitive yolk sac development in humans are yet to be clarified. In mice, a fraction of visceral endoderm cells persists at least until the formation of the early gut tube.[120] Other gastrulating cells migrate bilaterally from the PS and then cranially or laterally between the endoderm and epiblast, coalescing to form the embryonic mesoderm. epiblast cells that do not ingress through the PS are fated to become the embryonic ectoderm. Thus, through gastrulation, the epiblast in human embryos transforms into a trilaminar germ disc structure.

Current molecular understanding of mammalian gastrulation is primarily derived from mouse studies, emphasizing how interactions between the epiblast and surrounding extraembryonic tissues lead to gene expression patterns that initiate symmetry breaking and body axis formation.[121] In mouse embryos, signals from the anterior visceral endoderm inhibit epiblast differentiation. Developmental signaling, involving BMP, WNT and NODAL pathways, at the posterior epiblast prompts epithelial-mesenchymal transition (EMT) and cell ingression through the PS, acquiring mesendoderm identities. The precise molecular mechanisms for symmetry breaking in human epiblast at the onset of gastrulation remain unclear. Recent studies on monkey and human embryos reveal a population of putative visceral endoderm cells at the anterior end expressing WNT and NODAL antagonists,[122-124] akin to the mouse anterior visceral endoderm, suggesting shared mechanisms in mammalian species for epiblast symmetry breaking during gastrulation.

During mouse gastrulation, PGCs develop from somatic gastrulating epiblast cells due to BMP signals from adjacent extraembryonic tissues.[125-127] Knowledge about early PGC development in primate embryos is limited.[123,128] Unlike mice, *cynomolgus* monkey PGCs seem to emerge in the nascent amniotic ectoderm compartment before gastrulation.[123] Observations from *in vitro* cultured human embryos, *in vivo* post-implantation human and monkey embryos, and human embryo models also support the emergence of human PGCs firstly in nascent amniotic ectoderm prior to the gastrulation.[105,128,129] This observation requires further confirmation using other peri-gastrulation human and monkey embryonic tissues. It remains to be elucidated the molecular and cellular differences between human PGCs originated in the amniotic ectoderm compartment *vs.* those from somatic gastrulating epiblast cells.

Human gastrulation remains a profound mystery.[130] Prior to gastrulation, the human epiblast compartment is surrounded by two extraembryonic tissues, dorsal amniotic ectoderm and ventral visceral endoderm. Data from human embryo models support a possible inductive role of posterior amniotic ectoderm in triggering the onset of gastrulation in the posterior epiblast compartment.[105] How the amniotic ectoderm and visceral endoderm coordinate to mediate symmetry breaking, body axis formation, and PS development in human gastrula remains an important question to address in the future. Additionally, the mechanisms governing how gastrulating human cells segregate and give rise to organized germ layer lineages, as well as the development of human PGCs – including their origin and underlying genetic and molecular mechanisms - during human gastrulation, remain largely unresolved. These fundamental questions have profound implications for reproductive and regenerative medicine.

During gastrulation, germ layer subpopulations in the trilaminar embryonic disc come together, facilitating interactions that shape tissue layers, specify cell types, and initiate organ rudiment development (**Figure 2A[iii]**). A critical event in embryonic ectoderm is neural induction,[131] where it divides into the neuroectoderm (central) and surface ectoderm (lateral, future epidermis). The neuroectoderm forms the neural plate, which subsequently folds into the neural tube, covered by the surface ectoderm through the process of primary neurulation.[132] The rostral neural tube,from the brain to the rostral part of the spinal cord up to its mid-thoracic region is formed through primary neurulation. Caudal spinal cord, in contrast, is developed during the elongation of the embryo, through a less characterized secondary neurulation process. It is hypothesized that during gastrulation, caudal epiblast cells first ingress to give rise to a part of the tail bud mesenchyme, which contains a population of bipotent NMPs that give rise to both caudal spinal cord and paraxial mesoderm derivatives during the elongation of the embryo.[133] This tail bud mesenchyme subsequently epithelializes and undergoes cavitation, leading to the formation of one or several lumens (*i.e.*, secondary neurulation). Both primary and secondary neurulation have been observed in human embryos;[134,135] however, their exact contribution to human neural tube formation is still a matter of debate.

During neural tube formation, neural crest cells arise from the neural plate's edges.[136] These cells delaminate from the closing neural tube and migrate to various locations to generate diverse cell types. The identity of neural crest derivatives correlates with their position along the rostral-caudal body axis, with cranial neural crest cells preferentially generating mesenchymal

derivatives in the head, and trunk neural crest cells giving rise to sympathoadrenal cells. It remains poorly understood how mammalian neural crest cells are regionalized with different differentiation potentials along the rostral-caudal axis.[137] Within the neural tube,cells differentiate into distinct classes of neuronal progenitors at defined positions along both the rostral-caudal and dorsal-ventral body axes under the influence of inductive factors emanating from adjacent tissues, including two organizer regions that extend along the dorsal and ventral midlines of the embryo: dorsal surface ectoderm and ventral notochord.

Gastrulation organizes embryonic mesodermal cells into various regions, including cardiogenic mesoderm, axial mesoderm of the prechordal plate and notochord, paraxial mesoderm, intermediate mesoderm and lateral plate mesoderm. Each of these mesodermal regions undergoes some form of segmentation. The most notable segmentation occurs in the trunk and tail paraxial mesoderm, leading to somite formation, which contributes to skeletal muscles, axial skeleton, and dermis. This process, known as somitogenesis, is accompanied by a molecular oscillator called the segmentation clock.[138,139] The interaction of the segmentation clock with a signal wave traveling in the paraxial mesoderm along the cranial-caudal axis (the clock-and-wavefront model) is generally believed to control somite number, size, and axial identity in developing embryos.[140-142]

After gastrulation, the trilaminar embryonic disc undergoes folding due to differential growth rates. As a result, the cranial, lateral, and caudal edges of the embryonic disc converge along the ventral midline. The endodermal, mesodermal, and ectodermal layers fuse to their corresponding layers on the opposite side, creating the basic tube-within-a-tube body plan. This process transforms the flat embryonic endoderm into a primitive gut tube surrounded by mesoderm. Initially, the gut tube consists of foregut and hindgut separated by the midgut, which remains open to the definitive yolk sac. As the lateral edges of embryonic disc layers continue to join along the ventral midline, the midgut progressively transforms into a tube, and the definitive yolk sac neck narrows into a slender vitelline duct. Reciprocal interactions with mesoderm lead to regionalization of the gut tube along the rostral-caudal and dorsal-ventral body axes and the budding of endodermal organ domains. These organ buds develop as outgrowths of endodermal epithelium that intermingle with surrounding mesenchyme, and together they grow, branch, and eventually form functional endodermal organs. The foregut gives rise to the esophagus, trachea, stomach, lungs, thyroid, liver, biliary system, and pancreas; the midgut forms the small intestine, while the hindgut forms the large intestine.

After gastrulation, the cardiogenic mesoderm forms a cardiac crescent at the cranial end of the embryo, giving rise to a pair of lateral endocardial tubes through vasculogenesis. These tubes later fuse along the ventral midline in the future thoracic region to form a single heart tube, which consists of a single endocardial tube with adjacent mesoderm differentiating into contractile cardiomyocytes. The primary heart tube undergoes morphogenetic processes, like looping, remodeling, realignment, and septation, eventually leading to the development of a four-chamber heart, facilitating the separation of pulmonary and systemic circulations.

Modeling gastrulation and early organogenesis

The first human gastrulation model was created based on micropatterned two-dimensional (2D) colonies of primed hPSCs (**Table 1**), displaying a thickened PS-like ring structure and concentric regions of ectodermal, mesodermal, and endodermal tissues, surrounded by extraembryonic domains at colony boundaries.[143] The precision, reproducibility, and compatibility with high-resolution imaging of this model facilitate mechanistic investigations of molecular and cellular events involved in human gastrulation. Given its 2D topology, this model has been integrated with bioengineering tools, such as hydrogel substrates with tunable mechanical stiffnesses[144] and microfluidic gradient devices,[145] to study the roles of biophysical and biochemical signals in the gastrulation and aixal patterning of germ layer lineages (**Table 1**).

3D models of gastrulation and early organogenesis have been most successfully demonstrated using mouse stem cells (**Table 1**). In one such model, termed gastruloids, aggregated mESCs are embedded in culture medium containing diluted natural ECM molecules and are stimulated with exogeneous signals, typically WNT molecules, to induce cell differentiation and tissue patterning (**Table 1**).[146,147] Early mouse gastruloids were shown to model trunk development, exhibiting symmetry breaking, axial elongation, spinal cord-like structure and bilateral somite formation, a gut tube-like structure, a tail bud-like structure containing NMPs, and development of PGC-like cells (PGCLCs).[146-149] Recent mouse gastruloids showed features associated with cardiogenesis[150] and hematopoietic precursor- and erythroid-like cells spatially localized to a vascular-like structure,[151] mimicking *in vivo* blood cell development (**Table 1**). To promote development of anterior neural tissues, surrounding hydrogel signals in mouse gastruloids were modulated, together with WNT inhibition instead of WNT activation (**Table 1**).[152] When mESC aggregates were assembled with another mESC aggregate pre-treated with exogenous BMP4,

resulting mouse gastruloids developed organ primordia similar to those in neurula-stage mouse embryos, including patterned neural tube- and gut tube-like structures, somitic and intermediate mesodermal tissues, cardiac tissues, and a vasculature network (**Table 1**).[153] Moreover, including mTSCs or XEN cells in mouse gastruloids facilitated the development of neuroepithelial structures, such as regions resembling the anterior brain (**Table 1**).[154,155]

3D models of mouse gastrulation have also been developed by assembling mESCs and mTSCs (**Table 1**).[156] These models replicated morphogenetic events in embryonic and extraembryonic tissues during the mouse egg cylinder development. They also successfully induced the formation of definitive mesoderm and PGCLCs.[156] Further incorporation of XEN cells in these models resulted in the development of tissue structures resembling those in mouse gastrula.[20,21,157-159] Additionally, as previously discussed, *ex utero* culture of co-aggregated mESCs and mESC-derived TE- and extraembryonic endoderm-like cells in an improved rotating bottle culture system yielded advanced 3D mouse embryo models that could progress into early stages of organogenesis, albeit with a very low efficiency and organ primordia showing notable abnomalities.[20-22]

Significant progress has also been achieved in developing human 3D gastruloids (**Figure 2B[iii]** and **Table 1**). Using culture protocols similar to those for mouse gastruloids, free-floating aggregates of primed hPSCs under uniform chemical treatments break symmetry and form an A-P axis.[160,161] Human gastruloids undergo axial elongation with spatial cellular organizations of the three definitive germ layer lineages.[160,161] Under shaking cultures, human gastruloids demonstrate more organized trunk-like development, featuring spinal cord-like and gut tube-like structures integrated with peripheral neurons derived from neural crest cells.[161]

Interestingly, axial progenitor-like cells derived from primed hPSCs, which likely contain NMPs, could self-organize and exhibit *in vivo*-like co-morphogenesis of multiple tissues and their topographic organization in the trunk region, including spinal cord and bilateral somites (**Table 1**).[162-164] Furthermore, recent research further utilized primed hPSCs to specifically model somitogenesis[165-167] (**Figure 2B[iii]** and **Table 1**).

There are other human embryo models created to recapitulate early neural developmental events, such as the formation of the neural plate and neural fold, closure of neural folds, and neural tube regional patterning (**Table 1**). Following an early work using mESCs[168], dorsal-ventral neural patterning was imitated using hPSC-derived lumenal neural cysts in 3D cultures under caudalizing

and ventralizing chemical environments.[169-172] Many of the human neural developmental models were achieved using micropatterned 2D colonies of primed hPSCs subjected to chemical induction of ectodermal lineage development.[173-177] In one of these models, a self-organized ectodermal structure or "neuruloid" was generated, featuring a central lumenal neural epithelial structure overlaid by neural crest cells, with the entire structure covered with a layer of a prospective epidermis.[175] Thus, the tissue morphology and spatial cellular organization of the neuruloid is reminiscent of the ectodermal organization observed *in vivo* at the neurulation stage. Another neuruloid study further recapitulated the morphogenetic cellular events during the folding and closure of the neural plate in neurulation.[176] In addition, microfluidic gradient generation devices have been successfully utilized to superimpose exogenous patterning signals on hPSC-derived neural tissues to achieve their regional patterning.[178] In one pioneering study, patterned by microfluidic WNT signal gradients, hPSC-derived, planar neural tissues were generated that exhibit progressive caudalization from forebrain to midbrain to hindbrain, including formation of isthmic organizer characteristics.[179] Very recently, using two orthogonal and independently controllable microfluidic gradients, an hPSC-based, microfluidic neural tube-like structure (or μNTLS) was demonstrated, whose development recapitulates some critical aspects of neural patterning in both brain and spinal cord regions and along both rostral-caudal and dorsal-ventral axes[180] (**Figure 2B[iii]**). Studying neuronal lineage development using μNTLS revealed pre-patterning of axial identities of neural crest progenitors and functional roles of NMPs in spinal cord and trunk neural crest development.[180] The μNTLS approach is promising for studying interregional and long-range cellular interactions in neural development that are critical for complex network functions.

Interspecies chimeric contributions to early organogenesis

While there have been considerable advancements in intraspecies organogenesis *via* blastocyst complementation, success in the interspecies context remains limited even among closely related species like rats and mice, largely due to alleged xenogeneic barriers (discussed below). The challenges are even more pronounced in the realm of human-animal blastocyst complementation, where only a handful of attempts have been made, yielding variable outcomes.

Despite these obstacles, recent progress in human-animal interspecies organogenesis is encouraging[23] (**Figure 2C[iii]** and **Table 2**). The initial successful attempt at generating human

tissues in animals *via* blastocyst complementation came from a study by Garry and colleagues. This team successfully generated human endothelium in E17-E18 *ETV2*-null pig embryos from injected hPSCs.[181] Following this pioneering work, a subsequent study from the same group created human skeletal muscle tissue in *MYF5/MYOD/MYF6*-null pig embryos (E20 and E27) using hiPSCs.[182] In a major step forward, Lai and colleagues utilized multiple technologies to improve human chimerism in animal embryos and early-stage organs, leading to the production of a humanized pig mesonephros, comprising 40% to 60% human cells, within 3-4 weeks old pig fetuses.[183] These advances, in large part, can be attributed to continuous efforts in understanding and overcomeing the xenogeneic barriers that exist between donor hPSCs and animal embryo hosts.[23]

Challenges and Expectations

Challenges for organ generation using stem cell-based embryo models

There remains numerous challenges in stem cell-based embryo modeling for organ engineering. Addressing these challenges will require concerted and dedicated efforts in optimizing human stem cell cultures, standardizing protocols, and improving characterization methods and controllability of embryo modeling (**Figure 3**).

Efficiency, reproducibility and standardization. Despite significant strides in embryo modeling, achieving models with high fidelity, efficiency, controllability, and *in vivo*-like cellular organization and tissue architecture remains a substantial challenge. This difficulty is primarily attributed to the inherent variabilities in the self-organization and differentiation capabilities of human stem cells and their derivatives within the uncontrolled culture environments typical of most current embryo modeling efforts. As a result, embryo models are often influenced by transcriptional and epigenetic noise as well as unpredicatable cellular interactions within their local culture microenvironment. Moreover, the use of various stem cell types as starting populations for embryo modeling, each requiring different culture conditions to encourage differentiation and self-organization, introduces additional complexity. The establishment of cultures that accurately represent different human embryonic and extraembryonic cells are still in progress. The inherent variability and poorly understood characteristics of human stem cells further complicate the robust development of embryo models, thereby limiting their utility. Additionally, the absence of

standardized protocols for embryo modeling exacerbates variability in culture conditions and experimental results across different research laboratories, obstructing reproducibility and the ability to compare findings from embryo modeling studies effectively.

To overcome the challenges of limited efficiency and reproducibility in embryo modeling, it is imperative to harness advanced bioengineering tools capable of precisely managing tissue topological boundaries and dynamic chemical and mechanical signals in culture environments. These tools will be instrumental in creating high-fidelity, high-efficiency embryo models.[9,184] Recent advancements have yielded bioengineered human embryo models featured by enhanced precision, reproducibility, and compatibility with high-resolution imaging techniques, facilitating detailed mechanistic studies of the molecular and cellular processes underlying human development.[105,143,145,162,175,176,179,180] Looking ahead, the field of embryo modeling stands to gain substantially from integrative efforts that apply bioengineering strategies, including micropatterning, microfluidics, 3D bioprinting, and synthetic biology techniques like optogenetics, as well as cell-instructive biomaterials. These approaches aim to meticulously direct pattern formation, morphogenesis, and cell differentiation, thereby achieving more accurate control over the development of embryo models. This will enhance their efficiency, reproducibility, controllability, complexity, and *in vivo* relevance. Parallel efforts in establishing and thoroughly characterizing various human stem cell lines, especially those representing genuine human extraembryonic stem cells, will further advance these endeavors, making it possible to generate more accurate and useful models of human development.[30-36,78]

Recaptulating gastrulation and organogenesis in embryo models. Organ primordia in current embryo models often show notable structural defects and variations, are small in size, and lack organ-specific functionalities. *In vivo*, organogenesis occurs after gastrulation, a process where embryonic germ layers and their subpopulations within the trilaminar embryonic disc structure come together, promoting tissue-tissue interactions to specify cell types, drive morphogenetic events and initiate organ rudiment development. Thus, the most important outcome of gastrulation is the emergence of a recognizable structure containing organized germ layer lineages with spatially distinct identities in a fully-defined coordinate system.[130] Current 3D human embryo models fall short of replicating the intricate structural organization of embryonic germ layer lineages during peri-gastrulation development, posing a significant obstacle in accurately

modeling organogenesis. There are certain human embryo models containing axial progenitor-like cells that exhibit organized development of trunk regions, featuring the formation of structures such as the primitive gut tube, spinal cord, and bilateral somites.[162-164] These studies highlight the importance of future embryo modeling in promoting proper differentiation and spatial organization of embryonic germ layer lineages and their subpopulations, which will facilitate autonomous cellular interactions and provide an effective morphogenetic environment for organ formation.

The ongoing efforts in deriving *bona fide* human extraembryonic stem cells[30-36,78] and in developing *in vitro* implantation models[69,70,185] using endometrial cells will promote future development of more advanced human embryo models containing embryonic, extraembryonic and/or maternal components. The extraembryonic and maternal tissues will likely be pivotal in providing structural stability, managing topological boundaries, and facilitating endogenous, multidirectional tissue interactions. Together, they create a conductive morphogenetic environment that fosters cell differentiation and organization reminiscent of the gastrulation process. Continuous developments and refinements, particularly those incorporating bioengineering tools and cell-instructive biomaterial systems to precisely modulate dynamic biophysical and biochemical niche signals, will lead to more sophisticated human embryo models exhibiting proper organogenesis processes, with improved efficiency and controllability. Additionally, harnessing advanced bioreactor systems, including artificial placentas, is crucial for long-term culture of human embryo models. These systems, equipped with a continuous medium supply, automated sampling, real-time sensing, and meticulous control over culture conditions—including physiological and mechanical forces—might enable the growth of organ primordia into sizable, functional organs in embryo models.

Challenges for interspecies organogenesis

While successful in closely related rodent species like rats and mice, applying blastocyst complementation to humans remains challenging.[181,182] Key steps for successful human-animal blastocyst complementation include generating hPSCs that can robustly contribute to interspecies chimeras and overcoming developmental barriers between species to fully unlock this technique's potential for growing human organs in animals.

Despite various attempts using different hPSC types and host species, the chimeric contribution of human cells in interspecies chimeras remains markedly low. Furthermore, there

are inconsistent results about the efficiency and the extent to which hPSCs can integrate into embryos of evolutionarily distant host species. This uncertainty largely stems from the technical challenges in detecting and analyzing low levels of chimerism, especially in later stages of embryo and fetal development. To tackle this challenge, developing more effective quantification methods for low chimerism is crucial. Additionally, exploring how interspecies differences in early development contribute to the limited human chimerism observed in animal embryos, an issue often referred to as 'xenogeneic barriers',[23,186] is essential. A better understanding of xenogeneic barriers will be the key in addressing the challenge of low human chimerism, thereby advancing the use of interspecies blastocyst complementation for human organ generation in animals.

It necessitates a deeper understanding of the molecular and cellular events triggered by interspecies cell mixing in early development, in order to overcome xenogeneic barriers and translate the success of rat-mouse to human-animal blastocyst complementation. In contrast to chimera formation within the same species or between closely related species, numerous factors can differ significantly between humans and host animals of distant evolutionary origin, hindering efficient and extensive chimerism. Here, we discuss several key barriers that limit successful chimeric formation, including cell competition, incompatibility in cell adhesion, heterochrony, and ligand-receptor mismatches (**Figure 4**).

Cell competition. Cell competition describes a vital cell-cell interaction essential for multicellular life. It was initially studied in *Drosophila melanogaster* during wing disc development within genetic mosaics, where cells carrying a heterozygous *Minute* mutation are eliminated through apoptosis by surrounding wild-type cells.[187] More recently, cell competition has been observed in various mammalian tissues, supporting that this process is conserved.[188] During early mammalian development, epiblast cells undergo drastic changes in proliferation rate and reorganization of transcriptional, epigenetic, metabolic, and signaling networks. The complexity of these changes raises the likelihood of aberrant cells emerging, requiring intrinsic cellular mechanisms to detect and eliminate such cells to ensure normal development. In the context of interspecies chimera formation, xenogeneic hPSCs might be perceived as unfit or aberrant cells by neighboring host cells and targeted for elimination through cell competition. In aggreement, strategies to suppress hPSCs apoptosis improved human chimerism in mouse and pig embryos.[43,94,95,181,182,189]

To model cell competition in interspecies chimeras, researchers utilized an interspecies PSC co-culture strategy to uncover a previously unknown competive interaction between primed, but not naïve, PSCs from evolutionarily distant species (*e.g.*, humans *vs.* mice; humans *vs.* cows)[190] (**Figure 4A**). Comparative transcriptomic analysis of hPSCs in co-cultures *vs.* separate cultures revealed that genes related to the NF-κB signaling pathway, among others, were upregulated in "loser" hPSCs.[190] Genetic perturbation of the NF-κB signaling pathway by knocking out a core component of NF-κB complex, *P65* (also known as *RELA*), and an upstream adaptor *MyD88* in hPSCs prevented their apoptosis during co-culture with mEpiSCs and furthermore, improved their survival and chimerism in early mouse embryos[190] (**Figure 4A**). MyD88 is one of the primary adaptors for most mammalian Toll-like receptors (TLRs). The TLRs/MyD88/RELA-dependent loser cell apoptosis observed in human-mouse primed PSC competition is strikingly similar to the role Toll-related receptors (TRRs)-NF-κB played during cell competition in *Drosophila* wing disc development,[191] suggesting that the innate immunity pathway acts as a conserved gatekeeper to ensure normal development.

In contrast to loser cells, little is known regarding what enacts the winner status during interspecies PSC competition. A recent preprint study suggests that RNA sensing and innate immunity operates in "winner" cells during interspecies PSC competition.[192] By suppressing the retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) pathway in mouse embryos, researchers observed improved survival and chimerism from unmodified donor human PSCs (**Figure 4A**). This study suggests an alternative approach to promote interspecies chimerism of donor hPSCs by modifying host embryos.

Cell adhesion. Interspecies incompatibility may also result from mismatches in cell adhesion molecules (CAMs) between different species. During development, cell adhesion is crucial for the assembly of individual cells into 3D tissues, and differential cell adhesion is important for cell sorting and tissue boundary formation. For interspecies chimera formation, differential cell adhesion may impede donor hPSCs from effectively integrating with host counterparts and contributing to host development (**Figure 4B**). Mismatches of CAMs can result from structural and sequence differences between homologous adhesion proteins or from varying expression patterns and levels of adhesion molecules in embryos of different species. For donor hPSCs not expressing CAMs compatible with host embryos, they might not participate effectively in the

development of the epiblast lineage, ultimately leading to their expulsion from the embryo. To address this issue, strategies to modify key components of CAMs in hPSCs to render them more compatible with corresponding proteins from host species can be explored. For instance, the first extracellular loop of CLAUDIN, a tight junction (TJ) protein, plays a significant role in recognizing other CLAUDINs on neighboring cells. Thus, any divergence in its sequence may impair CLAUDIN binding and TJ formation. Consequently, it might serve as a useful strategy to replace this part of human CLAUDIN in hPSCs with the sequence from host species, thus allowing hPSCs to form proper TJs with host epiblast cells for more effective interspecies chimera formation.

The prospect of modifying each CAM involved in cell-cell adhesion incompatibility between species at different developmental stages can be very challenging. An alternative approach can employ synthetic biology to regulate adhesive interactions between cells through membrane-localized nanobody-antigen interactions[193,194] (**Figure 4B**). Nanobodies, which are single monomeric domain antibody fragments derived from camelid heavy chain IgG antibodies, offer several advantages, such as the ability to bind small antigens and robust expression in various model systems.[195] Recent studies have successfully utilized nanobody-antigen pairs to induce artificial cell adhesion in bacterial systems.[193] A recent study further expanded on this strategy for mammalian systems by developing synthetic CAMs (synCAMs) that combine orthogonal (nanobody-antigen) extracellular interactions with intracellular domains of native adhesion molecules.[194] This orthogonal system does not interfere with natural adhesion processes in mammalian cells and can be easily modified using multiple nanobody-antigen pairs or by altering the nanobody sequence to adjust adhesion strength. It will be intriguing to explore whether synCAMs can be utilized to enhance cell-cell adhesion between species, thus improving human cell chimerism in animal embryos.

Heterochrony. First proposed by Ernst Haeckel in 1875, heterochrony is a concept that encompasses any genetically regulated variations in the timing, rate, or duration of the developmental process in an organism, in comparison to its ancestral lineage or other species. Heterochrony can present a potential xenogeneic barrier to interspecies chimerism, as discrepancies in the developmental timing, rate, and duration between donor and host species may obstruct donor cells from effectively responding to environmental cues for proliferation and

differentiation in synchronization with host cells, thus hindering the harmonious integration of donor and host cells (**Figure 4C**).

Consistent with the concept of heterochrony, mammals exhibit considerable variation in the rate of embryonic development, which is often correlated with differences in body shape and size, age of sexual maturity, and lifespan. Interestingly, species-specific pace of development is often corroborated by directed differentiation of PSCs of various species outside the uterus. For example, one study showed that, using the same neural differentiation protocol, hPSCs took significantly longer to generate target neuronal cell types compared to mouse PSCs.[196] Intriguingly, human-specific neural differentiation rate could even persist in teratomas generated from hPSCs in a mouse host, suggesting that external host factors could not accelerate the developmental clock of donor human cells.[197] In addition to sequential gene regulation mediating developmental timing, oscillators, such as the "segmentation clock", can serve as timers controlling the tempo of morphogenesis and tissue formation. Recent studies show that the periodicity of the segmentation clock during somitogenesis *in utero* is retained in somite precursors derived from PSCs *in vitro*, adhering to the species-specific tempo.[198-201] These findings support that developmental timing requires a significant degree of cell autonomy, likely involving species-specific biochemical reaction speeds[196,198] and/or mitochondria metabolism.[202]

Despite inherent developmental timing differences among species, there are studies showing that some xenogeneic donor cells could adopt the developmental pace of host species when injected into preimplantation blastocysts. Successful generation of several human-animal chimeric embryos, as mentioned earlier, implies that a small portion of hPSCs accelerate their developmental rate to match that of their embryonic host species.[43,45,90] Supporting this notion, another study shows that PSCs from horses, which have a significantly longer gestation period (~11-12 months) compared to mice (~20 days), could contribute to chimera formation in early mouse embryos.[38] Adding to this evidence, a recent paper demonstrates that co-differentiation with the presence of mouse PSCs could accelerate the differentiation speed of hPSCs.[203] Additionally, two very recent preprint studies reveal that rat neurons could adjust to the developmental pace of their mouse hosts following blastocyst injection of rat PSCs into mouse blastocysts.[204,205] Together, these studies support that given their inherent plasticity, PSCs may be more adaptive in terms of differentiation pace than initially believed. Furthermore, non-cell-autonomous mechanisms may exist to regulate developmental timing of both donor and host cells during embryogenesis. This

847 highlights the need for future studies to improve foundamental understanding of how
848 developmental tempo is enacted during interspcies chimera formation.
849 A recent study conducted a comparative analysis of chimera formation success rates
850 following injection of primary neural crest cells (NCCs) into blastocysts or ESCs into E8.5 mouse
851 embryos (heterochronic injection), versus injecting ESCs into blastocysts or NCCs into E8.5
852 mouse embryos (isochronic injection).[206] Efficient chimera formation was observed under
853 isochronic injection conditions, and conversely, no functional chimeric contribution was detected
854 in heterochronic injections. Notably, human NCCs contribute to coat pigmentation in postnatal
855 mice chimeras after *in utero* injection into gastrulating mouse embryos, albeit at an very low
856 efficiency.[206] In agreement with this, primed hPSCs seldom contribute to chimera formation
857 following injection into mouse blastocysts but could successfully integrate and differentiate after
858 grafting into the epiblast of gastrulating mouse embryos[118,119] (**Figure 4C**). These findings support
859 that isochronic injection could improve successful engraftment of human cells into animal
860 embryos.
861
862 **Ligand-receptor incompatibility.** Another potential barrier is the interspecies incompatibility
863 between ligands and receptors, stemming from genetic diversification (**Figure 4D**). This often
864 results from ligand-receptor co-evolution aimed at refining binding affinity and specificity.
865 Consequently, ligands from one species might either fail to recognize or manifest reduced potency
866 in activating receptors from another species. For instance, while stem cell factor (SCF) across
867 diverse mammalian species shares over 75% sequence similarity, there's a marked difference in
868 their receptor activation across species. Specifically, human SCF displays restricted potency in
869 activating the mouse KIT, yet the efficacy of rodent SCF in engaging and activating the human
870 KIT nearly parallels that of human SCF.[207] It is a daunting task to identify and optimize all
871 mismatched ligand-receptor pairs across species. A strategic approach could be to pinpoint critical
872 signaling pathways hindered by such incompatibilities and the use of genetic replacement or
873 modification of pivotal receptors to help further improve interspecies chimerism.
874
875 **Current developments and future persectives in interspecies organogenesis**. As of now,
876 interspecies chimerism and blastocyst complementation remain inefficient. Even in experiments
877 between closely related species like rats and mice, chimeric efficiency is still notably lower than

878 intraspecies chimeras, despite a lack of PSC competition[190] and their closely aligned
879 developmental timing - differing by just 1-2 days in gestation period. Notably, a high degree of rat
880 chimerism in mice can lead to embryonic lethality due to developmental incompatibilities.[208]
881 These observations underscore the inherent challenges of cross-species chimerism, even among
882 evolutionary neighbors. Consequently, when considering chimerism and blastocyst
883 complementation between more distantly related species, such as humans and mice or humans and
884 pigs, expectations should be adjusted accordingly. Despite the substantial challenges, the vision of
885 generating human organs in animals - to mitigate the global organ donor shortage - persists with
886 renewed hope. A recent study achieved a significant advancement by successfully generating a
887 humanized mesonephros within pig fetuses.[183] This feat was accomplished by improving multiple
888 aspects of interspecies organogenesis, including an optimized human PSC culture, enhancing the
889 survival and competitiveness of human donor cells, and utilizing a genetically emptied host
890 developmental organ niche.[183]
891 It should be noted that interspecies organogenesis through the generation of chimeras is
892 different from the xenotransplantation approach, aiming to produce organs in pigs that are
893 predominantly human-cell derived. Future studies stand to benefit by merging these two strategies:
894 enriching genetically modified pig organs with human cells through blastocyst complementation.
895 This combination could further diminish immune barriers and render the organs more analogous
896 to human ones.
897
898 **Conclusion and Future Outlook**
899 In the past 25 years, we have made great strides since first capturing human embryonic
900 pluripotency in culture. Human PSCs have revolutionized regenerative medicine, paving the way
901 for fundamental discoveries and translations. Recently, the identification of a variety of human
902 pluripotency states has provided exciting opportunities to explore fresh, intriguing aspects of
903 human development and organ engineering. Human PSCs are notable for their ability to proliferate
904 indefinitely *in vitro*, coupled with their inherent developmental potential and exceptional capacity
905 for self-organization. These properties of hPSCs have granted us access to an extensive array of
906 human embryo models, some of which showing promising potential of generating different organ
907 primordia, such as the heart, gut tube, neural tube and somites. There is little doubt now that stem
908 cell-based embryo models have become useful experimental tools for advancing molecular and

cellular understanding of human development. Additionally, when used in interspecies chimeras and paired with gene-editing technologies and methods to overcome early interspecies developmental barriers, hPSCs could be used for the generation of human organ primordia within animal hosts. Given the rapid progress in embryo modeling and interspecies organogenesis, it is the authors' prediction that successful creations of human organ primordia, either *in vitro* using stem cell-derived embryo models or *in vivo* within interspecies chimeras, will be achieved in the near future.

Compared to embryo models and interspecies chimeras, tissue engineering, bioprinting, and organoid technologies are more established approaches for organ engineering. In this Perspective, we suggest that embryo models and interspecies chimeras offer alternative strategies promising for human organ engineering. Nonetheless, organ engineering remains a distant goal for both fields that requires careful strategic and integrative efforts to address the remaining numerous technical and ethical hurdles. Some of the technical difficulties have been discussed in previous sections. There is another critical challenge about how to grow hPSC-derived organ primordia from embryo models or animal hosts into fully functional and sizeable organs suitable for human transplants. Unfortunately, there is no direct solution currently available for this signifcant difficulty. We envision addressing this challenge will require parallel developments of related emerging technologies, such as advanced bioreactor-based culture systems or artificial placentas that can effectively connect with the vasculature of growing organs or embryo models to supply oxygen and nutrients while removing carbon dioxide and waste products. Such technological innovations are needed for prolonging the development of hPSC-derived organ primordia into fully functional organs. Another potential solution involves ectopical transplantation of hPSC-derived organ primordia, for example, into the kidney capsule or omentum of animal hosts, to integrate human organ primordia with the animal host's blood circulation for oxygen and nutrient supplies. Regarding interspecies organogenesis, the success observed in generating fully functional organs between mice and rats supports that the production of human organs in animals that are evolutionarily closer to humans could be technically more achievable. Needless to say, these proposed technological developments and chimera approaches themselves are technically challenging and ethically sensitive. Nonetheless, they hold the key for advancing embryo modeling and interspecies organogenesis towards the goal of creating complex, functional solid human organs in the laboratory.

Besides technical difficulties, there are abundant ethical challenges facing both embryo models and interspecies chimeras for human organ engineering. This is especially true when certain human tissues develop in embryo models and interspecies chimeras, particularly those involving neural cells in the central nervous system and germ cells - a situation often referred to as "moral humanization". To navigate these ethical considerations, precise genome engineering technologies such as CRISPR-Cas9 can be utilized to selectively deactivate genes necessary for neural development and germ cell specification. This way, hPSCs could be genetically modified to only differentiate into endodermal and mesodermal lineages - those responsible for the production of desired organs - thereby eliminating the risk of producing human neural cells derived from the ectodermal lineage or germ cells. In addition, there are several recent reviews and commentaries on current ethical considerations surrounding embryo modeling and interspecies organogenesis.[83,209] Readers are encouraged to consult these references to understand the complex ethical considerations and landscapes. Crucially, continuous and proactive ethical discussions involving scientists, bioethicists, policymakers, and the public are essential to establish, maintain and update ethical guidelines. These ethical guidelines should be in place before research on embryo models and interspecies organogenesis can proceed with due caution to prevent ethical dilemmas. Such ethical guidelines should be regularly updated, and in some cases, anticipate scientific and technological advances to ensure responsible research conduct.

Acknowledgements

J.W. is a New York Stem Cell Foundation – Robertson Investigator and Virginia Murchison Linthicum Scholar in Medical Research. Research in the Wu laboratory is supported by National Institutes of Health (UM1HG011996, R01HD103627, R01GM138565 and R21HD105349), NYSCF, ARSM, and the Welch Foundation (I-2088). Research in the Fu laboratory is supported by the Michigan-Cambridge Research Initiative, University of Michigan Mid-career Biosciences Faculty Achievement Recognition Award, National Institutes of Health (R21NS113518, R21HD100931, R21HD105126, R21HD105192, R21HD109635, R21NS127983, R01GM143297 and R01NS129850), National Science Foundation (CMMI1917304, CBET1901718 and PFI2213845), and the 21st Century Jobs Trust Fund received through the Michigan Strategic Fund from the State of Michigan (CASE315037). The authors apologize to colleagues whose work they could not cite owing to space restrictions.

Declaration of Interests

The authors declare no competing interests.

Figure Legends

Figure 1. A schematic overview of two innovative strategies for creating human organs from pluripotent stem cells (PSCs). (**A**) *In vitro* generation of organ via stem cell-derived embryo models. Such models, mimicking the initial stages of embryonic development, could potentially be advanced through cultivation in bioreactors and other ex vivo methods to nurture the growth of organ primordia into sizable, functional organs. (**B**) *In vivo* generation of organ via interspecies chimeras. Chimera competent human PSCs can be injected into animal embryos that lack essential genes for organ formation. This process facilitates the production of human organs in animal within the animal host as it undergoes its natural developmental processes.

Figure 2. A summary of human stem cell derived embryo models and the developmental stages *in vivo* they represent. (**A**) During early human development, the embryo develops from a zygote and proceeds through specific recognizable stages of (i) pre-implantation, (ii) peri-implantation, and (iii) organogenesis. During this process, cells in the human embryo differentiate and diversify while acting in a coordinated fashion to enact tissue morphogenesis and patterning programs to shape the body plan. (**B**) PSC-derived human embryo models are generated to mimic various *in vivo* developmental stages. (**C**) Chimera competent human PSCs are introduced into pre-implantation blastocysts or early post-implantation embryos of host animals. This process is designed to produce human-animal chimeras, along with tissues and organs enriched with humanb cells.

Figure 3. Challenges and future improvements in utilizing stem cell based embryo models for organ engineering.

Figure 4. Xenogeneic barriers. (**A**) A notable competitive interaction was identified between primed PSCs from evolutionarily distant species (e.g., human-mouse, human-cow, human-rat) based on interspecies PSC co-culture experiments. The elimination of the "loser" cells (e.g., human PSCs when co-cultured with mouse epiblast stem cells [EpiSCs]) is governed by the NF-κB signaling pathway. Disabling the *P65* gene (also known as *RELA*) or an upstream regulator (*MYD88*) of the NF-κB complex in human cells can overcome this competition, thus enhancing

the survival and chimerism of human cells within early mouse embryos. In "winner" cells (e.g., mouse EpiSCs), the retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) signaling pathway, an RNA sensor, appears to play an important role in determining the outcome of competitive interactions between co-cultured mouse and human PSCs. (**B**) Incompatibilities in cell adhesion, particularly among primed PSCs from different species, present a significant xenogeneic barrier. Employing 3D interspecies PSC co-cultures offers a valuable *in vitro* method to investigate this barrier. A notable approach to overcoming this issue involves engineering synthetic cell adhesion. This can potentially be achieved by leveraging membrane-anchored nanobody-antigen interactions to facilitate cell adhesion compatibility between PSCs from different species. (**C**) Heterochrony represents another xenogeneic barrier. Matching developmental timing of the donor PSCs with host embryos is an important consideration for the successful generation of intra- and inter-species chimeras. (**D**) Genomic evolution leading to mismatched ligand-receptor pairs poses another xenogeneic challenge.

Table 1: **Summary of available embryo models generated using pluripotent stem cells from different species.** Pluripotent stem cells (including both embryonic and induced pluripotent stem cells): PSCs; Extended / expanded pluripotent stem cells: EPSCs; Epiblast stem cells: EpiSCs; Trophoblast stem cells: TSCs; Extraembryonic endoderm stem cells: XENs; Inducible XEN cells (naïve PSCs transiently expressing *Gata4*/*6* or *SOX17*): iXENs; Inducible TSCs (naïve PSCs transiently expressing *CDX2* or *TFAP2C*): iTSCs; Totipotent blastomere-like cells: TBLCs; Trophectoderm: TE; Primitive endoderm: PE; Hypoblast: HYP; Extraembryonic cells: xEMs.

Human embryo models					
Starting cells	**Culture condition**	**Additional cells**	**Developmental stages to model**	**Model name**	**References**
Naïve PSCs	Aggregation of single cell type	N/A	Pre-implantation development	Blastoid	65,66,69,72
EPSCs	Aggregation of single cell type	N/A	Pre-implantation development	Blastoid	68
EPSCs	Aggregation of single cell type	TE-like cells	Pre-implantation development	EPS-blastoid	67
Somatic reprogramming intermediates	Aggregation of reprogramming intermediates	N/A	Pre-implantation development	iBlastoid	79
Primed-to-naïve intermediates	Aggregation during primed-to-naïve-state conversion	N/A	Pre-implantation development	Blastoid	80
Naïve PSCs	Aggregation of single cell type	N/A	Pre- and post-implantation development up to early gastrulation	Blastoid	71
Primed PSCs	Aggregation of single cell type	N/A	Early post-implantation development up to early gastrulation	Post-implantation amniotic sac embryoid	104,105
Primed PSCs	Aggregation of single cell type	N/A	Early post-implantation development up to early gastrulation	Epiblast model	106
Primed PSCs	Aggregation of different cell types	xEMs	Early post-implantation development up to early gastrulation	Post-attached embryo model	107
Naïve PSCs	Aggregation of different cell types	TSCs	Early post-implantation development up to early gastrulation	E-assembloid	60
Naïve PSCs	Aggregation of different cell types	PE/ExEM-like cells, TE-like cells	Early post-implantation development up to early gastrulation	Post-implantation stem-cell-based embryo model	108

PSCs with intermediate pluripotency	Aggregation of single cell type	N/A	Early post-implantation development up to early gastrulation	Extra-embryoid	[109]
Naïve PSCs	Aggregation of different cell types	iTSCs, iXENs	Early post-implantation development up to early gastrulation	Inducible embryoid	[110]
Naïve PSCs	Aggregation of different cell types	HYP-like cells, TE-like cells	Early post-implantation development up to early gastrulation	Bilaminoid	[111]
EPSCs	Aggregation of single cell type	N/A	Early post-implantation development up to early organogenesis	Peri-gastruloid	[113]
Primed PSCs	Co-culture of different cell types	HYP-like cells	Early post-implantation development up to early gastrulation and haematopoiesis	heX-embryoid	[112]
Primed PSCs	Patterned 2D cell colonies	N/A	Gastrulation	N/A	[143-145]
Primed PSCs	Aggregation of single cell type	N/A	Gastrulation	Gastruloid	[160]
Primed PSCs	Aggregation of single cell type	N/A	Gastrulation and early organogenesis	Elongating multi-lineage organized gastruloid	[161]
Primed PSCs	Aggregation of single cell type	N/A	Spinal cord and somite development in the trunk	Trunk-like structure	[163,164]
Primed PSCs	Aggregation of single cell type	N/A	Somitogenesis	Somitoid, segmentoid	[165]
Primed PSCs	Aggregation of single cell type	N/A	Somitogenesis	Somitoid	[166]
Primed PSCs	Aggregation of single cell type	N/A	Somitogenesis	Axioloid	[167]
Primed PSCs	Patterned 2D cell colonies	N/A	Neuroectoderm patterning	N/A	[173]
Primed PSCs	Patterned 2D cell colonies	N/A	Ectoderm patterning and neurulation	Neuruloid	[175]
Primed PSCs	Patterned 2D cell colonies	N/A	Germ layer patterning and neurulation	N/A	[177]
Primed PSCs	Patterned 2D cell colonies	N/A	Ectoderm patterning and neurulation	N/A	[176]
Primed PSCs	Aggregation of single cell type	N/A	Patterned spinal cord development	N/A	[170]
Primed PSCs	Aggregation of single cell type	N/A	Patterned spinal cord development	N/A	[171]
Primed PSCs	Patterned 2D cell colonies	N/A	Patterned neural tube development	N/A	[180]

1031

Mouse embryo models					
Starting cells	**Culture condition**	**Additional cells**	**Developmental stages to model**	**Model name**	**References**

Naïve PSCs	Aggregation of different cell types	TSCs	Pre-implantation development	Blastoid	[73]
Primed-to-naïve intermediates	Aggregation during primed-to-naïve-state conversion	N/A	Pre-implantation development	Blastocyst-like cyst	[76]
TBLCs	Aggregation of single cell type	N/A	Pre-implantation development	TBLC-blastoid	[77]
EPSCs	Aggregation of different cell types	TSCs	Pre- and early post-implantation development	EPS-blastoid	[74]
EPSCs	Aggregation of single cell type	N/A	Pre- and early post-implantation development	EPS-blastoid	[75]
Naïve PSCs	Assembly of two cell aggregates	TSCs	Early post-implantation development up to early gastrulation	ETS embryoid	[156]
Naïve PSCs	Aggregation of different cell types	TSCs, XENs	Early post-implantation development up to early gastrulation	ETX embryoid	[157,158]
Naïve PSCs	Aggregation of different cell types	TSCs, iXENs	Early post-implantation development up to early gastrulation	iETX embryoid	[159]
Naïve PSCs	Aggregation of different cell types	TSCs, iXENs	Post-implantation development up to early organogenesis	ETiX embryoid	[20]
Naïve PSCs	Aggregation of different cell types	iTSCs, iXENs	Post-implantation development up to early organogenesis	sEmbryo	[21]
Naïve PSCs	Aggregation of different cell types	iTSCs, iXENs	Post-implantation development up to early organogenesis	EiTiX embryoid	[22]
Naïve PSCs	Aggregation of single cell type	N/A	Gastrulation	Gastruloid	[146-148,150-152]
Naïve PSCs	Aggregation of single cell type	N/A	Gastrulation and early organogenesis	Trunk-like structure	[149]
Naïve PSCs	Assembly of two cell aggregates	N/A	Gastrulation and early organogenesis	Embryoid	[153]
Naïve PSCs	Assembly of two cell aggregates	TSCs	Gastrulation and early organogenesis	EpiTS embryoid	[154]
Naïve PSCs	Aggregation of different cell types	XENs	Gastrulation and early organogenesis	XEN enhanced gastruloid	[155]
Naïve PSCs	Single cell clonal assay	N/A	Patterned spinal cord development	N/A	[168]

1032

Embryo models of other species					
Starting cells	**Culture condition**	**Additional cells**	**Developmental stages to model**	**Model name**	**References**

Monkey naïve PSCs	Aggregation of single cell type	N/A	Pre- and post-implantation development up to early gastrulation	Blastoid	[84]
Bovine EPSCs	Aggregation of different cell types	TSCs	Pre-implantation development	Blastoid	[85]

1033
1034
1035
1036

Table 2: **A summary of human-animal interspecies chimera studies with different types of human pluripotent stem cells.**

Types of human PSCs	Host species	Level of chimerism
8CLCs	Mice	~1% (mice, E10.5)[42]
Extended/Expanded potental	Mice	~1% (mice, E10.5)[49]
	Monkeys	~7% (monkeys, E15, ex vivo)[114]
Naïve (2iLDOX, 5iLA and PXGL)	Mice, pigs, monkeys	Little to no chimerism[90-92]
Naïve (HENSM)	Mice	~1-2% (mice, E9.5-10.5)[43]
Naïve-like/Intermediate	Mice	~0.1-4% (mice, E17.5)[45]
		unknown (mice, 10.5)[44,89]
	Pigs	~0.001-0.01% (pigs, E28)[90]
Naïve (HENSM, apoptosis inhibited)	Mice	~1-20% (mice, E9.5-10.5)[43]
Naïve (4CL, apoptosis inhibited)	Pigs	unknown (pigs, E25 and E28)[183]
Primed	Mice, pigs, monkeys	Little to no chimerism
Primed (apoptosis inhibited)	Mice	~1% (mice, E10.5)[94,95,189,190]
	Pigs	~0.05 (pigs, E17)[182]
		0.001-0.1% (pigs, E20 and E27)[181]

References

1. Anand, R.P., Layer, J.V., Heja, D., Hirose, T., Lassiter, G., Firl, D.J., Paragas, V.B., Akkad, A., Chhangawala, S., Colvin, R.B., et al. (2023). Design and testing of a humanized porcine donor for xenotransplantation. Nature *622*, 393-401. 10.1038/s41586-023-06594-4.
2. Rafat, M., Jabbarvand, M., Sharma, N., Xeroudaki, M., Tabe, S., Omrani, R., Thangavelu, M., Mukwaya, A., Fagerholm, P., Lennikov, A., et al. (2023). Bioengineered corneal tissue for minimally invasive vision restoration in advanced keratoconus in two clinical cohorts. Nat Biotechnol *41*, 70-81. 10.1038/s41587-022-01408-w.
3. Hirsch, T., Rothoeft, T., Teig, N., Bauer, J.W., Pellegrini, G., De Rosa, L., Scaglione, D., Reichelt, J., Klausegger, A., Kneisz, D., et al. (2017). Regeneration of the entire human epidermis using transgenic stem cells. Nature *551*, 327-332. 10.1038/nature24487.
4. Ott, H.C., Matthiesen, T.S., Goh, S.K., Black, L.D., Kren, S.M., Netoff, T.I., and Taylor, D.A. (2008). Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. Nat Med *14*, 213-221. 10.1038/nm1684.
5. Lee, A., Hudson, A.R., Shiwarski, D.J., Tashman, J.W., Hinton, T.J., Yerneni, S., Bliley, J.M., Campbell, P.G., and Feinberg, A.W. (2019). 3D bioprinting of collagen to rebuild components of the human heart. Science *365*, 482-487. 10.1126/science.aav9051.
6. Grigoryan, B., Paulsen, S.J., Corbett, D.C., Sazer, D.W., Fortin, C.L., Zaita, A.J., Greenfield, P.T., Calafat, N.J., Gounley, J.P., Ta, A.H., et al. (2019). Multivascular networks and functional intravascular topologies within biocompatible hydrogels. Science *364*, 458-464. 10.1126/science.aav9750.
7. Clevers, H. (2016). Modeling Development and Disease with Organoids. Cell *165*, 1586-1597. 10.1016/j.cell.2016.05.082.
8. Kim, J., Koo, B.K., and Knoblich, J.A. (2020). Human organoids: model systems for human biology and medicine. Nat Rev Mol Cell Biol *21*, 571-584. 10.1038/s41580-020-0259-3.
9. Shao, Y., and Fu, J. (2022). Engineering multiscale structural orders for high-fidelity embryoids and organoids. Cell Stem Cell *29*, 722-743. 10.1016/j.stem.2022.04.003.
10. Das, S., Gordian-Velez, W.J., Ledebur, H.C., Mourkioti, F., Rompolas, P., Chen, H.I., Serruya, M.D., and Cullen, D.K. (2020). Innervation: the missing link for biofabricated tissues and organs. NPJ Regen Med *5*, 11. 10.1038/s41536-020-0096-1.
11. Sasai, Y. (2013). Cytosystems dynamics in self-organization of tissue architecture. Nature *493*, 318-326. 10.1038/nature11859.
12. Deglincerti, A., Croft, G.F., Pietila, L.N., Zernicka-Goetz, M., Siggia, E.D., and Brivanlou, A.H. (2016). Self-organization of the in vitro attached human embryo. Nature *533*, 251-254. 10.1038/nature17948.
13. Shahbazi, M.N., Jedrusik, A., Vuoristo, S., Recher, G., Hupalowska, A., Bolton, V., Fogarty, N.N.M., Campbell, A., Devito, L., Ilic, D., et al. (2016). Self-organization of the human embryo in the absence of maternal tissues. Nat Cell Biol *18*, 700-708. 10.1038/ncb3347.
14. Xiang, L., Yin, Y., Zheng, Y., Ma, Y., Li, Y., Zhao, Z., Guo, J., Ai, Z., Niu, Y., Duan, K., et al. (2020). A developmental landscape of 3D-cultured human pre-gastrulation embryos. Nature *577*, 537-542. 10.1038/s41586-019-1875-y.

15. Petropoulos, S., Edsgard, D., Reinius, B., Deng, Q., Panula, S.P., Codeluppi, S., Plaza Reyes, A., Linnarsson, S., Sandberg, R., and Lanner, F. (2016). Single-Cell RNA-Seq Reveals Lineage and X Chromosome Dynamics in Human Preimplantation Embryos. Cell *165*, 1012-1026. 10.1016/j.cell.2016.03.023.
16. Zhou, F., Wang, R., Yuan, P., Ren, Y., Mao, Y., Li, R., Lian, Y., Li, J., Wen, L., Yan, L., et al. (2019). Reconstituting the transcriptome and DNA methylome landscapes of human implantation. Nature *572*, 660-664. 10.1038/s41586-019-1500-0.
17. Tyser, R.C.V., Mahammadov, E., Nakanoh, S., Vallier, L., Scialdone, A., and Srinivas, S. (2021). Single-cell transcriptomic characterization of a gastrulating human embryo. Nature *600*, 285-289. 10.1038/s41586-021-04158-y.
18. Xu, Y., Zhang, T., Zhou, Q., Hu, M., Qi, Y., Xue, Y., Nie, Y., Wang, L., Bao, Z., and Shi, W. (2023). A single-cell transcriptome atlas profiles early organogenesis in human embryos. Nat Cell Biol *25*, 604-615. 10.1038/s41556-023-01108-w.
19. Xiao, Z., Cui, L., Yuan, Y., He, N., Xie, X., Lin, S., Yang, X., Zhang, X., Shi, P., Wei, Z., et al. (2024). 3D reconstruction of a gastrulating human embryo. Cell. 10.1016/j.cell.2024.03.041.
20. Amadei, G., Handford, C.E., Qiu, C., De Jonghe, J., Greenfeld, H., Tran, M., Martin, B.K., Chen, D.Y., Aguilera-Castrejon, A., Hanna, J.H., et al. (2022). Embryo model completes gastrulation to neurulation and organogenesis. Nature *610*, 143-153. 10.1038/s41586-022-05246-3.
21. Tarazi, S., Aguilera-Castrejon, A., Joubran, C., Ghanem, N., Ashouokhi, S., Roncato, F., Wildschutz, E., Haddad, M., Oldak, B., Gomez-Cesar, E., et al. (2022). Post-gastrulation synthetic embryos generated ex utero from mouse naive ESCs. Cell *185*, 3290-3306 e3225. 10.1016/j.cell.2022.07.028.
22. Lau, K.Y.C., Rubinstein, H., Gantner, C.W., Hadas, R., Amadei, G., Stelzer, Y., and Zernicka-Goetz, M. (2022). Mouse embryo model derived exclusively from embryonic stem cells undergoes neurulation and heart development. Cell Stem Cell *29*, 1445-1458 e1448. 10.1016/j.stem.2022.08.013.
23. Zheng, C., Ballard, E.B., and Wu, J. (2021). The road to generating transplantable organs: from blastocyst complementation to interspecies chimeras. Development *148*. 10.1242/dev.195792.
24. Chen, J., Lansford, R., Stewart, V., Young, F., and Alt, F.W. (1993). RAG-2-deficient blastocyst complementation: an assay of gene function in lymphocyte development. Proc Natl Acad Sci U S A *90*, 4528-4532. 10.1073/pnas.90.10.4528.
25. Kobayashi, T., Yamaguchi, T., Hamanaka, S., Kato-Itoh, M., Yamazaki, Y., Ibata, M., Sato, H., Lee, Y.S., Usui, J., Knisely, A.S., et al. (2010). Generation of rat pancreas in mouse by interspecific blastocyst injection of pluripotent stem cells. Cell *142*, 787-799. 10.1016/j.cell.2010.07.039.
26. Tanaka, S., Kunath, T., Hadjantonakis, A.K., Nagy, A., and Rossant, J. (1998). Promotion of trophoblast stem cell proliferation by FGF4. Science *282*, 2072-2075. 10.1126/science.282.5396.2072.
27. Evans, M.J., and Kaufman, M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature *292*, 154-156. 10.1038/292154a0.
28. Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A *78*, 7634-7638. 10.1073/pnas.78.12.7634.

29. Kunath, T., Arnaud, D., Uy, G.D., Okamoto, I., Chureau, C., Yamanaka, Y., Heard, E., Gardner, R.L., Avner, P., and Rossant, J. (2005). Imprinted X-inactivation in extra-embryonic endoderm cell lines from mouse blastocysts. Development *132*, 1649-1661. 10.1242/dev.01715.
30. Okae, H., Toh, H., Sato, T., Hiura, H., Takahashi, S., Shirane, K., Kabayama, Y., Suyama, M., Sasaki, H., and Arima, T. (2018). Derivation of Human Trophoblast Stem Cells. Cell Stem Cell *22*, 50-63 e56. 10.1016/j.stem.2017.11.004.
31. Dong, C., Beltcheva, M., Gontarz, P., Zhang, B., Popli, P., Fischer, L.A., Khan, S.A., Park, K.M., Yoon, E.J., Xing, X., et al. (2020). Derivation of trophoblast stem cells from naive human pluripotent stem cells. Elife *9*. 10.7554/eLife.52504.
32. Guo, G., Stirparo, G.G., Strawbridge, S.E., Spindlow, D., Yang, J., Clarke, J., Dattani, A., Yanagida, A., Li, M.A., Myers, S., et al. (2021). Human naive epiblast cells possess unrestricted lineage potential. Cell Stem Cell *28*, 1040-1056 e1046. 10.1016/j.stem.2021.02.025.
33. Io, S., Kabata, M., Iemura, Y., Semi, K., Morone, N., Minagawa, A., Wang, B., Okamoto, I., Nakamura, T., Kojima, Y., et al. (2021). Capturing human trophoblast development with naive pluripotent stem cells in vitro. Cell Stem Cell *28*, 1023-1039 e1013. 10.1016/j.stem.2021.03.013.
34. Linneberg-Agerholm, M., Wong, Y.F., Romero Herrera, J.A., Monteiro, R.S., Anderson, K.G.V., and Brickman, J.M. (2019). Naive human pluripotent stem cells respond to Wnt, Nodal and LIF signalling to produce expandable naive extra-embryonic endoderm. Development *146*. 10.1242/dev.180620.
35. Mackinlay, K.M., Weatherbee, B.A., Souza Rosa, V., Handford, C.E., Hudson, G., Coorens, T., Pereira, L.V., Behjati, S., Vallier, L., Shahbazi, M.N., and Zernicka-Goetz, M. (2021). An in vitro stem cell model of human epiblast and yolk sac interaction. Elife *10*. 10.7554/eLife.63930.
36. Wei, Y., Zhang, E., Yu, L., Ci, B., Sakurai, M., Guo, L., Zhang, X., Lin, S., Takii, S., Liu, L., et al. (2023). Dissecting embryonic and extraembryonic lineage crosstalk with stem cell co-culture. Cell *186*, 5859-5875 e5824. 10.1016/j.cell.2023.11.008.
37. Du, P., and Wu, J. (2024). Hallmarks of totipotent and pluripotent stem cell states. Cell Stem Cell *31*, 312-333. 10.1016/j.stem.2024.01.009.
38. Yu, L., Wei, Y., Sun, H.X., Mahdi, A.K., Pinzon Arteaga, C.A., Sakurai, M., Schmitz, D.A., Zheng, C., Ballard, E.D., Li, J., et al. (2021). Derivation of Intermediate Pluripotent Stem Cells Amenable to Primordial Germ Cell Specification. Cell Stem Cell *28*, 550-567 e512. 10.1016/j.stem.2020.11.003.
39. De Los Angeles, A., Ferrari, F., Xi, R., Fujiwara, Y., Benvenisty, N., Deng, H., Hochedlinger, K., Jaenisch, R., Lee, S., Leitch, H.G., et al. (2015). Hallmarks of pluripotency. Nature *525*, 469-478. 10.1038/nature15515.
40. Theunissen, T.W., Powell, B.E., Wang, H., Mitalipova, M., Faddah, D.A., Reddy, J., Fan, Z.P., Maetzel, D., Ganz, K., Shi, L., et al. (2014). Systematic identification of culture conditions for induction and maintenance of naive human pluripotency. Cell Stem Cell *15*, 471-487. 10.1016/j.stem.2014.07.002.
41. Guo, G., von Meyenn, F., Santos, F., Chen, Y., Reik, W., Bertone, P., Smith, A., and Nichols, J. (2016). Naive Pluripotent Stem Cells Derived Directly from Isolated Cells of the Human Inner Cell Mass. Stem Cell Reports *6*, 437-446. 10.1016/j.stemcr.2016.02.005.

42. Mazid, M.A., Ward, C., Luo, Z., Liu, C., Li, Y., Lai, Y., Wu, L., Li, J., Jia, W., Jiang, Y., et al. (2022). Rolling back human pluripotent stem cells to an eight-cell embryo-like stage. Nature *605*, 315-324. 10.1038/s41586-022-04625-0.
43. Bayerl, J., Ayyash, M., Shani, T., Manor, Y.S., Gafni, O., Massarwa, R., Kalma, Y., Aguilera-Castrejon, A., Zerbib, M., Amir, H., et al. (2021). Principles of signaling pathway modulation for enhancing human naive pluripotency induction. Cell Stem Cell *28*, 1549-1565 e1512. 10.1016/j.stem.2021.04.001.
44. Gafni, O., Weinberger, L., Mansour, A.A., Manor, Y.S., Chomsky, E., Ben-Yosef, D., Kalma, Y., Viukov, S., Maza, I., Zviran, A., et al. (2013). Derivation of novel human ground state naive pluripotent stem cells. Nature *504*, 282-286. 10.1038/nature12745.
45. Hu, Z., Li, H., Jiang, H., Ren, Y., Yu, X., Qiu, J., Stablewski, A.B., Zhang, B., Buck, M.J., and Feng, J. (2020). Transient inhibition of mTOR in human pluripotent stem cells enables robust formation of mouse-human chimeric embryos. Sci Adv *6*, eaaz0298. 10.1126/sciadv.aaz0298.
46. Ware, C.B., Nelson, A.M., Mecham, B., Hesson, J., Zhou, W., Jonlin, E.C., Jimenez-Caliani, A.J., Deng, X., Cavanaugh, C., Cook, S., et al. (2014). Derivation of naive human embryonic stem cells. Proc Natl Acad Sci U S A *111*, 4484-4489. 10.1073/pnas.1319738111.
47. Smith, A. (2017). Formative pluripotency: the executive phase in a developmental continuum. Development *144*, 365-373. 10.1242/dev.142679.
48. Kinoshita, M., Barber, M., Mansfield, W., Cui, Y., Spindlow, D., Stirparo, G.G., Dietmann, S., Nichols, J., and Smith, A. (2021). Capture of Mouse and Human Stem Cells with Features of Formative Pluripotency. Cell Stem Cell *28*, 453-471 e458. 10.1016/j.stem.2020.11.005.
49. Yang, Y., Liu, B., Xu, J., Wang, J., Wu, J., Shi, C., Xu, Y., Dong, J., Wang, C., Lai, W., et al. (2017). Derivation of Pluripotent Stem Cells with In Vivo Embryonic and Extraembryonic Potency. Cell *169*, 243-257 e225. 10.1016/j.cell.2017.02.005.
50. Yang, J., Ryan, D.J., Wang, W., Tsang, J.C., Lan, G., Masaki, H., Gao, X., Antunes, L., Yu, Y., Zhu, Z., et al. (2017). Establishment of mouse expanded potential stem cells. Nature *550*, 393-397. 10.1038/nature24052.
51. Shen, H., Yang, M., Li, S., Zhang, J., Peng, B., Wang, C., Chang, Z., Ong, J., and Du, P. (2021). Mouse totipotent stem cells captured and maintained through spliceosomal repression. Cell *184*, 2843-2859 e2820. 10.1016/j.cell.2021.04.020.
52. Hu, Y., Yang, Y., Tan, P., Zhang, Y., Han, M., Yu, J., Zhang, X., Jia, Z., Wang, D., Yao, K., et al. (2023). Induction of mouse totipotent stem cells by a defined chemical cocktail. Nature *617*, 792-797. 10.1038/s41586-022-04967-9.
53. Yang, M., Yu, H., Yu, X., Liang, S., Hu, Y., Luo, Y., Izsvak, Z., Sun, C., and Wang, J. (2022). Chemical-induced chromatin remodeling reprograms mouse ESCs to totipotent-like stem cells. Cell Stem Cell *29*, 400-418 e413. 10.1016/j.stem.2022.01.010.
54. Xu, Y., Zhao, J., Ren, Y., Wang, X., Lyu, Y., Xie, B., Sun, Y., Yuan, X., Liu, H., Yang, W., et al. (2022). Derivation of totipotent-like stem cells with blastocyst-like structure forming potential. Cell Res *32*, 513-529. 10.1038/s41422-022-00668-0.
55. Yu, X., Liang, S., Chen, M., Yu, H., Li, R., Qu, Y., Kong, X., Guo, R., Zheng, R., Izsvak, Z., et al. (2022). Recapitulating early human development with 8C-like cells. Cell Rep *39*, 110994. 10.1016/j.celrep.2022.110994.

56. Taubenschmid-Stowers, J., Rostovskaya, M., Santos, F., Ljung, S., Argelaguet, R., Krueger, F., Nichols, J., and Reik, W. (2022). 8C-like cells capture the human zygotic genome activation program in vitro. Cell Stem Cell *29*, 449-459 e446. 10.1016/j.stem.2022.01.014.
57. Johnson, M. (2019). Human in vitro fertilisation and developmental biology: a mutually influential history. Development *146*, dev183145. 10.1242/dev.183145.
58. O'Rahilly, R., and Muller, F. (2010). Developmental stages in human embryos: revised and new measurements. Cells Tissues Organs *192*, 73-84. 10.1159/000289817.
59. Shahbazi, M.N., Scialdone, A., Skorupska, N., Weberling, A., Recher, G., Zhu, M., Jedrusik, A., Devito, L.G., Noli, L., Macaulay, I.C., et al. (2017). Pluripotent state transitions coordinate morphogenesis in mouse and human embryos. Nature *552*, 239-243. 10.1038/nature24675.
60. Ai, Z., Niu, B., Yin, Y., Xiang, L., Shi, G., Duan, K., Wang, S., Hu, Y., Zhang, C., Zhang, C., et al. (2023). Dissecting peri-implantation development using cultured human embryos and embryo-like assembloids. Cell Res *33*, 661-678. 10.1038/s41422-023-00846-8.
61. Shahbazi, M.N. (2020). Mechanisms of human embryo development: from cell fate to tissue shape and back. Development *147*. 10.1242/dev.190629.
62. Rossant, J., and Tam, P.P.L. (2022). Early human embryonic development: Blastocyst formation to gastrulation. Dev Cell *57*, 152-165. 10.1016/j.devcel.2021.12.022.
63. Rossant, J., and Tam, P.P.L. (2017). New Insights into Early Human Development: Lessons for Stem Cell Derivation and Differentiation. Cell Stem Cell *20*, 18-28. 10.1016/j.stem.2016.12.004.
64. Shahbazi, M.N., and Zernicka-Goetz, M. (2018). Deconstructing and reconstructing the mouse and human early embryo. Nat Cell Biol *20*, 878-887. 10.1038/s41556-018-0144-x.
65. Yu, L., Wei, Y., Duan, J., Schmitz, D.A., Sakurai, M., Wang, L., Wang, K., Zhao, S., Hon, G.C., and Wu, J. (2021). Blastocyst-like structures generated from human pluripotent stem cells. Nature *591*, 620-626. 10.1038/s41586-021-03356-y.
66. Yanagida, A., Spindlow, D., Nichols, J., Dattani, A., Smith, A., and Guo, G. (2021). Naive stem cell blastocyst model captures human embryo lineage segregation. Cell Stem Cell *28*, 1016-1022 e1014. 10.1016/j.stem.2021.04.031.
67. Fan, Y., Min, Z., Alsolami, S., Ma, Z., Zhang, E., Chen, W., Zhong, K., Pei, W., Kang, X., Zhang, P., et al. (2021). Generation of human blastocyst-like structures from pluripotent stem cells. Cell Discov *7*, 81. 10.1038/s41421-021-00316-8.
68. Sozen, B., Jorgensen, V., Weatherbee, B.A.T., Chen, S., Zhu, M., and Zernicka-Goetz, M. (2021). Reconstructing aspects of human embryogenesis with pluripotent stem cells. Nat Commun *12*, 5550. 10.1038/s41467-021-25853-4.
69. Kagawa, H., Javali, A., Khoei, H.H., Sommer, T.M., Sestini, G., Novatchkova, M., Scholte Op Reimer, Y., Castel, G., Bruneau, A., Maenhoudt, N., et al. (2022). Human blastoids model blastocyst development and implantation. Nature *601*, 600-605. 10.1038/s41586-021-04267-8.
70. Yu, L., Logsdon, D., Pinzon-Arteaga, C.A., Duan, J., Ezashi, T., Wei, Y., Ribeiro Orsi, A.E., Oura, S., Liu, L., Wang, L., et al. (2023). Large-scale production of human blastoids amenable to modeling blastocyst development and maternal-fetal cross talk. Cell Stem Cell *30*, 1246-1261 e1249. 10.1016/j.stem.2023.08.002.

71. Karvas, R.M., Zemke, J.E., Ali, S.S., Upton, E., Sane, E., Fischer, L.A., Dong, C., Park, K.M., Wang, F., Park, K., et al. (2023). 3D-cultured blastoids model human embryogenesis from pre-implantation to early gastrulation stages. Cell Stem Cell *30*, 1148-1165 e1147. 10.1016/j.stem.2023.08.005.
72. Guo, M., Wu, J., Chen, C., Wang, X., Gong, A., Guan, W., Karvas, R.M., Wang, K., Min, M., Wang, Y., et al. (2024). Self-renewing human naive pluripotent stem cells dedifferentiate in 3D culture and form blastoids spontaneously. Nat Commun *15*, 668. 10.1038/s41467-024-44969-x.
73. Rivron, N.C., Frias-Aldeguer, J., Vrij, E.J., Boisset, J.C., Korving, J., Vivie, J., Truckenmuller, R.K., van Oudenaarden, A., van Blitterswijk, C.A., and Geijsen, N. (2018). Blastocyst-like structures generated solely from stem cells. Nature *557*, 106-111. 10.1038/s41586-018-0051-0.
74. Sozen, B., Cox, A.L., De Jonghe, J., Bao, M., Hollfelder, F., Glover, D.M., and Zernicka-Goetz, M. (2019). Self-Organization of Mouse Stem Cells into an Extended Potential Blastoid. Dev Cell *51*, 698-712 e698. 10.1016/j.devcel.2019.11.014.
75. Li, R., Zhong, C., Yu, Y., Liu, H., Sakurai, M., Yu, L., Min, Z., Shi, L., Wei, Y., Takahashi, Y., et al. (2019). Generation of Blastocyst-like Structures from Mouse Embryonic and Adult Cell Cultures. Cell *179*, 687-702 e618. 10.1016/j.cell.2019.09.029.
76. Kime, C., Kiyonari, H., Ohtsuka, S., Kohbayashi, E., Asahi, M., Yamanaka, S., Takahashi, M., and Tomoda, K. (2019). Induced 2C Expression and Implantation-Competent Blastocyst-like Cysts from Primed Pluripotent Stem Cells. Stem Cell Reports *13*, 485-498. 10.1016/j.stemcr.2019.07.011.
77. Zhang, P., Zhai, X., Huang, B., Sun, S., Wang, W., and Zhang, M. (2022). Highly efficient generation of blastocyst-like structures from mouse totipotent blastomere-like cells. Cold Spring Harbor Laboratory.
78. Gao, X., Nowak-Imialek, M., Chen, X., Chen, D., Herrmann, D., Ruan, D., Chen, A.C.H., Eckersley-Maslin, M.A., Ahmad, S., Lee, Y.L., et al. (2019). Establishment of porcine and human expanded potential stem cells. Nat Cell Biol *21*, 687-699. 10.1038/s41556-019-0333-2.
79. Liu, X., Tan, J.P., Schroder, J., Aberkane, A., Ouyang, J.F., Mohenska, M., Lim, S.M., Sun, Y.B.Y., Chen, J., Sun, G., et al. (2021). Modelling human blastocysts by reprogramming fibroblasts into iBlastoids. Nature *591*, 627-632. 10.1038/s41586-021-03372-y.
80. Tu, Z., Bi, Y., Zhu, X., Liu, W., Hu, J., Wu, L., Mao, T., Zhou, J., Wang, H., Wang, H., et al. (2023). Modeling human pregastrulation development by 3D culture of blastoids generated from primed-to-naive transitioning intermediates. Protein Cell *14*, 337-349. 10.1093/procel/pwac041.
81. Zhao, C., Reyes, A.P., Schell, J.P., Weltner, J., Ortega, N.M., Zheng, Y., Björklund, Å.K., Rossant, J., Fu, J., Petropoulos, S., and Lanner, F. (2021). Reprogrammed blastoids contain amnion-like cells but not trophectoderm. Cold Spring Harbor Laboratory.
82. Zheng, Y., Yan, R.Z., Sun, S., Kobayashi, M., Xiang, L., Yang, R., Goedel, A., Kang, Y., Xue, X., Esfahani, S.N., et al. (2022). Single-cell analysis of embryoids reveals lineage diversification roadmaps of early human development. Cell Stem Cell *29*, 1402-1419 e1408. 10.1016/j.stem.2022.08.009.
83. Clark, A.T., Brivanlou, A., Fu, J., Kato, K., Mathews, D., Niakan, K.K., Rivron, N., Saitou, M., Surani, A., Tang, F., and Rossant, J. (2021). Human embryo research, stem

cell-derived embryo models and in vitro gametogenesis: Considerations leading to the revised ISSCR guidelines. Stem Cell Reports *16*, 1416-1424. 10.1016/j.stemcr.2021.05.008.

84. Li, J., Zhu, Q., Cao, J., Liu, Y., Lu, Y., Sun, Y., Li, Q., Huang, Y., Shang, S., Bian, X., et al. (2023). Cynomolgus monkey embryo model captures gastrulation and early pregnancy. Cell Stem Cell *30*, 362-377 e367. 10.1016/j.stem.2023.03.009.
85. Pinzon-Arteaga, C.A., Wang, Y., Wei, Y., Ribeiro Orsi, A.E., Li, L., Scatolin, G., Liu, L., Sakurai, M., Ye, J., Hao, M., et al. (2023). Bovine blastocyst-like structures derived from stem cell cultures. Cell Stem Cell *30*, 611-616 e617. 10.1016/j.stem.2023.04.003.
86. Wang, Y., Ming, H., Yu, L., Li, J., Zhu, L., Sun, H.X., Pinzon-Arteaga, C.A., Wu, J., and Jiang, Z. (2023). Establishment of bovine trophoblast stem cells. Cell Rep *42*, 112439. 10.1016/j.celrep.2023.112439.
87. Zhao, L., Gao, X., Zheng, Y., Wang, Z., Zhao, G., Ren, J., Zhang, J., Wu, J., Wu, B., Chen, Y., et al. (2021). Establishment of bovine expanded potential stem cells. Proc Natl Acad Sci U S A *118*. 10.1073/pnas.2018505118.
88. Wu, J., and Izpisua Belmonte, J.C. (2015). Dynamic Pluripotent Stem Cell States and Their Applications. Cell Stem Cell *17*, 509-525. 10.1016/j.stem.2015.10.009.
89. Taei, A., Kiani, T., Taghizadeh, Z., Moradi, S., Samadian, A., Mollamohammadi, S., Sharifi-Zarchi, A., Guenther, S., Akhlaghpour, A., Asgari Abibeiglou, B., et al. (2020). Temporal activation of LRH-1 and RAR-gamma in human pluripotent stem cells induces a functional naive-like state. EMBO Rep *21*, e47533. 10.15252/embr.201847533.
90. Wu, J., Platero-Luengo, A., Sakurai, M., Sugawara, A., Gil, M.A., Yamauchi, T., Suzuki, K., Bogliotti, Y.S., Cuello, C., Morales Valencia, M., et al. (2017). Interspecies Chimerism with Mammalian Pluripotent Stem Cells. Cell *168*, 473-486 e415. 10.1016/j.cell.2016.12.036.
91. Aksoy, I., Rognard, C., Moulin, A., Marcy, G., Masfaraud, E., Wianny, F., Cortay, V., Bellemin-Menard, A., Doerflinger, N., Dirheimer, M., et al. (2021). Apoptosis, G1 Phase Stall, and Premature Differentiation Account for Low Chimeric Competence of Human and Rhesus Monkey Naive Pluripotent Stem Cells. Stem Cell Reports *16*, 56-74. 10.1016/j.stemcr.2020.12.004.
92. Theunissen, T.W., Friedli, M., He, Y., Planet, E., O'Neil, R.C., Markoulaki, S., Pontis, J., Wang, H., Iouranova, A., Imbeault, M., et al. (2016). Molecular Criteria for Defining the Naive Human Pluripotent State. Cell Stem Cell *19*, 502-515. 10.1016/j.stem.2016.06.011.
93. Takashima, Y., Guo, G., Loos, R., Nichols, J., Ficz, G., Krueger, F., Oxley, D., Santos, F., Clarke, J., Mansfield, W., et al. (2014). Resetting transcription factor control circuitry toward ground-state pluripotency in human. Cell *158*, 1254-1269. 10.1016/j.cell.2014.08.029.
94. Huang, K., Zhu, Y., Ma, Y., Zhao, B., Fan, N., Li, Y., Song, H., Chu, S., Ouyang, Z., Zhang, Q., et al. (2018). BMI1 enables interspecies chimerism with human pluripotent stem cells. Nat Commun *9*, 4649. 10.1038/s41467-018-07098-w.
95. Wang, X., Li, T., Cui, T., Yu, D., Liu, C., Jiang, L., Feng, G., Wang, L., Fu, R., Zhang, X., et al. (2018). Human embryonic stem cells contribute to embryonic and extraembryonic lineages in mouse embryos upon inhibition of apoptosis. Cell Res *28*, 126-129. 10.1038/cr.2017.138.
96. Ross, C., and Boroviak, T.E. (2020). Origin and function of the yolk sac in primate embryogenesis. Nat Commun *11*, 3760. 10.1038/s41467-020-17575-w.

97. Luckett, W.P. (1978). Origin and differentiation of the yolk sac and extraembryonic mesoderm in presomite human and rhesus monkey embryos. Am J Anat *152*, 59-97. 10.1002/aja.1001520106.
98. Shao, Y., Taniguchi, K., Gurdziel, K., Townshend, R.F., Xue, X., Yong, K.M.A., Sang, J., Spence, J.R., Gumucio, D.L., and Fu, J. (2017). Self-organized amniogenesis by human pluripotent stem cells in a biomimetic implantation-like niche. Nat Mater *16*, 419-425. 10.1038/nmat4829.
99. Pham, T.X.A., Panda, A., Kagawa, H., To, S.K., Ertekin, C., Georgolopoulos, G., van Knippenberg, S., Allsop, R.N., Bruneau, A., Chui, J.S., et al. (2022). Modeling human extraembryonic mesoderm cells using naive pluripotent stem cells. Cell Stem Cell *29*, 1346-1365 e1310. 10.1016/j.stem.2022.08.001.
100. Gerri, C., Menchero, S., Mahadevaiah, S.K., Turner, J.M.A., and Niakan, K.K. (2020). Human Embryogenesis: A Comparative Perspective. Annu Rev Cell Dev Biol *36*, 411-440. 10.1146/annurev-cellbio-022020-024900.
101. New, D.A., Coppola, P.T., and Terry, S. (1973). Culture of explanted rat embryos in rotating tubes. J Reprod Fertil *35*, 135-138. 10.1530/jrf.0.0350135.
102. Aguilera-Castrejon, A., Oldak, B., Shani, T., Ghanem, N., Itzkovich, C., Slomovich, S., Tarazi, S., Bayerl, J., Chugaeva, V., Ayyash, M., et al. (2021). Ex utero mouse embryogenesis from pre-gastrulation to late organogenesis. Nature *593*, 119-124. 10.1038/s41586-021-03416-3.
103. Taniguchi, K., Shao, Y., Townshend, R.F., Tsai, Y.H., DeLong, C.J., Lopez, S.A., Gayen, S., Freddo, A.M., Chue, D.J., Thomas, D.J., et al. (2015). Lumen Formation Is an Intrinsic Property of Isolated Human Pluripotent Stem Cells. Stem Cell Reports *5*, 954-962. 10.1016/j.stemcr.2015.10.015.
104. Shao, Y., Taniguchi, K., Townshend, R.F., Miki, T., Gumucio, D.L., and Fu, J. (2017). A pluripotent stem cell-based model for post-implantation human amniotic sac development. Nat Commun *8*, 208. 10.1038/s41467-017-00236-w.
105. Zheng, Y., Xue, X., Shao, Y., Wang, S., Esfahani, S.N., Li, Z., Muncie, J.M., Lakins, J.N., Weaver, V.M., Gumucio, D.L., and Fu, J. (2019). Controlled modelling of human epiblast and amnion development using stem cells. Nature *573*, 421-425. 10.1038/s41586-019-1535-2.
106. Simunovic, M., Metzger, J.J., Etoc, F., Yoney, A., Ruzo, A., Martyn, I., Croft, G., You, D.S., Brivanlou, A.H., and Siggia, E.D. (2019). A 3D model of a human epiblast reveals BMP4-driven symmetry breaking. Nat Cell Biol *21*, 900-910. 10.1038/s41556-019-0349-7.
107. Simunovic, M., Siggia, E.D., and Brivanlou, A.H. (2022). In vitro attachment and symmetry breaking of a human embryo model assembled from primed embryonic stem cells. Cell Stem Cell *29*, 962-972 e964. 10.1016/j.stem.2022.05.001.
108. Oldak, B., Wildschutz, E., Bondarenko, V., Comar, M.Y., Zhao, C., Aguilera-Castrejon, A., Tarazi, S., Viukov, S., Pham, T.X.A., Ashouokhi, S., et al. (2023). Complete human day 14 post-implantation embryo models from naive ES cells. Nature *622*, 562-573. 10.1038/s41586-023-06604-5.
109. Pedroza, M., Gassaloglu, S.I., Dias, N., Zhong, L., Hou, T.J., Kretzmer, H., Smith, Z.D., and Sozen, B. (2023). Self-patterning of human stem cells into post-implantation lineages. Nature *622*, 574-583. 10.1038/s41586-023-06354-4.

110. Weatherbee, B.A.T., Gantner, C.W., Iwamoto-Stohl, L.K., Daza, R.M., Hamazaki, N., Shendure, J., and Zernicka-Goetz, M. (2023). Pluripotent stem cell-derived model of the post-implantation human embryo. Nature *622*, 584-593. 10.1038/s41586-023-06368-y.
111. Okubo, T., Rivron, N., Kabata, M., Masaki, H., Kishimoto, K., Semi, K., Nakajima-Koyama, M., Kunitomi, H., Kaswandy, B., Sato, H., et al. (2023). Hypoblast from human pluripotent stem cells regulates epiblast development. Nature. 10.1038/s41586-023-06871-2.
112. Hislop, J., Song, Q., Keshavarz, F.K., Alavi, A., Schoenberger, R., LeGraw, R., Velazquez, J.J., Mokhtari, T., Taheri, M.N., Rytel, M., et al. (2023). Modelling post-implantation human development to yolk sac blood emergence. Nature. 10.1038/s41586-023-06914-8.
113. Liu, L., Oura, S., Markham, Z., Hamilton, J.N., Skory, R.M., Li, L., Sakurai, M., Wang, L., Pinzon-Arteaga, C.A., Plachta, N., et al. (2023). Modeling post-implantation stages of human development into early organogenesis with stem-cell-derived peri-gastruloids. Cell *186*, 3776-3792 e3716. 10.1016/j.cell.2023.07.018.
114. Tan, T., Wu, J., Si, C., Dai, S., Zhang, Y., Sun, N., Zhang, E., Shao, H., Si, W., Yang, P., et al. (2021). Chimeric contribution of human extended pluripotent stem cells to monkey embryos ex vivo. Cell *184*, 2020-2032 e2014. 10.1016/j.cell.2021.03.020.
115. Pastor, W.A., Chen, D., Liu, W., Kim, R., Sahakyan, A., Lukianchikov, A., Plath, K., Jacobsen, S.E., and Clark, A.T. (2016). Naive Human Pluripotent Cells Feature a Methylation Landscape Devoid of Blastocyst or Germline Memory. Cell Stem Cell *18*, 323-329. 10.1016/j.stem.2016.01.019.
116. Liu, X., Nefzger, C.M., Rossello, F.J., Chen, J., Knaupp, A.S., Firas, J., Ford, E., Pflueger, J., Paynter, J.M., Chy, H.S., et al. (2017). Comprehensive characterization of distinct states of human naive pluripotency generated by reprogramming. Nat Methods *14*, 1055-1062. 10.1038/nmeth.4436.
117. Liu, B., Chen, S., Xu, Y., Lyu, Y., Wang, J., Du, Y., Sun, Y., Liu, H., Zhou, H., Lai, W., et al. (2021). Chemically defined and xeno-free culture condition for human extended pluripotent stem cells. Nat Commun *12*, 3017. 10.1038/s41467-021-23320-8.
118. Wu, J., Okamura, D., Li, M., Suzuki, K., Luo, C., Ma, L., He, Y., Li, Z., Benner, C., Tamura, I., et al. (2015). An alternative pluripotent state confers interspecies chimaeric competency. Nature *521*, 316-321. 10.1038/nature14413.
119. Mascetti, V.L., and Pedersen, R.A. (2016). Human-Mouse Chimerism Validates Human Stem Cell Pluripotency. Cell Stem Cell *18*, 67-72. 10.1016/j.stem.2015.11.017.
120. Kwon, G.S., Viotti, M., and Hadjantonakis, A.K. (2008). The endoderm of the mouse embryo arises by dynamic widespread intercalation of embryonic and extraembryonic lineages. Dev Cell *15*, 509-520. 10.1016/j.devcel.2008.07.017.
121. Takaoka, K., and Hamada, H. (2012). Cell fate decisions and axis determination in the early mouse embryo. Development *139*, 3-14. 10.1242/dev.060095.
122. Nakamura, T., Okamoto, I., Sasaki, K., Yabuta, Y., Iwatani, C., Tsuchiya, H., Seita, Y., Nakamura, S., Yamamoto, T., and Saitou, M. (2016). A developmental coordinate of pluripotency among mice, monkeys and humans. Nature *537*, 57-62. 10.1038/nature19096.
123. Sasaki, K., Nakamura, T., Okamoto, I., Yabuta, Y., Iwatani, C., Tsuchiya, H., Seita, Y., Nakamura, S., Shiraki, N., Takakuwa, T., et al. (2016). The Germ Cell Fate of

Cynomolgus Monkeys Is Specified in the Nascent Amnion. Dev Cell *39*, 169-185. 10.1016/j.devcel.2016.09.007.

124. Mole, M.A., Coorens, T.H.H., Shahbazi, M.N., Weberling, A., Weatherbee, B.A.T., Gantner, C.W., Sancho-Serra, C., Richardson, L., Drinkwater, A., Syed, N., et al. (2021). A single cell characterisation of human embryogenesis identifies pluripotency transitions and putative anterior hypoblast centre. Nat Commun *12*, 3679. 10.1038/s41467-021-23758-w.

125. Ohinata, Y., Payer, B., O'Carroll, D., Ancelin, K., Ono, Y., Sano, M., Barton, S.C., Obukhanych, T., Nussenzweig, M., Tarakhovsky, A., et al. (2005). Blimp1 is a critical determinant of the germ cell lineage in mice. Nature *436*, 207-213. 10.1038/nature03813.

126. Saitou, M., Barton, S.C., and Surani, M.A. (2002). A molecular programme for the specification of germ cell fate in mice. Nature *418*, 293-300. 10.1038/nature00927.

127. Lawson, K.A., Dunn, N.R., Roelen, B.A., Zeinstra, L.M., Davis, A.M., Wright, C.V., Korving, J.P., and Hogan, B.L. (1999). Bmp4 is required for the generation of primordial germ cells in the mouse embryo. Genes Dev *13*, 424-436. 10.1101/gad.13.4.424.

128. Chen, D., Sun, N., Hou, L., Kim, R., Faith, J., Aslanyan, M., Tao, Y., Zheng, Y., Fu, J., Liu, W., et al. (2019). Human Primordial Germ Cells Are Specified from Lineage-Primed Progenitors. Cell Rep *29*, 4568-4582 e4565. 10.1016/j.celrep.2019.11.083.

129. Castillo-Venzor, A., Penfold, C.A., Morgan, M.D., Tang, W.W., Kobayashi, T., Wong, F.C., Bergmann, S., Slatery, E., Boroviak, T.E., Marioni, J.C., and Surani, M.A. (2023). Origin and segregation of the human germline. Life Sci Alliance *6*. 10.26508/lsa.202201706.

130. Sheng, G., Martinez Arias, A., and Sutherland, A. (2021). The primitive streak and cellular principles of building an amniote body through gastrulation. Science *374*, abg1727. 10.1126/science.abg1727.

131. Munoz-Sanjuan, I., and Brivanlou, A.H. (2002). Neural induction, the default model and embryonic stem cells. Nat Rev Neurosci *3*, 271-280. 10.1038/nrn786.

132. Colas, J.F., and Schoenwolf, G.C. (2001). Towards a cellular and molecular understanding of neurulation. Dev Dyn *221*, 117-145. 10.1002/dvdy.1144.

133. Wymeersch, F.J., Wilson, V., and Tsakiridis, A. (2021). Understanding axial progenitor biology in vivo and in vitro. Development *148*. 10.1242/dev.180612.

134. Santos, C., Murray, A., Marshall, A.R., Metcalfe, K., Narayan, P., de Castro, S.C.P., Maniou, E., Greene, N.D.E., Galea, G.L., and Copp, A.J. (2023). Spinal neural tube formation and regression in human embryos. eLife. 10.7554/eLife.88584.1.

135. Saitsu, H., and Shiota, K. (2008). Involvement of the axially condensed tail bud mesenchyme in normal and abnormal human posterior neural tube development. Congenit Anom (Kyoto) *48*, 1-6. 10.1111/j.1741-4520.2007.00178.x.

136. Simoes-Costa, M., and Bronner, M.E. (2015). Establishing neural crest identity: a gene regulatory recipe. Development *142*, 242-257. 10.1242/dev.105445.

137. Rocha, M., Beiriger, A., Kushkowski, E.E., Miyashita, T., Singh, N., Venkataraman, V., and Prince, V.E. (2020). From head to tail: regionalization of the neural crest. Development *147*. 10.1242/dev.193888.

138. Dubrulle, J., McGrew, M.J., and Pourquie, O. (2001). FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. Cell *106*, 219-232. 10.1016/s0092-8674(01)00437-8.

139. Dubrulle, J., and Pourquie, O. (2004). fgf8 mRNA decay establishes a gradient that couples axial elongation to patterning in the vertebrate embryo. Nature *427*, 419-422. 10.1038/nature02216.
140. Cooke, J., and Zeeman, E.C. (1976). A clock and wavefront model for control of the number of repeated structures during animal morphogenesis. J Theor Biol *58*, 455-476. 10.1016/s0022-5193(76)80131-2.
141. Harima, Y., Takashima, Y., Ueda, Y., Ohtsuka, T., and Kageyama, R. (2013). Accelerating the tempo of the segmentation clock by reducing the number of introns in the Hes7 gene. Cell Rep *3*, 1-7. 10.1016/j.celrep.2012.11.012.
142. Schroter, C., and Oates, A.C. (2010). Segment number and axial identity in a segmentation clock period mutant. Curr Biol *20*, 1254-1258. 10.1016/j.cub.2010.05.071.
143. Warmflash, A., Sorre, B., Etoc, F., Siggia, E.D., and Brivanlou, A.H. (2014). A method to recapitulate early embryonic spatial patterning in human embryonic stem cells. Nat Methods *11*, 847-854. 10.1038/nmeth.3016.
144. Muncie, J.M., Ayad, N.M.E., Lakins, J.N., Xue, X., Fu, J., and Weaver, V.M. (2020). Mechanical Tension Promotes Formation of Gastrulation-like Nodes and Patterns Mesoderm Specification in Human Embryonic Stem Cells. Dev Cell *55*, 679-694 e611. 10.1016/j.devcel.2020.10.015.
145. Manfrin, A., Tabata, Y., Paquet, E.R., Vuaridel, A.R., Rivest, F.R., Naef, F., and Lutolf, M.P. (2019). Engineered signaling centers for the spatially controlled patterning of human pluripotent stem cells. Nat Methods *16*, 640-648. 10.1038/s41592-019-0455-2.
146. van den Brink, S.C., Baillie-Johnson, P., Balayo, T., Hadjantonakis, A.K., Nowotschin, S., Turner, D.A., and Martinez Arias, A. (2014). Symmetry breaking, germ layer specification and axial organisation in aggregates of mouse embryonic stem cells. Development *141*, 4231-4242. 10.1242/dev.113001.
147. Beccari, L., Moris, N., Girgin, M., Turner, D.A., Baillie-Johnson, P., Cossy, A.C., Lutolf, M.P., Duboule, D., and Arias, A.M. (2018). Multi-axial self-organization properties of mouse embryonic stem cells into gastruloids. Nature *562*, 272-276. 10.1038/s41586-018-0578-0.
148. van den Brink, S.C., Alemany, A., van Batenburg, V., Moris, N., Blotenburg, M., Vivie, J., Baillie-Johnson, P., Nichols, J., Sonnen, K.F., Martinez Arias, A., and van Oudenaarden, A. (2020). Single-cell and spatial transcriptomics reveal somitogenesis in gastruloids. Nature *582*, 405-409. 10.1038/s41586-020-2024-3.
149. Veenvliet, J.V., Bolondi, A., Kretzmer, H., Haut, L., Scholze-Wittler, M., Schifferl, D., Koch, F., Guignard, L., Kumar, A.S., Pustet, M., et al. (2020). Mouse embryonic stem cells self-organize into trunk-like structures with neural tube and somites. Science *370*. 10.1126/science.aba4937.
150. Rossi, G., Broguiere, N., Miyamoto, M., Boni, A., Guiet, R., Girgin, M., Kelly, R.G., Kwon, C., and Lutolf, M.P. (2021). Capturing Cardiogenesis in Gastruloids. Cell Stem Cell *28*, 230-240 e236. 10.1016/j.stem.2020.10.013.
151. Rossi, G., Giger, S., Hübscher, T., and Lutolf, M.P. (2021). Gastruloids asin vitromodels of embryonic blood development with spatial and temporal resolution. Cold Spring Harbor Laboratory.
152. Girgin, M.U., Broguiere, N., Mattolini, L., and Lutolf, M.P. (2021). Gastruloids generated without exogenous Wnt activation develop anterior neural tissues. Stem Cell Reports *16*, 1143-1155. 10.1016/j.stemcr.2021.03.017.

153. Xu, P.F., Borges, R.M., Fillatre, J., de Oliveira-Melo, M., Cheng, T., Thisse, B., and Thisse, C. (2021). Construction of a mammalian embryo model from stem cells organized by a morphogen signalling centre. Nat Commun *12*, 3277. 10.1038/s41467-021-23653-4.
154. Girgin, M.U., Broguiere, N., Hoehnel, S., Brandenberg, N., Mercier, B., Arias, A.M., and Lutolf, M.P. (2021). Bioengineered embryoids mimic post-implantation development in vitro. Nat Commun *12*, 5140. 10.1038/s41467-021-25237-8.
155. Bérenger-Currias, N.M.L.P., Mircea, M., Adegeest, E., Van Den Berg, P.R., Feliksik, M., Hochane, M., Idema, T., Tans, S.J., and Semrau, S. (2020). Extraembryonic endoderm cells induce neuroepithelial tissue in gastruloids. Cold Spring Harbor Laboratory.
156. Harrison, S.E., Sozen, B., Christodoulou, N., Kyprianou, C., and Zernicka-Goetz, M. (2017). Assembly of embryonic and extraembryonic stem cells to mimic embryogenesis in vitro. Science *356*, eaal1810. 10.1126/science.aal1810.
157. Sozen, B., Amadei, G., Cox, A., Wang, R., Na, E., Czukiewska, S., Chappell, L., Voet, T., Michel, G., Jing, N., et al. (2018). Self-assembly of embryonic and two extra-embryonic stem cell types into gastrulating embryo-like structures. Nat Cell Biol *20*, 979-989. 10.1038/s41556-018-0147-7.
158. Zhang, S., Chen, T., Chen, N., Gao, D., Shi, B., Kong, S., West, R.C., Yuan, Y., Zhi, M., Wei, Q., et al. (2019). Implantation initiation of self-assembled embryo-like structures generated using three types of mouse blastocyst-derived stem cells. Nat Commun *10*, 496. 10.1038/s41467-019-08378-9.
159. Amadei, G., Lau, K.Y.C., De Jonghe, J., Gantner, C.W., Sozen, B., Chan, C., Zhu, M., Kyprianou, C., Hollfelder, F., and Zernicka-Goetz, M. (2021). Inducible Stem-Cell-Derived Embryos Capture Mouse Morphogenetic Events In Vitro. Dev Cell *56*, 366-382 e369. 10.1016/j.devcel.2020.12.004.
160. Moris, N., Anlas, K., van den Brink, S.C., Alemany, A., Schroder, J., Ghimire, S., Balayo, T., van Oudenaarden, A., and Martinez Arias, A. (2020). An in vitro model of early anteroposterior organization during human development. Nature *582*, 410-415. 10.1038/s41586-020-2383-9.
161. Olmsted, Z.T., and Paluh, J.L. (2021). Co-development of central and peripheral neurons with trunk mesendoderm in human elongating multi-lineage organized gastruloids. Nat Commun *12*, 3020. 10.1038/s41467-021-23294-7.
162. Yaman, Y.I., Huang, R., and Ramanathan, S. (2022). Coupled organoids reveal that signaling gradients drive traveling segmentation clock waves during human axial morphogenesis. Cold Spring Harbor Laboratory.
163. Yaman, Y.I., and Ramanathan, S. (2023). Controlling human organoid symmetry breaking reveals signaling gradients drive segmentation clock waves. Cell *186*, 513-527 e519. 10.1016/j.cell.2022.12.042.
164. Gribaudo, S., Robert, R., van Sambeek, B., Mirdass, C., Lyubimova, A., Bouhali, K., Ferent, J., Morin, X., van Oudenaarden, A., and Nedelec, S. (2023). Self-organizing models of human trunk organogenesis recapitulate spinal cord and spine co-morphogenesis. Nat Biotechnol. 10.1038/s41587-023-01956-9.
165. Miao, Y., Djeffal, Y., De Simone, A., Zhu, K., Silberfeld, A., Lee, J.G., Rao, J., Tarazona, O.A., Mongera, A., Rigoni, P., et al. (2022). Reconstruction and deconstruction of human somitogenesis in vitro. Cold Spring Harbor Laboratory.

166. Sanaki-Matsumiya, M., Matsuda, M., Gritti, N., Nakaki, F., Sharpe, J., Trivedi, V., and Ebisuya, M. (2022). Periodic formation of epithelial somites from human pluripotent stem cells. Nat Commun *13*, 2325. 10.1038/s41467-022-29967-1.
167. Yamanaka, Y., Hamidi, S., Yoshioka-Kobayashi, K., Munira, S., Sunadome, K., Zhang, Y., Kurokawa, Y., Ericsson, R., Mieda, A., Thompson, J.L., et al. (2023). Reconstituting human somitogenesis in vitro. Nature *614*, 509-520. 10.1038/s41586-022-05649-2.
168. Meinhardt, A., Eberle, D., Tazaki, A., Ranga, A., Niesche, M., Wilsch-Brauninger, M., Stec, A., Schackert, G., Lutolf, M., and Tanaka, E.M. (2014). 3D reconstitution of the patterned neural tube from embryonic stem cells. Stem Cell Reports *3*, 987-999. 10.1016/j.stemcr.2014.09.020.
169. Duval, N., Vaslin, C., Barata, T.C., Frarma, Y., Contremoulins, V., Baudin, X., Nedelec, S., and Ribes, V.C. (2019). BMP4 patterns Smad activity and generates stereotyped cell fate organization in spinal organoids. Development *146*, dev175430. 10.1242/dev.175430.
170. Ogura, T., Sakaguchi, H., Miyamoto, S., and Takahashi, J. (2018). Three-dimensional induction of dorsal, intermediate and ventral spinal cord tissues from human pluripotent stem cells. Development *145*, dev162214. 10.1242/dev.162214.
171. Zheng, Y., Xue, X., Resto-Irizarry, A.M., Li, Z., Shao, Y., Zheng, Y., Zhao, G., and Fu, J. (2019). Dorsal-ventral patterned neural cyst from human pluripotent stem cells in a neurogenic niche. Sci Adv *5*, eaax5933. 10.1126/sciadv.aax5933.
172. Abdel Fattah, A.R., Daza, B., Rustandi, G., Berrocal-Rubio, M.A., Gorissen, B., Poovathingal, S., Davie, K., Barrasa-Fano, J., Condor, M., Cao, X., et al. (2021). Actuation enhances patterning in human neural tube organoids. Nat Commun *12*, 3192. 10.1038/s41467-021-22952-0.
173. Xue, X., Sun, Y., Resto-Irizarry, A.M., Yuan, Y., Aw Yong, K.M., Zheng, Y., Weng, S., Shao, Y., Chai, Y., Studer, L., and Fu, J. (2018). Mechanics-guided embryonic patterning of neuroectoderm tissue from human pluripotent stem cells. Nat Mater *17*, 633-641. 10.1038/s41563-018-0082-9.
174. Britton, G., Heemskerk, I., Hodge, R., Qutub, A.A., and Warmflash, A. (2019). A novel self-organizing embryonic stem cell system reveals signaling logic underlying the patterning of human ectoderm. Development *146*, dev179093. 10.1242/dev.179093.
175. Haremaki, T., Metzger, J.J., Rito, T., Ozair, M.Z., Etoc, F., and Brivanlou, A.H. (2019). Self-organizing neuruloids model developmental aspects of Huntington's disease in the ectodermal compartment. Nat Biotechnol *37*, 1198-1208. 10.1038/s41587-019-0237-5.
176. Karzbrun, E., Khankhel, A.H., Megale, H.C., Glasauer, S.M.K., Wyle, Y., Britton, G., Warmflash, A., Kosik, K.S., Siggia, E.D., Shraiman, B.I., and Streichan, S.J. (2021). Human neural tube morphogenesis in vitro by geometric constraints. Nature *599*, 268-272. 10.1038/s41586-021-04026-9.
177. Sahni, G., Chang, S.Y., Meng, J.T.C., Tan, J.Z.Y., Fatien, J.J.C., Bonnard, C., Utami, K.H., Chan, P.W., Tan, T.T., Altunoglu, U., et al. (2021). A Micropatterned Human-Specific Neuroepithelial Tissue for Modeling Gene and Drug-Induced Neurodevelopmental Defects. Adv Sci (Weinh) *8*, 2001100. 10.1002/advs.202001100.
178. Sun, S., Xue, X., and Fu, J. (2023). Modeling development using microfluidics: bridging gaps to foster fundamental and translational research. Curr Opin Genet Dev *82*, 102097. 10.1016/j.gde.2023.102097.

179. Rifes, P., Isaksson, M., Rathore, G.S., Aldrin-Kirk, P., Møller, O.K., Barzaghi, G., Lee, J., Egerod, K.L., Rausch, D.M., Parmar, M., et al. (2020). Modeling neural tube development by differentiation of human embryonic stem cells in a microfluidic WNT gradient. Nat Biotechnol *38*, 1265-1273. 10.1038/s41587-020-0525-0.
180. Xue, X., Kim, Y.S., Ponce-Arias, A.I., O'Laughlin, R., Yan, R.Z., Kobayashi, N., Tshuva, R.Y., Tsai, Y.H., Sun, S., Zheng, Y., et al. (2024). A Patterned Human Neural Tube Model Using Microfluidic Gradients. Nature. 10.1038/s41586-024-07204-7.
181. Das, S., Koyano-Nakagawa, N., Gafni, O., Maeng, G., Singh, B.N., Rasmussen, T., Pan, X., Choi, K.D., Mickelson, D., Gong, W., et al. (2020). Generation of human endothelium in pig embryos deficient in ETV2. Nat Biotechnol *38*, 297-302. 10.1038/s41587-019-0373-y.
182. Maeng, G., Das, S., Greising, S.M., Gong, W., Singh, B.N., Kren, S., Mickelson, D., Skie, E., Gafni, O., Sorensen, J.R., et al. (2021). Humanized skeletal muscle in MYF5/MYOD/MYF6-null pig embryos. Nat Biomed Eng *5*, 805-814. 10.1038/s41551-021-00693-1.
183. Wang, J., Xie, W., Li, N., Li, W., Zhang, Z., Fan, N., Ouyang, Z., Zhao, Y., Lai, C., Li, H., et al. (2023). Generation of a humanized mesonephros in pigs from induced pluripotent stem cells via embryo complementation. Cell Stem Cell *30*, 1235-1245 e1236. 10.1016/j.stem.2023.08.003.
184. Fu, J., Warmflash, A., and Lutolf, M.P. (2021). Stem-cell-based embryo models for fundamental research and translation. Nat Mater *20*, 132-144. 10.1038/s41563-020-00829-9.
185. Shibata, S., Endo, S., Nagai, L.A.E., E, H.K., Oike, A., Kobayashi, N., Kitamura, A., Hori, T., Nashimoto, Y., Nakato, R., et al. (2024). Modeling embryo-endometrial interface recapitulating human embryo implantation. Sci Adv *10*, eadi4819. 10.1126/sciadv.adi4819.
186. Wu, J., Greely, H.T., Jaenisch, R., Nakauchi, H., Rossant, J., and Belmonte, J.C. (2016). Stem cells and interspecies chimaeras. Nature *540*, 51-59. 10.1038/nature20573.
187. Morata, G., and Ripoll, P. (1975). Minutes: mutants of drosophila autonomously affecting cell division rate. Dev Biol *42*, 211-221. 10.1016/0012-1606(75)90330-9.
188. Baker, N.E. (2020). Emerging mechanisms of cell competition. Nat Rev Genet *21*, 683-697. 10.1038/s41576-020-0262-8.
189. Zhu, Y., Zhang, Z., Fan, N., Huang, K., Li, H., Gu, J., Zhang, Q., Ouyang, Z., Zhang, T., Tang, J., et al. (2022). Generating functional cells through enhanced interspecies chimerism with human pluripotent stem cells. Stem Cell Reports *17*, 1059-1069. 10.1016/j.stemcr.2022.03.009.
190. Zheng, C., Hu, Y., Sakurai, M., Pinzon-Arteaga, C.A., Li, J., Wei, Y., Okamura, D., Ravaux, B., Barlow, H.R., Yu, L., et al. (2021). Cell competition constitutes a barrier for interspecies chimerism. Nature *592*, 272-276. 10.1038/s41586-021-03273-0.
191. Meyer, S.N., Amoyel, M., Bergantinos, C., de la Cova, C., Schertel, C., Basler, K., and Johnston, L.A. (2014). An ancient defense system eliminates unfit cells from developing tissues during cell competition. Science *346*, 1258236. 10.1126/science.1258236.
192. Hu, Y., Sun, H.X., Sakurai, M., Jones, A.E., Liu, L., Cheng, T., Zheng, C., Li, J., Ravaux, B., Luo, Z., et al. (2023). RNA Sensing and Innate Immunity Constitutes a Barrier for Interspecies Chimerism. bioRxiv, 2023.2003.2007.531624. 10.1101/2023.03.07.531624.

193. Glass, D.S., and Riedel-Kruse, I.H. (2018). A Synthetic Bacterial Cell-Cell Adhesion Toolbox for Programming Multicellular Morphologies and Patterns. Cell *174*, 649-658 e616. 10.1016/j.cell.2018.06.041.
194. Stevens, A.J., Harris, A.R., Gerdts, J., Kim, K.H., Trentesaux, C., Ramirez, J.T., McKeithan, W.L., Fattahi, F., Klein, O.D., Fletcher, D.A., and Lim, W.A. (2023). Programming multicellular assembly with synthetic cell adhesion molecules. Nature *614*, 144-152. 10.1038/s41586-022-05622-z.
195. Beghein, E., and Gettemans, J. (2017). Nanobody Technology: A Versatile Toolkit for Microscopic Imaging, Protein-Protein Interaction Analysis, and Protein Function Exploration. Front Immunol *8*, 771. 10.3389/fimmu.2017.00771.
196. Rayon, T., Stamataki, D., Perez-Carrasco, R., Garcia-Perez, L., Barrington, C., Melchionda, M., Exelby, K., Lazaro, J., Tybulewicz, V.L.J., Fisher, E.M.C., and Briscoe, J. (2020). Species-specific pace of development is associated with differences in protein stability. Science *369*. 10.1126/science.aba7667.
197. Barry, C., Schmitz, M.T., Jiang, P., Schwartz, M.P., Duffin, B.M., Swanson, S., Bacher, R., Bolin, J.M., Elwell, A.L., McIntosh, B.E., et al. (2017). Species-specific developmental timing is maintained by pluripotent stem cells ex utero. Dev Biol *423*, 101-110. 10.1016/j.ydbio.2017.02.002.
198. Matsuda, M., Yamanaka, Y., Uemura, M., Osawa, M., Saito, M.K., Nagahashi, A., Nishio, M., Guo, L., Ikegawa, S., Sakurai, S., et al. (2020). Recapitulating the human segmentation clock with pluripotent stem cells. Nature *580*, 124-129. 10.1038/s41586-020-2144-9.
199. Diaz-Cuadros, M., Wagner, D.E., Budjan, C., Hubaud, A., Tarazona, O.A., Donelly, S., Michaut, A., Al Tanoury, Z., Yoshioka-Kobayashi, K., Niino, Y., et al. (2020). In vitro characterization of the human segmentation clock. Nature *580*, 113-118. 10.1038/s41586-019-1885-9.
200. Chu, L.F., Mamott, D., Ni, Z., Bacher, R., Liu, C., Swanson, S., Kendziorski, C., Stewart, R., and Thomson, J.A. (2019). An In Vitro Human Segmentation Clock Model Derived from Embryonic Stem Cells. Cell Rep *28*, 2247-2255 e2245. 10.1016/j.celrep.2019.07.090.
201. Lazaro, J., Costanzo, M., Sanaki-Matsumiya, M., Girardot, C., Hayashi, M., Hayashi, K., Diecke, S., Hildebrandt, T.B., Lazzari, G., Wu, J., et al. (2023). A stem cell zoo uncovers intracellular scaling of developmental tempo across mammals. Cell Stem Cell *30*, 938-949 e937. 10.1016/j.stem.2023.05.014.
202. Iwata, R., Casimir, P., Erkol, E., Boubakar, L., Planque, M., Gallego Lopez, I.M., Ditkowska, M., Gaspariunaite, V., Beckers, S., Remans, D., et al. (2023). Mitochondria metabolism sets the species-specific tempo of neuronal development. Science *379*, eabn4705. 10.1126/science.abn4705.
203. Brown, J., Barry, C., Schmitz, M.T., Argus, C., Bolin, J.M., Schwartz, M.P., Van Aartsen, A., Steill, J., Swanson, S., Stewart, R., et al. (2021). Interspecies chimeric conditions affect the developmental rate of human pluripotent stem cells. PLoS Comput Biol *17*, e1008778. 10.1371/journal.pcbi.1008778.
204. Huang, J., He, B., Yang, X., Long, X., Wei, Y., Li, L., Tang, M., Gao, Y., Fang, Y., Ying, W., et al. (2024). Generation of rat forebrain tissues in mice. Cell *187*, 2129-2142 e2117. 10.1016/j.cell.2024.03.017.

205. Throesch, B.T., Khadeesh bin Imtiaz, M., Muñoz-Castañeda, R., Sakurai, M., Hartzell, A.L., James, K.N., Rodriguez, A.R., Martin, G., Lippi, G., Kupriyanov, S., et al. (2023). Building functional circuits in multispecies brains. bioRxiv, 2023.2004.2013.536815. 10.1101/2023.04.13.536815.
206. Cohen, M.A., Wert, K.J., Goldmann, J., Markoulaki, S., Buganim, Y., Fu, D., and Jaenisch, R. (2016). Human neural crest cells contribute to coat pigmentation in interspecies chimeras after in utero injection into mouse embryos. Proc Natl Acad Sci U S A *113*, 1570-1575. 10.1073/pnas.1525518113.
207. Jiang, X., Gurel, O., Mendiaz, E.A., Stearns, G.W., Clogston, C.L., Lu, H.S., Osslund, T.D., Syed, R.S., Langley, K.E., and Hendrickson, W.A. (2000). Structure of the active core of human stem cell factor and analysis of binding to its receptor kit. EMBO J *19*, 3192-3203. 10.1093/emboj/19.13.3192.
208. Yamaguchi, T., Sato, H., Kobayashi, T., Kato-Itoh, M., Goto, T., Hara, H., Mizuno, N., Yanagida, A., Umino, A., Hamanaka, S., et al. (2018). An interspecies barrier to tetraploid complementation and chimera formation. Sci Rep *8*, 15289. 10.1038/s41598-018-33690-7.
209. Hyun, I., Clayton, E.W., Cong, Y., Fujita, M., Goldman, S.A., Hill, L.R., Monserrat, N., Nakauchi, H., Pedersen, R.A., Rooke, H.M., et al. (2021). ISSCR guidelines for the transfer of human pluripotent stem cells and their direct derivatives into animal hosts. Stem Cell Reports *16*, 1409-1415. 10.1016/j.stemcr.2021.05.005.

Figure 1

Click here to access/download;Figure;Wu and Fu_Figure 1.pdf

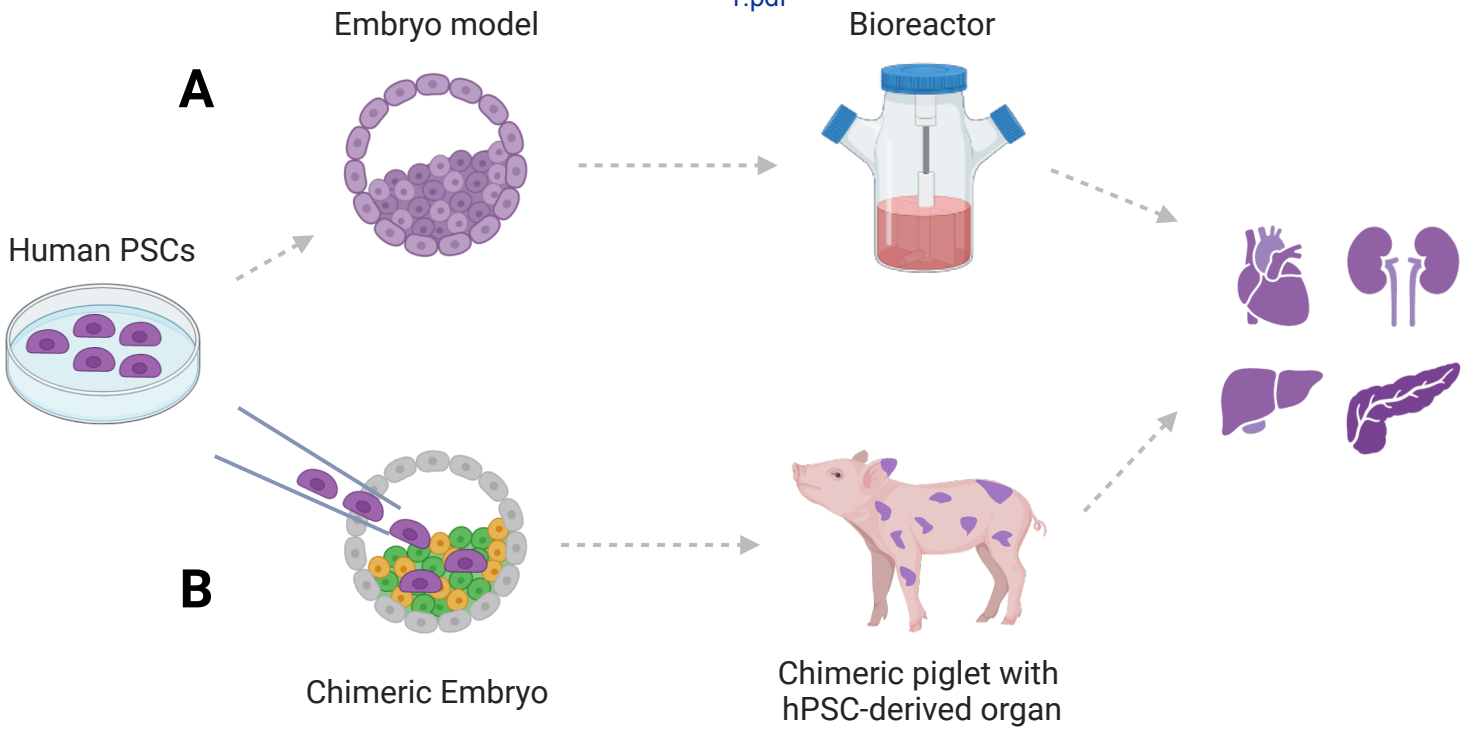


Figure 2

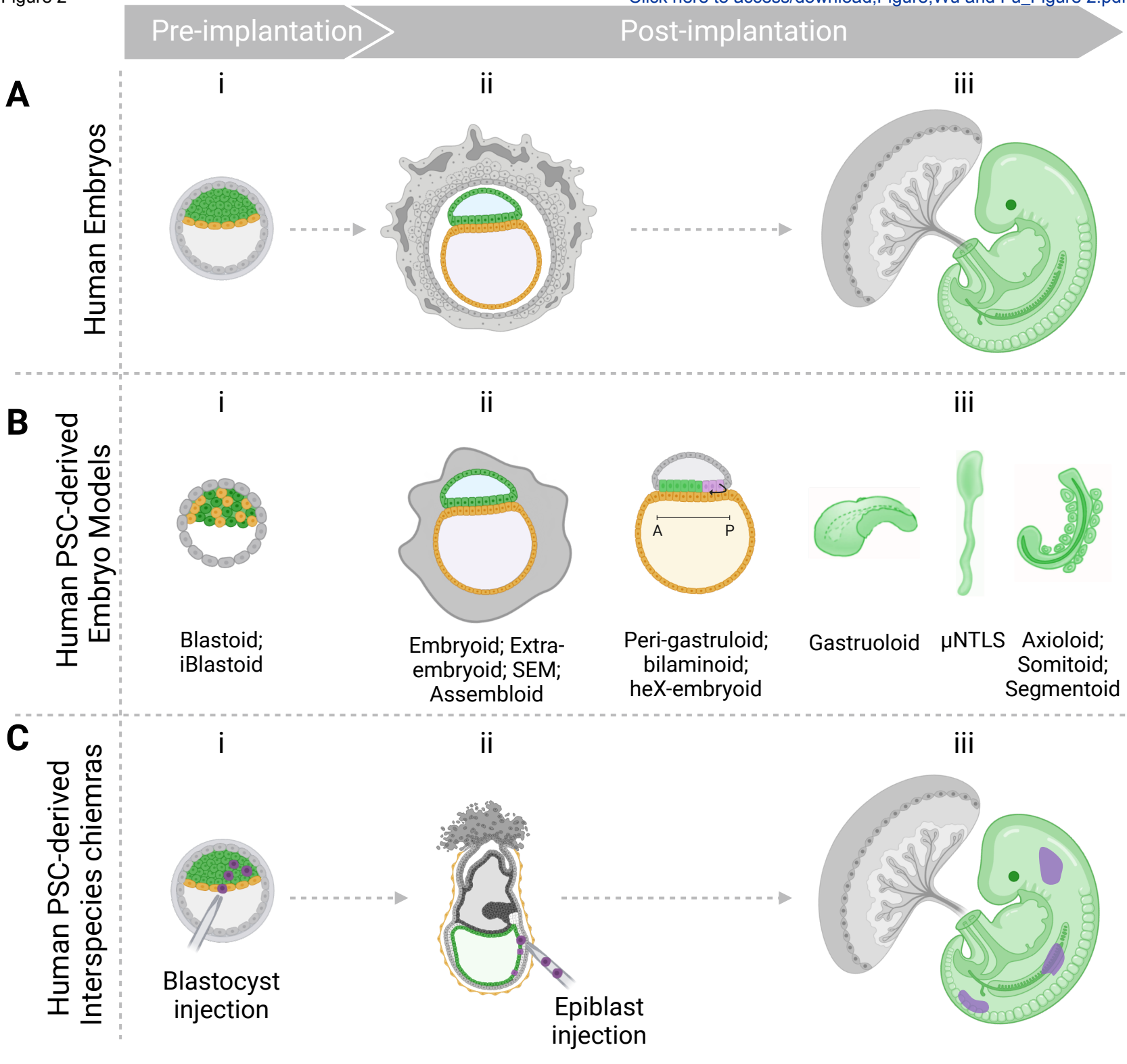


Figure 3

Click here to access/download;Figure;Wu and Fu_Figure 3.pdf

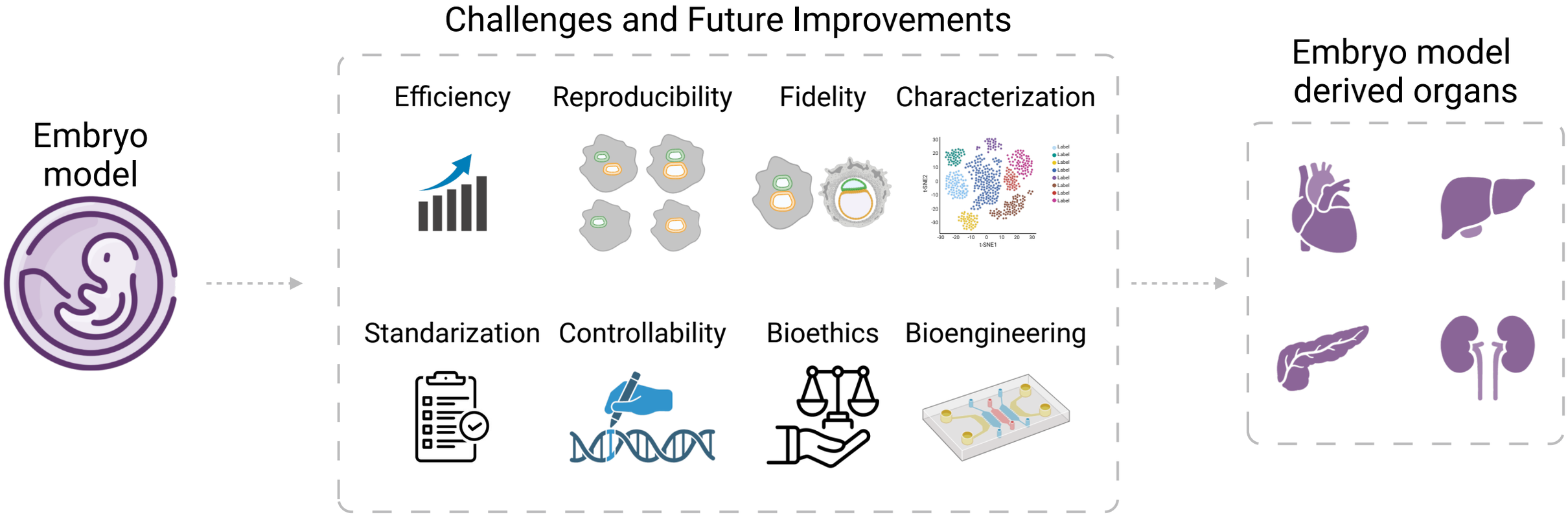


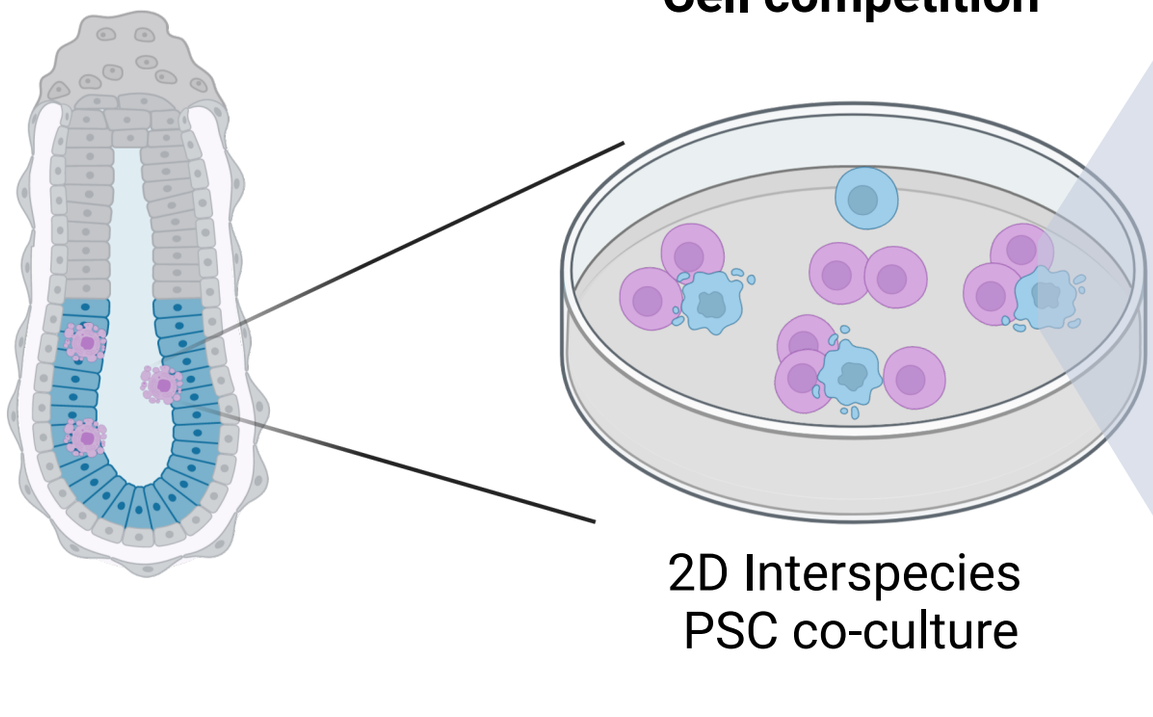
Figure 4

Click here to access/download;Figure;Wu and Fu_Figure 4.pdf

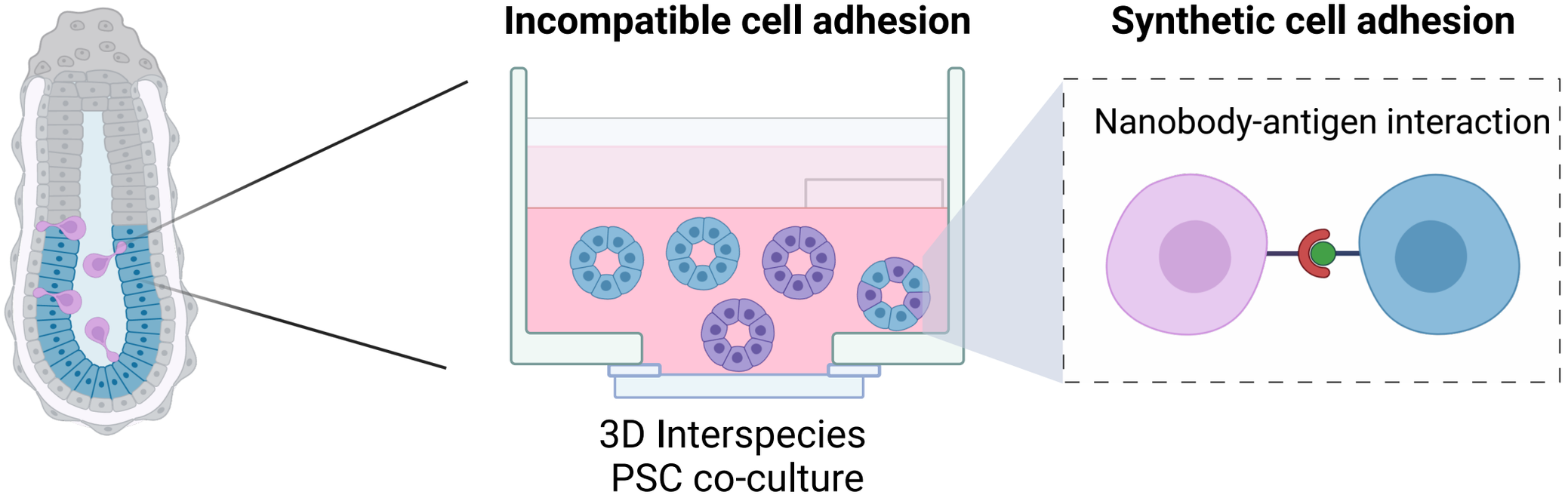
Donor cells (human)

Host cells (mouse)

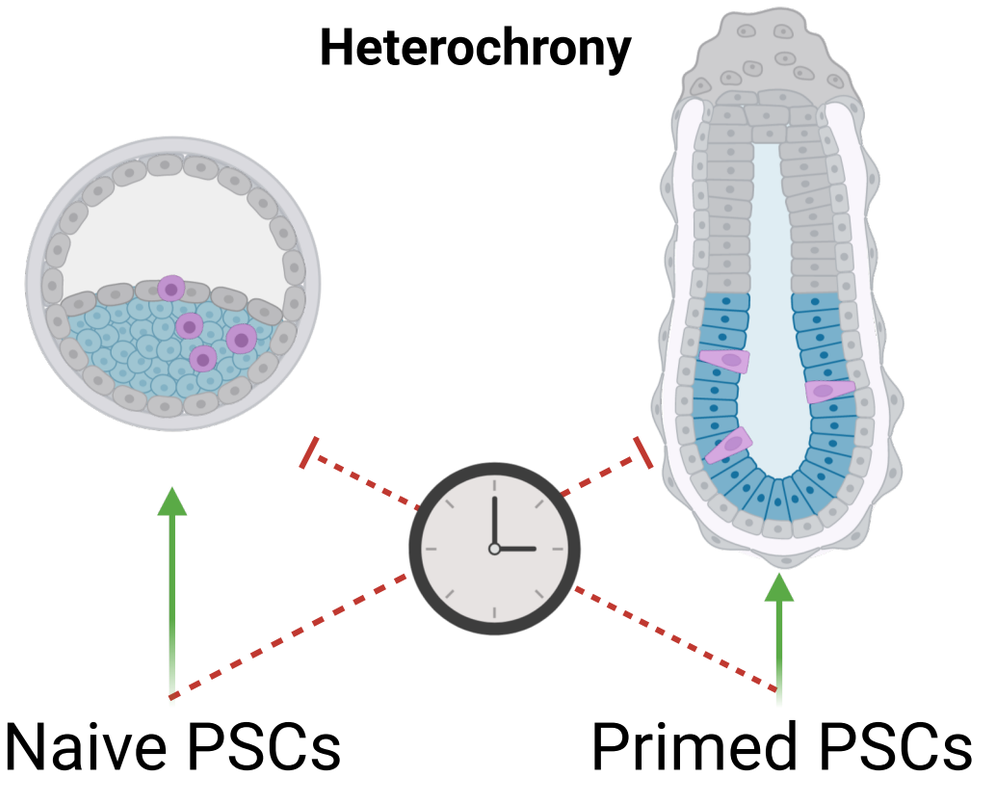
A



B



C



D