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Modeling Human Embryo Development with Embryonic and Extra-Embryonic Stem Cells

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Abstract

Early human post-implantation development involves extensive growth combined with a series of complex morphogenetic events. The lack of precise spatial and temporal control over these processes leads to pregnancy loss. Given the ethical and technical limitations in studying the natural human embryo, alternative approaches are needed to investigate mechanisms underlying this critical stage of human development. Here, we present an overview of the different stem cells and stem cell-derived models which serve as useful, albeit imperfect, tools in understanding human embryogenesis. Current models include stem cells that represent each of the three earliest lineages: human embryonic stem cells corresponding to the epiblast, hypoblast-like stem cells and trophoblast stem cells. We also review the use of human embryonic stem cells to model complex aspects of epiblast morphogenesis and differentiation. Additionally, we propose that the combination of both embryonic and extra-embryonic stem cells to form three-dimensional embryo models will provide valuable insights into cell-cell chemical and mechanical interactions that are essential for natural embryogenesis.

Introduction

Human embryo development has been a topic of great interest for millennia, yet much of our knowledge comes from the study of mouse embryogenesis and non-human primates due to limitations in studying human embryos. Nevertheless, following successful human oocyte fertilization and subsequent development *ex vivo*[1], we have a basic picture of human embryology until day 7 post-fertilization[2]. More recently, the development of new culture methods has allowed us to expand our knowledge of human development up to day 13/14[3–5], the legal limit of human embryo culture.

During the first 7 days of development, the embryo develops from a fertilized zygote to a blastocyst comprised of the epiblast (precursor to the embryo proper), the hypoblast (precursor to the yolk sac) and the trophectoderm (precursor to the embryonic portion of the placenta)[6–8] (Fig.1). During the second week of development, the blastocyst implants into the maternal uterus via the trophectoderm and undergoes subsequent complex morphogenetic events. This includes the polarization of the epiblast to form a central lumen (precursor to the amniotic cavity), formation of the amniotic epithelium (together with future amniotic mesoderm will form amniotic sac membrane), and possibly the differentiation of primordial germ cells (PGCs), the precursors of eggs or sperm, the origin of which is

unknown[9–11]. Further, extra-embryonic mesenchyme cells emerge, possibly from the hypoblast, and fill the luminal space between the trophoblast and other lineages[5,9,12]. On day 13/14, the primitive streak will form at the posterior of the epiblast, giving rise to the formation of three germ layers of the embryo proper. In-depth descriptions of human embryo development during these stages are reviewed elsewhere[2,8].

Many questions remain unanswered concerning these first 14 days of human embryo development. For example, what is the role and function of the key transcription factors that underlie lineage segregation? How is the amniotic epithelium specified? What is the origin of PGCs in the human embryo? Is human epiblast pre-patterned to specify the anterior-posterior axis prior to gastrulation? The ability to achieve molecular and mechanistic understanding of human development is limited when using natural human embryos due to the technical and ethical barriers in using genetic tools. Additionally, many donated embryos from patients undergoing *in vitro* fertilization treatment may harbor chromosomal translocations, aneuploidies, or harmful mutations[13]. Thus, an alternative approach is needed. While some insights have been gleaned from non-human primates, strong ethical limitations still exist while conservation of gene function and related mechanisms with the human context is not guaranteed. Further, in-depth mechanistic understanding is aided by the use of simplified models. Here, we discuss how stem cells resembling the first three lineages will provide promising complementary tools to model aspects of pre-gastrulation human development and dissect their inner-workings.

Human Stem Cells: Simple Models of Individual Tissues

Stem cells cultured under conventional conditions are, themselves, simple models of different tissues of the embryo. Using genetic perturbation, reporter assays that allow for the visualization of gene expression and signaling dynamics, and small molecule screens, stem cells can provide valuable insights into the mechanisms underlying their identity maintenance, differentiation potential, signaling dynamics and more. However, it has proven challenging to benchmark different stem cells against the embryo to understand how accurately, and at what stage, they recapitulate their respective tissues. Here we will discuss the derivation of different stem cells and their recapitulation of specific tissues in the early post-implantation human embryo.

Human Embryonic Stem Cells

Human embryonic stem cells (hESCs) which can be cultured *in vitro* indefinitely and retain key epiblast marker expression, such as the transcription factors OCT4 and SOX2, were first derived from the blastocyst-stage epiblast in 1998[14]. Conventionally, hESCs are cultured in media including factors that activate FGF and TGFB/Activin A signaling pathways. Overexpression of key epiblast genes in adult somatic cells also resulted in reprogrammed cells with hESC-like identities (human induced pluripotent stem cells; hiPSCs), which can also be propagated in FGF/ActivinA conditions[15–17]. Following their derivation, hESCs have become a valuable tool in understanding stem cell differentiation, and their clinical applications are expanding[18]. hESCs are also a valuable model to study pluripotency and differentiation potential, providing a useful tool in both modeling disease and investigating aspects of the human epiblast.

hESCs have been validated in their ability to give rise to cells derived from all three germ layers[19,20]. The potency of hESCs in standard culture conditions does not match that of mouse ESCs (mESCs), which exhibit features that more closely recapitulate a “naïve” state of pluripotency of the pre-implantation epiblast. In contrast, hESCs exhibit a primed state of pluripotency reminiscent of later post-

implantation epiblast and display higher levels of DNA methylation, expression of primed markers such as the signaling factor *FGF2*, and less compact cell colony morphology. Thus, many groups sought to capture the earlier, naïve pluripotency state exhibited by the blastocyst epiblast which is characterized by decreased DNA methylation and expression of distinct markers such as KLF4, *Xist* (representing the initiation of X-chromosome silencing in XX embryos) and certain transposable elements[21–24]. Many protocols have been developed to generate naïve hESCs (Table 1). Some rely on converting conventional, primed hESCs or hiPSCs with small molecules and varied media conditions. These conditions commonly involve inhibition of GSK3, ERK, PKC, and Rock pathways, LIF supplementation, and are sometimes coupled with targeted overexpression of specific proteins. Additionally, naïve hESCs can be derived directly from the blastocyst[24-35]. Each of these protocols results in different "naïve" cells, all of which tend to have a transcriptional profile distinct from primed hESCs and an increase in global demethylation. However, many lack the transposable elements expression profile matching that of the pre-implantation epiblast and instead more closely resemble the morula[22]. Others still seem to be more transcriptionally similar to the post-implantation epiblast rather than pre-implantation, including "Extended Potential" and "NHSM" cells[36].

Comparative work using naïve hiPSCs derived using different protocols has highlighted differences in transcriptional identity, methylation patterns, and chromosomal stability[37]. Specifically, two types of naïve cells showed the most similarity to the human pre-implantation epiblast both transcriptionally and epigenetically: 5i/L/A(F)[27] cells and Reset (t2iLGö/PXGL) cells[28,31,33,37]. Naïve cells vary in their capacity to respond to germ layer differentiation cues: Reset (t2iLGö/PXGL) cells require "capacitation" while other cells, such as the recently reported "HENSM" cells, do not[38,39]. The distinctions between different "naïve" hESCs raise questions about the nature of human naïve pluripotency, and how well these different protocols generate cells that recapitulate the pluripotency state of the pre-implantation epiblast. Limitations in using hESCs as a model of the epiblast also include variation across different hESC lines in gene expression levels, epigenetic properties, and more[40]. While these different cell lines are useful in investigating aspects of pluripotency state and differentiation, more robust comparative work is needed to understand the nature of pluripotency transitions/states – both in how well different hESC types correspond to the epiblast *in vivo* and how they compare to each other *in vitro*.

Human Hypoblast-Like Stem Cells

While hESCs can be used as a model of the human epiblast, hypoblast-like stem cells are useful in modeling the human hypoblast (Table 2). Hypoblast cells emerge from the inner cell mass and give rise to the yolk sac. During pre-gastrulation development, the hypoblast cells in contact with the overlying epiblast cells form a bilaminar disk. Whether the human hypoblast contains important signaling centers involved in signaling to the epiblast and shielding the anterior region from mesendoderm differentiation, analogous to the mouse Anterior Visceral Endoderm (AVE)[41], remains unknown. Models of human hypoblast cells could reveal how this may occur.

While successful derivation of mouse extraembryonic endoderm cells has been previously achieved[42], a hypoblast stem cell line has not yet been directly derived from human embryos. Alternatively, transcription factor reprogramming approaches applied to hESCs have been promising, albeit with some shortcomings (Table 2). It has been reported that constitutive expression of transcription factor SOX7 in hESCs leads to differentiation of hypoblast-like progenitors, although, expression of the hESC/epiblast markers OCT4 and NANOG was maintained[43], suggesting a failure in full trans-differentiation. Additionally, GATA6 induction in hESCs results in the downregulation of pluripotency gene expression and upregulation of hypoblast-associated genes. These cells also exhibit increased expression of trophoblast, mesoderm and other lineage markers[44], again suggesting a failure in full

hypoblast-like lineage commitment. Importantly, overexpression of these hypoblast transcription factors results in cell lines that could not self-renew beyond a few passages[43,44]. More recently, an expandable hypoblast-like stem cell line, so-called naïve extra-embryonic endoderm (nEnd), has been derived from naïve hESCs through culture with Activin, ChIR99021 and LIF[45]. Activation of FGF signaling was essential in this induction, suggesting a conserved role of this pathway in hypoblast specification, as in the mouse[46,47]. However, studies of the human blastocyst stage embryos indicate that FGF signaling is dispensable for hypoblast specification *ex vivo*[48,49]*,* suggesting that nEnd cells may be specified through distinct pathways. Nevertheless, transcriptomic analysis showed a strong correlation between nEnd and hypoblast of the human blastocyst, suggesting that it might be a promising initial *in vitro* model resembling the *in vivo* hypoblast[45]. Markers to distinguish the hypoblast from definitive endoderm, which forms following gastrulation, have not been identified in the human embryo, and such markers will be essential for evaluation of hypoblast-like cell lines. Direct isolation of hypoblast-like stem cells from human embryos still has not been accomplished.

Human Trophoblast-Like Stem Cells

The human placenta consists of three major trophoblast cell types: cytotrophoblast (CTB), syncytiotrophoblast (STB), and extravillous trophoblast (EVT). Following implantation, cytotrophoblast precursor cells expand and differentiate into STB in floating villi or EVT in anchoring villi[50]. Only in 2018, human trophoblast stem cells (hTSCs) were derived from the blastocyst trophectoderm and first-trimester placental isolates[51]. Both derivations required culture conditions supporting WNT and EGF signaling as well as TGFB, Histone Deacetylase, and Rock inhibition, and both derivations resulted in a self-renewing cytotrophoblast-like cell line which can give rise to both STB and EVT. Molecular, transcriptomic, and epigenetic characterizations demonstrate that these hTSCs are similar to both primary trophoblast isolates and day 10/12 *ex vivo* cultured human trophoblast[51,52].

Prior to the derivation of trophoblast cells directly from the embryo, trophoblast-like cells were derived by differentiation of hESCs using the signaling factor BMP4[53–55]. Genome-wide expression analysis showed a close correlation between the resulting cells and human placental cells[54], and they can give rise to STB- and EVT-like populations[56]. Subsequent work demonstrated that the transcription factors GATA2, GATA3, TFAP2A, and TFAP2C are part of the network activated in this protocol and that GATA3 overexpression in hESCs could mimic BMP4-driven differentiation[57]. Further characterization regarding key hallmarks defining temporally pooled first-trimester trophoblast cells has shown that BMP4-differentiated hESCs do not fulfill these criteria[58]. It has been proposed that these cells may actually be extraembryonic mesoderm derivatives[59] or amnion-like cells[36], or a mixed population. This highlights the need for robust characterization of stem cell lines.

Recently, new methods for differentiating hESCs to trophoblast-like stem cells have emerged. One study used chemically defined media to derive two distinct trophoblast-like cells from hESCs: transcription factor CDX2-negative, tumor suppressor $P63^{low}$ stem cells equivalent to primary hTSCs, and another novel CDX2-positive, $P63^{high}$ putative human trophectoderm stem cell (hTESC), which display distinct culture requirements and transcriptome differences compared with hTSCs[56,60]. Another study showed that ERK and TGFB inhibition of naïve hESCs results in differentiation to trophoblast-like cells in a BMP-independent manner[36]. Additionally, culture of naïve hESCs in hTSC media leads to a heterogeneous population of trophoblast-like cells, which can be further purified through cell-sorting[29,52,61]. In these conditions, conventional primed hESCs were not able to give rise to stable TS-like cells, but instead produced cells that, based on bioinformatic analysis, may possess amnion-like qualities[36,52]. A comparative analysis of human trophoblast-like cells generated from different protocols and different sources (Table 2), whether embryo- or hESC-derived, would be informative for

understanding the extent to which these trophoblast-like cells model the *in vivo* trophoblast niche. Specifically, such an assessment could determine transcriptional similarity to the *in vivo* niche as well as the fulfillment of previously described stringent criteria, including expression of key trophoblast markers such as GATA3 and TFAP2C, expression of HLA class I molecules, demethylation of the *ELF5* promoter, and expression of the chromosome 19 miRNA cluster expression[58].

Complex ESC-Derived Models of Early Human Development

Stem cell lines in conventional two-dimensional (2D) culture are powerful tools for investigating properties relevant to the human embryo. However, 2D culture is limited in its ability to recapitulate the natural tissue niche, including 3D spatial organization of different cell types, and the localized paracrine signaling between different structures and tissues. To better capture these properties, different approaches have been developed to model more complex aspects of embryogenesis using hESCs (Fig. 2). Here we discuss different hESC-derived models of the embryo, their utility, and their limitations.

Embryoid Bodies and Gastruloids

Similar to previous work in mESCs, hESCs can be aggregated into 3D structures and cultured in conditions permissive for differentiation. The resulting structures (embryoid bodies), demonstrated that hESCs can form derivatives of all three germ layers *in vitro*[62]. Many methodologies have emerged to generate embryoid bodies including aggregation in suspension, hanging drops, microwells, and Aggrewells as well as addition of factors promoting survival such as Rock inhibitor[63–67]. Each of these platforms allows for cells to aggregate to each other, rather than attach to the underlying dish as in conventional 2D culture conditions. Embryoid bodies offer a 3D model with complex differentiation behaviors that are useful in identifying different signaling factors required for differentiation of specific cell types, and in examining cellular behaviors key to germ layer differentiation during gastrulation, including epithelial-mesenchymal transition (EMT) and cell migration. Embryoid bodies can be induced to form a functional gastrula organizer, which drives secondary axis formation when grafted to frog embryos[68]. They are also often a starting point for directed differentiation and formation of complex organoids[63,69,70]. Embryoid bodies represent an opportunity to test the effects of different compounds or drugs on 3D structures undergoing cell fate decisions and behaviors reminiscent of the human embryo[64,66] and to test the potency of stem cells to give rise to specific lineages[52]. However, they lack a controlled organization of differentiation and tissue localization and do not directly model a specific stage or morphological structure within the embryo.

Recently, embryoid bodies treated with GSK-inhibitor CHIR99021 before and after aggregation in low-adherence plates have demonstrated asymmetric axial elongation over a four-day period[71]. At the elongating tip of these structures, termed gastruloids, markers of the three germ layers (BRA-positive (+) mesoderm, SOX17+ endoderm, and SOX2+ ectoderm) are present in distinct regions. Overall, the anterior region of gastruloids expresses cardiac development markers, while the most posterior, elongating tip resembles the mammalian tailbud. Similar to work with mouse gastruloids, human gastruloids represent post-primitive streak structures that include the transcriptional signature of somite formation, though they lack gene signatures of anterior structures[71]. This 3D model, therefore, represents stages of human development that lie beyond the legal boundaries of *ex vivo* culture offering an exciting opportunity to investigate transcriptional dynamics, cell differentiation, and signaling in the posterior human embryo. Given that no *ex vivo* confirmation of these events can be achieved in the natural embryo, it is not yet possible to know if divergences from model organisms represent bona fide species differences or artifacts of the system. This human gastruloid model is particularly valuable

to uncover mechanisms of axial elongation and patterning. Given the presence of a somitogenesis gene signature along the anterior-posterior axis, this model may complement existing 2D models to understand aspects of somitogenesis including segmentation timing and gene expression patterns[72]. However, these gastruloids do not mimic pre-gastrulation human development or the formation of germ cells at gastrulation.

Micropatterned hESCs

While hESCs cultured in conventional 2D environment lack defined organization, confinement of hESCs on 1000μm-diameter 2D micropatterns and exposure to BMP4 were shown to give rise to spatially organized rings of different cell types in a highly reproducible fashion, reminiscent of gastrulation[73]. Specifically, an outer CDX2+, SOX17 and SOX2-negative(-) "extra-embryonic" ring that shares characteristics of the trophoblast and amnion[74], a SOX17+NANOG+SOX2- "endodermal" ring, a BRA+NANOG+SOX17-SOX2- "mesoderm" ring, and a SOX2+NANOG-BRA-SOX17- "ectoderm" center[73]. Additional exposure of these 2D micropatterns to WNT and NODAL signaling drives the formation of a human "organizer" that is functional in inducing a secondary body axis when grafted into chick embryos[75]. This highly reproducible model of the germ layers normally established through gastrulation is extremely useful for investigating the signaling dynamics, morphogen and receptor behaviors, and their effect on cell fate as well as the self-organization potential of stem cells. These micropatterned hESCs have been used to demonstrate the complex interplay of BMP, WNT, and NODAL signaling dynamics[76–79]. This entails the exposure of exogenous BMP initially activating WNT expression, which in turn activates NODAL. The initial BMP exposure also drives receptor relocalization for cells towards the center of the colony, decreasing their sensitivity to BMP signaling, and the activation of its own inhibitor, NOGGIN, which also diffuses towards the center, eventually resulting in edge-restricted BMP activity that drives the CDX2+ extraembryonic fate. The WNT and NODAL signaling domains move as a wave toward the center of the colony, independent of any cell migration. The duration of the wave of WNT and NODAL, in addition to the rate of concentration change of NODAL, drives primitive streak-like mesendodermal identity, with the NODAL inhibitor CER1 acting downstream of WNT to specify endoderm versus mesoderm, and the maintained low levels of WNT at the dense colony center promoting ectodermal identity[76–80]. These discoveries have been possible through the use of reporter and knock-out cell lines to demonstrate the role of a given factor, which would not be possible in the natural human embryo. While powerful tools in modeling signaling dynamics in germ layer differentiation, these micropatterns lack key morphological features of the epiblast morphogenesis, such as formation of a central lumen to establish the pro-amniotic cavity and EMT to induce gastrulation.

Spheroids and Post-Implantation Amniotic Sac Embryoids

It has been demonstrated that hESCs, when exposed to an extracellular matrix, such as Matrigel, polarize and form a lumen[81,82]. The resulting structure is similar to a day 10 human epiblast in cell number, size and morphology[83]. This peri/post-implantation epiblast model has been used to investigate the mechanisms behind embryo polarization and lumenogenesis and the coupling of changes in the pluripotency state with morphogenesis. It was found that hESCs maintained in naïve conditions can polarize but are unable to open a lumen, while naïve cells allowed to undergo pluripotency transition undergo lumenogenesis successfully. This process is relevant to *in vivo* development as no embryos cultured in naïve conditions were able to exit naïve pluripotency or open a lumen, demonstrating the utility of hESCs to recapitulate key aspects of embryogenesis[81] and complement work in the human embryo. Moreover, when exposed to a 48-hour pulse of BMP4, these structures break symmetry, segregate SOX2 and BRA expression and induce EMT in the BRA+

domain[83]. These spheroids provide a model of the peri- and post-implantation epiblast and can recapitulate lumenogenesis and at least some aspects of symmetry breaking. However, they are relatively stagnant in their developmental potential and do not undergo amnion specification on one side of the lumen.

Additional work has generated hESC-derived models that recapitulate amniogenesis. hESCs cultured on a gel matrix, with a softer gel surrounding, were shown to form a squamous cyst of amnion-like cells[84]. At optimized cell density, a small subset of structures formed a cyst with amnion-like cells on one side of the lumen and columnar epiblast-like cells on the other[85]. This post-implantation amniotic sac embryoid (PASE) also exhibits EMT events in the columnar compartment such as the upregulation of SNAI1, downregulation of E-CADHERIN, and cell migration – similar to the formation of the primitive streak[85]. PASE formation has been optimized to an impressive extent by the use of microfluidic technology and exposure to BMP4 at the "amniotic" pole, providing an improved experimental system[86]. This also leads to the formation of PGC-like cells within the PASE structure and more robust EMT[86]. An anteriorized PASE lacking primitive streak-like formation can be achieved with the introduction of WNT and BMP inhibition at the "epiblast" pole[86]. Further, WNT signaling was shown to be instrumental in the inductive ability of amnion-like cells to drive primitive streak-like cell differentiation[86]. Thus, the PASE and its applications demonstrate the potential of hESCs to model the post-implantation, pre-gastrulation epiblast. Yet, questions remain of how closely these amnion-like cells resemble the amnion in natural embryos as current *ex vivo* culture systems do not robustly specify amnion, limiting the possibilities for an accurate comparison. Overall, the PASE offers a promising platform to investigate aspects of the post-implantation development such as amnion formation and its potential inductive roles in primitive streak and PGC specification.

Future Perspectives: Inclusion of Extra-embryonic Cells in Human Stem Cell Models

Thus far, modeling the human embryo with stem cell-derived structures has relied on hESCs cultured in the absence of extra-embryonic stem cells. Although cells isolated from first-trimester placentas can form 3D placental organoids *in vitro*[87], models of human pre-gastrulation including extra-embryonic tissues have not yet been reported. Interestingly, mouse ESCs and TSCs can form structures morphologically similar to the post-implantation mouse embryo[88]. Further addition of mouse extra-embryonic endoderm stem cells results in improved post-implantation structures[89]. Also, blastocyst-like structures have been derived from mouse ESCs, EpiSCs (primed mouse cells), or ESCs in combination with TSCs[90–92]. Comparisons of human versus mouse stem-cell derived models of development are reviewed elsewhere[93].

Given the success of generating more complete stem cell-derived embryo models in the mouse, is it possible to build human pre-gastrulation models comprising human epiblast, hypoblast, and trophoblast-like lineages (Fig.3)? If these different types of cells can self-organize into structures that acquire the embryo's architecture and molecular features of pre-implantation or post-implantation embryos, they would present powerful tools to investigate lineage crosstalk or differential lineage response to perturbation difficult to investigate in the natural embryo. This will likely require characterization of the different kinds of extra-embryonic stem cells regarding their potential to develop with hESCs. The development of stem cell models that include all three cell type precursors to the conceptus – the fetus and its supporting tissues – will require a reconsideration of ethical guidelines on how to regulate stem cell-based models of the embryo[94]. Some argue that future models encompassing all tissues of a conceptus have the potential to form a fetus and thus should be subjected to the 14-day

rule as natural human embryos aree[94-97]. Others argue that given some structures may begin at a stage past primitive streak formation, the framework of the 14-day rule is not suitable to address the ethical questions raised by stem cell-derived embryo-like structures[98] and other facts about these structures should be used in ethical argumentation[99]. The development of ethical guidelines regarding the limitations and funding restrictions on related work will be crucial as the field moves closer to developing models encompassing all cell types of the early human conceptus.

Conclusion: Synthetic Embryos Rooted in the Natural Embryo

It is estimated that 30-60% of pregnancies end in pre-clinical miscarriage during the "black-box" period of pre-gastrulation development[100,101]. In 2016, the "black box" of pre-gastrulation development was opened with *ex vivo* culture of human embryos in pre- and post-implantation stages[3,4]. By nature, this work has been largely descriptive. Recently, the single-cell transcriptional profile of a single gastrulating human embryo has been reported[102]. This resource, along with other profiles of *ex vivo* cultured embryos[5,7,103], will provide a transcriptional blueprint for the stem cell-based models leading to and encompassing gastrulation-like events. They provide opportunities to identify markers for hypoblast versus definitive endoderm, as well as other tissues. Using the natural embryo as a guide, stem cells offer remarkable opportunities to dissect molecular and signaling events, both within and between tissues, and interrogate the importance of different factors which is exceedingly difficult to determine *in vivo.* This includes understanding molecular mechanisms[104], self-organization principles[105,106], tissue mechanics and mechanobiology[107], and signaling activity[108]. Thus, models of human development will begin to elucidate why pregnancies fail at such high rates during this period.

Aneuploidy is thought to be a major cause of pregnancy loss, and a recent study has characterized the developmental potential of embryos harboring specific chromosome aneuploidies[109]. Single-cell RNA-sequencing studies have highlighted that the rate of aneuploidy in mosaic embryos is higher in the trophoblast compared to the epiblast during post-implantation, despite no difference during pre-implantation[110], raising the question if aneuploid cells are preferentially depleted in the epiblast as in mouse models of mosaic aneuploidy[111,112]. Building stem cell models including aneuploid embryonic and extra-embryonic stem cells will be useful in investigating mechanisms underlying phenotypes associated with specific aneuploidies, and the behavior of euploid versus aneuploid cells in a mosaic context. Also, the susceptibility of the embryo to novel pathogens – such as SARS-CoV-2 virus – could be further investigated *in vitro*[113].

The development of novel stem cell-based models of the embryo provides a simplified system with which we can understand complex processes underlying the beginning of our lives. Each model provides unique insights and opportunities, and continued work will expand our tools and knowledge to illuminate the mysterious black box of human development.

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Figure 1: Pre-Gastrulation Development of the Human Embryo. Following fertilization, the zygote undergoes a series of cleavage stages. Between the 4- and 8-cell stage, embryonic genome activation (EGA) occurs[114]. At the 8-to-16-cell stage the embryo compacts and undergoes polarization[115], and subsequently the morula forms in which the outside cells will give rise to the trophectoderm and the inside to the inner cell mass. At day 5 the embryo reaches the blastocyst stage[36,116], and at the late blastocyst stage the epiblast, hypoblast, and trophoblast cells have undergone lineage commitment[6–8]. The mature blastocyst then implants in the maternal uterus, and the epiblast exits pluripotency and undergoes lumenogenesis to form the amniotic cavity. Meanwhile, the hypoblast proliferates and begins to form the visceral endoderm surrounding the yolk sac cavity. The epiblast cells in contact with the overlying trophoblast give rise to the amnion while the cells in contact with the hypoblast will go on to form the embryo proper. The trophectoderm gives rise to cytotrophoblast and multinucleated syncytiotrophoblast which will in turn generate the embryonic portion of the placenta. On day 13/14 it is thought that PGCs are specified, extraembryonic mesenchyme has formed, and that the primitive streak forms at the posterior epiblast. ICM, Inner Cell Mass; EPI, Epiblast; HYPO, Hypoblast; TE, Trophectoderm; PGCs, Primordial Germ Cells.

Figure 2: Derivation of Human Stem Cells and Embryonic Stem Cell Models. Human embryonic stem cells (hESCs) and human trophoblast stem cells (hTSCs) have been derived directly from the human blastocyst while hypoblast-like cells (HBLCs) can be derived from naïve hESCs. Naïve hESCs can also give rise to trophoblast-like cells. hESCs can be used to form embryoid bodies and gastruloids, micropatterned hESC cultures, spheroids and post-implantation amniotic sac embryoids (PASE). Each can be used to investigate specific aspects of embryogenesis.

Figure 3: Theoretical Stem Cell Models with Embryonic and Extra-Embryonic Stem Cells. Embryonic, trophoblast, and hypoblast-like (hESC, hTSC, HBLCs) cells may be co-cultured to form either blastocyst or post-implantation-like structures containing both embryonic and extraembryonic cells. Ideally, these models would be able to mature and undergo transitions between stages that recapitulate the embryo in morphology, cell identity progression, and molecular characteristics.

Table 1: Naïve hESC Protocols

Paper	*Cell Name*	*Overexpression*	*Small Molecules*	*Source*
Hanna et al., 2010	N/A	OCT4, KLF4, KLF2	PD0325901, CHIR99021, LIF, (Forskolin)	hESCs
Gafni et al., 2013	NHSM (basis for commercially available Rset)	N/A	PD0325901, CHIR99021, LIF, FGF2, TGFB1, SP600125, SB204590	hESCs iPSCs
Chan et al., 2013	3iL	N/A	PD0325901, BIO, Dorsomorphin, LIF	hESCs
Ware et al., 2014	2i+FGF2	N/A	HDACi → PD0325901, FGF2, CHIR99021	Blastocyst ICM
Theunissen et al., 2014	5i/L/A	N/A	PD0325901, IM-12, SB590885 WH-4-023, Y-27632, LIF, Activin A	hESCs
Qin et al., 2016	Y-2iLF	YAP	PD0325901, CHIR99021, LIF, Forskolin	iPSCs
Takashima et al., 2016	Reset (hESC)	NANOG, KLF2	PD0325901, CHIR99021, LIF, Gö6983 (t2iLGö)	hESCs
Guo et al., 2016	Reset (Blastocyst)	N/A	t2iLGö	Blastocyst ICM
Yang et al., 2017	Extended Potential	N/A	LIF, CHIR99021, Dimethidene maleate, Minocycline hydrochloride, IWR-endo-1, Y-27632	iPSCs
Guo et al., 2017	Chemically (c)Reset	N/A	PD0325901, LIF, VPA, Sodium butyrate → PD0325901, LIF, Gö6983 → t2iLGö	hESCs
Gao et al., 2019	Expanded Potential	N/A	CHIR99021, A419259, XAV939, IWR, vitaminC, LIF	hESCs
Bayerl et al., 2020	HENSM	N/A	PD0325901, IWR-1, BIRB0796, Gö6983, CGP77675	hESCs

Table 2: Protocols for Derivation of Extra-Embryonic Stem Cell Types

Paper	***Model***	***Overexpression***	***Small Molecules***	***Source***
Draper et al., 2008	SOX7-OE hESCs (hypoblast-like)	SOX7	N/A	Conventional hESCs
Wamaitha et al., 2015	GATA6-OE hESCs (hypoblast-like)	GATA6	N/A	Conventional hESCs
Linneberg-Agerholm et al., 2019	nEnd cells from naïve hESCs (cReset + NHSM/Rset) (hypoblast-like)	N/A	Activin A, CHIR99021, LIF	Naïve hESCs (cReset and NHSM/Rset)
Xu et al., 2002; Amita et al., 2013; Li et al., 2013; Horii et al., 2016	BMP4-Differentiated ESCs (trophoblast-like)	N/A	BMP4	Conventional hESCs
Krendl et al., 2017	GATA3-OE hESCs (trophoblast like)	GATA3	N/A	Conventional hESCs
Okae et al., 2018	hTSCs (trophoblast-like)	N/A	EGF, CHIR99021, A83-01, SB431542, Y-27632, VPA	Blastocyst Trophectoderm or first-trimester placental isolates
Mischler et al., 2019	Converted hTESCs and TSCs (trophoblast-like)	N/A	BMP4, SB431542, CYM5541 → CYM5541, A83-01, FGF10, CHIR99021 (hTESCs) or Okae et al., 2018 media (TSCs)	Conventional hESCs
Guo et al., 2020	Converted TS-like (MEK/ERKi) (trophoblast-like)	N/A	PD0325901 (+ A83-01)	Naïve hESCs (cReset)
Dong et al., 2020; Cinkornpumin et al., 2020	Converted TS-like (Okae et al., 2018 media) (trophoblast-like)	N/A	Okae et al., 2018 media	Naïve hESCs (cReset and 5i/L/A)

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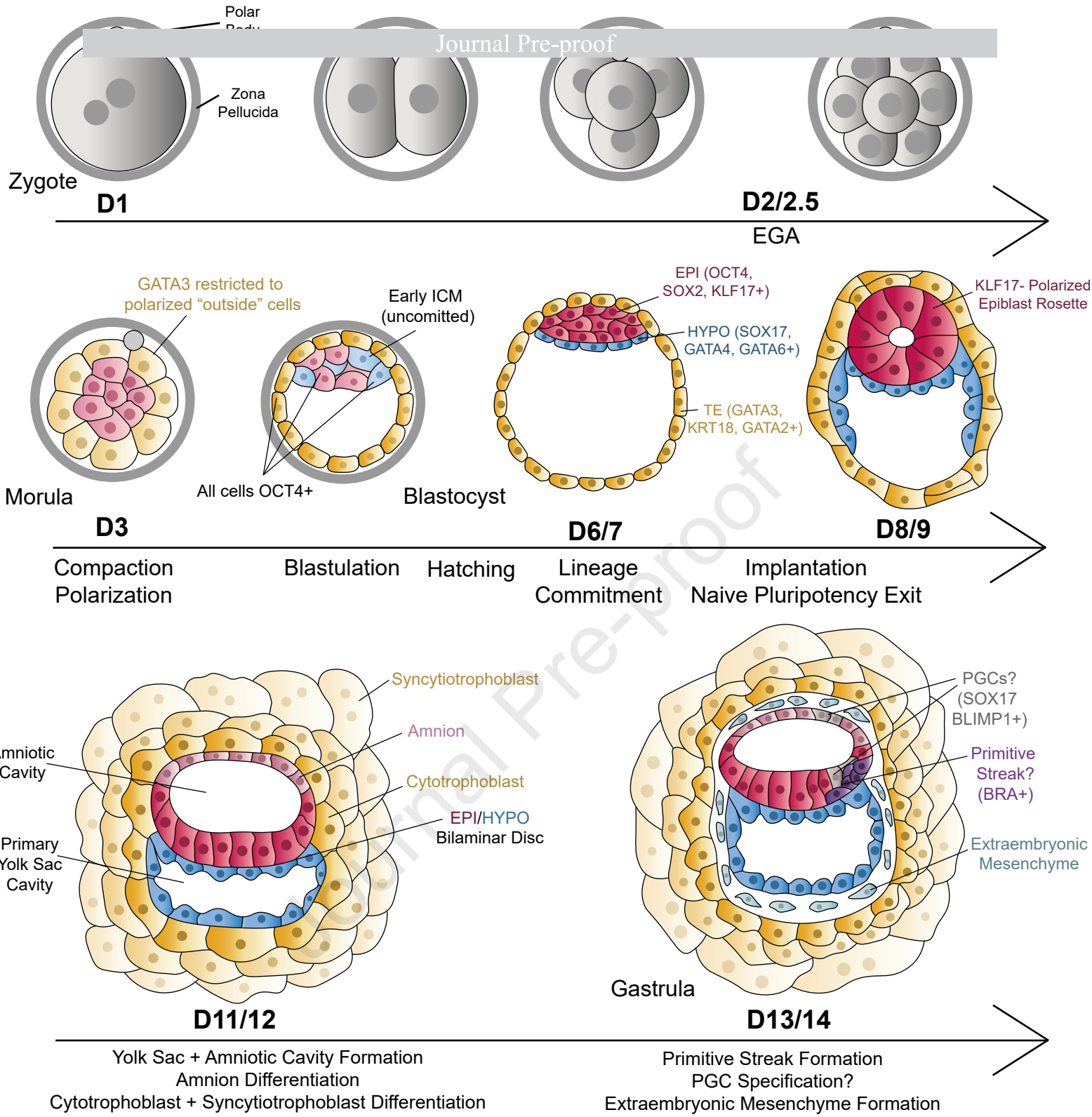
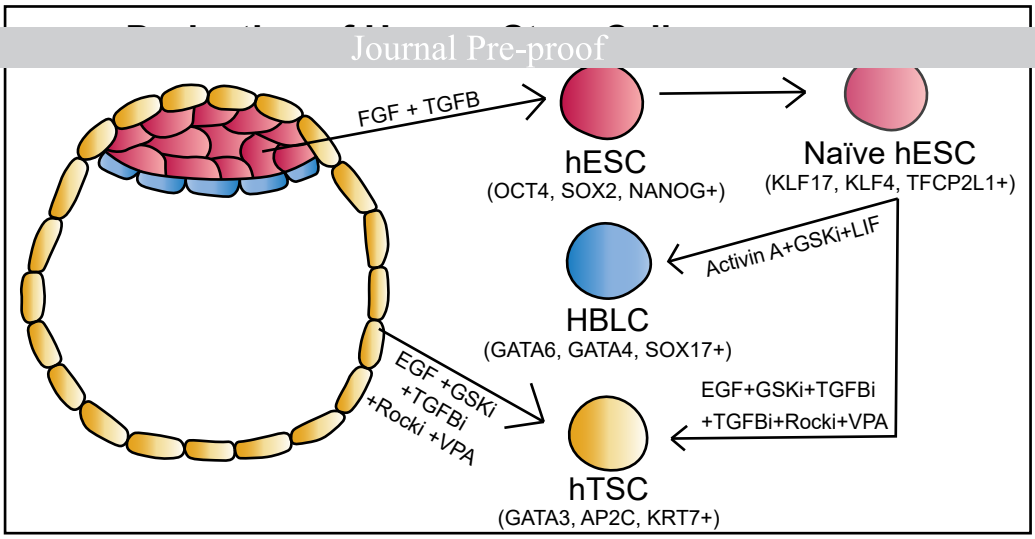
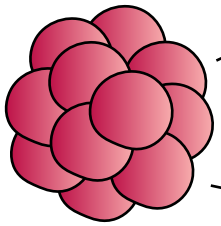




Embryoid Body

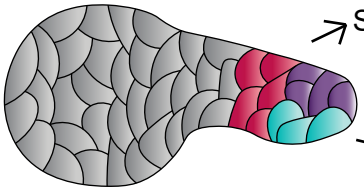


Specific Cell Types

Drug + Molecule Screen

Organoid Culture

ChIR99021



Signaling in A-P Axis

Axial Patterning

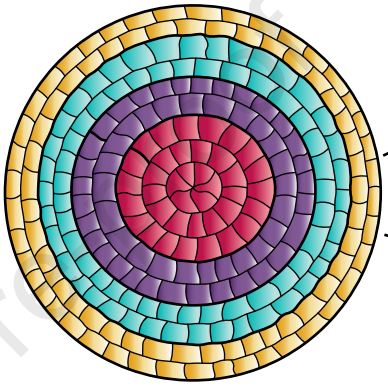
Endoderm-like (SOX17+)

Mesoderm-like (BRA+)

Ectoderm-like (SOX2+)

Gastruloid

Micropatterned hESCs



Signaling Dynamics

Gastrulation Patterning

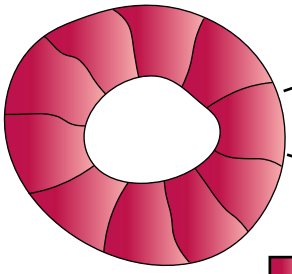
Trophoblast/Amnion-like
(CDX2+ SOX17- SOX2-)

Endoderm-like
(SOX17+ NANOG+ SOX2-)

Primitive Streak/Mesoderm-like
(BRA+ NANOG+ SOX17- SOX2-)

Ectoderm-like
(SOX2+ NANOG- BRA- SOX17-)

Hydrogel-Embedded Spheroid



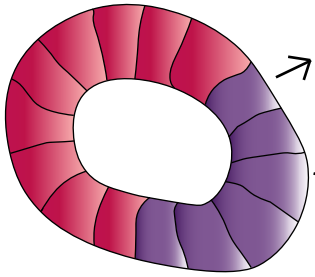
Naive Pluripotency Exit

Lumenogenesis

Epiblast-like

Primitive Streak-like

BMP4



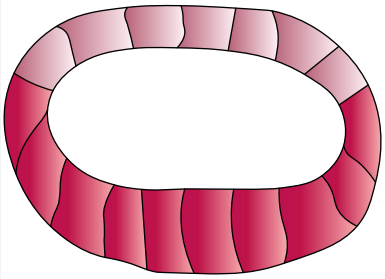
Signaling in A-P Axis

Symmetry Breaking Principles

Asymmetric Spheroid

Post-implantation Amniotic Sac Embryoid

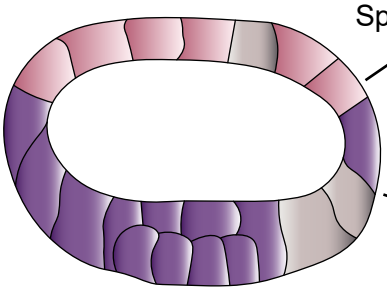
Anteriorized



Epiblast Amnion Split

Posteriorized

PGC Specification



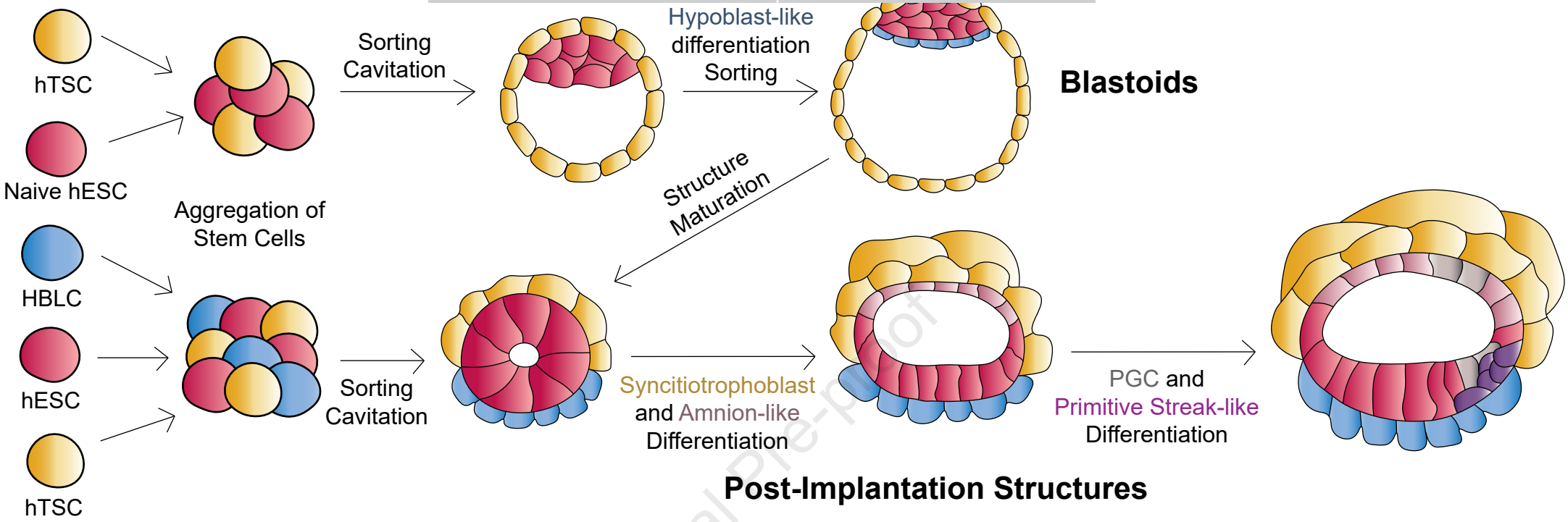
Primitive Streak Formation

Epiblast-like

Primitive Streak-like

Amnion-like

PGC-like



Modeling Human Embryo Development with Embryonic and Extra-Embryonic Stem Cells

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Highlights (85 Characters per bullet point; 3-5 Bullet points)

- Human stem cells are complementary tools to study early embryogenesis
- Embryonic stem cells can mimic aspects of epiblast development
- 3D culture of embryonic stem cells recapitulates aspects of epiblast morphology
- Prospect of combining embryonic & extra-embryonic stem cells to model early embryo