Modeling human embryo development with embryonic and extra-embryonic stem cells

Bailey A.T. Weatherbee[#a], **Tongtong Cui**[#b], **Magdalena Zernicka-Goetz**[a,b,*]

[a]Mouse and Human Embryo and Stem Cell Group, University of Cambridge, Department of Physiology, Development and Neuroscience, Downing Street, Cambridge, CB2 3EG, UK

[b]Plasticity and Synthetic Embryology Group, California Institute of Technology, Division of Biology and Biological Engineering, 1200 E. California Boulevard, Pasadena, CA, 91125, USA

[#] These authors contributed equally to this work.

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.

1. 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* (Edwards et al., 1969), we have a basic picture of human embryology until day 7 post-fertilization (Wamaitha and Niakan, 2018). More recently, the development of new culture methods has allowed us to expand our knowledge of human development up to day 13/14 (Deglincerti et al., 2016; Shahbazi et al., 2016; Xiang et al., 2020), 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

*Corresponding author. magdaz@caltech.edu (M. Zernicka-Goetz).

(precursor to the yolk sac) and the trophectoderm (precursor to the embryonic portion of the placenta) (Petropoulos et al., 2016; Stirparo et al., 2018; Molè et al., 2020) (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 (Sasaki et al., 2016; Chen et al., 2019; Kobayashi and Surani, 2018). Further, extra-embryonic mesenchyme cells emerge, possibly from the hypoblast, and fill the luminal space between the trophoblast and other lineages (Xiang et al., 2020; Sasaki et al., 2016; Nakamura et al., 2016). 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 (Wamaitha and Niakan, 2018; Molè et al., 2020).

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 (Baart et al., 2006). Thus, an alternative approach is needed. While some insights have been gleaned from non-human primates, strong ethical limitations still exist andconservation 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.

2. 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.

3. 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 (Thomson et al., 1998). 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 (Yu et al., 2007, 2009; Takahashi et al., 2007). Following their derivation, hESCs have become a valuable tool in understanding stem cell differentiation, and their clinical applications are expanding (Ilic and Ogilvie, 2017). 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 (James et al., 2006; Mascetti and Pedersen, 2016). 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 (Nichols and Smith, 2009; Theunissen et al., 2016; Pastor et al., 2018; Messmer et al., 2019). 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 (Messmer et al., 2019; Gafni et al., 2013; Chan et al., 2013; Theunissen et al., 2014; Guo et al., 2016, 2017; Gao et al., 2019; Hanna et al., 2010; Takashima et al., 2014; Ware et al., 2014; Qin et al., 2016; Yang et al., 2017). 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 (Theunissen et al., 2016). Others still seem to be more transcriptionally similar to the post-implantation epiblast rather than pre-implantation, including "Extended Potential" and "NHSM" cells (Guo et al., 2020).

Comparative work using naïve hiPSCs derived using different protocols has highlighted differences in transcriptional identity, methylation patterns, and chromosomal stability (Liu et al., 2017). Specifically, two types of naïve cells showed the most similarity to the human pre-implantation epiblast both transcriptionally and epigenetically: 5i/L/A(F) (Theunissen et al., 2014) cells and Reset (t2iLGö/PXGL) cells (Guo et al., 2016, 2017; Takashima et al.,

2014; Liu et al., 2017). 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 (Bayerl et al., 2020; Rostovskaya et al., 2019). 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 (Allegrucci and Young, 2007). 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*.

4. 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 endoderm. 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) (Perea-Gomez et al., 2001), 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 (Kunath et al., 2005), 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 (Draper et al., 2008), 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 (Wamaitha et al., 2015), 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 (Draper et al., 2008; Wamaitha et al., 2015). 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 (Linneberg-agerholm et al., 2019). Activation of FGF signaling was essential in this differentiation, suggesting a conserved role of this pathway in hypoblast specification, as in the mouse (Anderson et al., 2017; Yamanaka et al., 2010). However, studies of the human blastocyst stage embryos indicate that FGF signaling is dispensable for hypoblast specification *ex vivo* (Kuijk et al., 2012; Roode et al., 2012), 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 (Linneberg-agerholm et al., 2019). 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.

5. 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 (James et al., 2012). Only in 2018, human trophoblast stem cells (hTSCs) were derived from the blastocyst trophectoderm and first-trimester placental isolates (Okae et al., 2018). 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 (Okae et al., 2018; Dong et al., 2020).

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 (Li et al., 2013), and they can give rise to STB- and EVT-like populations (Horii et al., 2016). Subsequent work demonstrated that the transcription factors GATA2, GATA3, AP-2α, and AP-2γ are part of the network activated in this protocol and that GATA3 overexpression in hESCs could mimic BMP4-driven differentiation (Krendl et al., 2017). Further characterization regarding key hallmarks defining temporally pooled first-trimester trophoblast cells has shown that BMP4-differentiated hESCs do not fulfill these criteria (Lee et al., 2016). It has been proposed that these cells may actually be extraembryonic mesoderm derivatives (Bernardo et al., 2011) or amnion-like cells (Guo et al., 2020), 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 (Horii et al., 2016; Mischler et al., 2019). Another study showed that ERK and TGFB inhibition of naïve hESCs results in differentiation to trophoblast-like cells in a BMP-independent manner (Guo et al., 2020). 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 (Gao et al., 2019; Dong et al., 2020; Cinkornpumin et al., 2020). 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 (Guo et al., 2020; Dong et al., 2020). 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 AP-2γ, expression of HLA class I molecules, demethylation of the *ELF5* promoter, and expression of the chromosome 19 miRNA cluster expression (Lee et al., 2016).

6.   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.

7.   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 (Sun et al., 2006; Ungrin et al., 2008; Hwang et al., 2009; Flamier et al., 2017; Steven et al., 2012). 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 (Sharon et al., 2011). They are also often a starting point for directed differentiation and formation of complex organoids (Sun et al., 2006; Lancaster et al., 2013; Nakano et al., 2012). 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 (Flamier et al., 2017; Itskovitz-eldor et al., 2000; Ungrin et al., 2008) and to test the potency of stem cells to give rise to specific lineages (Dong et al., 2020). 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 (Moris et al., 2020). 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 (Moris et al., 2020). 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 (Matsuda et al., 2020). However, these gastruloids do not mimic pre-gastrulation human development or the formation of germ cells at gastrulation.

8. Micropatterned hESCs

While hESCs cultured in conventional 2D environment lack defined organization, confinement of hESCs on 1000 μm-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 (Warmflash et al., 2014). Specifically, an outer CDX2+, SOX17 and SOX2-negative (−) "extra-embryonic" ring that shares characteristics of the trophoblast and amnion (Minn et al., 2020), a SOX17+NANOG + SOX2- "endodermal" ring, a BRA + NANOG + SOX17-SOX2- "mesoderm" ring, and a SOX2+NANOG-BRA-SOX17- "ectoderm" center (Warmflash et al., 2014). 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 (Martyn et al., 2018). 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 (Chhabra et al., 2019; Martyn et al., 2019; Yoney et al., 2018; Etoc et al., 2016). 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 (Chhabra et al., 2019; Martyn et al., 2019; Yoney et al., 2018; Etoc et al., 2016; Heemskerk et al., 2019). 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.

9. 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 (Shahbazi et al., 2017; Taniguchi et al., 2015). The resulting structure is similar to a day 10 human epiblast in cell number, size and morphology (Simunovic et al., 2019). 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 (Shahbazi et al., 2017), demonstrating the utility of hESCs to recapitulate key aspects of embryogenesis and complement work in the human embryo. Moreover, when exposed to a 48-h pulse of BMP4, these structures break symmetry, segregate SOX2 and BRA expression and induce EMT in the BRA + domain (Simunovic et al., 2019). 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 (Shao et al., 2017a). 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 (Shao et al., 2017b). 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 (Shao et al., 2017b). 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 (Zheng et al., 2019). This also leads to the formation of PGC-like cells within the PASE structure and more robust EMT (Zheng et al., 2019). An anteriorized PASE lacking primitive streak-like formation can be achieved with the introduction of WNT and BMP inhibition at the "epiblast" pole (Zheng et al., 2019). Further, WNT signaling was shown to be instrumental

in the inductive ability of amnion-like cells to drive primitive streak-like cell differentiation (Zheng et al., 2019). 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.

10.   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* (Turco et al., 2018), 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 (Harrison et al., 2017). Further addition of mouse extra-embryonic endoderm stem cells results in improved post-implantation structures (Sozen et al., 2018). Also, blastocyst-like structures have been derived from mouse ESCs, EpiSCs (primed mouse cells), or ESCs in combination with TSCs (Rivron et al., 2018a; Sozen et al., 2019; Li et al., 2019). Comparisons of human versus mouse stem-cell derived models of development are reviewed elsewhere (Shahbazi and Zernicka-goetz, 2018).

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 (Rivron et al., 2018b). 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 (Rivron et al., 2018b; Hyun et al., 2020; Pereira Daoud et al., 2020; Sawai et al., 2020). Others argue that given some structures may begin at a stage beyond 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 (Aach et al., 2017) and other facts about these structures should be used in ethical argumentation (Piotrowska, 2019). 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.

11.   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 (Macklon et al., 2002; Wilcox et al., 1999). In 2016, the "black box" of pre-gastrulation development was opened with *ex vivo* culture of human embryos in pre- and post-implantation stages (Deglincerti et al., 2016; Shahbazi et al., 2016). By nature, this work has been largely descriptive. Recently, the single-cell transcriptional profile of a single gastrulating human embryo has been reported (Tyser et al., 2020). This resource, along with other profiles of *ex vivo* cultured embryos (Xiang et al., 2020; Stirparo et al., 2018; Zhou et al., 2019), 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 (Taniguchi et al., 2019), self-organization principles (Shahbazi et al., 2019; Martinez Arias and Lutolf, 2018), tissue mechanics and mechanobiology (Vianello and Lutolf, 2019), and signaling activity (Morgani and Hadjantonakis, 2020). 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 (Shahbazi et al., 2020). 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 (Starostik et al., 2020), raising the question if aneuploid cells are preferentially depleted in the epiblast as in mouse models of mosaic aneuploidy (Bolton et al., 2016; Singla et al., 2020). 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* (Weatherbee et al., 2020).

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.

Acknowledgments

References

Aach J, Lunshof J, Iyer E, Church GM, 2017. Addressing the ethical issues raised by synthetic human entities with embryo-like features. Elife 6, 1–20.

Allegrucci C, Young LE, 2007. Differences between human embryonic stem cell lines. Hum. Reprod. Update 13, 103–120. [PubMed: 16936306]

Amita M, et al., 2013. Complete and unidirectional conversion of human embryonic stem cells to trophoblast by BMP4. Proc. Natl. Acad. Sci. Unit. States Am. 10.1073/pnas.1303094110/-/ DCSupplemental. www.pnas.org/cgi/doi/10.1073/pnas.1303094110.

Anderson KGV, et al., 2017. Insulin fine-tunes self-renewal pathways governing naive pluripotency and extra-embryonic endoderm. Nat. Cell Biol. 19.

Baart EB, et al., 2006. Preimplantation Genetic Screening Reveals a High Incidence of Aneuploidy and Mosaicism in Embryos from Young Women Undergoing IVF, 21, pp. 223–233.

Bayerl J, et al., 2020. Tripartite Inhibition of SRC-WNT-PKC Signalling Consolidates Human Naive Pluripotency. bioRxiv.

Bernardo AS, et al., 2011. BRACHYURY and CDX2 mediate BMP-induced differentiation of human and mouse pluripotent stem cells into embryonic and extraembryonic lineages. Stem Cell. 9, 144–155.

Bolton H, et al., 2016. Mouse model of chromosome mosaicism reveals lineage-specific depletion of aneuploid cells and normal developmental potential. Nat. Commun. 1–12. 10.1038/ncomms11165.

Braude P, Bolton V, Moore S, 1988. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. Nature 332, 459–461. [PubMed: 3352746]

Chan Y, et al., 2013. Induction of a human pluripotent state with distinct regulatory circuitry that resembles preimplantation epiblast. Cell Stem Cell 13, 663–675. [PubMed: 24315441]

Chen D, et al., 2019. Human primordial germ cells are specified from human primordial germ cells are specified from lineage-primed progenitors. Cell Rep. 29, 4568–4582. [PubMed: 31875561]

Chhabra S, Liu L, Goh R, Kong X, Warmflash A, 2019. Dissecting the dynamics of signaling events in the BMP, WNT, and NODAL cascade during self-organized fate patterning in human gastruloids. PloS One 17, 1–38.

Cinkornpumin JK, et al., 2020. Naive human embryonic stem cells can give rise to cells with a trophoblast-like transcriptome and methylome. Stem Cell Reports 15, 198–213. [PubMed: 32619492]

Deglincerti A, et al., 2016. Self-organization of the in vitro attached human embryo. Nature 533, 251–254. [PubMed: 27144363]

Dong C, et al., 2020. Derivation of trophoblast stem cells from naive human pluripotent stem cells. Elife 9, 1–26.

Draper JS, Nagy A, Rossant J, Se CA, 2008. Establishment of endoderm progenitors by SOX transcription factor expression in human embryonic stem cells. Cell Stem Cell 3.

Edwards RG, Bavister BD, Steptoe PC, 1969. Early stages of fertilization in vitro of human oocytes in vitro. Nature 221, 632–635. [PubMed: 4886881]

Etoc F, et al., 2016. A balance between secreted inhibitors and edge sensing controls. Dev. Cell 39, 302–315. [PubMed: 27746044]

Flamier A, Singh S, Rasmussen TP, 2017. A standardized human embryoid body platform for the detection and analysis of teratogens. PloS One 12, 1–17.

Gafni O, et al., 2013. Derivation of novel human ground state naive pluripotent stem cells. Nature 504, 282–286. [PubMed: 24172903]

Gao X, et al., 2019. Establishment of porcine and human expanded potential stem cells. Nat. Cell Biol. 21, 687–699. [PubMed: 31160711]

Guo G, et al., 2016. Naive pluripotent stem cells derived directly from isolated cells of the human inner cell mass. Stem Cell Reports 6, 437–446. [PubMed: 26947977]

Guo G, et al., 2017. Epigenetic resetting of human pluripotency. Development 144, 2748–2763. [PubMed: 28765214]

Guo G, et al., 2020. Trophectoderm Potency Is Retained Exclusively in Human Naïve Cells. bioRxiv.

Hanna J, et al., 2010. Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. Proc. Natl. Acad. Sci. Unit. States Am. 107, 9222–9227.

Harrison SE, Sozen B, Christodoulou N, Kyprianou C, Zernicka-goetz M, 2017. Assembly of embryonic and extraembryonic stem cells to mimic embryogenesis in vitro. Science 80, 356.

Heemskerk I, et al., 2019. Rapid changes in morphogen concentration control self-organized patterning in human embryonic stem cells. Elife 8, 1–28.

Horii M, et al., 2016. Human pluripotent stem cells as a model of trophoblast differentiation in both normal development and disease. Proc. Natl. Acad. Sci. Unit. States Am. 10.1073/pnas.1604747113.

Hwang Y, et al., 2009. Microwell-mediated control of embryoid body size regulates embryonic stem cell fate via differential expression of WNT5a and WNT11. Proc. Natl. Acad. Sci. Unit. States Am. 106, 16978–16983.

Hyun I, Munsie M, Pera MF, Rivron NC, Rossant J, 2020. Toward guidelines for research on human embryo models formed from stem cells. Stem Cell Reports 14, 169–174. [PubMed: 31951813]

Ilic D, Ogilvie C, 2017. Concise review: human embryonic stem cells — what have we done? What are we doing? Where are we going? Stem Cell. 35, 17–25.

Itskovitz-eldor J, et al., 2000. Differentiation of Human Embryonic Stem Cells into Embryoid Bodies Comprising the Three Embryonic Germ Layers, 6, pp. 88–95.

James D, Noggle SA, Swigut T, Brivanlou AH, 2006. Contribution of human embryonic stem cells to mouse blastocysts. Dev. Biol. 295, 90–102. [PubMed: 16769046]

James JL, Carter AM, Chamley LW, 2012. Human placentation from nidation to 5 weeks of gestation. Part I : what do we know about formative placental development following implantation? Placenta 33, 327–334. [PubMed: 22374510]

Kobayashi T, Surani MA, 2018. On the Origin of the Human Germline. Development. 10.1242/dev.150433.

Krendl C, et al., 2017. GATA2/3-TFAP2A/C transcription factor network couples human pluripotent stem cell differentiation to trophectoderm with repression of pluripotency. Proc. Natl. Acad. Sci. Unit. States Am. 10.1073/pnas.1708341114.

Kuijk EW, et al., 2012. The roles of FGF and MAP kinase signaling in the segregation of the epiblast and hypoblast cell lineages in bovine and human embryos. Development 139, 871–882. [PubMed: 22278923]

Kunath T, et al., 2005. Imprinted X-inactivation in extra-embryonic endoderm cell lines from mouse blastocysts. Development 7, 1649–1661.

Lancaster MA, et al., 2013. Cerebral organoids model human brain development and microcephaly. Nature 501, 373–379. [PubMed: 23995685]

Lee CQE, et al., 2016. What is trophoblast? A comination of criteria define human first-trimester trophoblast. Stem Cell Reports 6, 257–272. [PubMed: 26862703]

Li Y, et al., 2013. BMP4-directed trophoblast differentiation of human embryonic stem cells is mediated through a Np63 + cytotrophoblast stem cell state. Stem Cells Regen 3976, 3965–3976.

Li R, et al., 2019. Generation of blastocyst-like structures from mouse embryonic and adult cell cultures. Cell 179, 687–702. [PubMed: 31626770]

Linneberg-agerholm M, et al., 2019. Naïve human pluripotent stem cells respond to Wnt, Nodal and LIF signalling to produce expandable naïve extra-embryonic endoderm. Development 146.

Liu X, et al., 2017. Comprehensive characterization of distinct states of human naive pluripotency generated by reprogramming. Nature 14.

Macklon NS, Geraedts JPM, Fauser BCJM, 2002. Conception to ongoing pregnancy : the 'black box' of early pregnancy loss. Hum. Reprod. Update 8, 333–343. [PubMed: 12206468]

Martinez Arias A, Lutolf MP, 2018. Mammalian body plan engineering: lessons and challenges. Curr. Opin. Struct. Biol. 11, 50–56.

Martyn I, Kanno TY, Ruzo A, Siggia ED, Brivanlou AH, 2018. Self-organization of a human organizer by combined Wnt and Nodal signalling. Nature. 10.1038/s41586-018-0150-y.

Martyn I, Brivanlou AH, Siggia ED, 2019. A wave of WNT signaling balanced by secreted inhibitors controls primitive streak formation in micropattern colonies of human embryonic stem cells. Development 246.

Mascetti VL, Pedersen RA, 2016. Human-mouse chimerism validates human stem cell pluripotency. Stem Cell. 18, 67–72.

Matsuda M, et al., 2020. Recapitulating the human segmentation clock with pluripotent stem cells. Nature 580, 124–129. [PubMed: 32238941]

Messmer T, et al., 2019. Transcriptional heterogeneity in naive and primed human pluripotent stem cells at single-cell resolution. Cell Rep. 26, 815–824. [PubMed: 30673604]

Minn KT, et al., 2020. High-resolution transcriptional and morphogenetic profiling of cells from micropatterned human embryonic stem cell gastruloid cultures. eLife, e59445. 10.7554/eLife.59445. [PubMed: 33206048]

Mischler A, et al., 2019. Two Distinct Trophectoderm Lineage Stem Cells from Human Pluripotent Stem Cells. bioRxiv.

Molè MA, Weberling A, Zernicka-goetz m, 2020. Comparative analysis of human and mouse development: from zygote to pre-gastrulation. Curr. Top. Dev. Biol. 136, 113–138. [PubMed: 31959285]

Morgani SM, Hadjantonakis AK, 2020. Signaling Regulation during Gastrulation: Insights from Mouse Embryos and in Vitro Systems. Current Topics in Developmental Biology, 137. Elsevier Inc.

Moris N, et al., 2020. An in Vitro Model for Anteroposterior Organisation during Human Development. 10.1038/s41586-020-2383-9.

Nakamura T, et al., 2016. A developmental coordinate of pluripotency among mice, monkeys and humans. Nature 537, 57–62. [PubMed: 27556940]

Nakano T, et al., 2012. Self-formation of optic cups and storable stratified neural retina from human ESCs. Cell Stem Cell 10, 771–785. [PubMed: 22704518]

Nichols J, Smith A, 2009. Perspective naive and primed pluripotent states. Stem Cell. 4, 487–492.

Nikas G, Ao A, Winston RML, Handyside AH, 1996. Compaction and surface polarity in the human embryo in vitro. Biol. Reprod. 55, 32–37. [PubMed: 8793055]

Okae H, et al., 2018. Derivation of human trophoblast stem cells. Stem Cell. 22, 1–14.

Paepe C. De, et al., 2013. Human trophectoderm cells are not yet committed. Hum. Reprod. 28, 740–749. [PubMed: 23257394]

Pastor WA, et al., 2018. TFAP2C regulates transcription in human naive pluripotency by opening enhancers. Nat. Cell Biol. 20.

Perea-Gomez A, Rhinn M, Ang SL, 2001. Role of the anterior visceral endoderm in restricting posterior signals in the mouse embryo. Int. J. Dev. Biol. 45, 311–320. [PubMed: 11291861]

Pereira Daoud AM, et al., 2020. Modelling human embryogenesis: embryo-like structures spark ethical and policy debate. Hum. Reprod. Update 1–20. [PubMed: 31803930]

Petropoulos S, et al., 2016. Single-cell RNA-seq reveals lineage and X chromosome dynamics in human preimplantation resource single-cell RNA-seq reveals lineage and X chromosome dynamics in human preimplantation embryos. Cell 165, 1012–1026. [PubMed: 27062923]

Piotrowska M, 2019. Avoiding the potentiality trap: thinking about the moral status of synthetic embryos. Monash Bioeth. Rev. 10.1007/s40592-019-00099-5.

Qin H, et al., 2016. YAP induces human naive pluripotency. Cell Rep. 14, 2301–2312. [PubMed: 26947063]

Rivron NC, et al., 2018a. Blastocyst-like structures generated solely from stem cells. Nature 557.

Rivron N, et al., 2018b. Debate ethics of embryo models from stem cells. Nature 564, 183–185. [PubMed: 30542177]

Roode M, et al., 2012. Human hypoblast formation is not dependent on FGF signalling. Dev. Biol. 361, 358–363. [PubMed: 22079695]

Rostovskaya M, Stirparo GG, Smith A, 2019. Capacitation of human naïve pluripotent stem cells for multi-lineage differentiation. Development 146, 1–15.

Sasaki K, et al., 2016. The germ cell fate of cynomolgus monkeys is specified in the nascent amnion article the germ cell fate of cynomolgus monkeys is specified in the nascent amnion. Dev. Cell 39, 169–185. [PubMed: 27720607]

Sawai T, et al., 2020. The moral status of human embryo-like structures: potentiality matters? EMBO Rep. 21, 1–6.

Shahbazi MN, Zernicka-goetz M, 2018. Deconstructing and reconstructing the mouse and human early embryo. Nat. Cell Biol. 20.

Shahbazi MN, et al., 2016. Self-organization of the Human Embryo in the Absence of Maternal Tissues, 18.

Shahbazi MN, et al., 2017. Pluripotent state transitions coordinate morphogenesis in mouse and human embryos. Nature 552, 239–243. [PubMed: 29186120]

Shahbazi MN, Siggia ED, Zernicka-Goetz M, 2019. Self-organization of stem cells into embryos: a window on early mammalian development. Science 364, 948–951. [PubMed: 31171690]

Shahbazi MN, et al., 2020. Developmental potential of aneuploid human embryos cultured beyond implantation. Nat. Commun. 1–15. 10.1038/s41467-020-17764-7. [PubMed: 31911652]

Shao Y, et al., 2017b. A pluripotent stem cell-based model for post-implantation human amniotic sac development. Nat. Commun. 8, 208. 10.1038/s41467-017-00236-w. [PubMed: 28785084]

Shao Y, et al., 2017a. Self-organized amniogenesis by human pluripotent stem cells in a biomimetic implantation-like niche. Nat. Mater. 10.1038/NMAT4829.

Sharon N, Mor I, Golan-lev T, Fainsod A, Benvenisty N, 2011. Molecular and functional characterizations of gastrula organizer cells derived from human embryonic stem cells. Stem Cell. 29, 600–608.

Simunovic M, et al., 2019. A 3D model of a human epiblast reveals BMP4-driven symmetry breaking. Nat. Cell Biol. 10.1038/s41556-019-0349-7.

Singla S, Iwamoto-Stohl LK, Zhu M, Zernicka-goetz M, 2020. Autophagy-mediated apoptosis eliminates aneuploid cells in a mouse model of chromosome mosaicism. Nat. Commun 1–15. 10.1038/s41467-020-16796-3. [PubMed: 31911652]

Sozen B, et al., 2018. Self-assembly of embryonic and two extra-embryonic stem cell types into gastrulating embryo-like structures. Nat. Cell Biol. 20.

Sozen B, et al., 2019. Self-organization of mouse stem cells into an extended potential blastoid article self-organization of mouse stem cells into an extended potential blastoid. Dev. Cell 51, 698–712. [PubMed: 31846649]

Starostik MR, Sosina OA, Mccoy RC, 2020. Single-cell Analysis of Human Embryos Reveals Diverse Patterns of Aneuploidy and Mosaicism, pp. 814–825. 10.1101/gr.262774.120.Freely.

Steven D, et al., 2012. Analysis of embryoid bodies derived from human induced pluripotent stem cells as a means to assess pluripotency. Stem Cell. Int. 10.1155/2012/738910.

Stirparo GG, et al., 2018. Integrated analysis of single-cell embryo data yields a unified transcriptome signature for the human pre-implantation epiblast. Development 145.

Sun B, Seung Y, Yoo J, Eun J, 2006. Enhanced differentiation of human embryonic stem cells into cardiomyocytes by combining hanging drop culture and 5-azacytidine treatment. Differentiation 74, 149–159. [PubMed: 16683985]

Takahashi K, et al., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861–872. [PubMed: 18035408]

Takashima Y, et al., 2014. Resetting transcription factor control circuitry toward ground-state pluripotency in human. Cell 158, 1254–1269. [PubMed: 25215486]

Taniguchi K, et al., 2015. Lumen formation is an intrinsic property of isolated human pluripotent stem cells. Stem Cell Reports 5, 954–962. [PubMed: 26626176]

Taniguchi K, Heemskerk I, Gumucio DL, 2019. Opening the black box: stem cell-based modeling of human post-implantation development. J. Cell Biol. 218, 410–421. [PubMed: 30552099]

Theunissen TW, et al., 2014. Systematic identification of culture conditions for induction and maintenance of naive human pluripotency. Cell Stem Cell 15, 471–487. [PubMed: 25090446]

Theunissen TW, et al., 2016. Molecular criteria for defining the naive human pluripotent state. Cell Stem Cell 19, 502–515. [PubMed: 27424783]

Thomson JA, et al., 1998. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145–1148. [PubMed: 9804556]

Turco MY, et al., 2018. Trophoblast organoids as a model for maternal-fetal interactions during human placentation. Nature 564, 263–267. [PubMed: 30487605]

Tyser RCV, et al., 2020. A Spatially Resolved Single Cell Atlas of Human Gastrulation. bioRxiv.

Ungrin MD, Joshi C, Nica A, Bauwens C, Zandstra PW Reproducible, 2008. Ultra high-throughput formation of multicellular organization from single cell suspension-derived human embryonic stem cell aggregates. PloS One 3.

Vianello S, Lutolf MP, 2019. Understanding the mechanobiology of early mammalian development through bioengineered models. Dev. Cell 48, 751–763. [PubMed: 30913407]

Wamaitha SE, Niakan KK, 2018. Human Pre-gastrulation Development. Current Topics in Developmental Biology, 128. Elsevier Inc.

Wamaitha SE, et al., 2015. Gata6 potently initiates reprograming of pluripotent and differentiated cells to extraembryonic endoderm stem cells. Genes Dev. 29, 1239–1255. [PubMed: 26109048]

Ware CB, et al., 2014. Derivation of naïve human embryonic stem cells. Proc. Natl. Acad. Sci. Unit. States Am. 111.

Warmflash A, Sorre B, Etoc F, Siggia ED, Brivanlou AH, 2014. A Method to Recapitulate Early Embryonic Spatial Patterning in Human Embryonic Stem Cells, 11.

Weatherbee BAT, Glover DM, Zernicka-goetz M, Glover DM, 2020. Expression of SARS-CoV-2 receptor ACE2 and the protease TMPRSS2 suggests susceptibility of the human embryo in the first trimester. Open Biol 10, 4–7.

Wilcox AJ, Baird DD, Weinberg CR, 1999. Time of implantation of the conceptus and loss of pregnancy. N. Engl. J. Med. 340, 1796–1799. [PubMed: 10362823]

Xiang L, et al., 2020. A developmental landscape of 3D-cultured human pre-gastrulation embryos. Nature 577.

Xu R, et al., 2002. BMP4 initiates human embryonic stem cell differentiation to trophoblast. Nat. Biotechnol. 20, 1261–1264. [PubMed: 12426580]

Yamanaka Y, Lanner F, Rossant J, 2010. FGF signal-dependent segregation of primitive endoderm and epiblast in the mouse blastocyst. Development 137, 715–724. [PubMed: 20147376]

Yang Y, et al., 2017. Derivation of pluripotent stem cells with in vivo embryonic and extraembryonic potency. Cell 169, 243–257. [PubMed: 28388409]

Yoney A, et al., 2018. WNT signaling memory is required for ACTIVIN to function as a morphogen in human gastruloids. Elife 7, 1–28.

Yu J, et al., 2007. Induced pluripotent stem cell lines derived from human somatic cells. Science 80, 318.

Yu J, et al., 2009. Human induced pluripotent stem cells free of vector and transgene sequences. Science 80, 324.

Zheng Y, et al., 2019. Controlled modelling of human epiblast and amnion development using stem cells. Nature. 10.1038/s41586-019-1535-2.

Zhou F, et al., 2019. Reconstituting the transcriptome and DNA methylome landscapes of human implantation. Nature. 10.1038/s41586-019-1500-0.
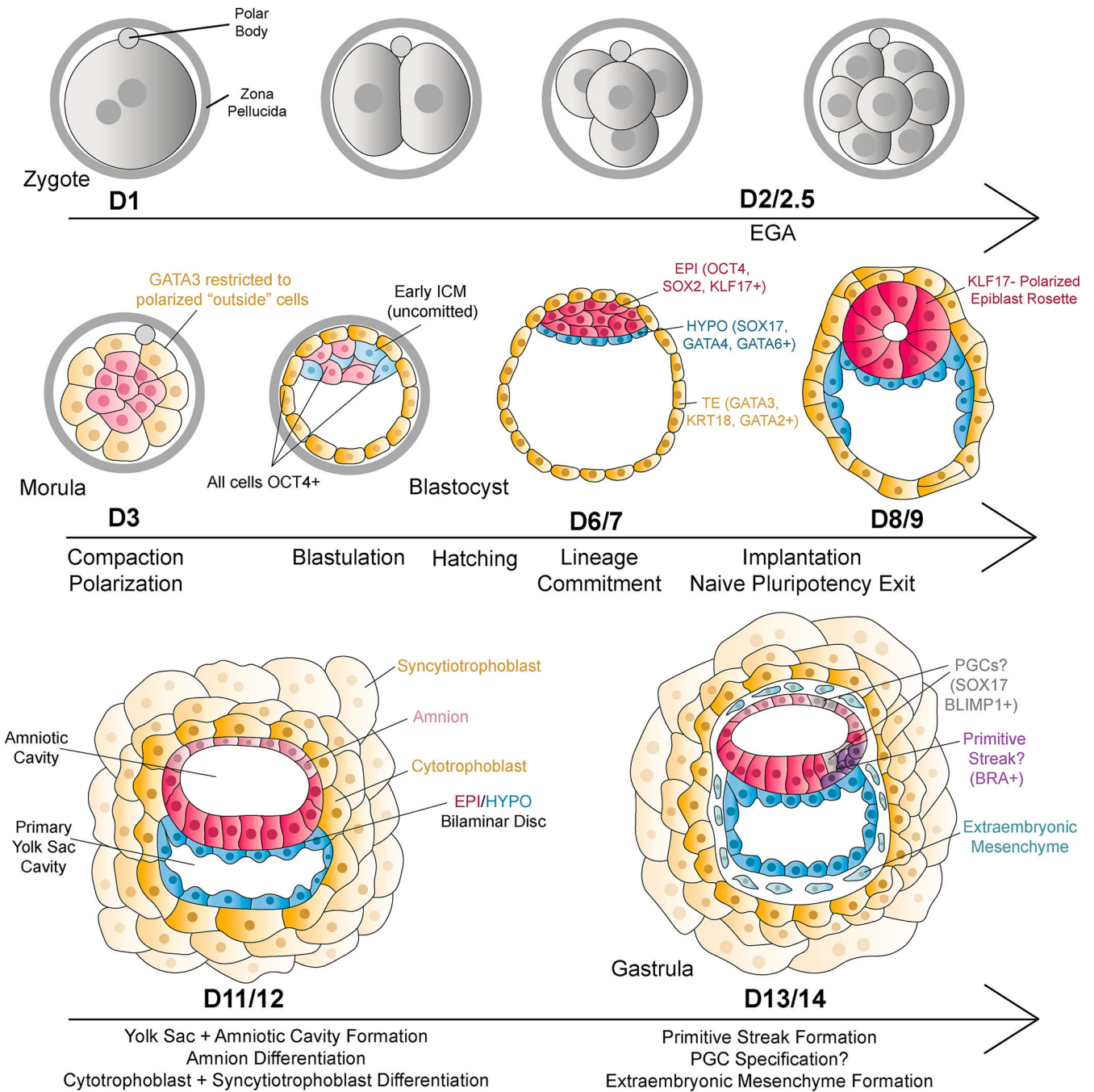
Fig. 1. Pre-Gastrulation Development of the Human Embryo.

Following fertilization, the zygote undergoes a series of cleavage stages. Between the 4- and 8-cell stage, embryonic genome activation (EGA) occurs (Braude et al., 1988). At the 8-to-16-cell stage the embryo compacts and undergoes polarization (Nikas et al., 1996), 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 (Guo et al., 2020; Paepe et al., 2013), and at the late blastocyst stage the epiblast, hypoblast, and trophoblast cells have undergone lineage commitment (Petropoulos et al., 2016; Stirparo et

al., 2018; Molè et al., 2020). 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.
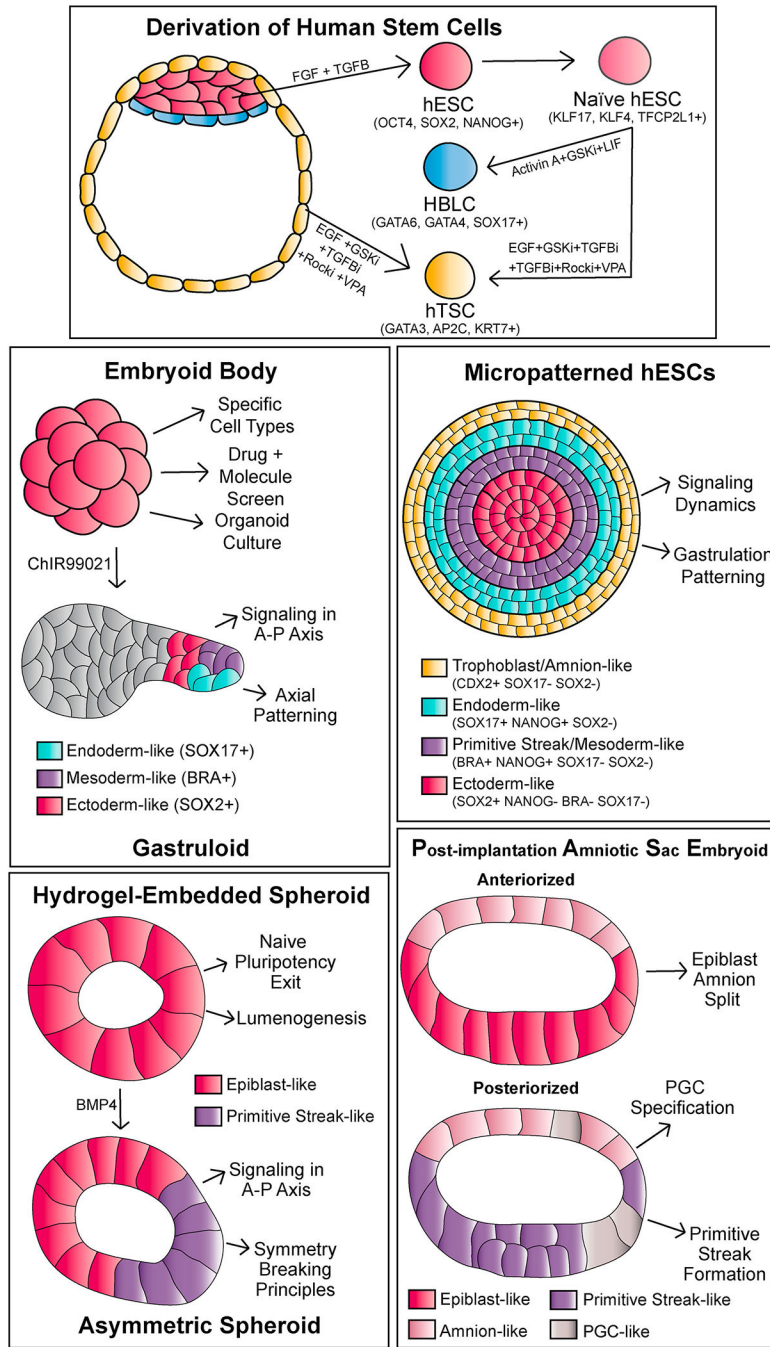
Fig. 2. Derivation of Human Stem Cells and Embryonic Stem Cell Models.
Human embryonic stem cells (hESCs) and human trophoblast stem cells (hTSCs) have been derived directly from the human blastocyst while hypoblast-like cells (HBLCs) can be derived from naïve hESCs. Naïve hESCs can also give rise to 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.
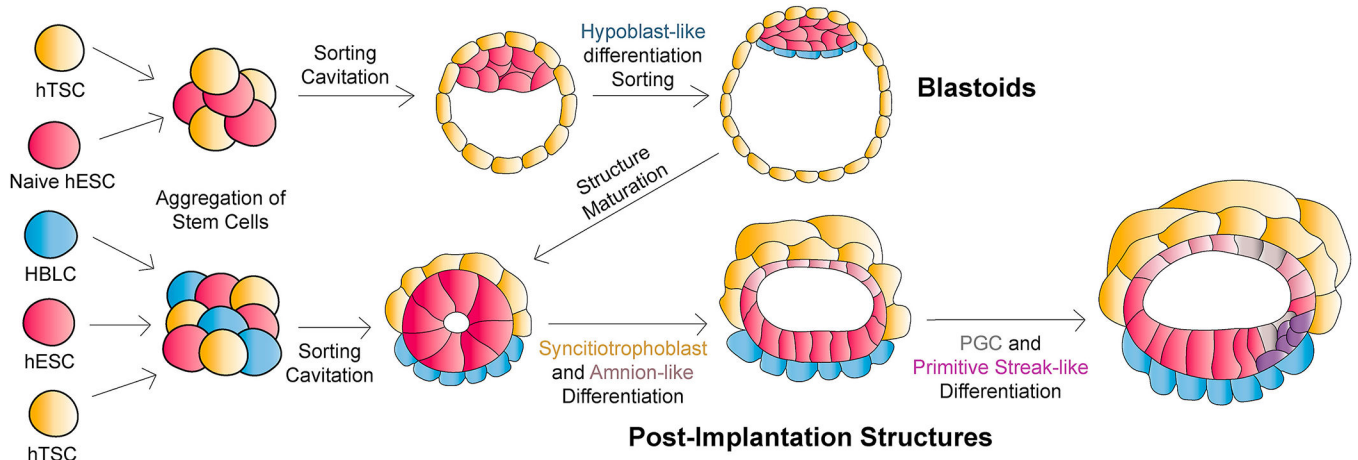
Fig. 3. Theoretical Stem Cell Models with Embryonic and Extra-Embryonic Stem Cells.
Embryonic, trophoblast, and hypoblast-like (hESC, hTSC, HBLCs) cells may be co-cultured to form either blastocyst or post-implantation-like structures containing both embryonic and extraembryonic cells. Ideally, these 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., 2014	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)